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Segregated Regulatory Elements Direct β-Myosin Heavy Chain Expression in Response to Altered Muscle Activity*

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Our previous transgenic analyses revealed that a 600-base pair β-myosin heavy chain (βMyHC) promoter conferred mechanical overload (MOV) and non-weight-bearing (NWB) responsiveness to a chloramphenicol acetyltransferase reporter gene. Whether the same DNA regulatory element(s) direct βMyHC expression following MOV or NWB activity *in vivo* remains unknown. We now show that a 293-base pair βMyHC promoter fused to chloramphenicol acetyltransferase (β293) responds to MOV, but not NWB activity, indicating a segregation of these two diverse elements. Inclusion of the βMyHC negative regulatory element (−332 to −300; βNRE) within transgene β350 repressed expression in all transgenic lines. Electrophoretic mobility shift assays showed highly enriched binding activity only in NWB soleus nuclear extracts that was specific to the distal region of the βNRE sense strand (dβNRE-S; −332 to −311). Supershift electrophoretic mobility shift assay revealed that the binding at the distal region of the βNRE sense strand was antigenically distinct from cellular nucleic acid-binding protein and Y-box-binding factor 1, two proteins shown to bind this element. Two-dimensional UV cross-linking and shift Southwestern blotting analyses detected two proteins (50 and 52 kDa) that bind to this element. These *in vivo* results demonstrate that segregated βMyHC promoter elements transcriptionally regulate βMyHC transgene expression in response to two diverse modes of neuromuscular activity.

The sarcomeric myosin heavy chain (MyHC)[1] is a major contractile protein that is encoded by a multigene family constituted by eight members (1). Each member has been shown to be responsive to a complex set of intrinsic and extrinsic signals that collectively regulate their expression in a defined developmental stage- and muscle fiber type-specific pattern. Within the MyHC gene family, the βMyHC is the one member whose regulated expression in both cardiac and skeletal muscle has been most extensively studied. Expression of the βMyHC gene has been detected in the ventricular myocardium and skeletal muscles comprising the hind limb of fetal mice, whereas in the adult, its expression is primarily restricted to skeletal muscles or muscle regions composed predominately of slow twitch type I fibers (2, 3). *In vitro* investigations of the control of βMyHC gene transcription have led to the identification of a control region located within the proximal promoter (nucleotides −300 to −188 of the human βMyHC gene) region (4–7). This control region was found to be composed of three discrete DNA regulatory elements termed MCAT-like, C-rich and βe3, which are highly conserved in location and sequence across species (4–7). The absolute requirement for these elements to obtain high levels of gene expression was demonstrated in transient transfection studies where the independent disruption of any of these conserved elements decreased and/or abolished muscle-specific expression of βMyHC reporter genes (4–7).

Located just upstream from the βMyHC control region is a highly conserved negative regulatory element (βNRE; nucleotides −332 to −300) that represses transcription of βMyHC reporter genes and heterologous promoters in transient expression assays (4, 8). Several recent studies have provided evidence that two distinct proteins interact with this element: cellular nucleic acid-binding protein (CNBP) and the Y-box binding factor, YB-1 (9, 10). In co-transfection experiments, CNBPα, but not CNBPβ, was shown to repress βMyHC reporter gene expression in rat fetal heart cells. Although the regulatory effects of YB-1 binding at the βNRE have not been determined, existing evidence suggests that YB-1 plays a role in mediating tissue-specific gene expression in a variety of tissue types including striated muscle (11–14). While the βNRE has been shown to regulate βMyHC gene expression in primary cardiocytes (9), its regulatory role *in vivo* has not been identified, nor has this element been shown to play a regulatory role in mediating βMyHC gene expression in skeletal muscle.

A more complex picture of the regulatory mechanisms directing βMyHC gene expression has emerged from recent transgenic investigations. These investigations revealed that decreased muscle-specific βMyHC transgene expression was observed only when all three DNA regulatory elements comprising the βMyHC control region were simultaneously mutated (15, 16). Furthermore, it was also shown that additional sequences located upstream of nucleotide −600 are required for high levels of muscle-specific βMyHC transgene expression and that these element(s) can compensate for the loss of any one or two control region elements (15, 16). Clearly, these *in vivo* findings indicate that complex combinatorial interactions between distal and proximal DNA regulatory elements are required for high levels of striated muscle-specific expression.

An important and relatively unexplored area concerns the

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[1] The abbreviations used are: MyHC, myosin heavy chain; βMyHC, β-myosin heavy chain; βNRE, βMyHC negative regulatory element; dβNRE, distal region of βNRE; dβNRE-S, distal region of the βNRE sense strand; CNBP, cellular nucleic acid-binding protein; YB-1 and -3, Y-box-binding factor 1 and 3, respectively; MOV, mechanical overload; NWB, non-weight-bearing; bp, base pair(s); CS, control-soleus; NWB-S, NWB-soleus; CP, control-plantaris; MOV-P, MOV-plantaris; CAT, chloramphenicol acetyltransferase; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay.

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malleable nature of βMyHC gene expression in response to a broad spectrum of physiological perturbations including changes in the levels of circulating thyroid hormone, modified patterns of electrical stimulation, and altered mechanical work loads. We have focused our efforts on delineating DNA regulatory element(s) that direct βMyHC gene expression in response to diverse modes of neuromuscular activity such as mechanical overload (MOV) and non-weight-bearing (NWB) activity (17, 18). Our transgenic deletion analysis revealed that 293 base pairs (bp) of proximal promoter region is minimally sufficient to confer MOV induction to a CAT reporter gene, while 600 bp of proximal promoter region is sufficient to direct NWB responsiveness (17, 18). The regulation of βMyHC expression in response to MOV and NWB is correlated with a specific fiber phenotype change, where under normal conditions βMyHC expression is primarily restricted to slow twitch type I fibers but can be induced in fast twitch type II fibers following MOV and decreased in slow twitch type I fibers by NWB activity. Given the associated fiber type shift with the antithetic nature of βMyHC expression in response to these two diverse perturbations, several queries arise. First, do MOV and NWB activity direct βMyHC gene transcription via the same or distinct cis-acting element(s)? Second, do MOV and/or NWB element(s) serve an independent or overlapping role in specifying fiber type-specific expression? Third, does the βNRE contribute to the decrease in βMyHC expression seen under NWB conditions *in vivo*? As an initial step toward resolving these critical regulatory mechanism(s), we have set out to delimit a βMyHC gene promoter region that is responsive to NWB activity by generating transgenic lines harboring transgenes composed of either 293 or 350 bp of the human βMyHC gene proximal promoter region (β293, β350) fused to the 5′-end of the CAT gene. Notable findings resulting from this transgenic study are that the DNA regulatory sequence(s) that mediate βMyHC gene expression in response to either MOV or NWB activity are segregated to different regions within the proximal promoter of the βMyHC gene and that a negative regulatory element (dβNRE-S) that interacts with two unidentified protein(s), may play an *in vivo* role in NWB regulation of βMyHC.

EXPERIMENTAL PROCEDURES

Generation and Screening of Transgenic Mice—Transgenic mice were generated by microinjection of purified transgene DNA into pronuclei of single cell fertilized embryos as described previously (19). Transgene-positive mice (founders) were identified by Southern blot analysis. Subsequent offspring derived from mating the founders were screened for transgene incorporation using the polymerase chain reaction. In the present study, multiple independent transgenic lines representing transgenes β350 and β293 (Fig. 1) were studied, and all lines were maintained in a heterozygous state by continual outbreeding to non-transgenic FVB/n mice.

Animal Care and MOV and NWB Procedures—The NWB and MOV procedures used in this study were approved by the Animal Care Committee for the University of Missouri-Columbia, and the NWB and MOV mice were housed in an AAALAC (Association for the Assessment and Accreditation of Laboratory Animal Care International) accredited animal facility. All mice were provided with food and water *ad libitum* and were housed at room temperature (24 °C) with a 12-h light-dark cycle in either standard filter top cages (control and MOV mice) or cages designed for head-down tilt suspension (hind limb suspension), as described previously (18). Adult β293 transgenic mice, 12 weeks of age, were randomly assigned to one of four groups: 1) a NWB group that used hind limb suspension to impose NWB conditions (NWB; $n = 46$); 2) a NWB control group that served as cage ambulatory controls for the NWB group (C, $n = 46$); 3) a MOV group where MOV was imposed on the fast twitch plantaris muscle bilaterally by surgically removing the gastrocnemius and soleus muscles (17, 20) (MOV; $n = 17$); and 4) a MOV control group that represented a sham-operated control for the MOV group (C; $n = 17$). Mice were prepared for the NWB experiment by an inexpensive modification of the noninvasive tail traction procedure, as described previously (18). For MOV studies, mice weighing an average of 25.7 g (see Tables II and III) were anesthetized with 0.017 ml of 2.5% (w/v) avertin/g of body weight (17, 20). Following a 2-week NWB period and an 8-week MOV period, control (NWB-C, MOV-C) and experimental (NWB, MOV) animals were anesthetized and weighed, and control-soleus (CS), NWB-soleus (NWB-S), control-plantaris (CP), and MOV-plantaris (MOV-P) muscles were collected for further study. All muscles were trimmed clean of fat and connective tissue, weighed, and stored at −80 °C until assayed. Prior to the initiation of the NWB study, a 4-week time course analysis was undertaken to establish the time period over which the greatest decrease in mouse muscle mass, transgene expression (composed of 5600 bp of βMyHC 5′ promoter sequence upstream of the CAT gene (15–17)), and endogenous βMyHC mRNA and protein levels occurs post-NWB activity. Our results indicated that the greatest magnitude of change for each of the aforementioned parameters occurred after 2 weeks of NWB activity; therefore, the present NWB study was conducted with a 2-week termination time point.[2]

Isolation and Analysis of RNA—Isolation of total cellular RNA was performed as described previously (20) using the acid guanidinium thiocyanate phenol-chloroform method as described by Chomczynaski and Sacchi (22). The purity of RNA was judged by the ratio of the absorbances (260/280 nm). Due to the small size of mouse muscle, it was necessary to pool muscle from 3–5 mice (CS = 6–8 mg; NWB-S = 5–6 mg; CP = 16 mg; MOV-P = 30 mg). Six micrograms of total RNA from CS and NWB-S or 8 μg from CP and MOV-P muscles was denatured at 65 °C in a solution containing 5 μl of deionized formamide and 1 μl of 10× running buffer. The inclusion of ethidium bromide allowed visualization of the RNA samples. Total RNA was fractionated on a 1.5% (w/v) agarose, 2.2 M formaldehyde-containing gel, transferred to a nylon filter (Duralon, Stratagene) by capillary blotting, and immobilized on the membrane by ultraviolet (UV) irradiation (UV Stratalinker, Stratagene).

