Regulation of Murine Cytochrome *c* Oxidase Vb Gene Expression during Myogenesis

YY-1 AND HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN D-LIKE PROTEIN (JKTBP1) RECIPROCALLY REGULATE TRANSCRIPTION ACTIVITY BY PHYSICAL INTERACTION WITH THE BERF-1/ZBP-89 FACTOR*

Ettickan Boopathi‡, Nibedita Lenka‡§, Subbuswamy K. Prabu‡, Ji-Kang Fang‡,
Frank Wilkinson‡, Michael Atchison‡, Agata Giallongo¶, and Narayan G. Avadhani‡‖

*From the ‡Department of Animal Biology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia,
Pennsylvania 19104 and the ¶Istituto di Biomedicina e Immunologia Molecolare, Consiglio Nazionale delle Ricerche,
Via Ugo La Malfa 153, Palermo 90146, Italy*

A transcription suppressor element (sequence −481 to −320) containing a G-rich motif (designated GTG) and a newly identified CAT-rich motif (designated CATR) was previously shown to modulate expression of the mouse cytochrome *c* oxidase Vb gene during myogenesis. Here, we show that the GTG element is critical for transcription activation in both undifferentiated and differentiated myocytes. Mutations of the CATR motif abolished transcription repression in myoblasts while limiting transcription activation in differentiated myotubes, suggesting contrasting functional attributes of this DNA motif at different stages of myogenesis. Results show that the activity of the transcription suppressor motif is modulated by an orchestrated interplay between ubiquitous transcription factors: ZBP-89, YY-1, and a member of the heterogeneous nuclear ribonucleoprotein D-like protein (also known as JKTBP1) family. In undifferentiated muscle cells, GTG motif-bound ZBP-89 physically and functionally interacted with CATR motif-bound YY-1 to mediate transcription repression. In differentiated myotubes, heterogeneous nuclear ribonucleoprotein D-like protein/JKTBP1 bound to the CATR motif exclusive of YY-1 and interacted with ZBP-89 in attenuating repressor activity, leading to transcription activation. Our results show a novel mechanism of protein factor switching in transcription regulation of the cytochrome *c* oxidase Vb gene during myogenesis.

Cytochrome *c* oxidase (COX)[1] is an essential component of the mitochondrial electron transport chain in eukaryotes and is considered to be a major site of regulation of mitochondrial oxidative phosphorylation. Located on the mitochondrial inner membrane, this rate-limiting enzyme is also implicated in the

production of reactive oxygen species under chemical/oxidative stress conditions and during ischemia/reperfusion injury (1–4). The oligomeric complex consists of three large primarily catalytic subunits encoded by the mitochondrial genome and 10 smaller subunits encoded by nuclear genes (5). The nuclear encoded subunits are thought to modulate enzyme function, although the precise structural or biochemical details remain unclear. The nuclear encoded COX Vb is a ubiquitously expressed subunit of the complex, although its level of expression in different tissues varies markedly, ranging from very low levels in liver to very high levels in skeletal and cardiac muscle. A previous study has shown that expression of the COX Vb mRNA is increased by 5–7-fold during myogenesis of C2C12 skeletal and H9C2 cardiac myocytes (6). Recent studies also suggest a role for the COX Vb subunit in modulating the kinetic properties of the enzyme complex (4, 7).

The structural organization and transcription regulation of a number of nuclear encoded COX genes from different mammalian sources have been characterized (8–12). A common emerging theme among these and other mitochondrially destined oxidative phosphorylation genes is that a majority of them are positively regulated by transcription factors NRF1 and NRF2, the latter also known as GA-binding protein (13), although some are modulated by more general factors such as YY-1 and Sp1 (14–16). The regulation of murine COX Vb gene expression is particularly interesting since it involves an array of transcription factors, including tissue-specific or tissue predominant factors (10, 16–20). First, the COX Vb mRNA shows extensive 5′ heterogeneity due to transcription initiation at multiple sites. Region −8 to +40 of the promoter contains at least five sites for protein binding, each functioning as a putative transcription start site. The initiation of RNA at position +1 has been shown to depend on YY-1 factor binding, and the site at position +17 is dependent on Ets family factor NRF2 binding (17, 18). Additionally, expression of the COX Vb gene is subject to regulation by NRF1 and NRF2, which bind to the immediate upstream regions of the promoter (20).

Although the COX Vb gene is constitutively expressed, its transcription is induced 5–7-fold during induced differentiation of C2C12 myoblasts into myotubes (6). Another characteristic of the COX Vb gene is the transcription suppressor activity of a DNA motif spanning sequence −481 to −320 of the promoter (6). This sequence consists of four putative protein-binding motifs, including a G-rich motif (designated GTG), a motif showing partial consensus to YY-1 factor-binding sites Nuclear Factor E1′, a newly identified CAT-rich motif (designated CATR) showing no significant homology to previously de-

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scribed transcription factor-binding DNA motifs, and a cryptic serum response factor-binding CArG motif (ψCArG). Although the precise nature of individual protein-binding motifs is not known, a previous study from our laboratory showed that the suppressor region DNA binds to nuclear factors and yields multiple bands in gel mobility shift assays (6). Furthermore, discrete differences in the antibody supershifts of DNA-bound complexes with nuclear extracts from undifferentiated and differentiated C2C12 myocytes and a markedly reduced repressor activity of the transcription suppressor motif in C2C12 myotubes suggest a role for this DNA motif in modulating transcription activity during myogenesis (6).

