Structural Characterization and Regulatory Element Analysis of the Heart Isoform of Cytochrome c Oxidase VIa*

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In order to investigate the mechanism(s) governing the striated muscle-specific expression of cytochrome c oxidase VIaH, we have characterized the murine gene and analyzed its transcriptional regulatory elements in skeletal myogenic cell lines. The gene is a single copy, spans 689 base pairs (bp), and is comprised of three exons. The 5'-ends of transcripts from the gene are heterogeneous, but the most abundant transcript includes a 5'-untranslated region of 30 nucleotides. When fused to the luciferase reporter gene, the 3.5-kilobase 5'-flanking region of the gene directed the expression of the heterologous protein selectively in differentiated Sol8 cells and transgenic mice, recapitulating the pattern of expression of the endogenous gene. Deletion analysis identified a 300-bp fragment sufficient to direct the myotube-specific expression of luciferase in Sol8 cells. The region lacks an apparent TATA element, and sequence motifs predicted to bind NRF-1, NRF-2, ox-box, or PPAR factors known to regulate other nuclear genes encoding mitochondrial proteins are not evident. Mutational analysis, however, identified two cis-elements necessary for the high level expression of the reporter protein: a MEF2 consensus element at -90 to -81 bp and an E-box element at -147 to -142 bp. Additional E-box motifs at closely located positions were mutated without loss of transcriptional activity. The dependence of transcriptional activation of cytochrome c oxidase VIaH on cis-elements similar to those found in contractile protein genes suggests that the striated muscle-specific expression is coregulated by mechanisms that control the lineage-specific expression of several contractile and cytosolic proteins.

Cytochrome c oxidase (COX) is the terminal enzyme of the electron transport chain (1). It catalyzes the coupled reactions of electron transfer from ferrocytochrome c to water (2) and proton translocation across the inner mitochondrial membrane (eukaryotes) or the cytoplasmic membrane (prokaryotes) (3-5). Energy conserved in the form of an electrochemical gradient across the membrane provides the driving force for the ATP synthase to phosphorylate ADP (6, 7). Not surprisingly, alterations of the activity of cytochrome c oxidase can exert significant control over the flux of aerobic ATP production (8).

Eukaryotic COX is a multicomponent enzyme consisting of 12 polypeptides in Saccharomyces cerevisiae (9) and 13 polypeptides in mammals (10). The three largest subunits (I, II, and III) of the eukaryotic enzyme are encoded by mitochondrial genes (11, 12), and the remainder (IV, Vα, Vb, VIa, VIb, VIc, VIIa, VIIb, VIIc, and VIII (nomenclature of Kadenbach et al. (10)) are encoded by nuclear genes (11, 13). The isolation of catalytically active cytochrome c oxidase from Paracoccus denitrificans (14–17) and the finding that it comprises only three subunits that show significant amino acid homology to subunits I, II, and III of the eukaryotic protein (18, 19) have led to the view that these subunits constitute the catalytic core of the eukaryotic enzyme (2, 20, 21). The functions of the 9 or 10 nuclear encoded subunits in the eukaryotic cytochrome c oxidase are less well understood. Gene disruption studies in S. cerevisiae (22–29) indicate that most of these are essential components for the assembly or maintenance of a functional protein. However, a null mutation in the gene encoding subunit VIII (homologous to mammalian subunit VIa) reduces cellular respiration and cytochrome c oxidase activity to 80% of the wild type levels (30). In addition, a yeast strain with a null mutation in the gene encoding subunit VIa (homologous to mammalian subunit VIa) exhibited altered responsiveness to ATP and potassium phosphate (31, 32). Thus, subunits VIII and VIa may function to modulate enzyme activity in response to changes in metabolic conditions. It is uncertain whether conclusions about subunit function derived from gene disruption experiments in yeast can be extended to mammals (21, 25) since there are no known corresponding mutations in mammalian subunits.
The regulatory effect of ADP (54), indicating that COXVIaH may be required for mediating the tissue-specific allosteric effect of ADP on the heart enzyme.