The specific cDNA probes used in this study were as follows: mouse-specific CNBP probe (23), rat-specific YB-1 (10), and human 18 S rRNA (24). For detection of CAT mRNA, a *Hin*dIII/*Bam*HI fragment from pSVOCAT was used. All cDNA probes were labeled with [α-^{32}P]dCTP (3000 Ci/mmol) using a random primer kit (Stratagene) to a specific activity of $7–8 \times 10^8$ cpm/μg of DNA. An oligonucleotide probe corresponding to the mouse βMyHC 3′-untranslated region was used to detect mouse βMyHC-specific transcripts (20) and was end-labeled to a specific activity of 6×10^8 cpm/ng of DNA with γ-^{32}P (6000 Ci/mmol)-labeled ATP using a KinAse-It kit (Stratagene). Filters were washed twice in 2× sodium chloride/sodium citrate, 0.1% (w/v) SDS for 15 min at room temperature followed by two 30-min washes in 0.1× SSC, 0.1% (w/v) SDS at 60 °C. Filters were dried and exposed to x-ray film (XAR-5, Eastman Kodak Co.) overnight at −80 °C. Differences between intensities of signal in control and experimental lanes were quantified using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA) and expressed as relative percentage difference of arbitrary units. All Northern blots were hybridized with an 18 S rRNA cDNA probe to verify that each lane contained equal (±5%) amounts of RNA and for the purpose of normalizing all RNA values.

CAT Assay—CAT assays were performed as described by Tsika *et al.* (20) with the following modifications. Extracts were prepared from transgenic tissues using a glass tissue homogenizer to disrupt tissues in 250 mM Tris-HCl, pH 7.8, and 5 mM EDTA. All muscle extracts were prepared from frozen tissue, and each n value represents pooled muscles from one mouse. The protein concentration of each extract was determined by the method of Bradford (21). Tissue extracts were heated at 65 °C for 10 min followed by centrifugation for 10 min at $10{,}000 \times g$. Since each transgenic line exhibits inherently different transgene expression levels, it was necessary to use different amounts of tissue extract and variable incubation times so that the CAT enzyme activities could be determined within a linear range (30% conversion) as described previously (17, 20). Direct comparisons between and within transgenic lines representing both control and experimental groups (CS, NWB-S, CP, and MOV-P) were facilitated by presenting the data as specific CAT activity (pmol/μg of protein/min).

Isolation of Nuclear Protein Extracts—Nuclear extracts were isolated from adult rat CS and NWB-S muscle. The sequential isolation of myonuclei and nuclear extract protein was performed according to the protocol of Lichtstein *et al.* (25) with minor modifications as described by Mar *et al.* (26) and Larkin *et al.* (27). All procedures were carried out on ice. All buffers contained 2 μg/ml of each protease inhibitor: aprotinin and leupeptin and 0.5 mM phenylmethylsulfonyl fluoride. Eight grams of either CS or NWB-S muscle, harvested from adult female Sprague-Dawley rats (200 g), were minced in phosphate-buffered sa-

[2] J. McCarthy and R. W. Tsika, manuscript in preparation.

TABLE I
Oligonucleotide probes

Name[a]	Sequence[b]	Position	Reference
βNRE-S	5′-GTGGTCTTGGTGGTCGTGGTCAGTTCCCTCTC-3′	−332/−301	4
βNRE-AS	5′-GGAGAGGGAACTGACCACGACCACCAAGACCAC-3′	−332/−300	
pβNRE-S	5′-GTCAGTTCCCTCTC-3′	−314/−301	
dβNRE-S	5′-GTGGTCTTGGTGGTCGTGGTCA-3′	−332/−311	
dβNRE-Smt1	5′-GTGtgCgTcaTatgCcTcaTgA-3′		
dβNRE-Smt2	5′-aTatgCgTcGTGGTCGTGGTCA-3′		
HMG-CoA (S)	5′-GAAGCTT**GTGCGGTG**GAATTCTGCA-3′	SREoctamer	30
HF-1 (S)	5′-GCCAAAAGTGGTCATGGGGTTATTTTTA-3′	−72/−44	14
C-rich (S)	5′-GCACTTTGAGCCACCCCGCCCC-3′	−254/−233	

[a] βNRE, negative regulatory element of βMyHC gene; p, proximal; d, distal; mt, mutation of βNRE with base pair changes in lowercase letters; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A reductase gene sterol regulatory element (SRE) octamer (boldface type); HF-1 element of myosin light chain 2 gene; S, sense strand; AS, anti-sense strand.
[b] Underlined base pairs show the greatest homology to βNRE as determined by the GCG Wisconsin package BestFit program.

line. Minced muscle tissue was incubated for two 15-min intervals in relaxation buffer 1 (100 mM KCl, 5 mM $MgCl_2$, 5 mM EGTA, 5 mM sodium pyrophosphate, pH 6.8), followed by two 10-min washes in RBII (50 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM sodium pyrophosphate, pH 6.8). The muscle tissue was then washed with 100 ml of homogenization buffer A (0.3 M sucrose, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM HEPES, pH 7.5, 0.5 mM EGTA, 2 mM EDTA, 10 mg/ml bovine serum albumin, 1 mM dithiothreitol (DTT)), followed by homogenization in 300 ml of fresh homogenization buffer A. Muscle tissue was disrupted with an Omni homogenizer (20-mm saw-tooth generator) at 60% power for three 20-s pulses. Muscle homogenates were then centrifuged (1000 × *g*, 4 °C, 10 min) in a Beckman JA-25–50 rotor, and the pellets were solubilized in homogenization buffer B (0.3 M sucrose, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM HEPES, pH 7.5, 0.1 mM EGTA, 0.1 mM EDTA, 10 mg/ml bovine serum albumin, 1 mM DTT) containing 0.5% (v/v) Triton X-100. The solubilized pellet was homogenized (7-mm saw-tooth generator) at 60% power for one 15-s pulse, followed by homogenization with a hand-held Teflon glass Dounce homogenizer. Percoll (Amersham Pharmacia Biotech) was added to the homogenate to a final concentration of 27% (v/v), and the mixture was centrifuged at 27,000 × *g* at 4 °C for 15 min. The nuclear pellet was solubilized in 10 ml of lysis buffer (10 mM HEPES, pH 7.5, 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 10% (w/v) glycerol). Nuclei were lysed by dropwise addition of 3 M NH_4SO_4 (pH 7.9) to a final concentration of 0.4 M, and the resulting extract was gently shaken for 30 min at 4 °C. The lysate was ultracentrifuged at 126,000 × *g* for 1 h using a Beckman Ti-70 rotor, and the supernatant was collected. Solid NH_4SO_4 (0.3 g/ml) was added slowly to the supernatant, and nuclear proteins were allowed to precipitate on ice for 30 min. Precipitated proteins were pelleted by ultracentrifugation at 126,000 × *g* for 30 min and resuspended in 500 μl of dialysis buffer (25 mM HEPES, pH 7.6, 40 mM KCl, 0.1 mM EDTA, 10% (w/v) glycerol, 1 mM DTT). The muscle nuclear protein extract was dialyzed twice for 1 h each time against dialysis buffer, and aliquots were quick frozen and stored at −80 °C. Once thawed, nuclear protein extracts were not refrozen for use. Protein concentration was determined according to Bradford (21).

Electrophoretic Mobility Shift Assay—All oligonucleotide probes used in this study are listed in Table I. The double-stranded βNRE oligonucleotide probe (nucleotides −332 to −300) was labeled by fill-in reaction using the Klenow fragment of *Escherichia coli* DNA polymerase I and [α-^{32}P]dCTP. Single-stranded βNRE oligonucleotide probes (antisense, sense, sense-proximal, and sense-distal) were end-labeled by T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and [γ-^{32}P]dATP (6000 Ci/mmol) and gel-purified. Binding reactions were performed for 20 min at room temperature in a 25-μl total volume. The binding reactions contained binding buffer (50 mM Tris-HCl, pH 7.9, 50 mM KCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, and 5% (w/v) glycerol), 20,000 cpm of labeled DNA probe, 1 μg of either CS or NWB-S nuclear extract, and 0.1 μg of poly(dI·dC) (Amersham Pharmacia Biotech) as a nonspecific competitor. Specificity of DNA binding was assessed by the addition of a 100-fold molar excess of unlabeled homologous or heterologous competitor DNA to the binding reaction. In some binding reactions, either HeLa nuclear extract (250 ng) or partially purified recombinant rat YB-1 protein extract (100 ng) was used in place of soleus nuclear extracts. Following incubation, DNA-protein complexes were electrophoretically resolved from unbound oligonucleotide probe on a 5% (w/v) nondenaturing polyacrylamide gel using 0.5% (w/v) Tris borate/EDTA buffer, pH 8.3, at 220 V for 2.5 h at 4 °C. Supershift experiments were performed by preincubation of nuclear extracts or partially purified YB-1 protein with polyclonal antibody to human CNBP or *Xenopus* YB-3 (contains 89% overall amino acid identity with rat YB-1 (14)) for 30 min at 4 °C prior to the addition of the labeled DNA probe. Following electrophoresis, gels were dried, and DNA-protein complexes were visualized by autoradiography.