In this study, using a combination of mutational analysis and protein purification by DNA affinity chromatography, we found that the GTG motif and the downstream CATR motif are critical for the function of the transcription suppressor and for modulating its activity during myogenesis. The GTG motif bound a ubiquitously expressed 95-kDa factor (BERF-1/ZBP-89), whereas the downstream CATR motif bound either YY-1 or hnRNP-D (a homolog of the human JKTBP1 protein) exclusive of each other. Interestingly, a high negative modulation of COX Vb promoter activity in undifferentiated myocytes involved physical interaction of CATR motif-bound YY-1 with adjacently bound BERF-1/ZBP-89. In differentiated myotubes, the suppressor activity was reversed by hnRNP-D/hJKTBP1 protein binding to this site in place of YY-1 and its physical interaction with BERF-1/ZBP-89. These results show a new mechanism of transcription factor switching during induced skeletal myogenesis.

MATERIALS AND METHODS

COX Vb Promoter Constructs and cDNA Constructs—The mouse COX Vb promoter constructs used in this study were derived from the −481/+40 chloramphenicol acetyltransferase promoter DNA (6), and PCR-amplified DNA was subcloned in the XhoI and SacI sites of the pGL3 basic vector (Promega Corp). Mutations targeted to each of the four possible protein-binding motifs within this region (see Fig. 1*A* and Table I) were generated by overlapping PCR using primers carrying mutations and subcloned in the pGL3 basic vector. The details of ZBP-89 cDNA (21) and the various wild-type and mutant YY-1 cDNA constructs (22) were described previously.

Cell Growth, Transfection, and Assay of Luciferase Activity—Murine skeletal C2C12 myocytes and 3T3 fibroblast cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum as described (6). Cells were seeded in 6-well culture plates 24 h prior to transient transfection. A ratio of 1 μg of DNA to 3 μl of FuGENE 6 (Roche Applied Science) was used in all transfections. Cells were transfected in triplicates with wild-type or mutant COX Vb-luciferase reporter constructs (2.5 μg/well) and the pRL-SV40 plasmid (0.2 μg/well; Promega Corp.), which expresses *Renilla* luciferase, as an internal control. The luciferase activity was normalized by comparison with *Renilla* luciferase activity, which was used as an internal control. In cotransfection experiments YY-1, ZBP-89, and hJKTBP1 cDNA expression plasmids (0.5 μg each) were used along with different mutant or wild-type COX Vb promoter constructs. The cells were harvested 48 h after transfection and washed three times in phosphate-buffered saline. The cells were lysed in 500 μl of lysis buffer (0.1 M potassium phosphate (pH 7.8), 1% Triton X-100, 1 mM dithiothreitol, and 2 mM EDTA) by continuous shaking at 4 °C for 15 min. The cell lysate was centrifuged in a microcentrifuge for 20 s to pellet large membranes and cell debris. Luciferase activity was measured using the Dual Luciferase assay system (Promega Corp.). Luciferase assays were performed with 10 μl of cell lysate in 100 μl of assay buffer. The luminometer was set for a 30-s integration period at high gain. Luciferase activities were normalized to *Renilla* luciferase activity, which was used as an internal control. The transcription rates are presented as relative luciferase activities.

Preparation of Nuclear Extracts—Miniprep nuclear extracts were prepared from transfected C2C12 cells by the method of Schreiber *et al.* (23) with minor modifications. Transfection was done using 1–2 μg of effector plasmid DNA/plate. Cells from five different plates (~7–10 × 10⁶ cells) were used to prepare nuclear extracts. Pooled cells were washed with phosphate-buffered saline and resuspended in ice-cold buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM pepstatin, and 1 mM leupeptin). The cells were allowed to swell on ice for 15 min, lysed with 1% (v/v) Nonidet P-40, and centrifuged. The nuclear pellet was resuspended in 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride and rocked on a mixer at 4 °C for 15 min. The nuclear extract was centrifuged, and the supernatant was frozen in aliquots and stored at −70 °C. Large-scale nuclear extracts from differentiated and undifferentiated C2C12 myoblasts were prepared by the method of Dignam *et al.* (24).

Purification and Characterization of Negative Enhancer-binding Proteins—Nuclear extract (~600 μg of protein) from fully differentiated C2C12 myocytes was subjected to $(NH_4)_2SO_4$ fractionation. Protein fraction precipitating between 25 and 50% $(NH_4)_2SO_4$ was enriched for DNA-binding proteins by heparin agarose chromatography (25). Briefly, 1 ml of heparin-agarose (Sigma) pre-equilibrated with buffer B (5 mM HEPES (pH 7.5), 30 mM KCl, 0.1 mM dithiothreitol, 15% glycerol, 1 mM phenylmethanesulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml antipain) was mixed with extract in the same buffer by gentle rocking at 4 °C for 1 h. The mixture was poured into a 1-ml column, and unbound proteins were eluted by washing with 15 ml of buffer B. Protein fraction eluted with buffer B containing 0.5–1 M KCl (~135 μg of protein) was pooled, dialyzed against buffer B, and used for DNA affinity chromatography. Region −481 to −391 of the negative enhancer DNA (~40 μg) was conjugated to CNBr-activated Sepharose using a standard procedure (26). The protein in 3 ml of buffer B containing 5% glycerol was incubated with 1 ml of DNA resin at 4 °C for 2 h by gentle rocking on a mixer, and the mixture was poured into a 0.8-cm diameter column. The column was washed with 20 ml of buffer B and the last 5 ml of wash was collected, concentrated, and used for analysis. The bound proteins were eluted sequentially with 3 ml each of loading buffer containing 200 mM, 400 mM, 600 mM, and 1 M KCl. The eluates were concentrated by centrifugation through Centricon filters (Amicon, Inc.) and used for electrophoresis.