Although the tissue and developmental specific expression of COXVIaH has been described, and the genes encoding this subunit isoform in bovine and rat have been isolated (55, 56), no report has been published analyzing the regulatory mechanisms of its expression. While studies of the mechanisms of the lineage-specific expression of cytosolic and contractile proteins in striated muscle have progressed rapidly in recent years (57), our understanding of the lineage-dependent expression of mitochondrial proteins is still at a rudimentary stage. Elucidation of the mechanisms of muscle-specific expression of COXVIaH, a regulatory subunit of a key mitochondrial enzyme, will expand the current understanding of the regulatory mechanism(s) governing the differentiation of specialized oxidative myotubes. In the present study, we have cloned and characterized the gene encoding murine COXVIaH and analyzed its transcriptional regulatory elements in murine skeletal muscle cell lines. The results indicate that the skeletal myotube-specific expression of COXVIaH is, in large part, regulated by the same myogenic factors that control the muscle-specific expression of contractile proteins. No regulatory elements essential for the transcription of genes encoding ubiquitously expressed mitochondrial proteins can be identified by sequence comparisions in the functional COXVIaH promoter region. This evidence and the developmental switch that occurs soon after birth suggest that there must be concerted mechanisms employed by specialized oxidative myotubes to coordinate COXVIaH expression with other mitochondrial proteins.

**EXPERIMENTAL PROCEDURES**

**Isolation and Characterization of the Murine COXVIaH Gene**—A partial cDNA of murine COXVIaH spanning nucleotides 78–306 (provided by Dr. Bill Parsons) was used to screen a 129/Sv mouse genomic library (Stratagene); four independent ρ clones were isolated. An 11-kb KpnI/SalI and a 9-kb XbaI fragment encompassing the entire cDNA sequence were subcloned into pSP72 (Stratagene) and characterized by restriction digestion and Southern hybridization analyses using standard procedures (58). Bidirectional sequencing of genomic fragments covering nucleotides 698 to 1212 relative to the transcriptional start site of the gene was performed with the dideoxynucleotide chain termination method using a commercial kit (Sequenase v2.1, Stratagene).

Southern Blot Hybridization—Genomic DNA was isolated from J1 embryonic stem cells, digested with various restriction enzymes, size-fractionated on a 0.8% agarose gel, and membranes (GeneScreen, DuPont NEN) (58). The membrane was probed with 32P-labeled DNA synthesized using the Klenow fragment of DNA polymerase I. Hybridization was performed in a 50% formamide containing solution at 42 °C overnight. After sequential washes in 2 × SSC, 1% SDS at room temperature for 30 min and 0.2 × SSC, 1% SDS at 68 °C for 1 h, the membranes were subjected to autoradiography.

Northern Blot Hybridization, Ribonuclease Protection Assay, and Rapid Amplification of 5′ cDNA Ends (5′-RACE)—Total RNA was isolated from various mouse organs or cultured cells using the methods of Gilsin et al. (59) and Ullrich et al. (60). For Northern blot analysis, the RNA was size-fractionated on a 1.1% formaldehyde agarose gel, transferred to a nylon membrane, and probed with 32P-labeled DNA synthesized using the partial cDNA of COXVIaH as template. Conditions for hybridization and posthybridization washes were the same as those described for Southern analysis. The ribonuclease protection assay was performed essentially as described (58). A 789-bp HindIII/NcoI genomic fragment spanning nucleotides 728 to 1212 relative to the transcriptional start site of the gene was subcloned and used as the template for transcription of a 32P-labeled antisense riboprobe. The probe was hybridized to 30 μg of total RNA isolated from various mouse organs in a solution containing 80% formamide at 50 °C overnight. Following RNase H and T1 digestion, protected fragments were fractionated on a 6% denaturing polyacrylamide gel, and protected bands were visualized following autoradiography. 5′-RACE was performed by the method described by Frohman (61). Briefly, poly(A)-1 RNA from mouse heart was reverse-transcribed using an antisense oligonucleotide homologous to nucleotides +184 to +203 relative to the transcriptional start site as the initial primer. Homopolymeric dC was added to the 3′-end of the first strand cDNA using terminal deoxynucleotidyl transferase in the presence of dCTP. The d-tailed cDNA of COXVIaH was amplified with PCR using a poly-dG containing oligonucleotide as the 5′-primer and an antisense oligonucleotide complementary to base pair +80 to +103 of COXVIaH cDNA as the 3′-primer. Following gel purification, the PCR products were subcloned into Bluescript II SK (Stratagene) and sequenced.