Western Analysis—Muscle extracts, nuclear (50 μg) or cytoplasmic (50 μg), were fractionated by 10% (w/v) SDS-polyacrylamide gel electrophoresis. Proteins were electrophoretically transferred to a nitrocellulose membrane in transfer buffer containing 25 mM Tris-HCl, pH 8.3, 192 mM glycine, and 20% (v/v) methanol. The membrane was blocked by incubation for 16 h at 4 °C in Tris-buffered saline/Tween (TBS-T; 10 mM Tris-HCl, pH 7.9, 150 mM NaCl, 0.05% (v/v) Tween 20) containing 5% (w/v) nonfat milk. The filter was then incubated for 2 h at 25 °C with a 1:1000 dilution of primary antibody, corresponding to either polyclonal human anti-CNBP or rat anti-YB-1. The filter was then washed three times for 5 min each with TBS-T and incubated for 1 h at 25 °C with a 2000-fold dilution of donkey anti-rabbit IgG horseradish peroxidase-linked secondary antibody (Amersham Pharmacia Biotech) in TBS-T containing 5% (w/v) nonfat milk. Following three 5-min washes with TBS-T, immunocomplexes were visualized using a chemiluminescent detection kit (Amersham Pharmacia Biotech) according to the manufacturer's recommendation.

Two-dimensional UV Cross-linking Analysis—The first dimension of this assay involved EMSA using the dβNRE-S probe and NWB-S nuclear extracts, since only this reaction revealed the formation of a highly enriched DNA-protein complex. EMSA was performed as described above, except that the reaction mixture was scaled up 10-fold and run on a 0.75-mm-thick nondenaturing gel. Immediately following electrophoresis, the top gel glass plate was removed, and the gel was covered with Saran Wrap and placed on a transluminator (312 nm) for 30 min at 4 °C. The gel was dried for 20 min and exposed to film for an additional 30 min. Following autoradiography, the specific band corresponding to the cross-linked DNA-protein complex was excised and soaked in 2× sample buffer (125 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (w/v) glycerol, 28 mM β-mercaptoethanol, 0.01% (w/v) bromphenol blue) for 30 min at room temperature. The gel slice was transferred to a sample well of a 1.5 mm SDS-polyacrylamide gel (4% (w/v) stacking gel, 12% (w/v) resolving gel) and electrophoresed at constant voltage (125 V) through the stacking gel and at 200 V through the resolving gel for 90 min at room temperature (28). Following electrophoresis, the gel was placed on Whatman filter paper and dried, and DNA-protein complexes were visualized by autoradiography.

Shift Southwestern Analysis—The specific DNA-protein complex formed when the distal portion of the βNRE sense strand (−332 to −311) was incubated in a binding reaction with NWB soleus nuclear extracts and was separated by EMSA. EMSA was performed essentially as described above except that the binding reaction was scaled up 10-fold, and 13 independent reaction mixtures were electrophoresed in a 0.75-mm-thick gel. Following EMSA, the section of the gel containing the DNA-protein complex was electrophoretically transferred to membranes (nitrocellulose and DEAE) placed in series using conditions described above for Western blot analysis. During transfer, the protein component of the DNA-protein complex bound to the nitrocellulose membrane and the dβNRE-S DNA probe bound to the DEAE membrane. Following localization of the bound protein using the DEAE membrane, the protein was eluted from the nitrocellulose membrane by incubation in a 20% (v/v) acetonitrile solution for 3 h at 37 °C. The eluate was centrifuged for 10 min to remove particulate material, lyophilized to remove solvent, and resuspended in 20 μl of 50 mM Tris-HCl, pH 7.5. The recovered protein was solubilized in 6× sample

TABLE II
Body and soleus weights from control and NWB groups
Values are means ± S.E.

Weight	*n*	Control	NWB	Change
				%
Body (initial), g	46	25.4 ± 0.7	25.7 ± 0.5	
Body (final), g	46	25.7 ± 0.5	22.7 ± 0.4	−12[a]
Soleus, mg	92	7.47 ± 0.15	5.18 ± 0.09	−31[a]
Soleus, mg/g	92	0.28 ± 0.00	0.23 ± 0.00	−19[a]

[a] $p < 0.01$.

TABLE III
Body and plantaris weights from control and MOV groups
Values are means ± S.E.

Weight	*n*	Control	MOV	Change
				%
Body (final), g	17	29.4 ± 1.8	30.5 ± 1.6	
Plantaris, mg	34	14.6 ± 0.4	29.5 ± 0.8	102[a]
Plantaris, mg/g	34	0.51 ± 0.01	0.97 ± 0.02	92[a]

[a] $p < 0.01$.

buffer (350 mM Tris-HCl, pH 6.8, 30% (w/v) glycerol, 10% (w/v) SDS, 0.93 mM DTT, 0.012% (w/v) bromphenol blue) and electrophoretically resolved by 12% (w/v) SDS-polyacrylamide gel electrophoresis at constant voltage (200 V) for 45 min at room temperature. The protein was electrophoretically transferred to a nitrocellulose membrane as described above for Western analysis. The membrane was incubated for 10 h at 4 °C in a blocking solution composed of EMSA binding reaction buffer (minus glycerol) containing 5% (w/v) nonfat milk. DNA-protein interaction occurred during incubation of the membrane in a blocking solution containing 0.25% nonfat milk and labeled dβNRE-S probe (2×10^6 cpm/ml) for 10 h at 4 °C. Following hybridization, the membrane was washed three times for 5 min at room temperature in a solution consisting of 50 mM Tris-HCl, pH 7.9, 30 mM KCl, 1 mM $MgCl_2$, 0.5 mM EDTA, and 0.5 mM DTT, air-dried, and exposed to film overnight.

Statistical Analysis—All statistical analyses were performed using the StatView SE + Graphics program (Version 4.1; Abacus Concepts, Inc.). Student's *t* tests were used to assess differences between group means. All data are reported as means ± S.E. The lowest significance level accepted was $p < 0.05$. Given the large number of transgenic mice required for mRNA analysis (5 mice/*n*), we restricted our analysis to include three independent measurements ($n = 3$) following the 2-week NWB period, which precluded a statistical analysis.

RESULTS

Changes in Morphology and Phenotype following NWB Activity or MOV—NWB activity imposed by hind limb suspension results in muscle weight loss (atrophy) and a slow to fast myofiber transition accompanied by decreased βMyHC expression (18). There were no significant differences measured in the initial body weight of mice representing either the control ($n = 46$) or NWB ($n = 46$) groups (Table II). However, 2 weeks of NWB activity resulted in a significant decrease in body weight (NWB; −11.7%) when compared with body weight values of mice representing the control group (Table II). Importantly, the initial and final body weights of mice comprising the control group did not differ significantly over the 2-week NWB period, indicating that body weight loss for the NWB group was the result of NWB activity induced by hind limb suspension and not the inhibition of normal growth. In agreement with our previous study (18), 2 weeks of NWB activity resulted in a significant decrease in both absolute (mg) (−30.7%) and normalized (−19.2%) weights of the NWB-S muscle as compared with CS muscle weight (Table II). To assess whether NWB decreased endogenous βMyHC expression in the NWB-S, Northern hybridization, SDS-polyacrylamide gel electrophoresis analysis of MyHC content, and myosin ATPase histochemistry were performed. In agreement with our previous findings, Northern hybridization showed a 38% decrease in endogenous βMyHC mRNA in the NWB-S following a 2-week period of NWB activity (Fig. 2) (18). The NWB-induced decrease in endogenous βMyHC expression was also seen at the protein level as assessed by myosin ATPase histochemistry and myosin heavy chain separation (data not shown; see Ref. 18).

To evaluate the effect of MOV on muscle enlargement (hypertrophy), normalized plantaris weight (mg/g of body weight) was obtained. Over the 8-week experimental period, no significant differences were measured in body weights between adult transgenic mice representing sham-operated control ($n = 17$) and MOV ($n = 17$) groups (Table III). As expected, 8 weeks of MOV resulted in a significant increase (92%) in normalized (mg/g of body weight) MOV-P weight as compared with CP values (Table III). As reported previously, the hypertrophic growth of the MOV-P was accompanied by a concomitant increase in βMyHC mRNA and protein expression (data not shown; see Ref. 17).