DNA-Protein Binding by Electrophoretic Mobility Shift Assay—Binding assays were carried out as described (27) using 5 μg of nuclear protein extract and 0.4 ng (10,000 cpm) of ³²P-labeled gap-filled DNA probe. Competition for DNA binding was carried out as described (19, 28) using the indicated amounts of unlabeled double-stranded DNA. Competitor DNA was preincubated with the reaction mixture for 15 min at 25 °C prior to the addition of the labeled DNA probe. Binding reactions were carried out for 25 min at 25 °C. For antibody supershift assays, 1–2 μg of preimmune IgG or antibody specific for YY-1, the GTG motif, or hJKTBP1 (hnRNP-D) was added to the reaction mixture and incubated for 20 min at 25 °C, followed by incubation with the added DNA probe as described above. Samples were electrophoresed on 4% polyacrylamide gels in 0.25× Tris/glycine/EDTA (1× Tris/glycine/EDTA = 25 mM Tris base, 100 mM glycine, and 1 mM EDTA) or 0.25× Tris/boric acid/EDTA (1× Tris/boric acid/EDTA = 89 mM Tris base, 89 mM boric acid, and 2 mM EDTA) at 160 V for 1.5 h. Gels were dried under vacuum and exposed to x-ray film overnight at −80 °C with intensifying screens.

Preparation of Antibody to JKTBP1, the Human Homolog of Mouse hnRNP-D—The partially characterized expressed sequence tag cDNA clone encoding the human JKTBP1 protein was obtained from the Genome Systems Inc., fully sequenced, and submitted to the NCBI Protein Database (accession number AY453824). The 301-amino acid-long reading frame with an N-terminally added His₈ tail was cloned into the pET-28a(+) vector (Novagen), expressed in *Escherichia coli* cells, and induced with isopropyl-β-D-thiogalactopyranoside. The protein was purified by affinity binding to Ni²⁺ chelate resin (29) and used for raising polyclonal antibody in rabbits. Details of immunization, blood sampling, and titer determination were as described previously (30). The IgG-rich fraction was isolated by $(NH_4)_2SO_4$ fractionation of serum proteins, and monospecific antibody was purified by affinity adsorption to Sepharose-conjugated hJKTBP1.

Antibody Pull-down Assays—For testing the *in vitro* interaction, YY-1, ZBP-89, and hJKTBP1 cDNA constructs were used as templates for generating ³⁵S-labeled *in vitro* translation products in a TnT coupled rabbit reticulocyte lysate system (Promega Corp.). The extent of ³⁵S incorporation and the integrity of the translation products were verified by electrophoresis on SDS-polyacrylamide gels, followed by autoradiography. Co-immunoprecipitation was carried out as described by Anderson and Blobel (31). Briefly, both ZBP-89 and YY-1 translation products were incubated in 20 mM phosphate buffer (pH 7.2) at 30 °C for 20 min. The reaction mixture was then incubated with 2 μl (1 mg/ml) of anti-ZBP-89 antibody overnight at 4 °C. The immune complex was precipi-

tated using protein A-agarose beads. The beads were washed five times (1 ml/wash) with 0.1% Triton X-100, 0.02% SDS, 150 mM NaCl, 5 mM EDTA, and 10 units/ml Nonidet P-40 and suspended in 2× Laemmli buffer. The proteins were identified by Western blotting using anti-YY-1, anti-hJKTBP1, and anti-ZBP-89 antibodies.

For testing the *in vivo* interaction, co-immunoprecipitation was carried out as described (31) with nuclear extracts from transfected cells. The nuclear extract was also analyzed for the ectopic expression of ZBP-89, hJKTBP1, YY-1, and its mutants by Western blot analysis.

Immunoblot Analysis—Proteins were separated by 10% SDS-PAGE and transferred onto nitrocellulose membrane. After blocking with 5% nonfat dry milk in phosphate-buffered saline (20 mM sodium phosphate buffer) containing 0.3% Tween 20, the blots were incubated with the desired polyclonal antibody and developed with horseradish peroxidase-conjugated secondary antibody. The bands were visualized by enhanced chemiluminescence using a Super-Glo chemiluminescence kit (Pierce) and visualized with a Bio-Rad VersaDoc chemiluminescence imager.

Chromatin Immunoprecipitation Assays—Chromatin immunoprecipitation was carried out as described (32). Briefly, C2C12 myoblasts as well as myotubes (2×10^7 cells each) were cross-linked in growth medium (1.1% (v/v) formaldehyde, 100 mM NaCl, 0.5 mM EGTA, and 50 mM Tris-HCl (pH 8.0)), and cross-linking was terminated with 0.125 M glycine. Nuclei were prepared and sonicated as described by Orlando *et al.* (32) with 15 pulses of 30 s each at 4 °C to obtain an average length of ~0.5 kilobase pairs of DNA. Chromatin samples (0.6 units each at $A_{260\ nm}$) were subjected to immunoprecipitation with primary antibodies (2.5 μg of IgG); the antigen-antibody complexes were separated using protein A-coated beads; and both the immunoprecipitates and supernatant fractions were collected. Chromatin fractions were incubated at 65 °C for 16 h to reverse the cross-links, and DNA was extracted from both immunoprecipitated and non-precipitated supernatant fractions. DNA samples (20 ng in each case) were subjected to PCR amplification using the suppressor region-specific primers 5-GTTC-GAGACTGCAGACAGCT-3′ (sense) and 5′-AACAGAGTGGAGGAGG-GAAT-3′ (antisense). The PCR products were analyzed on 2% agarose gels.

