Regulation of Murine Cytochrome Oxidase Vb Gene Expression in Different Tissues and during Myogenesis

ROLE OF A YY-1 FACTOR-BINDING NEGATIVE ENHANCER

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The mouse cytochrome oxidase (COX) Vb promoter contains three sequence motifs with partial or full consensus for YY-1 and GTG factor binding and a CArG box, located between positions −480 and −390. Individually, all three motifs stimulated transcription of the TKCAT promoter, and bound distinctly different proteins from the liver and differentiated C2C12 nuclear extracts. Collectively, these motifs, together with the downstream flanking sequence, −378 to −320, suppressed the transcription activity of heterologous promoters, thymidine kinase-chloramphenicol acetyltransferase (TKCAT) and COXIV/CAT. The transcription activities of both TKCAT and COXIV/CAT constructs were induced 3–4-fold during induced myogenesis of C2C12 cells. The downstream CArG-like motif binds transcription factor YY-1, while the upstream YY-1-like motif binds to a yet unidentified factor. Co-expression with intact YY-1, but not that lacking the DNA binding domain suppressed the transcriptional activity. Mutations targeted to the CArG-like motif abolished the suppressive effect of the negative enhancer and the inducibility of the promoter during myogenic differentiation. Our results suggest that the activity of the negative enhancer may determine the level of expression of the COX Vb gene in different tissues.

The immediate upstream region of the mouse COX Vb gene consists of a TATA-less GC-rich sequence characteristic of a number of “housekeeping” genes. The basal promoter region has been mapped to the −8 to +40 region of the gene, which contains several protein-binding motifs. At least five of these protein-binding motifs correspond to discrete transcription initiation sites (10). Initiation of RNA mapping to the +1 position of the promoter has been shown previously to depend upon a YY-1-binding initiator motif (12). Recently we have found that the GA-binding protein-factor-binding Ets repeat sequence GTTCCCGGAAG at the +17 to +26 position also functions as an independent initiator for transcription of two clusters of RNAs mapping to the +18/19 and +23/24 regions (10).

Although the COX Vb gene is constitutively expressed, its level of expression varies in different tissues. COX Vb mRNA is 10–20-fold more abundant in the heart and kidney as compared to the liver. Furthermore, the COX Vb mRNA level increases markedly during induced differentiation of C2C12 myoblasts into myotubes. In the present study we have investigated the contributions of various protein-binding motifs from the 5′ distal region of the promoter in the overall transcriptional rates of the promoter. We have identified a negative regulatory region, which encompasses a YY-1-like (YY-1′) and CArG-like (CArG′) consensus motifs flanking a GTG element (13), a novel enhancer found in the new oncogene promoter. Results presented in this paper show that factor YY-1 binding to the downstream CArG′ motif is important for the activity of the negative enhancer. Our results therefore suggest that in addition to the multiple positive cis acting elements (7–11), a negative enhancer region is involved in the complex regulatory circuit of the COX Vb gene expression.

MATERIALS AND METHODS

Plasmid Constructs—The 5′ deletion promoter constructs were derived from the −574 CAT DNA (5), which contains the mouse COX Vb sequence −574 to +40, cloned in pCAT basic plasmid (Promega Biotech Corp.) at the HindIII and blunt ended XhoI sites. A series of 5′ deletions of the −574 CAT DNA were generated by the exonuclease III digestion of linearized plasmid, using the “Erase a Base System” (Promega Biotech Corp.). The authenticity of all mutations were determined by dideoxy sequencing (14).