Transgene β293 Expression Pattern and Regulation in Response to NWB Activity and MOV—Our previous work revealed that an NWB-responsive element(s) resides within a 600-bp region located immediately 5′ to the βMyHC gene transcription start site (Fig. 1) (18). To further delimit the location of this element(s), we have generated multiple independent transgenic mouse lines for each of two different transgenes (β293 and β350) (Fig. 1). Since adult-stage βMyHC gene expression is primarily restricted to slow type I muscle fibers (Fig. 1, *schematic diagram*), we first examined the levels of CAT specific activity (pmol/μg of protein/min) in protein extracts obtained from muscles containing different proportions of type I fibers, as well as nonmuscle tissue. As shown in Fig. 3*A*, transgene β293 was not expressed in the adult heart but was expressed in a muscle-specific manner in accordance to type I fiber composition (soleus > gastrocnemius > plantaris). Collectively, these results show that transgene β293 expression qualitatively mimics the normal adult-stage expression pattern of the endogenous βMyHC gene.

We have previously shown that NWB activity decreases βMyHC expression in the slow twitch soleus muscle (18). To determine if an NWB element(s) resides within transgene β293, CAT specific activity was measured in CS and NWB-S muscle following a 2-week period of NWB activity. Surprisingly, CAT specific activity measured in NWB-S extract revealed an unexpected increase (1.2–3.7-fold) as compared with CS extract (Fig. 3*B*, Table IV). To examine whether these results were due to post-transcriptional regulation, we analyzed transgene β293 expression at the level of mRNA accumulation (Fig. 2). In contrast to the 38% decrease in endogenous βMyHC mRNA levels measured after 2 weeks of NWB activity, exogenous CAT mRNA levels measured for transgenic line β293:L7 increased, confirming our CAT assay results (Figs. 2 and 3*B*; Table IV).

In contrast to NWB activity, MOV induces endogenous βMyHC gene expression in the fast twitch plantaris muscle of rodents (17) (Fig. 1, *schematic diagram*). To determine if transgene β293 responds to MOV, the plantaris muscles of mice representing each of six independent lines were overloaded and assayed for CAT specific activity 8 weeks later. Expression assays showed that CAT specific activity measured in MOV-P muscle extracts was 3–16-fold higher than that measured in sham-operated CP muscle extracts (Fig. 3*B*, Table IV), which agrees with our previous findings for this transgene (17). Taken together, these data provide convincing evidence that NWB and MOV element(s) are segregated to different regions within the βMyHC-proximal promoter.

Transgene β350 Expression Pattern and Regulation in Response to NWB Activity and MOV—In striking contrast to transgene β293, under control conditions no detectable levels of

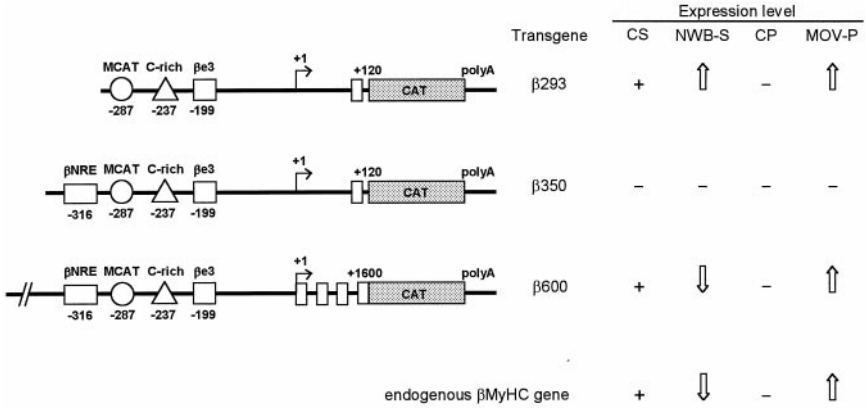

FIG. 1. **Response of various βMyHC transgenes to NWB and MOV activity.** Transgenes consist of either 293 bp (β293) or 350 bp (β350) of human βMyHC 5′-flanking sequence and 120 bp of 5′-untranslated region fused to the CAT reporter gene. Transgene β600 contains 600 bp of mouse βMyHC 5′-flanking sequence and 1600 bp of 5′-untranslated region linked to the CAT reporter gene. Relative location of the highly conserved proximal promoter *cis*-elements (MCAT, C-rich, βe3, and βNRE) comprising the βMyHC control region are as designated. +, moderate to high levels of expression; −, barely detectable to no expression; ⇑, increased expression; ⇓ decreased expression. Data are compiled from Refs. 17 and 18 and data herein.

TABLE IV
Response of β293 transgene CAT specific activity to NWB and MOV

Values are means ± S.E. CAT specific activity (pmol/μg of protein/min) was measured by incubation of protein extracts with 20 mM acetyl-CoA and ^{14}C-chloramphenicol (0.2 μCi/0.35 μM) in 250 mM Tris-HCl, pH 7.8, at 37 °C. Incubation of muscle protein extracts representing each transgenic line was performed as follows: MOV study using transgene β293 (line β293:L4, 5 μg/h; β293:L5, 3 μg/h; β293:L6, 30 μg/21 h; β293:L7 30 μg/17 h; β293:L96, 7.5 μg/30 min; β293:L99, 7.5 μg/30 min); NWB study using transgene β293 (β293:L4, 6 μg/h; β293:L5, 3 μg/h; β293:L6, 30 μg/17 h; β293:L7, 10 μg/17 h; β293:L96, 7.5 μg/15 min; β293:L99, 2 μg/30 min). Note: CP and MOV-P CAT specific activities for lines 4–6 were previously reported (Ref. 17 and references within) and are shown for ease of comparison.

Line	Copy no.	NWB				MOV			
		n	CS	NWB-S	Increase	*n*	CP	MOV-P	Increase
					-fold				*-fold*
4	17	8	68 ± 19	252 ± 48	3.7[a]	8	12.66 ± 2.52	185 ± 35	14.6[a]
5	36	8	167 ± 38	392 ± 75	2.4[a]	8	26.4 ± 4.24	420 ± 68	15.9[a]
6	4	6	6.55 ± 2.11	7.65 ± 3.12	1.2	8	0.11 ± 0.02	1.28 ± 0.21	11.5[a]
7	5	8	5.53 ± 0.88	14.29 ± 0.95	2.6[a]	6	0.12 ± 0.03	0.59 ± 0.13	4.9[a]
96	64	8	1254 ± 54	2172 ± 147	1.7[a]	6	279 ± 38	832 ± 16	2.9[a]
99	84	8	490 ± 57	1111 ± 135	2.3[a]	5	13.68 ± 5.04	102 ± 18.2	7.5[a]

[a] $p < 0.05$.

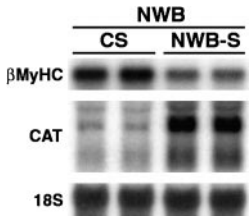

FIG. 2. **Northern blot analysis of NWB soleus muscle from transgenic mice harboring transgene β293 line 7 (β293:L7).** Total RNA isolated from CS and NWB-S (6 μg) muscle pooled from 3–5 animals was fractionated on a 1.5% agarose denaturing gel. Intensity of the hybridization signal was quantitated using a PhosphorImager and normalized to 18 S (24) values to account for loading differences between lanes.

CAT specific activity could be measured in muscle or non-muscle tissues of mice representing each of 11 independent transgenic lines harboring transgene β350. Since transgene β293 was up-regulated by NWB activity, we tested transgene β350 expression following 2 weeks of NWB activity; however, as under basal conditions, detectable levels of transgene β350 expression could not be measured in the NWB-S muscle. Furthermore, 8 weeks of MOV did not result in the induction of transgene β350 expression in the MOV-P muscle. These results could be accounted for if transgene β350 integrated into an inactive chromosomal site. However, due to the large number (11) of β350 lines tested for CAT expression, it is highly improbable that chromosomal position effects can account solely for the lack of transgene expression. Therefore, we interpret these *in vivo* results to implicate the βNRE (−332 to −300) as a negative element responsible for the transcriptional silencing of transgene β350 and suggest that the βNRE may also play a role in NWB-induced decreases in βMyHC gene transcription.

Enriched DNA-Protein Interaction at βNRE—Our transgenic results point to a correlative link between the βNRE and the transcriptional repression of transgene β350. Therefore, to investigate the protein binding properties of the βNRE under control and NWB conditions, we performed direct and competitive electrophoretic mobility shift assays (EMSAs) using nuclear extract isolated from CS and NWB-S muscle (Fig. 4, *A* and *B*). Incubation of a double-stranded ^{32}P-labeled βNRE probe (DS, −332 to −300; Table I) with NWB-S nuclear extracts resulted in a barely detectable DNA-protein complex that was not discernible when CS nuclear extracts were used (Fig. 4*A*, *lanes 1–3*). Previous studies have shown that βNRE interacts with proteins (*e.g.* CNBP and YB-1) that bind double- or single-stranded nucleic acid (9, 10); therefore, we next examined DNA-protein interactions using various single-stranded forms of the βNRE. Interestingly, when an ^{32}P-labeled βNRE sense strand (βNRE-S, -332 to -300) was incubated with NWB-S nuclear extracts, a binding complex was formed that was highly enriched in comparison with that formed when CS nuclear extracts were used (Fig. 4*A*, *lanes 4–6*). In contrast, a binding complex was not formed when ^{32}P-βNRE antisense (βNRE-AS, −332 to −300) or ^{32}P-βNRE sense-proximal (pβNRE-S, −317 to −300) strands were incubated with either CS or NWB-S nuclear extracts (Fig. 4*A*, *lanes 7–12*). However,