**Plasmid Constructions**—The promoterless plasmid, pBST-Luc (kindly provided by Dr. R. Bassel-Duby) served as the vector backbone for the luciferase expression constructs. In this study, the unique HindIII site of pBST-Luc was bluntned and converted to an XhoI site by linker amplification. Several fragments of 5′-DNA of the COXVIaH gene were generated via convenient restriction enzyme sites and subcloning into pBS-5K (Stratagene); pBS6aHC was constructed by inserting a HindIII/blunted-MaeI fragment containing nucleotides 698 to +17 of the COXVIaH gene into the HindIII/HindIII sites; a HindIII fragment containing nucleotide −1290 to −699 of the gene was inserted into the HindIII site of pBS6aHC to generate pBS6aHB; pBS6aHA was constructed by subcloning an XbaI/HindIII fragment containing nucleotide −3500 to −1291 of the gene into the XbaI/HindIII sites of pBS6aHC and subsequent insertion of the HindIII fragment containing nucleotide −1290 to −699 of the gene into the HindIII site; pBS6aHD was generated by removing a HindIII/BglII fragment spanning nucleotide −698 to −284 of the gene from pBS6aHC, blunting the ends, and religating the plasmid; pBS6aHFE was constructed by insertion of a HindIII/Taq fragment containing nucleotides −698 to −23 of the gene between the HindIII/AccI sites of pBS SK; a BamHI/PvuI fragment from pBS6aHC containing nucleotide −698 to −142 of the gene was subcloned into the BamHI/PvuI sites of pSP72 to generate pSP72C6aHC. C6aHA, -B, -C, -D, -F, and -G fragments were then 5′-terminally cloned into the BamH1/XhoI sites of pBS-Luc between the upstream SV40 poly(A) capture cassette (positioned to reduce nonspecific background luciferase activity) and the cDNA of luciferase. Construction of pBS6aHE was accomplished by subcloning a PvuI/HindIII fragment from pBS6aHC containing nucleotide −698 to −144 of the gene into the Smal/XhoI sites of pBS-Luc. Mutations of potential transcriptional regulatory elements in C6aHD were generated by directed mutagenesis using sequential PCR as described (62). Two oligonucleotides, with the desired mutation in an overlapping region antisense to each other, were paired with either T3 or T7 primers to amplify two fragments of C6aHD overlapping each other at the mutational region using pBS6aHC as the template. The entire C6aH bearing the desired mutation was then generated by annealing the two overlapping fragments together, followed by PCR amplification using the two universal primers. After digestion with BamHI and XhoI, the mutated C6aHD region was subcloned into pBST-Luc as described. The identity of each promoter-reporter construct was confirmed by bidirectional DNA sequence analysis.

**Cell Culture and Transfection**—Myoblasts (Sol8 and C2C12) and fibroblasts (10T1/2 and NIH3T3) were grown in growth medium (Dulbecco’s modified Eagle’s medium with high glucose supplemented with 20% (Sol8) or 10% (all others) fetal bovine serum, 200 units/ml penicillin and 20 μg/ml streptomycin). Myotubes were derived from myoblasts by growing subconfluent cultures (~80% confluent) in differentiation medium (same as growth medium except 2% horse serum was substituted for the 20% fetal bovine serum). Transfection was performed by trypsinization and electroporation of approximately 105 cells in 0.5 ml of growth medium containing 80 μg of sonicated salmon sperm DNA, 25 μg of test DNA, and 25 μg of cytomegalovirus-β-galactosidase expression vector (as an internal control of transfection efficiency). Electroporation was conducted with a Bio-Rad Gene Pulser using field strengths from 950 to 1400 volts/cm and a capacitance of 960 microfarads. Cells were immediately plated on 6-cm (myotubes) or 10-cm (myoblasts) gelatin-coated plates in growth medium containing 5 mM butyric acid. The medium was replaced with butyrate-free medium after transfection. One experimental group of 10T1/2 cells was harvested 30–40 h after transfection, and the other was maintained in growth medium and harvested 92–110 h after transfection.