The following synthetic double-stranded DNA oligomers were used for cloning in different plasmid vectors, for PCR amplification of promoter regions, or for competition in gel mobility shift experiments. These double-stranded DNAs were synthesized either with 5′ HindIII and 3′ SalI linkers (YY-1′, GTG, and CArG′) or 5′ HindIII and 3′ XhoI linkers (Mut1 and Mut2): upstream YY-1-like motif (sequence −480 to −461), referred to as YY-1′, 5′-aggtCTAGAGTTGAGGGAATTg-3′; GTG element (sequence −456 to −431), 5′-aggtACTGTTGGGGGGGGGGTG-3′; downstream CArG-like motif (sequence −412 to −390), referred to as CArG′, 5′-aggtGGCCCAATTCGCAATTTGGTGTA-CAACTAAGCg-3′; mutated YY-1 (+1 position) at the initiator site (sequence −8 to +31), referred to as MUT1, 5′-tgcagGTCCCGC-CAATCTTGCTCAGCCTGTTCCCGGAAGTGCAT-3′; mutated YY-1

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‡ The abbreviations used are: COX, cytochrome oxidase; CAT, chloramphenicol acetyltransferase; TKCAT, thymidine kinase-CAT; bp, base pair(s); PCR, polymerase chain reaction; CMV, cytomeglovirus.
**RESULTS**

**Tissue-dependent and Cell-specific Variations of COX Vb mRNA Levels**—Northern blot hybridization of RNA from different mouse tissues and during myogenesis. A, 20 μg of total RNA from different mouse tissues were resolved by electrophoresis, blotted to a Nytran membrane and hybridized with either 32P-labeled COX Vb cDNA probe or 18 S rRNA probe as described under “Materials and Methods.” B, 20 μg of each total RNA from the subconfluent C2C12 myoblasts (50–60% confluent), 100% confluent myoblast cells, and fully differentiated myotubes were hybridized with the COX Vb cDNA probe.

The hybridization pattern in Fig. 1A is consistent with the S1 nuclease protection data showing differences in COX Vb mRNA levels in different tissues (10). Moreover, the level of Vb mRNA increased nearly 5-fold during induced differentiation of murine C2C12 myoblasts into myotubes (Fig. 1B). Thus, although the COX Vb mRNA is expressed ubiquitously, its level of expression varies markedly in different tissues.

Identification of a Negative Enhancer Sequence in the 5' Upstream Region of the COX Vb Promoter—Fig. 2 shows the nucleotide sequence of the −574 to +40 region of the mouse COX Vb gene, and potential protein-binding motifs both at the immediate upstream and distal regions of the promoter. Previous studies (5, 10, 11) showed that the −319 to +20 region of the promoter contains a number of sequence motifs that act as transcription activators, and initiators. In the present study, the positive or negative effects of the 5' proximal region of the COX Vb gene was studied by a similar 5' deletion analyses.

Fig. 3 shows that the −574/+40 CAT DNA exhibits only 20% CAT activity in 3T3 fibroblast cells as compared to the activity of the previously analyzed −319/+40 CAT DNA construct. Similar 4–5-fold lower activities were also obtained in COS cells, Hep 3B cells, and uninduced C2C12 myocytes. Progressive 5' deletions to −520 and −481 positions of the promoter had little effect on the transcriptional activity in all four cell types, while deletions to sequence −406 resulted in about 5-fold higher activity in all cells tested. As shown in the illustration at the bottom of Fig. 3, deletion to nucleotide −406 eliminates the upstream YY-1 and GTG motifs, in addition to disrupting the CarG' motif. Additionally, internal deletion of sequence −378 to −320 from the −520/+40CAT DNA showed activity near 100% in 3T3 and C2C12 myoblasts (results not shown). These results suggest that the −481 to −320 region of the promoter containing at least four different potential protein-binding motifs functions as a negative enhancer in different cell lines tested.