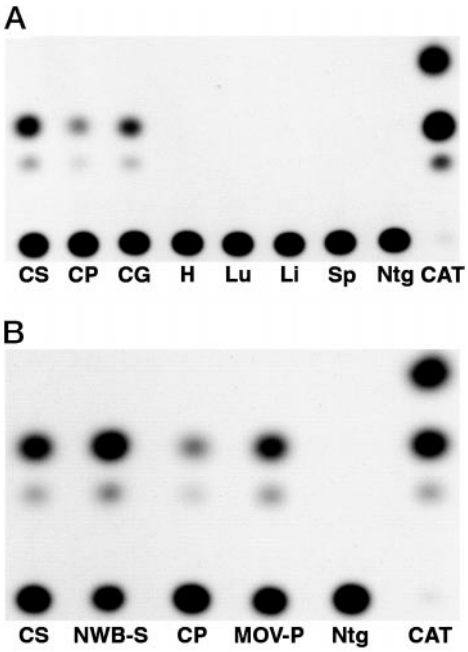

FIG. 3. *A*, representative CAT assay demonstrating the expression pattern of the β293:L96 transgene in various adult tissues. Protein extracts (7.5 μg) prepared from control muscle (soleus (CS), gastrocnemius (CG), plantaris (CP), and heart (*H*)) and nonmuscle (lung (*Lu*), liver (*Li*), and spleen (*Sp*)) tissues were incubated for 15 min at 37 °C. Extracts obtained from nontransgenic (*Ntg*) mouse soleus muscle served as a negative control, and purified CAT protein (70 ng) was used as a positive control. *B*, representative CAT assay showing transgene β293:L96 expression following 2 weeks of NWB activity or 8 weeks of MOV. Protein extracts (7.5 μg) were incubated for 15 min (CS and NWB-S) or 30 min (CP and MOV-P) at 37 °C. Ntg CS was used as negative control, and 70 ng of purified CAT protein was used as a positive control. Note the unexpected increase in CAT specific activity of NWB-S sample in comparison with CS. The increase in CAT specific activity in response to NWB activity was observed in all β293 transgenic lines tested (see Table IV).

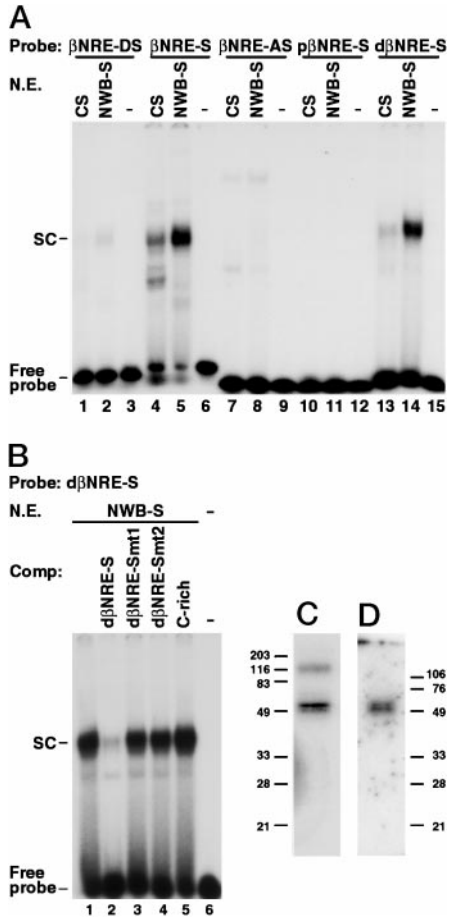

FIG. 4. *A*, EMSA of βNRE-protein interaction. A DNA-protein complex (*SC*, specific complex) was delimited to the distal portion (*lanes 13* and *14*) of the βNRE sense strand. −, no extract. *B*, competition EMSA. 100-fold molar excess of cold mutated dβNRE-S probe (*lanes 3* and *4*) or nonspecific probe (*lane 5*) was added to the binding reaction. *C*, two-dimensional UV cross-linking of DNA-protein complex. Three bands with apparent molecular masses of 50, 52, and 112 kDa were identified. *D*, shift-Southwestern of DNA-protein complex. Two bands were detected (50 and 52 kDa) that corresponded to those identified by UV cross-linking.

a highly enriched binding complex was detected when a ^{32}P-labeled βNRE sense-distal strand (dβNRE-S, −332 to −311) was incubated with NWB-S nuclear extracts (Fig. 4*A*, *lanes 13–15*). The formation of this binding complex was judged to be sequence-specific as determined by competition with a 100-fold molar excess of unlabeled wild type dβNRE-S (Fig. 4*B*, *lane 1 versus lane 2*) and the lack of competition seen when either a 100-fold molar excess of unlabeled mutant dβNRE-S probe (dβNRE-Smt1 and dβNRE-Smt2; Table I) or nonspecific DNA (sense strand of βMyHC C-rich element) are used in competition EMSAs (Fig. 4*B*, *lane 1 versus lanes 3–5*). These data demonstrate that highly enriched, sequence-specific DNA binding activity exists in NWB-S nuclear extracts that preferentially bind the dβNRE-S.

Biochemical Analysis of DNA-binding Factor(s) Interacting at dβNRE—As an initial characterization of the factor(s) in NWB-S nuclear extracts that bind to dβNRE-S, we performed two-dimensional UV cross-linking analysis. The dβNRE-S probe was incubated with NWB-S nuclear extract, and the binding complex was separated from unbound probe by EMSA. Subsequently, the EMSA gel was exposed to UV light (312 nm) for 30 min, and the enriched binding complex was excised from the gel, electroeluted, and resolved on a 12% (w/v) SDS-polyacrylamide gel. As shown in Fig. 4*C*, two-dimensional UV cross-linking analysis detected three bands with apparent molecular masses of 116, 52, and 50 kDa. To further study the protein components of this binding complex, we performed shift Southwestern blot analysis essentially as described by Demczuk *et al.* (29). Following electroblotting of the enriched NWB-S binding complex, the protein was eluted from the nitrocellulose membrane and subjected to Southwestern analysis using a ^{32}P-labeled dβNRE-S probe. Two bands were detected that correspond to the 52- and 50-kDa proteins detected by UV cross-linking analysis (Fig. 4, *C* and *D*). It is not clear why the 116-kDa band was not observed in shift Southwestern analysis, but one possibility is that this band represents a multimer of the binding complex formed during exposure to UV light. These results indicate that the enriched binding complex formed at the dβNRE-S site is composed of at least two distinct proteins whose identities are presently unknown.

Expression Pattern of CNBP and YB-1 in CS and NWB-S Muscle—Previous investigations have shown that the zinc finger-binding factor CNBP and the Y-box binding protein YB-1 bind the βNRE (9, 10). By Northern blot analysis, we show that both CNBP and YB-1 are expressed in CS and NWB-S muscle; however, only YB-1 mRNA levels decreased (−28%) following 2 weeks of NWB activity (Fig. 5*A*). To determine the subcellular location of CNBP and YB-1, CS and NWB-S nuclear and cytoplasmic extract was fractionated by SDS-polyacrylamide gel electrophoresis and analyzed by Western blot (Fig. 5, *B* and *C*). Western analysis using anti-CNBP serum revealed that CNBP was detected in HeLa nuclear extracts but not in CS or NWB-S nuclear extract (Fig. 5*B*, *lanes 1–3*), whereas it was detected in

TABLE V

Sequence alignment of human, rabbit, mouse, and rat βNRE

βNRE sequences were aligned using the BestFit program of the GCG Wisconsin package. Boldface base pairs indicate a mismatch in comparison with the human βMyHC sequence. The italicized sequences represent the distal βNRE.

Human βMyHC	5′-*GTGGTCT__TGGTGGTCGTGGTCA*GTTCCCTCTCC-3′	−332/−300
Rabbit βMyHC	5′-*GAGGTCACACTGGTGGTCCTGGTCA*GTTCCCTCTCC-3′	−314/−278
Mouse βMyHC	5′-*GAGGTCG__TGGTGGTCGTGGTCA*G**CTTA**CTCTCT-3′	−323/−291
Rat βMyHC	5′-*GAGGTCA__TGGTGGTCGTGGTCA*G**CA**C**C**CTC**T**-3′	−322/−290

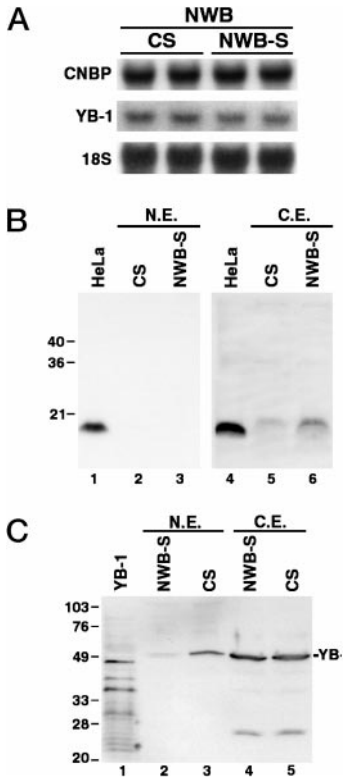

FIG. 5. *A*, Northern blot analysis of CNBP and YB-1 expression within NWB soleus muscle from transgenic mice harboring transgene β293 line 7 (β293:L7). Total RNA was isolated from CS and NWB-S (6 μg) muscle pooled from 3–5 animals, fractionated on a 1.5% agarose denaturing gel, and quantitated for hybridization intensity as described in the legend to Fig. 2. *B*, CNBP protein in skeletal muscle and HeLa cell nuclear and cytoplasmic extracts. Western blot analysis is shown of 50 μg of HeLa cell (*lanes 1* and *4*), CS (*lanes 2* and *5*), and NWB-S (*lanes 3* and *6*) nuclear (*N.E.*) or cytoplasmic extracts (*C.E.*) using a human polyclonal CNBP antibody. Note the absence of CNBP protein in NWB-S nuclear extracts. *C*, YB-1 protein in skeletal muscle nuclear and cytoplasmic extracts. Western blot analysis is shown of 50 μg of NWB-S (*lanes 2* and *4*) and CS (*lanes 3* and *5*) nuclear or cytoplasmic extracts (*N.E.* and *C.E.*, respectively) using a YB-1 polyclonal antibody. Partially purified rat YB-1 protein served as a positive control (*lane 1*). Note the barely detectable levels of YB-1 protein in NWB-S nuclear extracts.