**Enzyme and Protein Assays**—Cell extracts were prepared and assayed for luciferase activity and β-galactosidase activity as described (63). Luciferase activity was determined by measuring the amplitude of the peak chemiluminescence with a Berthold Biolumat LB 9500 C luminometer. Background luciferase activity was assessed in extracts from parallel cultures transfected with the promoterless plasmid pB-
Fig. 1. Organization of the gene encoding murine COXVIaH. A, partial restriction map of the 11-kb KpnI/SaiI fragment encoding COXVIaH. E1, E2, and E3 denote the positions of the three exons. B, schematic structure of the gene encoding COXVIaH. Presentation is based on bidirectional sequence analysis of a DNA fragment spanning nucleotides 699 to 1212 relative to the transcriptional start site. Open boxes and solid lines represent exons and introns, respectively. The schematic is proportional to actual length in base pairs. The direction of transcription and the start site are represented by an arrow. The positions of the polyadenylation site and the translational start site are indicated by numbers in parentheses.

STLuc. β-galactosidase activity was determined spectrophotometrically after incubation of the extracts with o-nitrophenyl-β-D-galactopyranoside. Luciferase activity in cell extracts transfected with various fusion constructs was expressed as chemiluminescence counts over that of background after correction for transfection efficiency with the β-galactosidase activity. Protein concentration was measured colorimetrically with the Bio-Rad reagent (Bio-Rad). All assays were linear with respect to protein concentration.

Transgenic Animal Production—Transgenic mice were generated by standard methods (64) using the KpnI/Not fragment of the promoter-reporter construct pBS-T Luc6CA-HA that was free of plasmid sequences. The presence of the transgene in F1 progeny was determined by Southern analysis of tail DNA using a 1.3-kb EcoRI fragment from the luciferase cDNA. Luciferase activity in various organs from transgene-positive mice was determined essentially as described (63) with the following modifications: organs were excised and quickly frozen in liquid nitrogen and then homogenized in a solution containing 70 mM HCl, 0.25 mg/ml soybean trypsin inhibitor (Sigma), and 0.1% Nonidet P-40. Following centrifugation at 12,000 rpm at 4°C for 10 min, 50 μl of the supernatant was immediately mixed with 250 μl of assay solution, and activity was determined by measuring peak chemiluminescence during a 10-s interval after injection of 100 μl of 5 μl luciferin.

RESULTS

Cloning and Structural Characterization of the COXVIaH Gene—Four independent λ phage clones were isolated by screening a 129Sv mouse genomic library with a partial cDNA of murine COXVIaH. Two overlapping fragments encompassing the entire gene of COXVIaH were subcloned and characterized by restriction digestion and Southern hybridization analysis. A partial restriction map of the 11-kb KpnI/SaiI fragment is shown in Fig. 1A. The restriction pattern of the genomic clones was then compared with that of the endogenous gene obtained by Southern blot analysis of genomic DNA from J1 embryonic stem cells. A DNA probe containing nucleotides 283 to +91 relative to the transcriptional start site of the COXVIaH gene hybridized to single restriction fragments in genomic DNAs digested with a variety of restriction enzymes, indicating that the gene was single copy and did not represent a pseudogene (as is common for several nuclear encoded mitochondrial proteins). The identical restriction digestion patterns between the cloned gene and the endogenous DNA isolated from tail biopsy samples verified that the structure of the gene was not altered during the cloning process (data not shown).

Genomic fragments covering nucleotides 699 to +1212 of the COXVIaH gene were bidirectionally sequenced. As illustrated in Figs. 1B and 2, the gene spans 689 bp and comprises three exons separated by two small introns. All of the exon-intron boundaries conform to consensus splice junction rules. The immediate 5′-region of the gene lacks both TATA and CAAT boxes.