To evaluate the activity of the negative regulatory region during muscle cell differentiation, unindifferentiated mouse C2C12 myoblasts were transfected with various 5' deletion CAT constructs and the relative CAT activities in unindifferentiated myoblasts and differentiated myotubes were assayed. Results of transient transfection in Fig. 4 show that the activities of the −520 and −481 deletion constructs are induced.
Negative Modulation of the COX Vb Gene Expression

Fig. 2. Nucleotide sequence of the 5′ upstream region of the mouse COX Vb gene. The nucleotide residues are numbered beginning with the major transcription start site at +1. Multiple sites of transcription initiation and the direction of transcription are marked with solid arrows. The double underlined regions indicate the positions of various sequence-specific protein-binding motifs. The boxed region represents the major negative enhancer sequence involved in the downregulation of COX Vb expression.

about 3.5-fold in differentiated C2C12 myocytes as compared to undifferentiated myoblasts. The results also show that deletion clones −406CAT, −319CAT and −174CAT exhibit nearly 100% activity in both differentiated and undifferentiated myocytes. The internal deletion clone Δ(−378/−320) showed a similar activity in the range of 99–100% in both undifferentiated and differentiated myocytes. These results show that the negative modulatory effect of the enhancer region is reduced severalfold during myogenesis, and provide a rational basis for the observed 5–6-fold higher mRNA levels in induced C2C12 myotubes (presented in Fig. 1B). Furthermore, the results show that in addition to the −481 to −406 sequence, the downstream flanking sequence, −378 to −319, is also necessary for the maximal effectiveness of the negative enhancer.

Effects of the Negative Enhancer Region on the Activities of Heterologous Promoters—An examination of nucleotide sequence from this region (see Fig. 2) shows the presence of a YY-1-like motif at position −480 to −464 (YY-1′), a GTG element at positions −456 to −431, and a CArG-like protein-binding motif (CArG′) at position −412 to −390. The effects of these individual protein-binding motifs on the transcription activity of a heterologous promoter, pTKCAT was tested in 3T3 fibroblasts, Hep 3B cells, and C2C12 myocytes. It is seen (Fig. 5A) that all three elements individually function as transcription activators, although the relative extent of stimulation in different cells varies markedly. For example, all three sequence motifs induced transcription by 10–20-fold in both 3T3 and C2C12 cells. In Hep 3B cells, however, both the YY-1′ and CArG′ motifs were minimally active, while the GTG sequence motif yielded over 15-fold increased activity. Interestingly, the 90-bp fragment (sequence −481 to −390) containing all three sequence motifs in the same order as they exist in the COX Vb promoter, exhibited significantly lower transcription stimulation in all three cell lines as compared to activities obtained with the individual sequence motifs (Fig. 5A). Moreover, the activity of the TKCAT plasmid was reduced 3–4-fold in C2C12 cells when the sequence −481 to −390 of the COX Vb gene was cloned upstream of the TK promoter. The same construct, however, showed a marginal reduction in activity in 3T3 and Hep 3B cells. In support of the internal deletion data in Fig. 5A, these results suggest that in addition to sequence −480 to 390, the downstream sequence up to −320 is also required for the effectiveness of the negative enhancer activity. Results also suggest that three different activator motifs, when placed in a tandem array with appropriate downstream flanking sequence up to −320, function as a transcription suppressor in both the COX Vb promoter as well as in heterologous promoters.

Since the negative enhancer effect of the COX Vb promoter is reduced during myogenesis (Fig. 4), we investigated the gener-
ality of this effect using heterologous COXIV and TKCAT promoters, both of which are ubiquitously expressed with no significant muscle predominance. Results in Fig. 5 show that both TKCAT and COXIV/CAT promoters show nearly similar transcription activities in both uninduced myoblasts and induced myotubes. Constructs containing the COX IVb 2481 to 2319 sequence at the 5′ upstream regions, however, show 5–6-fold reduced activities in undifferentiated C2C12 myoblasts. These results further confirm that the 2481 to 2319 region is involved in conferring the negative enhancer activity, and that the effectiveness of the negative enhancer is reduced during induced myogenesis.

