Structural Organization and Promoter Analysis of the Bovine Cytochrome c Oxidase Subunit VIIc Gene

A FUNCTIONAL ROLE FOR YY1

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Cytochrome c oxidase (COX) subunit VIIc is one of the nuclear encoded subunits of the 13-subunit holoenzyme that carries out the terminal step in the electron transport chain. We have isolated the gene for this subunit, previously shown to be ubiquitously expressed from a single copy gene in the genome, and show that 167 base pairs of DNA surrounding the transcriptional start site contain the minimal promoter of this gene. This basal promoter contains two YY1 sites and at least one site for NRF-2, which show binding to their cognate factors. Mutation of both YY1 sites eliminates most of the promoter activity. Mutation at the upstream YY1 site significantly reduces the efficiency of transcript initiation at the major start site and thus plays the dominant role in COX7C regulation. COX7C is, thus, the second nuclear gene of COX that is regulated by YY1, suggesting that it is a third common factor, along with NRF-1 and NRF-2, to be associated with COX gene regulation.

Mammalian cytochrome c oxidase (COX) is a 13-subunit protein complex located on the inner mitochondrial membrane that catalyzes the transfer of electrons from reduced cytochrome c to molecular oxygen and participates in the translocation of protons across the membrane. Proton translocation generates an electrochemical gradient that drives the synthesis of ATP. The complex consists of three primarily catalytic subunits encoded by mitochondrial DNA and 10 polypeptides encoded in the nucleus that are suggested to function in regulation and/or assembly (1–3). Three of the nuclear-encoded subunits (VIa, VIIa, and VIII) exist as a pair of isoforms (4–6) in most mammalian species: a liver (L) form that is present in most tissues and a heart/muscle (H) form specific for adult cardiac and skeletal muscle.

Several COX nuclear genes, including those of isoforms, have now been isolated and characterized and provide a means of assessing their role in function and regulation; in addition, defects in the expression of genes for COX nuclear subunits are likely to cause human disease. A detailed analysis of the basal promoters of three ubiquitously expressed COX genes thus far available (COX4, COX5B, and COX7AL) reveal that they have regulatory sites in common (7–12). COX4, COX5B, and COX7AL contain multiple, functional binding sites for nuclear respiratory factor-2 (NRF-2; Ref. 8); also called GAPBP, GA-binding protein). COX5B and COX7AL also harbor a functional site for NRF-1 (13, 14), which binds to a number of housekeeping genes; a functional site has also been detected in COX6C, although the promoter of this gene has not been extensively analyzed (15). Binding sites for the ubiquitous factor Spl are found in the basal promoters of COX4, COX5B, and COX7AL. Finally, the multifunctional factor Yin-Yang-1 (YY1) has only been found in COX5B, where it acts by initiating basal transcription. None of the above elements, however, is found in the basal promoter of COX6AH, the only H isoform COX promoter so far characterized (16). The basal promoter of this gene is dependent on muscle-specific elements such as the E-box (MyoD binding site) and MEF-2 (myocyte-specific enhancer-binding factor-2) binding sites.

To further elucidate the mechanisms of COX regulation and expression, we have characterized the bovine gene encoding the 63-amino acid precursor of subunit VIIc (COX7C), a gene encoding a subunit without isoforms and thus expressed in all tissues. We present here the sequence of the COX7C gene and identify the transcription factors that regulate its expression. We show that its basal promoter is regulated by two YY1 sites, one of which is required for efficient initiation of transcription from the major start site. COX7C is the second COX nuclear gene in which YY1 plays a role in promoter function, suggesting that YY1 may be a third common factor, along with NRF-1 and NRF-2, involved in COX gene regulation.