Mapping the 5′-End of COXVIaH Transcripts—The 5′-end of COXVIaH transcripts was determined by RNase protection assay as well as rapid amplification of 5′-end cDNAs (5′-RACE). The results are shown in Fig. 3. Total RNA from hearts and skeletal muscles of mice at various developmental ages protected multiple fragments of a riboprobe antisense to nucleotides –728 to +61 of the COXVIaH gene. The sizes of these fragments ranged from 72 to 154 bp, with the dominant fragment clustered at 91 bp (Fig. 3A). In order to exclude the possibility that there were upstream untranslated exons beyond the range of the riboprobe used in the RNase protection assay, upstream sequences of COXVIaH transcripts were determined from 12 independent subclones of the 5′-region of COXVIaH cDNA obtained from mouse heart poly(A) RNA by 5′-RACE, a procedure for amplification of nucleic acid sequences from an mRNA template between a defined internal site and unknown sequences at the 5′-end. Consistent with data from the RNase protection assay, the 5′-ends of COXVIaH cDNAs are heterogeneous, located at nucleotide –64 to +5 relative to the translational start site, and the majority of the cDNAs have their 5′-ends clustered at nucleotide –30 (Fig. 3B). Taken together, the results indicate that the 5′-ends of COXVIaH mRNAs are heterogeneous, with the predominant tran-
Expression of COXVIaH in Murine Cell Lines—The availability of tissue culture cell lines that undergo differentiation to fully functional myotubes has allowed investigations into the mechanisms that govern expression of some lineage-specific muscle genes. To determine if the tissue-specific expression of COXVIaH in striated muscle can be modeled in murine myogenic cell lines during differentiation, steady state levels of COXVIaH transcript were examined in the skeletal muscle cell lines Sol8 and C2C12 at the myoblast and myotube stages. For comparison, two fibroblast lines, 10T1/2 and NIH3T3, were also examined. Total RNA isolated from these cell lines, before and after the induction of myogenic differentiation by downshift into serum-free media, was probed with 32P-labeled DNA prepared with a partial cDNA of COXVIaH. As shown in Fig. 4, COXVIaH mRNA was not detected in 10T1/2 and NIH3T3 fibroblasts, Sol8, or C2C12 myoblasts. Abundant COXVIaH mRNA, however, was present in Sol8 and C2C12 myotubes 2 days after the induction of differentiation of confluent cultures of myoblasts in a serum-depleted medium. No COXVIaH mRNA was detected in 10T1/2 and NIH3T3 fibroblast cultures depleted of serum. Thus, the expression of COXVIaH in skeletal muscle cell lines was dramatically up-regulated during the transition from myoblasts to myotubes. The up-regulation of COXVIaH expression during myotube formation thus provided a suitable in vitro system to examine the mechanisms controlling the lineage-dependent expression of COXVIaH in skeletal muscle.

Regulatory Element Analysis of the COXVIaH 5'-Region—To locate the proximal promoter region of the COXVIaH gene and identify cis-elements essential for mediating its skeletal myotube-specific expression, fragments of 5'-flanking sequences of the gene with various deletions were fused to the cDNA of firefly luciferase. Promoter activity of these fragments was assessed by transient transfection of these promoter-reporter constructs into Sol8 and 10T1/2 cells followed by assays for luciferase activity in cell extracts before and after myogenic differentiation. The results are shown in Fig. 5. The region containing nucleotides -3500 to +17 of the COXVIaH gene directed the expression of the heterologous protein in Sol8 myotubes about 900-fold over the background. In comparison, the same construct was 5- and 9-fold less active in Sol8 myoblasts and 10T1/2 fibroblasts, respectively. The relatively high transcriptional activity of the 3500-bp region of the COXVIaH gene in Sol8 myoblasts and 10T1/2 fibroblasts was surprising, and may be due to nonspecific transcriptional initiation when present as episomal DNA. This notion is supported by the observation that when this promoter-reporter construct was microinjected into mouse oocytes to generate transgenic mice, it was capable of directing the tissue-specific expression of luciferase into the heart and skeletal muscle in a pattern virtually identical to the endogenous gene (Fig. 7).
Transcriptional Regulatory Element Analysis of COXVIaH

Deletion analysis of the expression of COXVIaH5'-luciferase gene constructs in mouse cell lines. Left, schematic illustration of the promoter-reporter fusion constructs. Various DNA fragments from the COXVIaH 5'-flanking region were fused directionally to the cDNA of firefly luciferase (open box). Numbers indicate the positions of the start and end points of the fragment relative to the transcriptional start site. Right, luciferase activity in Sol8 and 10T1/2 cells transfected with the promoter-reporter fusion constructs. Transfection, preparation of cell extracts, and enzymatic assay were performed as described under “Experimental Procedures.” Luciferase activity is expressed as chemiluminescence counts over that of background after correction for transfection efficiency. Solid and hatched bars represent data from Sol8 myoblasts and myotubes, respectively. Open bars represent data from 10T1/2 fibroblasts harvested at a time window parallel to that of Sol8 myoblasts and myotubes, respectively. Data are the means ± S.E. from three to seven separate experiments.