Unusual Sequence Requirements for YY-1 Binding to the Negative Enhancer Region—Previous studies have shown that the CArG sequence motif from the muscle-specific creatine kinase promoter binds to transcription factors YY-1 and SRF, exclusive of each other (25, 26). The 90-bp DNA from the negative enhancer region of COX IVb gene contains two potential YY-1-binding sites: the YY-1′ site at the −480 to −461 position and a CArG′ motif at the −412 to −390 position. We have therefore tested both the YY-1′ and CArG′ motifs for binding to bacterially expressed purified YY-1. Panel A represents the DNA-protein binding under the normal binding conditions (room temperature and use of poly(dI-dC)) described under “Materials and Methods.” Panel B shows binding under conditions that detect rapidly dissociable complex (binding at 4°C using 25 μg/ml poly(dG-dC)). Panel C represents the localization of the YY-1-binding site in the negative enhancer region by methylation interference analysis. The 32P-end-labeled antisense strand of the 90-bp DNA fragment (−480 to −390 sequence) was partially methylated by dimethyl sulfate and used for binding with 15 μg of purified YY-1 factor as described previously (12, 24). The protein-bound and unbound DNA were recovered by electroelution following polyacrylamide gel electrophoresis. The recovered DNA was subjected to piperidine cleavage, and the fragments were resolved by electrophoresis on 8% polyacrylamide gel containing 8 M urea. A Maxam-Gilbert sequence reaction for purine residues was run alongside. Partially protected nucleotides of the sequence are indicated with open circles. The sense strand does not show any protection and is not presented here.
The gel mobility shift pattern showed a major and a very minor slow migrating complex, which probably represent the single and double binding sites, respectively. The results also show that under conditions that detect low affinity binding (incubation at 4°C, with added dG-dC) the CArG' motif, but not the YY-1' DNA bound to purified YY-1 (Fig. 6B). In support of the gel mobility shift data, methylation interference analysis with the YY-1-bound 90-bp DNA (Fig. 6C) showed partial protection at nucleotides −403, −408, and −409, demonstrating that a region of the CArG' sequence motif is responsible for binding. These results suggest an interesting possibility that the formation of high affinity YY-1 complex with the 90-bp DNA requires some of the upstream sequences in addition to the putative CArG' region.

The gel mobility shift pattern in Fig. 7 shows that the 90-bp DNA probe forms six different complexes with the nuclear extract from differentiated C2C12 myotube, two of which competed with complexes formed with bacterially expressed YY-1. Consistent with the YY-1 binding affinities of individual motifs in Fig. 6, the YY-1' motif failed to compete with either of the complexes that comigrated with the YY-1-bound complexes, while the CArG' DNA competed with complex 1 but not with complex 3. However, this motif competed effectively with complexes 4, 5, and 6. Surprisingly, the GTG motif also competed effectively with complex 1 in addition to complexes 4–6. Finally, the YY-1-binding motif from the Igk 3' enhancer region (23) competed with both of the complexes that comigrated with the YY-1-bound complexes, in addition to complexes 4–6. Although not shown, the 90-bp DNA effectively competed out all of the complexes. These results suggest the possibility that some of the slow migrating complexes are higher order complexes formed due to protein-protein interaction.

The YY-1 factor binding to the 90-bp DNA was further ascertained by including a YY-1-specific antibody in the mobility shift experiment. As shown in Fig. 8A, affinity-purified antibody caused a supershift of the complex obtained with bacterially expressed purified YY-1, while preimmune serum had no effect on DNA-protein complex formation. Similarly, the formation of YY-1-bound complex with the C2C12 nuclear extract was affected by the antibody, although the supershift of the complex is not readily apparent because of the masking effect due to the presence of a number of slow migrating complexes in the region (Fig. 8B). The electrophoretic patterns in Figs. 7 and 8 show significant differences mainly due to the use of different buffers in these two experiments. The resolution of DNA-bound complexes was better with the Tris-glycine buffer system in Fig. 7, while the 0.25 × TBE buffer used in Fig. 8 was better suited for the antibody reactivity.