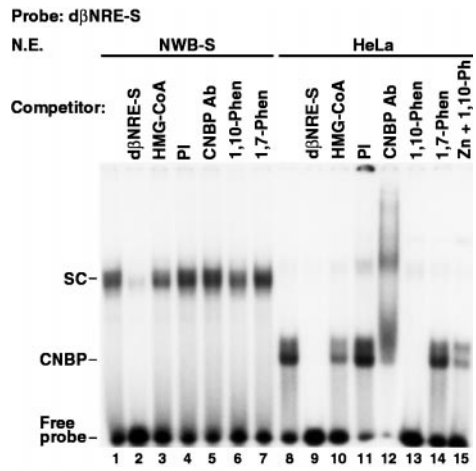

FIG. 6. **EMSA analysis for dβNRE-S binding to NWB-S and HeLa cell nuclear extracts.** ^{32}P-labeled dβNRE-S probe and nuclear extracts from NWB-S (1 μg) (*lanes 1–7*) and HeLa cells (250 ng) (*lanes 8–15*) were incubated for 20 min at room temperature in the absence (*lanes 1* and *8*) or presence of 100-fold molar excess of unlabeled dβNRE-S (*lanes 2* and *9*) or 3-hydroxy-3-methylglutaryl CoA probe (*lanes 3* and *10*). Thirty minutes prior to the addition of the dβNRE-S probe, preimmune serum (*PI*) (*lanes 4* and *11*), anti-CNBP antibody (*lanes 5* and *12*), 4 mM 1,10-phenanthroline (*1,10-Phen*; *lanes 6* and *13*), 4 mM 1,7-phenanthroline (*1,7-Phen*; *lanes 7* and *14*), or 4 mM 1,10-phenanthroline and 0.5 mM $ZnCl_2$ (*lane 15*) were incubated with nuclear extract at 4 °C. Binding complexes were resolved as described under "Experimental Procedures."

CS and NWB-S cytoplasmic extracts (Fig. 5*B*, *lanes 5* and *6*). Western analysis using a YB-1 polyclonal antibody detected a protein of approximately 50 kDa in CS and NWB-S cytoplasmic extracts that had a migration pattern similar to that of partially purified recombinant YB-1 protein (Fig. 5*C*, *lane 1 versus lanes 4* and *5*). A 50-kDa protein was also detected in CS nuclear extracts that was barely detectable in NWB-S nuclear extracts (Fig. 5*C*, *lanes 1 versus 2–5*). Collectively, these data provide evidence that CNBP and YB-1 are expressed in adult skeletal muscle; however, their absence in NWB-S nuclear extracts supports the notion that they are not a component of the binding activity in NWB-S nuclear extracts.

Is CNBP a Component of the Enriched dβNRE Binding Complex?—To directly establish whether CNBP is a component of the highly enriched binding activity identified in NWB-S nuclear extracts, competition and supershift EMSAs were performed (Fig. 6). The addition of a 100-fold molar excess of unlabeled 3-hydroxy-3-methylglutaryl CoA probe (previously shown to bind CNBP (30)) to the binding reaction did not interfere with complex formation, whereas a 100-fold molar excess of unlabeled dβNRE-S probe did (Fig. 6, *lanes 1–3*). Furthermore, the addition of either preimmune serum or anti-CNBP serum to the binding reaction neither supershifted nor inhibited binding complex formation when NWB-S nuclear extracts were used (Fig. 6, *lanes 4* and *5*). Since CNBP is a zinc finger protein, we tested whether the addition of the metal ion chelator 1,10-phenanthroline would inhibit binding complex formation. Interestingly, when 4 mM of either 1,10-phenanthroline (*1,10-Phen*) or the non-metal-chelating analog 1,7-phenanthroline (*1,7-Phen*) was added to the binding reaction, complex formation was not altered (Fig. 6, *lanes 1*, *6*, and *7*).

To further investigate whether CNBP interacts with the dβNRE-S probe, EMSAs were performed using HeLa cell nuclear extracts, since these extracts have been shown to be a rich source of CNBP (31). When using HeLa nuclear extracts, EMSAs revealed a binding complex with a higher mobility than that formed when using NWB-S nuclear extracts (*lane 1 versus lane 8*). The addition of a 100-fold molar excess of unlabeled dβNRE-S and 3-hydroxy-3-methylglutaryl CoA probes to the binding reaction as competitor led to either a complete or near complete inhibition of ^{32}P-labeled dβNRE-S binding to HeLa cell nuclear extracts, respectively (Fig. 6, *lanes 9* and *10*). In contrast to the dβNRE-S-protein complex formed with NWB-S nuclear extract, the addition of anti-CNBP serum to the HeLa binding reaction resulted in a supershifted binding complex, whereas the addition of preimmune serum had no effect (Fig. 6,

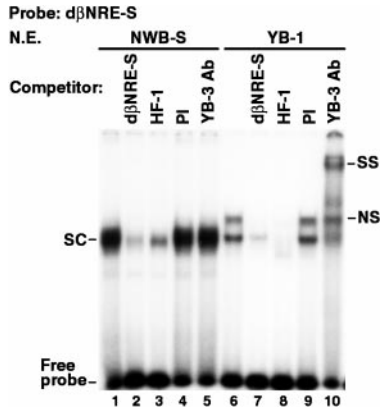

FIG. 7. **EMSA analysis for dβNRE-S binding to NWB-S nuclear extracts and recombinant YB-1.** A ^{32}P-labeled dβNRE-S probe was incubated with either NWB-S nuclear extract (1 μg) or partially purified rat YB-1 protein (100 ng). The specific DNA-protein complex (*SC*) was examined by competition with a 100-fold molar excess of unlabeled dβNRE-S probe (*lanes 2* and *7*) or the sense strand of HF-1 oligonucleotide (*lanes 3* and *8*). Preimmune serum (*PI*) (*lanes 4* and *9*) and polyclonal YB-3 antibody (*lanes 5* and *10*) were incubated for 30 min at 4 °C prior to the addition of the dβNRE-S probe. The YB-3 antibody produced a supershift (*SS*) of the lower YB-1 complex (*lane 10*). *NS*, nonspecific DNA-protein complex.

lane 11 versus lane 12). Furthermore, 1,10-phenanthroline, but not 1,7-phenanthroline, inhibited the formation of a binding complex that could be reconstituted by the addition of 0.5 mM $ZnCl_2$ to the binding reaction (Fig. 6, compare *lanes 13–15*). The results from our EMSAs provide compelling evidence that CNBP can bind the βNRE-S probe but is not a component of the specific binding complex formed when using NWB-S nuclear extracts.

Is YB-1 a Component of the Enriched dβNRE-S Binding Complex?—YB-1 has been shown to bind the rabbit βMyHC suppressor element (−311 to −294) (10) that has an 85% sequence identity to the dβNRE-S probe (Table V). Accordingly, we performed EMSAs to determine if YB-1 is a component of the NWB-S binding complex. Incubation of the ^{32}P-labeled dβNRE-S probe with NWB-S nuclear extract resulted in a binding complex that was competed away by the addition of a 100-fold molar excess of unlabeled dβNRE-S probe (Fig. 7, *lane 1 versus lane 2*). Although the addition of excess unlabeled HF-1 probe (containing the HF-1a element previously shown to bind YB-1 (14)) competed away ^{32}P-labeled dβNRE-S binding to NWB-S nuclear extracts (Fig. 7, *lane 1 versus lane 3*), it was not as effective as the unlabeled dβNRE-S probe (Fig. 7, *lane 1 versus lane 2*). Preincubation of NWB-S nuclear extracts with either preimmune serum or a polyclonal YB-3 antibody neither abolished ^{32}P-labeled dβNRE-S binding nor supershifted the specific binding complex (Fig. 7, *lanes 4* and *5*). To determine whether YB-1 could interact with the human βMyHC dβNRE-S sequence, bacterially expressed recombinant YB-1 protein was used in EMSAs. In contrast to NWB-S nuclear extracts, incubation of partially purified bacterially expressed recombinant YB-1 protein with the ^{32}P-labeled dβNRE-S probe resulted in the formation of two distinct binding complexes, one with an electrophoretic mobility equivalent to the complex formed with NWB-S nuclear extracts (Fig. 7, *lane 6*). The addition of a 100-fold molar excess of unlabeled dβNRE-S or HF-1 probe nearly abolished the formation of both YB-1 binding complexes (Fig. 7, *lanes 7* and *8*). Preincubation of recombinant YB-1 with preimmune serum did not interfere with YB-1 ^{32}P-labeled dβNRE-S complex formation; however, preincubation with YB-3 polyclonal antibody (YB-3 protein has 89% overall amino acid identity to YB-1) supershifted the YB-1 ^{32}P-labeled dβNRE-S binding complex with the highest mobility (*SC*), indicating that the other binding complex was due to nonspecific (*NS*) binding (Fig. 7, *lanes 7–10*). Collectively, these experiments show that recombinant YB-1 protein can bind the human βMyHC dβNRE-S probe. However, the lack of an anti-YB-3 supershifted complex when using NWB-S nuclear extracts strongly suggests that YB-1 is not a component of the specific DNA-protein complex formed between ^{32}P-labeled dβNRE-S and NWB-S nuclear extracts.