EXPERIMENTAL PROCEDURES

Materials—Ribonuclease A was obtained from Sigma; Ribonuclease T1 was from Life Technologies, Inc.; T3 and T7 RNA polymerases and avian myeloblastosis virus reverse transcriptase were from Promega Corp. [γ-32P]ATP (3000 Ci/mmol), [α-32P]CTP (800 Ci/mmol), and [α-35S]dATP (1500 Ci/mmol) were from DuPont NEN. pCH110 was from Pharmacia Biotech Inc. The CAT enzyme-linked immunosorbent assay kit, chlorophenol red β-n-galactopyranosidase, and yeast tRNA were purchased from Boehringer Mannheim. The Sequenase Version 2.0 DNA sequencing kit was from U. S. Biochemicals, and the ribonuclease protection assay kit (RPA II™) was from Ambion. Oligonucleotide primer synthesis was by the Center for Molecular Medicine and Genetics (Wayne State University) or Random Hill Biosciences (Ramona, CA). All other chemicals were from Fisher, Sigma, or Life Technologies, Inc.

Cell Culture—HeLa (2-CCL), HeLa S3 (CCL-2.2), and C6[C3] mouse myoblasts (1772-CRL) were obtained from American Type Culture Collection. Media and supplements were obtained from Life Technologies, Inc. HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) containing penicillin (40 units/ml) and streptomycin (40 μg/ml). HeLa S3 suspension cultures were grown in a similar medium containing 5% FBS.
C₄C₁₂₄ myoblasts were cultured in DMEM supplemented with 15% FBS, 0.5% chick embryo extract, and gentamicin (50 μg/ml). Myotubes were obtained by growing myoblasts in dishes coated with calf skin collagen (Calbiochem) and inducing them to differentiate in DMEM plus 10% horse serum and gentamicin. Transfection conditions are described below.

Characterization of λBGL-2Δ—λBGL-2Δ was isolated from a bovine genomic EMBL-3 Sp6/T7 library (17). The phage was purified through two rounds of screening (18) using labeled COX7C cDNA (19) as probe and the phage DNA purified (20). Restriction fragments of the genomic (phage) DNA that were positive upon Southern analysis with COX7C cDNA probe were subcloned into pBluescript KS(+) vector (Stratagene) and sequenced (21).

 Primer Extension Assay—Primer extension was carried out as described previously (22), using total RNA isolated from adult bovine heart tissues and a 23-nt antisense primer corresponding to nt 183–161 of the first exon (Fig. 3). The primer-extended products were resolved on 6% polyacrylamide, 6 M urea gels and autoradiographed for 1–2 weeks at ~70 °C. A pBluescript KS(−) plasmid sequenced with the universal forward primer served as a DNA size marker.

 Ribonuclease Protection Assay—A 395-bp SacII-HincII fragment comprising 121 bp of the first intron, at least 131 bp of the first exon, as deduced from the cDNA (19), and an additional contiguous region of 143 bp at the 5’ end, was cloned at the SacII-HincII site of pBluescript KS(−) and used for nuclease protection with 40 μg of total RNA as described previously (23).

 CAT Constructs and Transfection Analysis—EcoRI, PstI, or SacII sites located upstream of the transcriptional start site and a HpaII site located within the non-coding portion of the first exon were used to isolate fragments that were cloned into pGKOCAT (24) to generate 1.8-kb EcoRI–HpaII, 1.5-kb PstI–HpaII, and 93-bp SacII–HpaII. Recombinant plasmids were isolated, verified by sequencing, and purified through two CaCl₂ gradients.

 HeLa cells (8 × 10⁶/ml) were grown, transfected, and the normalized CAT activity determined as described (9). For each CAT construct 3–4 dishes were used and each experiment repeated at least four times.

 Electrophoretic Mobility Shift Assay (EMSA)—The 167-bp SacII–HpaII fragment (~93 to +74) containing the putative basal promoter was end-labeled with [γ-³²P]ATP and T4 polynucleotide kinase and used for EMSA. A typical binding reaction (25) in a 20-μl final volume contained 5–10 fmol of the labeled fragment, 2 μg of poly(dI-dC), 20 mM Hepes (pH 7.9), 1 mM MgCl₂, 4% Ficoll, 0.5 mM dithiothreitol, 50 mM KCl, and protein. The latter consisted of HeLa SS extract (26) (7 μg of protein), purified histidine-tagged YY1 (600–700 ng), or purified NFR-2 (45 ng each of subunits α and β). For competition assays, the binding reactions were preincubated with a 100- and 1000-fold molar excess of the unlabeled fragment for 30 min on ice prior to incubation with the labeled probe for an additional 20 min. Samples were electrophoresed on 4% PAGE in 0.25 TBE (1× TBE = 90 mM Tris borate, 2 mM EDTA, pH 8.0) for 3 h in the cold and autoradiographed. Complementary oligonucleotides 21–32 bases long spanning the SacII–HpaII fragment (Fig. 5) were annealed in TE buffer plus 150 mM NaCl and the duplex DNA was recovered from PAGE (22), end-labeled with [γ-³²P]ATP, and used in EMSA. Electrophoresis was as before but for 2 h.