**Fig. 7.** The YY-1 binding pattern with nuclear extract from C2C12 myotubes. Binding reactions were performed with the 32P-labeled 90-bp probe using either 10 μg of myotube nuclear extract or 0.2 μg of purified bacterially expressed YY-1 protein using the Tris-glycine buffer system. 25 ng of each of the competitor oligonucleotide YY-1', CArG', GTG element, and Igk 3'YY-1 factor-binding site (CCACCTC-CATCTT), YY-1Mut1, and YY-1Mut2 (see Ref. 12) were added as indicated at the top of lanes. The DNA-protein complexes are indicated by arrows.

**Fig. 8.** Evidence for YY-1 binding to the 90-bp negative regulatory region. **Panel A** represents the antibody supershift pattern of the −480−800 DNA-bound complexes with purified YY-1 protein. Additions are as indicated at the bottom of each lane. **Panel B** shows the antibody supershift pattern of complexes with the C2C12 nuclear extract. Gel shift was carried out using the 0.25 × TBE buffer. The arrows indicate the position of the YY-1-bound complex.
FIG. 9. Protein binding profiles with the individual sequence elements from the negative enhancer region. Binding reactions were performed using 10 μg each of the nuclear extract and the indicated 5'-end-labeled probes (20,000 cpm) using the Tris-glycine buffer. Panel A represents the binding of the GTG element (sequence −449/−435) to liver and differentiated C2C12 myocyte nuclear extract. Additions are indicated above the lanes. Numbers in parentheses represent the amount (in nanograms) of unlabeled competitor used. Distinct protein-DNA complexes referred to under "Results" are indicated by arrows at the left. The slowest migrating complex marked as NS represents a nonspecific DNA-protein interaction. Panel B shows the protein binding profile with the CArG' probe (sequence −412/−393) using nuclear extracts from liver and C2C12 myotube. Specific binding is shown by competition with 25 and 50 ng of self- and non-self-unlabeled synthetic double-stranded oligonucleotides. Panel C represents the gel mobility shift pattern of the YY-1' DNA motif (sequence −476/−464) with nuclear extracts from liver and differentiated C2C12 myocyte extract. Additions are as shown at the top of each lane.
hand, formed a complex that was competed by excess unlabeled GTG DNA, not by the CArG' or YY-1' DNAs. In some lanes, an additional nonspecific complex (NS) was also observed, which was not competed by excess competitor DNAs. Although the complexes obtained with the myotube extract appear to co-migrate with complex 1 formed with the liver extract, currently the precise protein compositions of these complexes remain unclear.

Fig. 9B shows the protein binding patterns of the CArG' motif with the liver and myotube nuclear extracts. The CArG' DNA formed two to three closely migrating complexes with the liver extract, all of which were competed by excess unlabeled CArG' DNA but very poorly by the YY-1' and GTG DNAs. The nuclear extract from C2C12 myotube, on the other hand, formed a single complex that co-migrated with the fastest migrating complex obtained with the liver extract. Additionally, the complex formed with the myotube extract was competed with excess CArG' DNA but not with the YY-1' and GTG DNAs. The major complex with the myotube extract migrated differently from the complex formed with bacterially expressed YY-1 (Fig. 9B); in addition, it is not supershifted by the YY-1 antiserum (results not shown), suggesting that it does not represent a YY-1-bound complex. Finally, as shown in Fig. 9C, the YY-1' DNA motif formed two weak complexes with both liver nuclear extract and myotube extract that were self-competed, but not with the CArG' or GTG DNAs. The slow migrating complexes with the liver and myotube extracts showed different migration on the gel, suggesting that different tissue-specific proteins may be involved in the formation of these complexes. These results together suggest tissue-specific differences in protein binding patterns with all of the three DNA motifs from the negative regulatory region.