RESULTS

Mutational Analysis of the Upstream Negative Enhancer Region of the COX Vb Promoter—In our previous study, we showed that region −481 to −320 of the mouse COX Vb gene suppresses the transcription activity of a heterologous thymidine kinase-chloramphenicol acetyltransferase promoter (6). This region is also responsible for the nearly 7-fold lower activity of the COX Vb promoter in undifferentiated myoblasts. Sequence comparison and protein binding properties of the ~160-bp DNA from this region indicated the occurrence of four possible protein-binding motifs (designated upstream NFE1′, GTG, CATR, and ψCArG) (Fig. 1*A*). In this study, the role of these individual motifs in modulating the transcription activity of the promoter during myogenesis was tested by mutational analysis. As shown before (6), the transcription activity of the promoter construct increased by ~7-fold in C2C12 cells induced to differentiate into myotubes compared with the activity in uninduced myocytes. Mutations targeted to the upstream NFE1′ and downstream ψCArG sites affected neither the activity of the promoter in uninduced myoblasts nor the inducibility of promoter activity in differentiated myotubes. However, mutations targeted to the GTG element reduced the promoter activity in both undifferentiated and differentiated myocytes, suggesting a functional role for this motif in modulating promoter activity. Mutations targeted to the CATR site caused a 2-fold increased activity in undifferentiated myoblasts, but a reduction in the overall transcription activity in myotubes. These results suggest that the CATR site may augment the suppressor activity in undifferentiated myocytes, whereas it may have an activator function in differentiated myotubes. These data suggest that the GTG and CATR sites are important for the differential activities of the suppressor element during myogenesis. The suppressor region DNA will hereafter be referred to as GTG/CATR DNA.
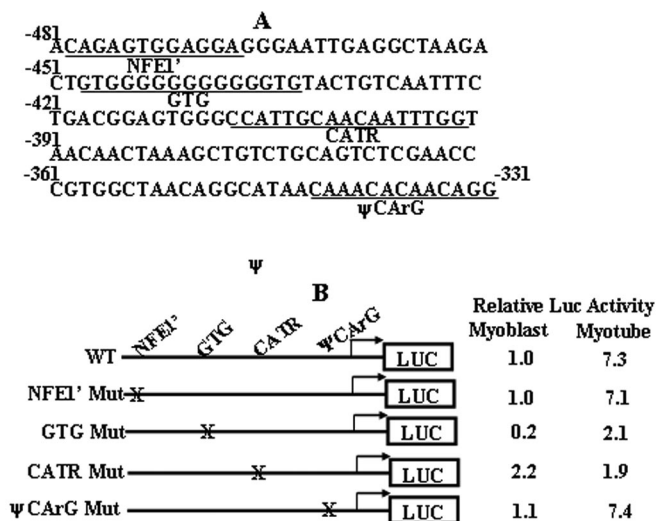

FIG. 1. **Functional analysis of DNA motifs showing partial homology to known protein-binding sequences.** *A*, the negative enhancer region of the COX Vb promoter (sequence −331 to −481) showing the predicted protein-binding motifs (*underlined*). *B*, transcription analysis of promoter constructs in the pGL3 basic vector. Mutations at individual motifs were introduced as shown in Table I. Transcription activities in undifferentiated C2C12 myoblasts and induced myotubes (average of three different transfections) are shown. *WT*, wild-type; *Mut*, mutant; *LUC*, luciferase.

Purification and Characterization of the Nuclear Proteins That Bind to GTG/CATR DNA—A previous study from our laboratory using a combination of gel mobility shift analysis and methylation interference analysis showed that the CATR motif binds to purified YY-1 protein under low off-rate conditions (6). In this study, we investigated the nature of the proteins that bind to GTG/CATR DNA by DNA affinity chromatography. Since mutations in the ψCArG region had no effect on the transcription activity of the promoter (Fig. 1*B*), we used a 105-bp DNA segment spanning sequence −376 to −481 (Fig. 1*A*) for conjugating to CNBr-activated Sepharose. The $(NH_4)_2SO_4$-fractionated nuclear extract from differentiated myotubes was first enriched for DNA-binding proteins by heparin-agarose binding and used for protein purification by DNA affinity chromatography. As shown in Fig. 2*A*, the protein fraction that eluted from the column with buffer containing 0.2 M KCl contained four distinct protein bands with apparent molecular masses of 95, 50, 41, and 39 kDa. The fraction that eluted with 0.4 M KCl contained three proteins, two of which comigrated with the 41- and 39-kDa species present in the 0.2 M KCl fraction. This fraction also contained an additional protein band at ~36 kDa that was not seen in proteins that eluted with the 0.2 M KCl-containing buffer. On the other hand, the fraction that eluted with 0.6 M KCl contained the 41- and 39-kDa proteins and lacked significant 36-kDa species. Finally, the faction that eluted with 1 M KCl did not show any stainable proteins. Although not shown, use of DNA carrying mutated GTG sequence for affinity purification abolished the binding of all the proteins except the 36-kDa protein. On the other hand, mutation of the CATR site selectively abolished purification of the 36-kDa protein.