Deletion of COXVIaH 5'-sequence from nucleotide −3500 to −283 slightly increased luciferase activity in Sol8 myotubes to 1250-fold over background but significantly reduced luciferase activity in Sol8 myoblasts and 10T1/2 fibroblasts to 1/20 and 1/60 of that found in Sol8 myotubes, respectively. This suggests there may be nonspecific transcriptional enhancer elements within this region. Further 5'-deletion from nucleotide −283 to −144 disrupted a consensus E-box sequence (CAGCTG) at −174 to −142 and resulted in a 9–12-fold decrease of luciferase activity in Sol8 myotubes as well as in Sol8 myoblasts and 10T1/2 fibroblasts, suggesting the existence of a positive element in this region, which may be regulated by a factor(s) common to myoblasts, myotubes, and fibroblasts. A 3'-deletion from nucleotide +17 to +23 of a fragment spanning nucleotides −698 to +17 of the COXVIaH gene resulted in a 3-fold decrease of luciferase activity in Sol8 myotubes, Sol8 myoblasts, and 10T1/2 fibroblasts. Further 3'-deletion of this fragment from nucleotide −23 to −142 caused a further 5-fold decrease of luciferase activity in Sol8 myoblasts but had no effect on luciferase activity in Sol8 myotubes and 10T1/2 fibroblasts, suggesting the existence of a positive element in this region, which is regulated by myotube specific factors. Together, the deletion analysis located a 300-bp fragment spanning nucleotide −283 to +17 of the COXVIaH gene that is sufficient to direct the high level, myotube-specific expression of luciferase in Sol8 cells.

Regulatory Element Analysis of the COXVIaH Promoter—The nucleotide sequence of the 300-bp COXVIaH promoter is presented in Fig. 5A. Several enhancer elements are present in this region that might be involved in its tissue-specific expression. Three E-box elements (CANNTG), which function as muscle-specific enhancers in the regulation of the expression of several muscle-specific proteins through their interaction with
the MyoD family of transcription factors (57), are present beginning at nucleotides –147, –75, and –60. A single MEF2 element, another muscle-specific enhancer sequence (65), is present beginning at nucleotide –90. Finally, a single GATA element (WGATAR), which may function as an enhancer in the regulation of the lineage-specific gene expressions in T-cells (66), erythroid cells (67), and cardiac myocytes (68), is present beginning at nucleotide –105. In order to determine the functional significance of these potential regulatory elements in the transcriptional regulation of COXVIaH, mutant constructs of the 300-bp COXVIaH promoter containing nucleotide substitutions in each of the three E-boxes or the MEF2 element or both (at their respective sites in the wild type promoter) were transfected into Sol8 and 10T1/2 cells. The effects of the mutations on the promoter activity were assessed by assaying luciferase activity in cell extracts. Mutation in the MEF2 element to a site incapable of DNA binding (65) completely abolished the expression of luciferase in myotubes to the level found in myoblasts (Fig. 6B) but had no effect on luciferase activity in Sol8 myoblasts and 10T1/2 fibroblasts. This indicates that this element is essential for the myogenic differentiation-dependent expression of COXVIaH in skeletal myotubes. Mutation in the E-box at nucleotide –147 reduced the expression of luciferase in myotubes to 20% of the wild type level. This effect, however, was not myotube-specific since the same mutation also resulted in a 10–20-fold reduction of the expression of the reporter gene in Sol8 myoblasts and 10T1/2 fibroblasts. Mutation in the E-box at nucleotide –60 decreased luciferase activity in myotubes, myoblasts, and fibroblasts to 65% of the wild type level, whereas mutation in the E-box at nucleotide –75 did not affect the expression of luciferase in any cell type.