Down-regulation of COX Vb Transcription by Coexpression with YY-1 cDNA—Since YY-1 is known to be involved both in positive as well as negative modulation of transcription activity with different promoters (27–34), the effects of YY-1 overexpression on the activity of the COX Vb promoter was studied. We have addressed the role of the initiator site YY-1-binding site and also distal upstream CArG' motif, which also binds YY-1, on the activity of the negative enhancer. The −520/ +40CAT construct (−520/CAT) and also constructs carrying mutations at the initiator YY-1 site (−520/MUT1 and −520/MUT2) were co-transfected with CMV/YY-1 expression construct in C2C12 myoblasts and the transcriptional activities were assayed. As shown in Fig. 10A, co-expression with 1 and 2 µg of YY-1 cDNA construct caused about 60% and 84% inhibition, respectively. Both MUT1 and MUT2 constructs yielded essentially similar patterns of inhibition, although the extent of inhibition was about 10% lower as compared to the wild type construct.

The role of negative enhancer region in the YY-1-mediated transcription down-regulation was further ascertained using heterologous promoter TKCAT and COXIV/CAT constructs, which lack YY-1-binding sites. Results in Fig. 10A show that YY-1 had negligible to marginal (0.7–10%) inhibitory effect on the transcription of both TKCAT and COXIV/CAT constructs. Activities of constructs containing the COX Vb −520 to −320 sequence in both cases were inhibited by YY-1 in a concentration-dependent manner. A control experiment in Fig. 10B shows the levels of immunodetectable YY-1 in untransfected 3T3 cells and cells transfected with 1–10 µg of YY-1 construct. The Western blot data show that the level of YY-1 in untransfected control cells is below the limit of immunodetection, while immunodetectable protein bands are seen in cells transfected with 1, 5, and 10 µg of YY-1 cDNA. These results provide evidence that the −520 to −319 region of the promoter contains a negative enhancer activity, which is modulated by YY-1.

To ascertain the role of the CArG' motif in the YY-1-mediated down-regulation of transcription, we generated two mutant constructs, −481/CArG'M1 and −481/CArG'M2. Mutations were targeted to the CArG' region of the −481/MUT1CAT plasmid, which has mutated YY-1-binding site at the +1 position. As shown in Fig. 10C, both M1 and M2 mutant forms of CArG' DNA motifs, whose sequences are shown below the gel shift pattern, failed to compete significantly with CArG' DNA binding to YY-1. In direct binding assays (Fig. 10C), the CArG'M1 DNA bound to YY-1 at a vastly reduced level, while the CArG'M2 DNA did not bind to YY-1. Transcriptional activities of the parent and mutant constructs were compared by transfection in C2C12 myoblasts as well as differentiated myotubes. As expected, the parent plasmid construct, −481/MUT1 exhibited about 8-fold higher transcriptional activity in induced myotubes as compared to myoblasts (see Fig. 10D). −481/CArG'M1 and −481/CArG'M2 constructs yielded 9–11-fold higher activities in uninduced myoblasts as compared to the −481/MUT1 construct, suggesting that mutations in the CArG' sequence drastically reduced or abolished the negative enhancer function of the −319 to −481 sequence of the promoter. Additionally, in C2C12 myotubes, co-transfection with 1 µg of CMV/YY-1 cDNA construct inhibited the activity of the −481/MUT1 construct by over 70%, while similar co-transfections had marginal inhibitory effects (10–18%) on the activities of the −481/CArG'M1 and −481/CArG'M2 constructs. Surprisingly, both of these mutant constructs showed 70–80% reduced transcription activity in induced C2C12 myotubes, suggesting that in addition to functioning as a component of the negative enhancer, this region might also function as a transcription enhancer under some cellular conditions. These results provide evidence that the YY-1-binding CArG' region of the negative enhancer plays an important role in modulating the transcriptional activity of the promoter.