 Site-directed Mutagenesis of the YY1 Motifs in COX7C—The 167-bp basal promoter fragment was resynthesized from a set of 6 overlapping oligonucleotides spanning both strands as described (27). Appropriate mutant or wild-type (WT) complementary oligonucleotides were used to create the desired upstream (U), downstream (D), and upstream-downstream (UD) YY1 mutant, or WT constructs. Mutations were created by introducing the sequence AAAAGGG for the YY1 core recognition motif NCCATN. For YY1–D, the introduced changes did not alter the overlapping NFR-2 site (Fig. 5). Briefly, the oligonucleotides were phosphorolyated, annealed, and ligated; the 167-bp fragment recovered by PAGE was cloned into the SmaI site of pGKOCAT vector. After transformation into Escherichia coli DH5α cells, recombinants harboring the desired inserts were identified by restriction enzyme digestion and sequencing.

 Transcriptional Analysis of YY1 Mutations in Transfected Cells—Total RNA from C₄C₁₂₄ myoblasts, myotubes, and HeLa cells transfected with the WT basal promoter, as well as those harboring the various YY1 mutations, was subjected to a ribonuclease protection assay. Two antisense probes were generated by transcription from the T3 promoter using appropriately digested pBluescript plasmid containing the WT 167-bp basal promoter fragment (WT probe) or one with a mutant YY1–D site (D-probe) (Fig. 9B). The WT probe was used to detect transcripts arising from transfected WT and YY1–U mutant basal promoter CAT constructs and the D-probe was used to detect transcripts arising from the YY1–D and YY1–UD mutated constructs. Both the probes and the transcripts arising from the transfected plasmids include a common 62-bp vector sequence (Fig. 9B) that serves to differentiate these transcripts from any endogenous ones that may be protected by the probe. Both probes will protect all transcripts initiating from the +1 site to the beginning of the CAT gene. Yeast tRNA and mock-transfected cells were used as a control for each probe. Individual transcripts were quantitated by densitometric scanning with a Molecular Dynamics PhosphorImager. The results from the ribonuclease protection assays were confirmed by primer-extension analysis (22).
using an antisense primer specific to the 5'-untranslated region of the CAT gene (5'-TAGCTCCTGAAAATCTCGCC) (data not shown).

**RESULTS**

**Isolation and Organization of the COX7C Gene**—The COX7C genomic region from λBGL-2J (see “Experimental Procedures”) was completely sequenced on both strands (Fig. 1A). The first exon (185 nt) encodes the complete 16-amino acid presequence and the first 9 amino acids of the mature protein; it also contains a 5'-non-coding region of 110 nt. The second exon (129 bp) encodes the remaining 38 amino acids of the mature protein and 15 nt of the 3'-non-coding region, whereas the third encodes 218 nt of the non-coding region. All the intron/exon junctions follow the GT-AG rule (28); the first and second introns are 838 and 915 bp, respectively.

The gene is located at a CpG island (29), based on the distribution of CpG residues. Of the 52 Cpgs that span the sequenced gene (Fig. 1C), 94% are found near the transcriptional start site and the first intron; by contrast, the distribution of Cpg residues appears to be fairly uniform (Fig. 1C).