N-terminal microsequencing of all four bands from the 0.2 M KCl-eluted fraction, three bands from the 0.4 M KCl-eluted fraction, and two bands from 0.6 M KCl-eluted fraction was carried out to gain insight into the nature of the proteins that bind to the suppressor DNA. Sequencing results yielded an identical N terminus of MNIDDKLEGLFKL- for the 95-kDa protein as well as the 50- and 39-kDa proteins from the 0.2 M KCl, 0.4 M KCl, and 0.6 M KCl elutes. In repeated attempts, the

A

B

Human JKTBP1

MEDMNEYSNIEEFAEGSKINASKMQQDDGKMFIGGLSWDTSKKDLT 46

YSRFGEVVDCTIKDDPVTGRS**RGFGFV**LFKDAASVDKVLVLKEHKL 92

DGKLIDPKRAKALAGKEPPKKVFVGGLSPTTSEEQIKEYFGAFGEIEN 138

IELPMDTKTNER**RGFCFI**TYTDEEPVKKLLESRYHQIGSGKCEIKVAQ 184

PKEPYR**QQQQQQ**K**GGRGAAAGGRGGTRGRG**RGQGQNWNQGFN 230

NYYDQGYGNYNSAYGGDQNYSGYGGYDYTGYNYGNYGYGQGQG 276

YADYSGQQSTYGKASRGGGNHQNNYQPY 301

C

FIG. 2. **Purification and characterization of GTG/CATR DNA-binding proteins.** *A*, the transcription suppressor region-binding protein was purified by DNA affinity chromatography using nuclear extract from fully differentiated C2C12 myotubes as described under "Materials and Methods." Column fractions eluted with loading buffer (*Wash 1*) and with buffer containing 0.2, 0.4, 0.6, and 1.0 M NaCl (*Wash 2*, *Wash 3*, *Wash 4*, and *Wash 5*, respectively) were resolved by electrophoresis on 12% SDS-polyacrylamide gels and stained with Coomassie Blue. Individual bands from the *second* through *fourth lanes* were excised and subjected to N-terminal sequencing. Complexes I, II, and IV from *Wash 2* yielded the sequence NH$_2$-MNIDDKLEGLFLK-. Complex III from *Wash 2* and similarly migrating complexes from *Wash 3* and *Wash 4* did not yield detectable residues. Complex II from *Wash 3* and a similarly migrating complex from *Wash 4* yielded an N-terminal sequence similar to complex IV from *Wash 2*. The fastest migrating complex from *Wash 3* (complex V) yielded the sequence NH$_2$-MEDMNEYSNIEEF—. *B*, a BLAST search with the N-terminal sequence of the 36-kDa protein (MEDMNEYSNIEEF-) identified the protein as hJKTBP1, which also shows 100% homology to the mouse hnRNP-DL protein. Putative DNA-binding RGF motifs and the Gln/Asn-rich putative transcription activation domains are indicated by *boldface letters*. *C*, DNA binding specificity of the ZBP-89 and JKTBP1 proteins purified by DNA affinity column purification. The 95-kDa protein (*Prot.*; complex I from *Wash 2* in *A*) and the 36-kDa protein (complex V from *Wash 3* in *A*) were eluted, renatured by dialyzing against steadily decreasing concentrations of guanidine hydrochloride (6 to 0 M)-containing buffers, and used for gel shift analysis with the GTG DNA or CATR DNA probe (Table I). *Extr.*, extract.