The specificity of the YY-1-mediated inhibition of transcription was further ascertained using various YY-1 deletions expressed as Gal4 fusion proteins. Recently, Bushmeyer et al. (18) have systematically mapped various functional domains of YY-1 using the Gal4 promoter system. The Gal4 fusion constructs (18) were co-expressed with the COX Vb −520/+40CAT construct in 3T3 cells. Results presented in Fig. 11A show that Gal4-YY-1 inhibits the transcription activity by about 80%, indicating that it is as effective as the parent YY-1 cDNA construct in down-regulating the COX Vb promoter activity. The Δ(16–99)Gal4-YY-1 construct (deleted amino acid residues 16–99), which contains almost all the functional domains, excepting the transcription activation domain, inhibits the activity by about 85%. The 1–256/Gal4-YY-1 construct (deleted residues 257–414), which lacks the DNA binding as well as transcription suppressor domains (18), has no inhibitory effect, suggesting that DNA binding is important for its negative regulatory activity. The construct 201–414/Gal4-YY-1 (deleted residues 1–200), which contains both intact DNA binding domain and transcription repressor domain inhibits the activity at a rate comparable to the intact Gal4-YY-1 construct. The Western blot presented in Fig. 11B shows that the various Gal4-YY-1 fusion proteins are indeed expressed in C2C12 myoblasts under the co-expression conditions described in Fig. 11A. As reported previously (18), the YY-1 N-terminal domain fusion protein, 201–414/Gal4-YY-1, shows an abnormally faster migration on SDS-polyacrylamide gels in comparison to other Gal4 fusion proteins. These results demonstrate that both DNA binding and transcription repressor domains of the YY-1 are essential for the negative enhancer activity of the protein.
DISCUSSION

Subunit Vb of the mammalian COX is classified as a ubiquitously expressed subunit of the enzyme complex (2). Results presented in this study, along with previous results (5, 10), demonstrate that the relative abundance of the COX Vb mRNA in different tissues varies markedly; the mouse kidney and heart contain about 10–20-fold higher level of the mRNA than the liver (Fig. 1A). Furthermore, a 4–6-fold higher level of the mRNA is detected during induced differentiation of C2C12 myocytes. Our results suggest that widely varying levels of Cox Vb mRNA transcription in different tissues are due to the presence of an unusual negative regulatory element at the 5'}
Results of deletion analyses (Figs. 3 and 4) show that the negative regulatory region spans sequences −481 to −321, and consists of two distinct domains. A region between −481 and −390 contains three different sequence motifs showing partial consensus to the YY-1-binding site (NF-E1'), a GTG enhancer element, and a CArG-like element (CArG'). The second region between −390 and −321 contains an Ets sequence motif, which binds to an unknown factor from 3T3, liver, and C2C12 nuclear extracts (results not shown). All three upstream region sequence motifs, YY-1', GTG, and CArG', stimulated transcription of the TKCAT promoter in 3T3, Hep3B, and C2C12 cells at different levels (Fig. 5). All three DNA motifs exhibited distinctly different patterns of protein binding with nuclear extracts from liver and C2C12 myotubes (Fig. 9), suggesting that the cell- or tissue-specific binding patterns may reflect upon the differing levels of transcription activation with these activator motifs in different cells. The three motifs placed together in a tandem array, as they exist in the COX Vb promoter, nearly completely reversed the transcriptional activity obtained with the individual sequence motifs. The transcriptional down-regulation of the heterologous promoter was more pronounced when further downstream sequences up to −320 were linked to the TKCAT promoter or the mouse COXIV promoter (Fig. 5). It is therefore likely that the 90-bp region, containing the three protein-binding motifs, together with the flanking downstream sequence is involved in conferring the negative regulatory activity.

An interesting observation of this study relates to the unusual nature of YY-1 binding to the negative regulatory region. Gel mobility shift patterns in Fig. 6 show that the YY-1' and CArG' sequences fail to bind to bacterially expressed purified YY-1 factor under conditions that detect only complexes with slow off rates. Under conditions that detect rapidly dissociating complexes, however, the CArG' sequence motif, but not the YY-1' motif binds to the YY-1 factor (Fig. 6). The 90-bp DNA probe containing all three sequence motifs, on the other hand, binds to the YY-1 factor under slow off rate conditions and the bound complex yields a single-site methylation footprint, which maps to the CArG' sequence region. Thus, although the CArG' sequence region appears to provide the site for binding, the overall binding efficiency appears to be modulated by a still unknown sequence or structure within the 90-bp DNA sequence. YY-1 is a multifunctional factor, which functions both as a transcriptional activator and a repressor (18, 27–35) of different promoters under different cellular conditions and in different cell systems. Additionally, YY-1 binding is known to induce DNA bending in some cases (35). In the present case, YY-1 binding may induce similar DNA bending, facilitating specific interactions between protein-bound complexes at both upstream and downstream regions of the COX Vb negative enhancer.