The transcriptional start site of the COX7C gene was determined by primer-extension and nucleotide protection analyses. A 23-nt antisense primer that anneals to the first exon, beginning 2n t5 (nt 183–161; Fig. 3) of the first intron, was used to reverse transcribe total bovine RNA. A major primer-extended product of 183 nt was obtained, suggesting that the first exon is 185 nt long. This product is clustered along with at least two minor products that all lie within 8–10 nt of each other (but not clearly evident in Fig. 2A). This result was consistent with a ribonuclease protection assay. In the latter, a cloned 395-bp fragment, extending from the SacII site (nt -293) to the HinclI site (nt -1306), containing at least 131 bp of the first exon as deduced from the cDNA, and an additional 143 bp of 5'-upstream sequence, was radioactively transcribed to yield sense and antisense transcripts and each was hybridized to total bovine RNA. Only the antisense transcript probe yielded protected fragments (Fig. 2B, lanes CO-AS, lane CO-AS), the largest of which was 180 nt. At least three protected bands (including the 180-nt band) were seen, consistent with the primer extension.
were made in pGKOCAT vector with 1.8 kb, 1.5 kb, and 93 bp 5′-untranslated region and immediately flanking the transcriptional start site (referred to two putative YY1 motifs (30, 31) between 2 and 32 nt upstream of the poly(A) tail, respectively (Fig. 3). The gene has two potential poly(A) addition signals, one matching the consensus AATAAA and another, AATTAA, located 132 nt downstream of the translational start site (at +1), where it acts as an initiator (10). Another notable motif present is for NRF-2 ((A/C)GGAA): two sites upstream of the translational start site (at 69 to 74) representing the basal promoter. The presence of two YY1 sites is consistent with the YY1-U and YY1-D motifs present within this fragment.

Analysis for the presence of known regulatory sites revealed two putative YY1 motifs (30, 31) between −17 and +69, one immediately flanking the transcriptional start site (referred to as the YY1-upstream site) and the other in the 5′-untranslated region, 55 bp downstream of the transcriptional start site (YY1-downstream site) (Figs. 3 and 5). The two sites match the reported consensus (Fig. 5) (32, 33). The presence of YY1 motifs is especially interesting since a YY1 site has also been found in COX5B, where it acts as an initiator (10). Another notable motif present is for NRF-2 (A/C/G/GA): two sites upstream of the translational start site (at +69 to +65 and −158 to −154; Fig. 3) and four sites in the first intron, including a pair of tandem motifs spaced 4 bp apart. Six more motifs can be found if the above consensus is extended to include a GGAT core: two in the upstream region (at −111 to −115 and −60 to −64), one at the start site (+6 to +2), and three in the first intron. There are no canonical TATA or CCAAT boxes or binding sites for NRF-1. There are also no GC-BOX (Sp1 binding sites) with the core sequence GGCGCG (34), but several that show partial homology are present. A perfect match to the Sp1 consensus (G/T/G/A/)GGCC/GT/GA/A/G/G/T; Ref. 35) is present between +579 and +587 in the first intron and two more, with a single mismatch (8/9), are located between +73 and +65 in the 5′-untranslated region and +691 and +683 in the first intron.

Promoter Analysis of Flanking DNA—Three CAT constructs were made in pGKOCAT vector with 1.8 kb, 1.5 kb, and 93 bp of flanking DNA (Fig. 4). These were transfected into HeLa and C2C12 cells, and their normalized CAT activity was compared with the largest CAT construct, EcoRI-7cCAT. In HeLa cells, significant activity (117 ± 26%) is retained with the smallest construct both with HeLa extracts (data not shown) and purified YY1 (Fig. 5). Hence, the putative basal promoter must reside within the 167-bp fragment defined by SacI and HpaII ends. This fragment contains two putative sites for YY1 and at least a single site for NRF-2. Similarly, in C2C12 cells, the SacI-7cCAT construct is as active as the largest construct both in myotubes (138 ± 26%) and myoblasts (138 ± 17%) (Fig. 4).

Binding of Transcription Factors to the Core Promoter—We used EMSA to examine the ability of YY1 to bind to the basal promoter both with HeLa extracts (data not shown) and purified YY1 (Fig. 6). Increasing amounts of YY1 (0.1–600 ng) were used for binding. Two complexes, Complex A (YY1 bound to a single site) and Complex B (YY1 bound to both sites) are seen. The presence of two YY1 sites is consistent with the YY1-U and YY1-D motifs present within this fragment.
activity were assessed in both undifferentiated and differenti-