DISCUSSION

One mechanism by which adult-stage skeletal muscle adapts to new functional demands is by modulating transcriptional activation and repression of select muscle genes. Because the βMyHC gene is regulated in a muscle type-specific fashion throughout development and in response to a broad range of physiological stimuli, it represents an excellent model system in which to study the mechanisms underlying differential gene transcription. Our approach used to gain insight into the mechanistic basis underlying βMyHC plasticity is to study its regulation in response to altered neuromuscular activity induced by altered mechanical loads. For example, under conditions of decreased mechanical loading imposed by NWB we have previously demonstrated a 40–50% decrease in the basal expression level of transgenes composed of either 5600 or 600 bp of βMyHC 5′-flanking DNA in the slow twitch soleus muscle of adult transgenic mice (18). As concerns the regulation of βMyHC expression in response to increased MOV, we have shown that a transgene composed of 293 bp of βMyHC 5′-flanking DNA (β293) was minimally sufficient to induce expression in both the fast twitch plantaris and slow twitch soleus muscles (17). In an effort to extend these findings, this study was undertaken to investigate whether distinct DNA element(s) regulate the antithetic expression pattern of the βMyHC gene in response to MOV and NWB activity. For this purpose, transgenic mice harboring transgenes composed of different lengths of βMyHC 5′-flanking sequence were generated, and their expression was analyzed in response to MOV and NWB activity. Our *in vivo* findings herein represent the first clear presentation of evidence that distinct βMyHC promoter sequence(s) mediate βMyHC expression in response to MOV and NWB activity and that these element(s) are segregated to different regions within the βMyHC proximal promoter.

In Vivo Determination of Segregated MOV and NWB Regulatory Elements—We initiated these studies by establishing the basal pattern of transgene β293 and β350 expression within muscle and nonmuscle tissues of adult mice and how these patterns change following either NWB activity or MOV. Although transgene β293 is not expressed at detectable levels in single fibers, measurements of CAT specific activity using whole tissue extracts qualitatively suggests that transgene β293 expression faithfully mirrored endogenous βMyHC expression, since its expression was not detected in adult ventricular muscles or nonmuscle tissues (Fig. 3, *A* and *B*; Table IV). Furthermore, following an 8-week period of MOV, the expression of transgene β293 was induced in the predominantly fast twitch plantaris muscle at levels that were 3–16-fold higher than control levels, clearly indicating that this transgene harbored MOV-responsive element(s) (Fig. 3*B*, Table IV). In striking contrast to the measured decrease in endogenous βMyHC mRNA (Fig. 2) and protein (data not shown (18)) expression following NWB activity, transgene β293 expression was not down-regulated, but instead, its expression level was unexpectedly increased by 1.2–3.7-fold above expression levels measured in control soleus muscle (Fig. 3, Table IV). At present, the precise molecular mechanism underlying transgene β293 up-regulation by NWB activity is unclear; however, it is clear that

sequences required for NWB regulation are missing. Importantly, this surprising result was obtained for each of six independent transgenic lines examined, and each line carries different relative transgene copy numbers; therefore, it is highly unlikely that transgene copy number or chromosomal position effects can account for these results (Table IV). Because transgene β293 responds to MOV, but not NWB activity, in a manner similar to the endogenous βMyHC gene, the regulatory elements required for MOV and NWB regulation must be segregated, thus eliminating the possibility that a single MOV/NWB DNA element exists for the βMyHC gene.

To locate regulatory element(s) involved in NWB-dependent regulation of the βMyHC gene, we examined a transgene containing 350 bp of human βMyHC 5′-flanking DNA (transgene β350) that harbored the βNRE at its 5′-terminal end. In contrast to the unexpected increase in transgene β293 expression following NWB, analysis of these transgenic lines revealed that expression had been completely abolished in control, NWB soleus, and MOV-P muscle. This result was unanticipated because two previous reports have demonstrated that rat βMyHC reporter constructs of comparable size and harboring the entire βNRE were expressed in permanent myogenic cell lines as well as following direct injection into rabbit ventricular muscle and rat control and pressure overloaded ventricular muscle (8, 32). A number of possibilities exist that may explain the disparity between our transgenic results and those of the aforementioned studies. First, it is possible that minor sequence differences between the rat and human βMyHC proximal promoter regions (>85% identity) used in these studies can account for the observed differences in expression. However, this possibility was eliminated when nine transgenic lines carrying a rat-derived construct (β333; −333 to +34 rat βMyHC/luciferase reporter) that harbored the entire βNRE also did not express.[3] Second, it is possible that transgenes β350 and β333 integrated into an inactive chromosomal site; however, due to the large number of β350 (eleven) and β333 (nine) transgenic lines tested for expression, it is unlikely that chromosomal position effects can account solely for the lack of transgene expression. An alternate and more likely explanation for previous results is that when cell culture systems or direct DNA injection was used, transgene β350 and β333 remained episomal, whereas in our study these transgenes were exposed to an additional level of regulation imposed by chromatin architecture or nuclear microenvironment, and perhaps in this context the 5′-terminal location of the βNRE conferred a dominant negative effect on transgene transcription. In fact, the βNRE has been reported to act in a positional manner in that its 5′ location more effectively suppressed expression of both heterologous and βMyHC promoter/reporter constructs (8). Similarly, the expression of a vascular smooth muscle α-actin reporter construct was shown to be repressed when a negative regulatory element was placed 5′ and adjacent to an enhancer element containing an MCAT sequence (33). It is noteworthy that in the latter experiments, the positioning of a negative element located at the 5′-terminal end and adjacent to a MCAT element is identical to the linear arrangement of elements within transgene β350 used in this study.

CNBP Is Not a Component of the Highly Enriched dβNRE-S Binding Complex—As a first step toward identifying the protein(s) that bind to the dβNRE-S binding site, and to determine if CNBP was a component of the binding complex, we performed EMSA using nuclear extracts isolated from control and NWB soleus muscle. At present, the cellular function of CNBP has not been defined; however, multiple lines of evidence implicate CNBP as a regulator of both transcription and translation. Convincing evidence that CNBP is involved in translational control has recently been provided by Pellizzoni *et al*. (34), who show that CNBP binds a highly conserved target sequence within the 5′-untranslated region of ribosomal protein mRNAs. It is thought that this type of interaction modulates the efficiency of ribosomal protein mRNA translation and thus ribosome production. Further support for a role for CNBP as a translational regulator comes from the studies with mouse liver cells indicating that CNBP was exclusively found in the cytoplasmic compartment (35). On the other hand, the notion that CNBP functions as a transcription factor is based on its documented binding to promoter elements of target genes that have high sequence similarities to the 3-hydroxy-3-methylglutaryl CoA reductase SRE (GTGC/GGGTG), and its ability to regulate different promoter/reporter gene constructs in diverse cell contexts (30). For example, a recent study has demonstrated that both CNBPα and CNBPβ bind the βMyHC gene βNRE and that overexpression of CNBPα in primary cardiocytes led to decreased βMyHC reporter gene expression (9).

In this study, using EMSAs we have identified dβNRE-S binding activity that is sequence-specific and highly enriched only in NWB-S nuclear extracts (Fig. 4, *A* and *B*). While CNBP has been shown to bind the βNRE and regulate the expression of βMyHC reporter gene constructs in cultured cardiac cells (9), our experiments confirm that CNBP can bind the βNRE; however, we provide multiple lines of evidence that CNBP is not a regulator of βMyHC expression during NWB activity. First, an antibody that recognizes CNBP detected its presence only in cytoplasmic extracts where it was more abundant in NWB-S than control muscles (Fig. 5*B*). Second, in mobility shift assays, the binding complex formed between dβNRE-S and NWB-S nuclear extracts had a low mobility, and the complex was neither abolished by the presence of the metal ion chelator 1,10-phenanthroline nor supershifted by antibody recognizing CNBP. In striking contrast, the binding complex formed with HeLa nuclear extracts, a known source of CNBP, had a high mobility, was abolished by the metal ion chelator 1,10-phenanthroline, was subsequently reconstituted by the addition of $ZnCl_2$, and was supershifted by antibody recognizing CNBP (Fig. 6). Third, by UV cross-linking and shift Southwestern analyses, single-stranded binding proteins with greater molecular mass (50 and 52 kDa) than the 19-kDa CNBP were identified (Fig. 4, *C* and *D*). An additional important finding that further characterizes the dβNRE-S binding activity found in NWB-S nuclear extracts is that metal ions are not required for binding to the dβNRE-S site.

YB-1 Is Not a Component of the Highly Enriched dβNRE-S Binding Complex—Like CNBP, various Y-box binding factors have been shown to regulate gene expression at the level of translation by binding to mRNA, while other members interact with single and double-stranded DNA to regulate gene transcription (11). Transcriptional control by Y-box proteins generally occurs by binding to regulatory elements containing a consensus inverted CCAAT box sequence located within the transcriptional control region of a gene. Accumulating evidence has implicated YB-1 as a general regulator of transcription and as playing a role in directing tissue-specific expression in a variety of cell contexts including muscle (11). For example, *in vitro* expression assays have shown that a highly conserved 27-bp region (−87 to −60), composed of a Y-box and an adjacent upstream MCAT element, was sufficient to confer muscle specific expression on the *c-fos* basal promoter (12). Also, *in vitro* overexpression assays showed that two Y-box-binding factors, MY1 and MY1a, led to decreased expression of the nicotinic acetylcholine receptor δ-subunit promoter/reporter constructs only when a 47-bp (−47 to −1) activity-dependent (innervation)

[3] D. Vyas and R. Tsika, manuscript in preparation.

enhancer element was present and that both Y-box factors bound specifically to the sense strand of this enhancer element (13). In addition to skeletal muscle, Y-box binding factors have also been implicated in directing cardiac specific expression. Specifically, YB-1 and an associated factor have been shown to bind to the HF-1a site, which contains an inverted CCAAT-like element and acts in conjunction with an adjacent HF-1b/MEF2 site to confer ventricular specific expression to MLC-2v reporter constructs and transgenes (14). Finally, independent experimental approaches have shown that the ubiquitously expressed Y-box binding factor YB-1 interacts with the βMyHC gene βNRE (10).