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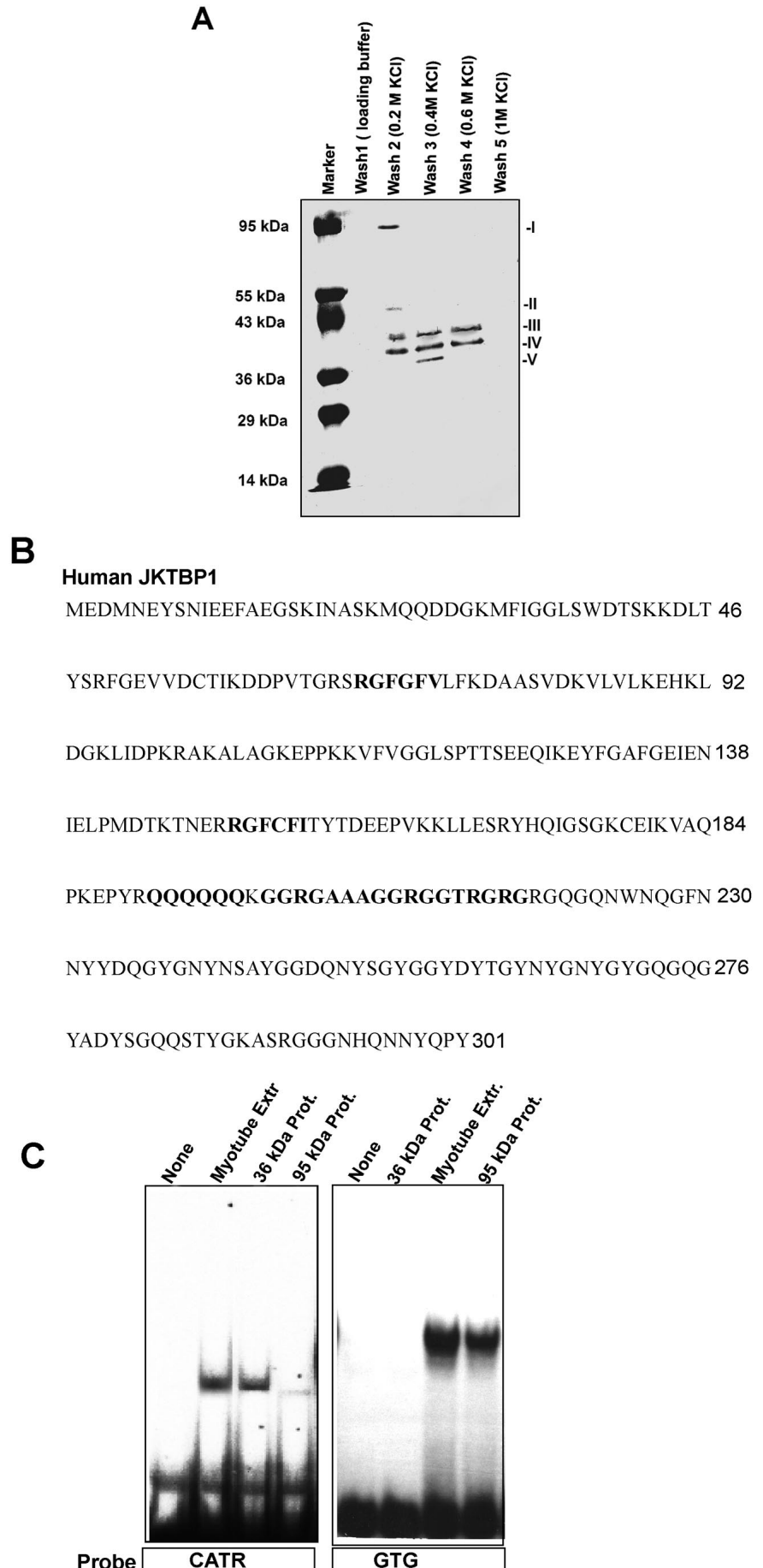
41-kDa protein in all three fractions did not yield detectable sequence, suggesting a blocked N terminus. The 36-kDa protein that eluted with 0.4 M KCl yielded an N-terminal sequence of MEDMNEYSNIEEF-. Protein data base searching revealed that the 95, 50, and 39-kDa proteins belong to a previously reported family of zinc finger proteins, termed BERF-1/ZBP-89 (33). This ubiquitous transcription factor is expressed as intact 95–115-kDa and C-terminally truncated forms of 64, 56, 49, and 32 kDa in different cell types and species (33–35). However, a recent study reported the expression of only the largest 115-kDa species in C2C12 myoblasts (21). Consistent with these latter data, immunoblot analysis of nuclear extracts from undifferentiated myoblasts and differentiated myotubes showed the presence of only the full-length 95-kDa BERF-1/ZBP-89 protein (data not shown). Additionally, use of antibodies against the N-terminal, C-terminal, and internal zinc finger domains (21) failed to identify any smaller protein components. Results also showed a nearly comparable level of ZBP-89 in both undifferentiated and differentiated myotubes (data not shown). Based on this, we believe that the faster migrating 50- and 39-kDa proteins in the affinity-purified fractions that show N-terminal sequence identical to the 95-kDa protein probably represent proteolytic degradation products generated during purification.

A BLAST search with the N-terminal sequence of the 36-kDa protein showed homology to a reading frame of an unidentified and partially sequenced expressed sequence tag from a human cDNA library. We obtained the corresponding human expressed sequence tag cDNA clone from Genome Systems Inc. and, as shown in Fig. 2*B*, fully sequenced the cDNA (NCBI accession number AY453824). The predicted reading frame shows 100% amino acid sequence homology to a recently reported human cDNA clone designated hJKTBP1 (36) and a mouse cDNA clone designated hnRNP-DL. hnRNP family proteins bind to DNA in a sequence-specific manner and activate promoter-specific transcription (37). hJKTBP1 and mouse hnRNP-DL show 65% homology to a CArG box-binding protein known as CArG-binding factor A (38). The 30-amino acid-long hnRNP-DL protein, which is similar to hJKTBP1, binds to double-stranded DNA and induces transcription activation (39). hJKTBP1 (Fig. 2*B*) contains two RGF motifs, which probably compose part of the DNA-binding domain. The proteins also contain a Gln/Gly/Arg-rich sequence, characteristic of transcription activation domains, and a Tyr/Gly-rich domain near the C terminus, probably representing phosphorylation sites. The N-terminal His-tagged protein was expressed in *E. coli* cells, purified, and used for generating antibody. Because of nearly 100% sequence homology, both human and mouse homologs will hereafter be referred to as JKTBP1.

The 95- and 36-kDa proteins that eluted from a companion gel were tested for DNA binding ability. As shown in Fig. 2*C*, the 36-kDa protein specifically bound to the CATR DNA probe, whereas the 95-kDa protein bound to the GTG DNA probe. Although not shown, the NFE1′ and ψCArG DNA motifs did not efficiently compete for protein binding to the CATR and GTG DNA probes.

Steady-state Levels of hnRNP-DL during Myogenesis—To gain further insight into the role of the JKTBP1 protein in the regulation of COX Vb gene expression, we determined its distribution in different tissues and also its steady-state levels during induced myogenesis. The immunoblot in Fig. 3*A* shows that nuclear extracts from mouse liver and kidney contained relatively low levels of JKTBP1, whereas extracts from skeletal muscle and heart contained very high levels. Nuclear extract from mouse brain contained a moderately high level, but a lower level compared with muscle tissue. The level of the nu-
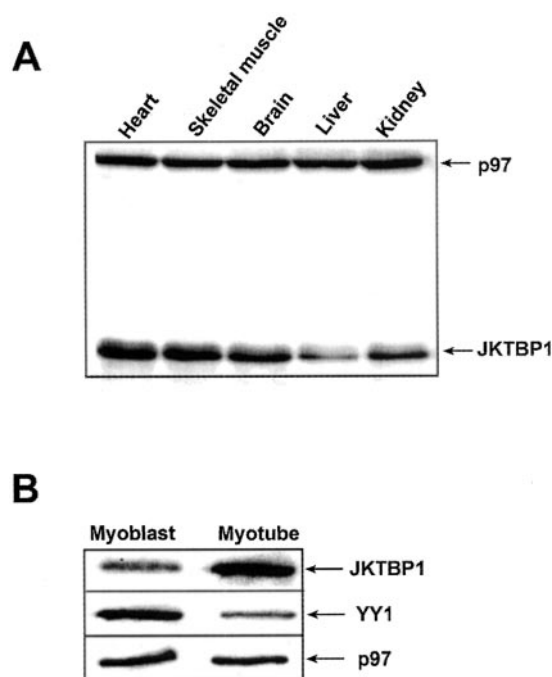