Experiments in Fig. 10 (A and D), demonstrating a 60% suppression of transcription in 3T3 and C2C12 myoblasts by co-expression with YY-1 cDNA, provide evidence for its involvement in the negative regulation of the COX Vb gene under transient transfection conditions. Several lines of evidence suggest that the YY-1-mediated inhibition is specific. First, as shown in Fig. 10, the YY-1-mediated inhibition is dose-dependent. Results of cotransfection with various deletion constructs of YY-1 (expressed as Gal-4 fusion proteins) in Fig. 11 demonstrate that the DNA binding and repressor domains of YY-1 are essential for transcription suppression of the COX Vb promoter, while the transcription activation domain is not necessary. Third, mutations targeted to the CArG' region of the promoter resulted in vastly reduced YY-1 binding and reduced effectiveness of the negative enhancer (Fig. 10, C and D). It is therefore likely that the relative levels of YY-1 in a given cell type may determine the level of expression of the COX Vb gene. It is also likely that other tissue-specific factors binding to the CArG' or other flanking motifs might affect the binding or the activity of the YY-1 factor. It was recently reported that although nuclear extract from differentiated chick embryonic myocytes contained YY-1 protein, its DNA binding efficiency was reduced, possibly due to interference from another muscle-specific factor (36). It is known that a number of different nuclear protein factors, including Sp1 (37), E1A (38), FKBP12 (39), and c-Myc (40), interact with YY-1 and modulate its functional properties. Our results showing a reduced transcription activity of the −481/CArG'M1 and −481/CArG'M2 constructs in differentiated myotube suggests that in the context of the
COX Vb promoter, the CArG sequence functions as a transcription activator in differentiated myocytes, and mutations in this region may affect the binding of myotube-specific transcriptional activator protein(s).

A number of different negative enhancer mechanisms have been reported (41, 42), including the repression due to competition for DNA-binding sites, the quenching mechanism, where the repressor interferes with the function of activator(s) without inhibiting its binding ability, and the direct repression where the negative regulatory factor blocks the activity of the basal transcription complex. It appears likely that the COX Vb negative regulatory region represents a mechanism similar to the quenching phenomenon. The activity of the negative enhancer may require specific interactions between proteins bound to the upstream 90-bp DNA sequence with those bound to sequences downstream of the −390 region, although the details of this interaction currently remain unknown. We also postulate that positive acting sequence motifs like YY-1, GTG, and CArG, when correctly juxtaposed to the downstream regulatory region, alter the activity of various transactivator proteins, with the net effect being the transcriptional repression of the promoter. Recent studies have shown that in a number of different promoters, regulatory regions consist of alternatively positioned positive and negative acting regulatory elements. In the case of the low density lipoprotein receptor gene, the sterol-dependent binding of a protein to a regulatory sequence inhibits or alters the activity of an adjacent Sp1 factor-binding site (43). In several other cases, including the mouse IgH (44), the T cell receptor gene locus (45), rat collagen II gene (46), and cystic fibrosis transmembrane conductance regulatory gene (47), transcriptional regulation involves interaction between both positive and negative elements. Currently the nature of proteins binding to the individual sequence motifs of the COX Vb negative regulatory region, the nature of interaction between various binding complexes, and the precise role of the YY-1 in mediating these interactions remain unknown. Further experiments are under way to elucidate these mechanisms.

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