promoter fragment. The effects of these mutations on CAT

lanes 3, incubation with YY1 alone; lanes 5 and 6, competition with 100- and 1000-fold molar excesses of specific competitor, respectively; lanes 5 and 6, competition with 100- and 1000-fold molar excesses of nonspecific competitor. B, binding of YY1 to the U site. ds7c-3.5, containing the YY1-U site, was used in EMSA and the experiment performed as in A. ds7c-6 is a nonspecific competitor. C, binding of NRF-2 to the basal promoter. The basal promoter contains an NRF-2 site overlapping YY1-D. ds7c-6, containing the NRF-2 site, was used in EMSA, and ds7c-3 was used as nonspecific DNA. Lane 2 represents incubation with NRF-2-a + β1 subunits; lanes 3 and 4 represent competition with 100- and 1000-fold molar excesses of specific competitor; lanes 5 and 6 represent similar competition with a nonspecific competitor.

FIG. 7. A, binding of YY1 to the D site. ds7c-6, a duplex oligonucleotide containing the YY1-D site, was used in EMSA. Lane 1, free probe; lane 2, incubation with YY1 alone; lanes 3 and 4, competition with 100- and 1000-fold molar excesses of specific competitor, respectively; lanes 5 and 6, competition with 100- and 1000-fold molar excesses of nonspecific competitor. B, binding of YY1 to the U site. ds7c-3.5, containing the YY1-U site, was used in EMSA and the experiment performed as in A. ds7c-6 is a nonspecific competitor. C, binding of NRF-2 to the basal promoter. The basal promoter contains an NRF-2 site overlapping YY1-D. ds7c-6, containing the NRF-2 site, was used in EMSA, and ds7c-3 was used as nonspecific DNA. Lane 2 represents incubation with NRF-2-a + β1 subunits; lanes 3 and 4 represent competition with 100- and 1000-fold molar excesses of specific competitor; lanes 5 and 6 represent similar competition with a nonspecific competitor.

FIG. 8. Site-directed mutagenesis of the YY1-motifs and their effect on COX7C promoter activity. Mutations (X) were introduced at the YY1-U, -D, and -UD motifs of the basal promoter (−93 to +74) as described under “Experimental Procedures.” The three mutant constructs and a WT construct were transfected into HeLa, C2C12 myoblasts, and myotubes. Promoter activity was determined by expressing normalized CAT activity as a percent of the WT value. The transcriptional start site at +1 is indicated.

For the wild-type transfected promoter (−93 to +74) in cultured cells is consistent with that obtained from mRNA isolated from tissue (data not shown). This suggests that the transfected CAT gene is driven by the COX7C basal promoter and not by cryptic sequences. In addition to the major start site, as already noted, a cluster of heterogeneous transcripts beginning immediately downstream of this site is seen. To examine if these initiation sites are affected by the two YY1 mutations, we transfected HeLa cells with the WT and mutant YY1-U, -D, and -UD basal promoter constructs. Total RNAs from these cells were subjected to ribonuclease protection assays with probes that would detect the major transcript initiating at the +1 site as well as the heterogeneous cluster of minor transcripts initiating downstream. In Fig. 9A, the major start site (+1) (larger arrows) and initiation sites for the three minor transcripts (smaller arrows) in the WT-transfected promoter are shown. The major transcript constitutes 47% of these transcripts by densitometric analysis. This pattern is retained in the D mutant, although the overall amount of transcripts is reduced. Significantly, the major transcript in WT cells is reduced in both the U and UD mutants (16 and 20%, respectively); one of the downstream transcripts now constitutes the largest fraction (32–33%, respectively). Overall, there is a net decrease in total transcript levels in the U, D, and UD mutants. Some of the decrease in the D lane is due to the lesser amount of total RNA used (Fig. 9A, legend). These observations hold true for both myoblasts and myotubes (data not shown). Thus, the binding of YY1 to the U site appears to correlate with initiating transcription from the +1 site.