Analysis of the COXVIaH Promoter in Transgenic Mice—In order to confirm that the 5′-flanking region of the COXVIaH gene functions as a striated muscle-specific promoter/enhancer element in vivo, the DNA construct from nucleotides –3500 to +17 fused to luciferase was used to generate transgenic mice. Three separate lines of mice were generated. One line failed to transmit the transgene to subsequent progeny, precluding further analysis as of this writing. Two lines transmitted the transgene in a Mendelian fashion and were available for analysis. Of these, only one line (Fig. 6) showed expression. Luciferase activity was detected in heart and skeletal muscle and at a low level in brain tissue. In contrast, no detectable luciferase activity was present in the liver, kidney, or stomach from this line. Although preliminary, these data suggest that all the information necessary for the striated muscle-specific expression of COXVIaH is present on this 3.5-kb 5′-flank. A detailed analysis of these promoter elements in transgenic animals is in progress and will be reported separately.

DISCUSSION

Remarkable insights into the regulatory mechanisms of skeletal muscle differentiation have been gained through the isolation of the basic helix-loop-helix (bHLH) myogenic determination factors and the MEF2 family of muscle-specific transcriptional factors. The MEF2 proteins are encoded by four separate genes, each of which undergoes alternative splicing. They bind to an A/T-rich motif found in many muscle genes and activate their transcription (65, 69–73). Most studies on the mechanisms of tissue-specific transcription in striated muscle have focused on proteins associated with the contractile apparatus, since these are the obvious unique components in the muscle lineage. More recently, it has been documented that muscle-specific isoforms are present for several mitochondrial proteins coupled to oxidative phosphorylation (33, 74), underscoring that in addition to contractile proteins, striated muscle must also acquire unique proteins associated with mitochondria for its special need in aerobic ATP production. This is particularly true for oxidative fibers. The 5′-flanking regions of genes encoding several of the muscle-specific isoforms of mitochondrial proteins contain E-box as well as MEF2 elements (55, 56, 74); however, the functional significance of these elements in the transcriptional regulation of these genes remains largely undefined.

The present study was undertaken to analyze the structure and transcriptional regulation of the gene encoding COXVIaH, a muscle-specific subunit isoform of cytochrome c oxidase. Characterization of the murine gene encoding COXVIaH revealed an exon-intron structure similar to that found in the genes from bovine and rat. The 5′-ends of transcripts from the murine gene are heterogeneous, with the majority of transcripts having a 30-bp 5′-untranslated region. This contrasts with the 181- and 203-bp 5′-untranslated regions reported for transcripts from the bovine and rat genes, respectively (55, 56). Whether this reflects true species differences, or whether the discrepancy is due to different interpretations of primer extension experiments is unclear. In contrast to bovine, but similar to rat, the 5′-region of the murine gene lacks both TATA and CAAT boxes. Thus, the proximal region of the murine COXVIaH promoter has structural features resembling those of housekeeping genes (75) but not those of regulated and tissue-specific genes. The TATA-less feature and the ability of the COXVIaH promoter to direct skeletal muscle specific transcription in transgenic mice (Fig. 7) thus represents an unusual case of muscle-specific transcriptional regulation in the absence of a consensus TATA element.

Using transient transfection, we have located a 300-bp 5′-flanking region of the COXVIaH gene, which is sufficient to direct high level, skeletal myotube-specific expression of a heterologous protein in Sol8 cells in a pattern similar to the expression of the endogenous gene. Sequence analysis revealed three potential E-boxes and an MEF2 element clustered at nucleotide –147 to –60 of this region. Mutation in the MEF2 element diminished luciferase activity in differentiated myotubes to levels found in myoblasts, and the effect was restricted to myotubes. Thus, interaction of this site with a myotube-specific factor is essential for myotube-specific transcriptional activation. There is compelling evidence to suggest this factor is MEF2, since MEF2 binding is rapidly induced following downshift of myoblasts to form myotubes, and presumptive MEF2 sites undergo DNase footprinting only upon conversion to myotubes (72). Finally, our data show that conversion of this
site to a site incapable of binding MEF2 completely abolishes the myotube-specific transactivation of the luciferase reporter cassettes (but not myoblasts). It follows that direct interaction with the skeletal muscle-specific bHLH proteins alone cannot account for the myotube specificity of transcription from the COXVIaH promoter. Since in the absence of the MEF2 site, the three E-boxes are unable to confer myotube-specific transcription. Mutations in the proximal two E-boxes at nucleotides –75 and –60 of the COXVIaH promoter did not promote large decreases of luciferase activity in myotubes, indicating that as individual elements they are likely dispensable. Mutation in the distal E-box at nucleotide –147 resulted in a dramatic decrease in luciferase activity. This occurred in the presence of the two downstream E-boxes, indicating that either the location or additional sequences outside the canonical E-box motif distinguished the distal E-box from the two upstream ones. The parallel decrease in luciferase activity in 10T1/2 fibroblasts caused by the same mutation, however, argues against muscle-specific bHLH factors interacting with this site since they are known to be absent in 10T1/2 cells. Although not strictly excluded, it is unlikely that the parallel effects caused by this mutation in myoblasts, myotubes, and fibroblasts are due to disruption of interactions of this site with distinct factors in different cell types. A plausible explanation is that this E-box is interacting with ubiquitously present factors to enhance transcription from the COXVIaH promoter.