Due to the relevance of recent findings demonstrating the involvement of YB-1 in directing cardiac and skeletal muscle-specific gene expression, we explored the possibility that YB-1 may be a component of the NWB-S single-stranded binding activity identified in our mobility shift assays. However, despite previous observations, our experiments provide evidence that eliminates YB-1 as a component of the enriched dβNRE-S binding activity in NWB-S extracts. Most compelling is that in supershift EMSAs, a polyclonal YB-3 antibody did not abolish the formation of a binding complex when using NWB-S nuclear extracts, whereas it effectively supershifted the specific complex formed when using partially purified YB-1 protein (Fig. 7, *lane 6 versus lane 10*). Additionally, in competition EMSAs, the use of mutant forms of the dβNRE-S site revealed the sequence-specific nature of the single-stranded binding activity in NWB nuclear extracts, since mutant forms did not compete with wild type dβNRE-S for complex formation (Fig. 4*B*). These results were further confirmed when the MLC2v HF-1 binding site was found to be qualitatively a less effective competitor of complex formation between dβNRE-S and NWB-S nuclear extracts, despite the high degree of sequence similarity (>85%) to the dβNRE-S site (Fig. 7, *lanes 1–3*). In addition, partially purified recombinant YB-1 protein bound to the βNRE antisense strand (data not shown), whereas this strand was not bound by NWB-S nuclear extracts (Fig. 4*A*). Furthermore, an antibody that recognizes YB-1 detected its presence in cytoplasmic extracts obtained from both NWB-S and CS muscles, where its abundance did not appear to differ qualitatively. Although YB-1 protein was present in CS nuclear extracts, albeit at lower levels than in cytoplasmic extracts, it was barely detectable in NWB-S nuclear extracts, effectively eliminating it as a potential regulator of gene transcription (Fig. 5*C*). The low abundance of detectable nuclear localized YB-1 protein is partially corroborated by our Northern hybridization, which showed a 28% decrease in YB-1-specific mRNA following 2 weeks of NWB activity (Fig. 5*A*).

The *in vivo* identification of DNA regulatory element(s) and their corresponding binding protein(s) that regulate βMyHC gene expression in response to MOV and NWB activity is important for a better understanding of skeletal muscle adaptation to altered neuromuscular activity and fiber type-specific expression. This study provides the first persuasive *in vivo* evidence that the regulatory element(s) directing βMyHC gene expression in response to the diverse physiological stimuli of MOV and NWB activity segregate to distinct regions within the βMyHC gene-proximal promoter. Although the exact mechanism underlying increased expression of transgene β293 in response to NWB activity is unclear at present, our findings suggest that the βNRE may be involved in NWB-dependent decreases in βMyHC gene expression. This conclusion is based on our transgenic analysis showing that a 57-bp region harboring the βNRE added to the 5′-terminal end of transgene β293 results in a transcriptionally repressed transgene (β350) (Fig. 1). This conclusion was further supported by results indicating that enriched binding activity at the dβNRE-S site was only seen under NWB conditions in EMSAs. While the exact components of the dβNRE-S binding complex remain to be identified, UV cross-linking and shift Southwestern analyses showed that two proteins of approximately 50 and 52 kDa comprised the dβNRE-S binding activity in NWB-S nuclear extracts. The identification of these components induced by NWB activity will not be trivial, since these pursuits will require extensive and careful investigation incorporating intact animal models to ensure the preservation of physiologically relevant regulatory pathways. Nevertheless, our results provide critical insights essential for determining how muscle cells transduce changing mechanical loads into changes in transcription levels of specific genes. The elucidation of these mechanisms will probably lead to the development of countermeasures aimed at preventing muscle wasting accompanying various disease states and zero gravity exposure.

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REFERENCES

1. Weiss, A., and Leinwand, L. A. (1996) *Annu. Rev. Cell Dev. Biol.* **12,** 417–439
2. Lyons, G. E., Ontell, M., Cox, R., Sassoon, D., and Buckingham, M. (1990) *J. Cell Biol.* **111,** 1465–1476
3. Lyons, G. E., Schiaffino, S., Sassoon, D., Barton, P., and Buckingham, M. (1990) *J. Cell Biol.* **111,** 2427–2436
4. Flink, R. L, Edwards, J. G., Bahl, J. J., Liew, C.-C., Sole, M., and Morkin, E. (1992) *J. Biol. Chem.* **267,** 9917–9924
5. Shimizu, N., Dizon, E., and Zak, R. (1992) *Mol. Cell. Biol.* **12,** 619–630
6. Shimizu, N., Prior, G., Umeda, P. K., and Zak, R. (1992) *Nucleic Acids Res.* **20,** 1793–1799
7. Thompson, W. R., Nadal-Ginard, B. and Mahdavi, V. (1991) *J. Biol. Chem.* **266,** 22678–22688
8. Edwards, J. G., Bahl, J. J., Flink, I., Milavetz, J., Goldman, S., and Morkin, E. (1992) *Biochem. Biophys. Res. Commun.* **189,** 504–510
9. Flink, I. L, and Morkin, E. (1995) *J. Biol. Chem.* **270,** 6959–6965
10. Gore, M. T., Sumbandam, T., and Umeda, P. K. (1993) *Circulation* **88,** 1261 (abstr.)
11. Matsumoto, K., and Wolffe, A. P. (1998) *Cell Biol.* **8,** 318–323
12. Ruiz-Lozano, P., de Leccea, L., Buesa, C., de la Osa, P. P., LePage, D., Gualberto, A., Walsh, K., and Pons, G. (1994) *Gene* (*Amst.*) **14,** 243–248
13. Sapru, M. K., Gao, J. P., Walke, W., Burmeister, M., and Goldman, D. (1996) *J. Biol. Chem.* **271,** 7203–7211
14. Zou, Y., and Chien K. R. (1995) *Mol. Cell Biol.* **15,** 2972–2982
15. Knotts, S., Rindt, H., Neumann, J., and Robbins, J. (1994) *J. Biol. Chem.* **269,** 31275–31282
16. Rindt, H., Gulick, J., Knotts, S., Neumann, J., and Robbins, J. (1993) *J. Biol. Chem.* **268,** 5332–5338
17. Tsika, G. L., Wiedenman, J. L., Gao, L., McCarthy, J. J., Sheriff-Carter, K., Rivera-Rivera, I. D., and Tsika, R. W. (1996) *Am. J. Physiol.* **271,** C690–C699
18. McCarthy, J. J., Fox, A. M., Tsika, G. L., Gao, L., and Tsika. R. W. (1997) *Am. J. Physiol.* **272,** R1552–R1561
19. Tsika, R. W. (1994) *Exercise Sport Sci. Rev.* **22,** 361–388
20. Tsika, R. W., Hauschka, S. D., and Gao, L. (1995) *Am. J. Physiol.* **269,** C665–C674
21. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
22. Chomczynaski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
23. Kingsley, P. D., Zinkin, A. S., Silver, L. S., Pendola, F. L., and Palis, J. (1996) *Dev. Growth Differ.* **38,** 697–706
24. Erickson, J. M, Rushford, C. L., Dorney, D. J., Wilson, G. N., and Schmickel, R. D. (1981) *Gene* (*Amst.*) **16,** 1–9
25. Lichtstein S. J., Wuarin, J., Schibler, U. (1987) *Cell* **51,** 963–973
26. Mar, J. H., Ordahl, C. P. (1990) *Mol. Cell. Biol.* **10,** 4271–4283
27. Larkin, S. B., Farrance, I. K. G., Ordahl, C. P. (1996) *Mol. Cell. Biol.* **16,** 3742–3755
28. Gray, T. A., Gumucio, D. L., and Collins, F. S. (1990) *Technique* **2,** 147–154
29. Demczuk, S., Harbers, M., and Vennstrom, B. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 2574–2578
30. Rajavashisth, T. B., Taylor, A. K., Andalibi, A., Svenson, K. L., Lusis, A. J. (1989) *Science* **245,** 640–643
31. Michelotti, E. F., Tomonaga, T., Krutzsch, H., and Levens, D. (1995) *J. Biol. Chem.* **270,** 9494–9499
32. Hasegawa, K., Lee, S. J., Jobe, S. M., Markham, B. E., Kitsis, R. N. (1997) *Circulation* **96,** 3943–3953
33. Kelm, R. J., Jr., Sun, S., Straugh, A. R., and Getz, M. J. (1996*) J. Biol. Chem.* **271,** 24278–24285
34. Pellizzoni, L., Lotti, F., Maras, B., and Pierandrei-Amaldi, P. (1997) *J. Mol. Biol.* **267,** 264–275
35. Warden, C. H., Krisans, S. K., Purcell-Huynh, D., Leete, L. M., Daluiski, A., Diep, A., Taylor, B. A., and Lusis, A. J. (1994) *Genomics* **24,** 14–19