FIG. 3. **Contrasting levels of the JKTBP1 and YY-1 proteins in undifferentiated and differentiated C2C12 myocytes.** *A*, immunoblot analysis of nuclear protein fractions (50 μg each) from the indicated mouse tissues with antibody to JKTBP1 (1:2000 dilution). The results show that heart and skeletal muscle contained the highest levels of 36-kDa JKTBP1. The blot was also co-developed with antibody to the nuclear marker protein p97. *B*, immunoblot analysis of nuclear proteins (50 μg each) from undifferentiated C2C12 myoblasts and differentiated myotubes. The blot was probed with a mixture of antibodies against hJKTBP1, YY-1, and the nuclear protein p97. The latter was used as a loading control.

clear transport factor p97, used as a loading control, showed no variations in these tissues. Consistent with published results (40), the differentially spliced 42-kDa human isoform (hJKTBP2) was not expressed in mouse cells or tissues at detectable levels. Fig. 3*B* shows that nuclear extract from undifferentiated C2C12 myoblasts contained a relatively low level of JKTBP1, but a high level of the YY-1 factor. However, the differentiated myotubes contained a nearly 10-fold higher JKTBP1 level and less than one-tenth of the YY-1 level compared with undifferentiated myocytes. The results on varying YY-1 levels are consistent with previous studies showing markedly reduced expression of YY-1 during myogenesis (41, 42). The level of the nuclear p97 protein used as a loading control in Fig. 3*B* was nearly comparable in both nuclear extracts. These results show the predominance of the JKTBP1 protein in the nuclear compartments of skeletal and cardiac muscle and also differentiated C2C12 myocytes.

The endogenous levels of YY-1 and mouse JKTBP1 and their binding to the 105-bp negative enhancer DNA were further tested by gel mobility shift assays using nuclear extracts from C2C12 myoblasts and differentiated myotubes. Nuclear extract from C2C12 myoblasts formed two major complexes with the 105-bp DNA probe (Fig. 4*A*). Both anti-YY-1 and anti-ZBP-89 antibodies yielded similarly migrating supershifted complexes. Preimmune IgG did not yield a detectable supershift. Also, antibody to hJKTBP1 (hnRNP-DL) yielded no visible supershifted complex with extract from myoblasts. Nuclear extract from differentiated myotubes yielded three different complexes (marked I, II, and III), of which complexes I and II migrated slower than the similarly marked complexes obtained from the undifferentiated myoblast extract. Complex III from the myotube extract migrated slower than any of the complexes from
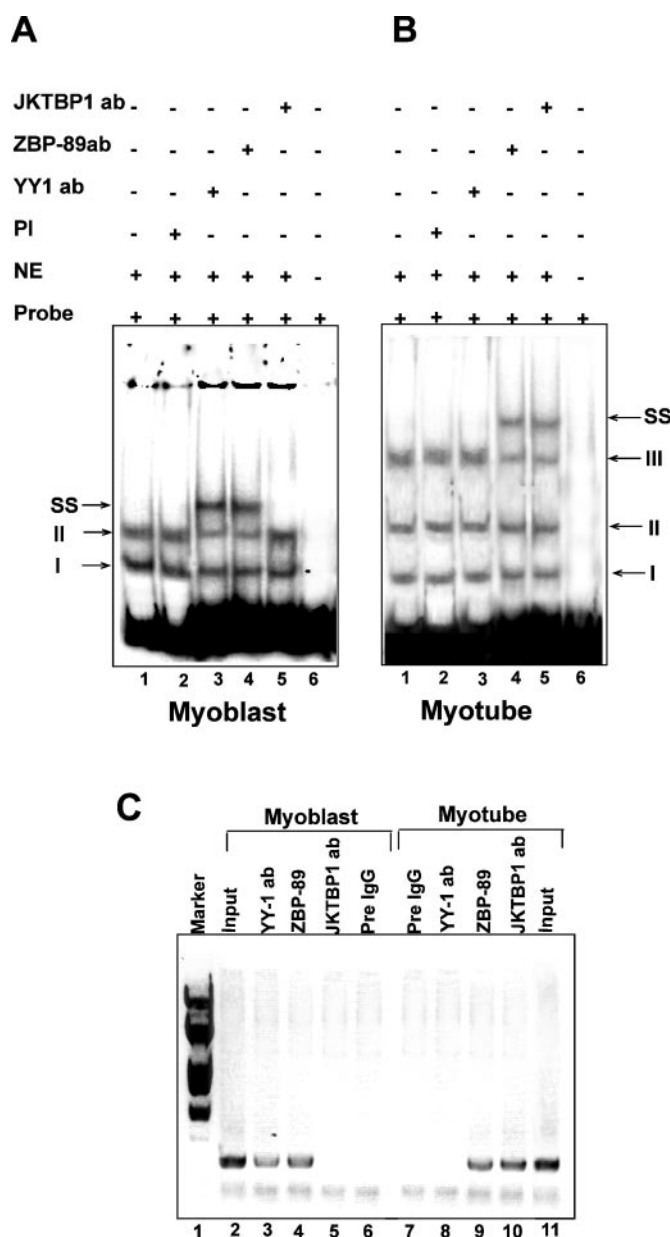
A