Taken together, the present study establishes the functional significance of the MEF2 and distal E-box sites in the skeletal muscle-specific transcription of COXVIaH. The dependence on common cis-elements in the transcriptional activation of both contractile proteins (69–71) and COXVIaH suggests that the striated muscle-specific expression of these two classes of molecules is co-regulated by common mechanisms. Additionally, since COXVIaH is required for the tissue-specific allosteric effect of ADP on bovine heart cytochrome c oxidase (54), such coregulation may be of selective advantage for the oxidative muscle lineage in signaling the energy demand of the contractile apparatus to the mitochondria.

As an isoform of a cytochrome c oxidase subunit, the expression of COXVIaH must be regulated in concert with other mitochondrial proteins. Coordinated up-regulation of the expression of mitochondrially encoded and nuclear encoded mitochondrial proteins has been documented in rabbit skeletal muscle after sustained contraction via nerve stimulation (76, 77) and in patients suffering from mitochondrial myopathy (78, 79). Recent in vitro experiments identified several cis-elements and trans-factors essential for the transcription of nuclear encoded mitochondrial proteins. Two sequence motifs with the consensus of YGCGCAYGCGC and MGGAAG have been identified in several ubiquitously expressed nuclear encoded mitochondrial proteins. Their cognate binding factors, nuclear respiratory factors 1 and 2, have been isolated. The functional significance of these cis-elements and trans-factors in the transcription of several nuclear encoded mitochondrial proteins including the mitochondrial transcription factor A has been demonstrated in HeLa cells (80–82). Promoter analysis in skeletal muscle myogenic cell lines, on the other hand, has identified the obox and rebox cis-elements essential for the transcription of the genes encoding the muscle-specific adenine nucleotide translocase and the p-subunit of ATP synthase (83–85). While these studies suggest that transcriptional regulation is an important mechanism in coordinating mitochondrial biogenesis, the physiological significance of these cis-elements and trans-factors in coordinated regulation of the expression of mitochondrial proteins has not been established. A search of the 300-bp COXVIaH promoter for NRF-1 and NRF-2 as well as the oxbox and rebox sequence motifs revealed no such sequences, suggesting that the coordination of the expression of COXVIaH with other mitochondrial proteins may be regulated by different but concerted mechanisms. The identification of promoter elements sufficient to direct expression of a reporter gene in striated muscle in transgenic animals and the availability of animal models to induce coordinated up-regulation of the expression of mitochondrial proteins, Nonetheless, will facilitate further experimentation aimed at the elucidation of the mechanisms coordinating the expression of COXVIaH during mitochondrial biogenesis.

Additional studies will be required to ascertain if these same elements are required for transcriptional regulation in cardiac myocytes. The GATA site present in the proximal region is an obvious candidate for this regulation, but the MEF2 and E-box motifs may also be indispensable. Finally, although not specifically addressed by this study, the question as to the physiological role of this subunit in the regulation of oxidative phosphorylation in striated muscle remains to be defined in mammals. Targeted mutagenesis of this gene in murine embryonic stem cells and creation of mutant mice that lack this subunit may provide insight into the functional significance of COXVIaH in oxidative myocytes.

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