**DISCUSSION**

The minimal promoter of COX7C can be localized to a sequence that contains 93 bp of 5′-flanking region and 74 bp of the 5′-untranslated region. This 167-bp region contains two sites for YY1 and at least one site for NRF-2. All three sites show binding to their cognate factors (Fig. 10). In addition to COX7C and COX5B (10), the nuclear encoded α-subunit of the mitochondrial F1F0 ATP synthase is also regulated by YY1 (37). Thus, three subunits of the respiratory chain are regulated by this factor, a multifunctional zinc-finger protein, variously referred to as NF-E1 (31), δ (38), UCRBP (39), CF-1 (40), F-Act 1 (41), and NMP-1 (42), that can activate, repress, or initiate transcription. The presence of two or more binding sites for YY1 and their location 3′ to the transcriptional start site have been frequently observed (32, 42–45).

**COX7C expression appears to be more critically dependent**...
on the relative proportion of WT transcripts but decreases their net amount. It is less clear how the interaction of YY1 to the D site affects promoter activity (but see below). We also note that the YY1 double mutation does not completely abolish CAT promoter activity. At least 20–45% of the CAT activity is still retained in these mutants, suggesting that the NRF-2 motif in the basal promoter could also contribute to promoter activity.

The close proximity of YY1-U to the transcriptional start site indicates that this binding site could function as an initiator, as seen for COX5B. YY1-U may therefore be involved in the transcriptional or stability of the preinitiation complex, perhaps by interaction with the basal transcription machinery. The ability of YY1 to recruit Pol II to the initiation site by interaction with TCP11B, or TBP, has been demonstrated (46). Consistent with this view, we see that mutating the U site nearly abolishes initiation at the major start site, which accounts for nearly half of the transcripts arising from the WT promoter.

The effect of mutating the D site is less clear. YY1-D overlaps an NRF-2 site. The core motif for YY1 (CCAT; top strand) and NRF-2 (GGAA; bottom strand) are spaced only 3 nt apart. However, the four zinc fingers of YY1 are presumed to interact with 12 nt of the bottom strand, with the core motif positioned at the center (93). This indicates that the binding of one factor would sterically hinder the binding of the other. As a repressor, YY1 is known to overlap the DNA binding motifs of a wide range of activator proteins by precluding their binding. For instance, overlapping binding sites have been observed for YY1 and NF-eB in the serum amyloid A1 gene promoter (47), YY1 and serum response factor in the skeletal α-actin gene promoter (41), YY1 and MGF in the β-casein promoter (48) and YY1 and GATA-1 in the e-globin gene promoter (49). In COX7c, the significance of the YY1/NRF-2 overlap is not clear, especially since NRF-2 is also a general activator of COX gene expression (8). NRF-2, a member of the ets family of proteins that regulate genes involved in development, growth control, and cell transformation, has been found in the promoters of all ubiquitously expressed COX nuclear genes so far characterized: COX4 (7, 11, 12), COX5B (10), COX7AL (9), and COX7c. It is conceivable that the binding of these factors to the overlapping YY1/NRF-2 motif in COX7c is dictated by the steady-state levels of YY1 and NRF-2 in different tissues. YY1 levels are known to be decreased during the onset of the myogenic program (41). Alternatively, YY1 binding at the D site may serve to alter the topology of the promoter by DNA bending (44), which would facilitate protein–protein interactions or enhance protein binding to DNA. In the e-fos serum response element, where the serum response factor and YY1 binding sites overlap, it has been shown that both proteins can co-occupy this element, apparently facilitated by a YY1-induced bending of the serum response element DNA to enhance binding of serum response factor (50). This possibility can be extended to the YY1/NRF-2 motif of the COX7c promoter, and may explain why a mutation at the YY1-D site decreases CAT activity. If a YY1-induced bend facilitates enhanced binding
of NRF-2 at the D site, it follows that a mutation would decrease the kinetics of NRF-2 binding, thus explaining the modest decrease in CAT activity.

**COX7C** (YY1/NRF-2), **COX5B** (YY1/NRF-1/NRF-2), **COX7AL** (NRF-1/NRF-2), and **COX4** (NRF-2) are four ubiquitously expressed genes whose promoters have been analyzed in some detail. At least three factors appear to contribute to their expression. It is not clear why some of these are NRF-1-dependent and others YY1-dependent. NRF-2 appears to be a common factor in these genes and its interaction, if any, with YY1 and NRF-1 is not known. It is conceivable that some of these factors may sense the redox state of the cell and coordinate their interactions with other factors. A clearer picture will emerge only when the promoters of additional COX genes are characterized.

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