B

Myoblast   **Myotube**

C

FIG. 4. **Variable DNA binding patterns of nuclear proteins at different stages of myogenesis.** Gel mobility shift assays were carried out with ^{32}P-labeled (10,000 cpm) 105-bp negative enhancer DNA probe (sequence -376 to -481) using nuclear extracts (15 μg of protein each) as described under "Materials and Methods." *A* and *B*, binding patterns with nuclear extract (*NE*) from undifferentiated myoblasts and differentiated myotubes, respectively. In both *A* and *B*, reaction mixtures were preincubated with the indicated antibodies (*ab*) or the control rabbit preimmune IgG fraction (*PI*; *Pre*) (200 ng each) for 10 min on ice before adding the labeled DNA probe. The same amount of bovine serum albumin was added in control reactions. *SS*, supershifted complex. *C*, chromatin immunoprecipitation assays with chromatin fractions from C2C12 myoblasts and myotubes. Input DNA (300 ng) and 20 ng of DNA from each of the immunoprecipitated fractions were amplified twice by PCR (35 cycles each) as described under "Materials and Methods." PCR products (20-μl aliquots from each reaction) were resolved on a 2% agarose gel and viewed by staining with ethidium bromide.

the undifferentiated myoblast extract. As with the extract from undifferentiated myoblasts, antibody to ZBP-89 yielded a supershifted band in the myotube extract. In contrast, however, the latter extract yielded complexes that were supershifted by antibody to hJKTBP1. Consistent with the low endogenous level of the protein (Fig. 3*B*), antibody to YY-1 yielded a very minor supershifted complex with extract from differentiated

myotubes. Furthermore, the anti-ZBP-89 and anti-hJKTBP1 antibody-supershifted complexes from the myotube extract migrated considerably slower than the supershifted complexes from the myoblast extract. Based on reduced band intensity, complex II from the myoblast nuclear extract (Fig. 4*A*) and complex III from the myotube nuclear extract (Fig. 4*B*) are the more likely targets of antibody supershift. The nature of complex I in Fig. 4*A* and of complexes I and II in Fig. 4*B* remains unclear, although they may represent the binding of additional but unknown proteins. In support of the immunoblot results in Fig. 3*B*, these results show increased levels of JKTBP1, but reduced levels of YY-1 protein in differentiated myotubes. The antibody supershift data suggest that ZBP-89 may exist as a higher order complex with YY-1 (Fig. 4*A*) or JKTBP1 (Fig. 4*B*). The results also confirm that both endogenously expressed YY-1 and JKTBP1 bind to the transcription suppressor region DNA.

The levels of ZBP-89, YY-1, and JKTBP1 binding to the 105-bp DNA under *in vivo* conditions at different stages of myogenesis were investigated by chromatin immunoprecipitation assay. As shown in Fig. 4*C*, immunoprecipitates with anti-YY-1 and anti-ZBP-89 antibodies yielded prominent PCR products in undifferentiated myoblasts (*lanes 2–6*), whereas immunoprecipitate with anti-hJKTBP1 antibody yielded no significant PCR product. In the case of myotubes (*lanes 7–11*), however, immunoprecipitates with anti-ZBP-89 and anti-hJK-TBP1 antibodies yielded prominent PCR products, whereas that with anti-YY-1 antibody yielded a minor PCR-amplified DNA band. Total input DNA (300 ng) yielded a PCR product of similar size and intensity. Although not shown, 20 ng of DNA not subjected to immunoprecipitation and similar amounts of DNA from the post-immunoprecipitation supernatant fraction in all cases yielded very low to marginal PCR amplification. In support of the immunoblot analysis data (Fig. 3*B*) and gel shift analysis (Fig. 4, *A* and *B*) with nuclear extracts, these results show that the negative enhancer DNA in myoblasts is bound to ZBP-89 and YY-1, whereas in differentiated myotubes, it is bound predominantly to ZBP-89 and JKTBP1.

Sequence Motifs of the Suppressor Region That Bind to the Three Proteins—Based on the gel mobility shift data (Fig. 2*C*) with affinity-purified proteins and the reported binding specificity of ZBP-89 (21), the ZBP-89 characterized here likely binds to the G-rich region of the negative enhancer (designated GTG). This was tested by gel shift analysis with the *in vitro* translated ZBP-89 factor (21). As shown in Fig. 5*A*, the protein yielded a single complex with the 105-bp DNA probe that was effectively competed by excess wild-type GTG DNA, but not by mutant GTG DNA with substituted G residues. It is known that the ZBP-89-responsive G-rich sequence motifs from the β-enolase promoter (21) also bind to the ubiquitous factor Sp1. However, purified Sp1 factor (Promega Corp.) failed to bind in a detectable level to the GTG motif from the COX Vb promoter, suggesting a selective specificity of this motif for ZBP-89 (data not shown).

A previous study from our laboratory showed that the YY-1 protein binds to the CATR motif under high off-rate binding conditions (6). Methylation interference analysis showed that three of the C residues and an A residue (Table I, asterisks) are the contact points for YY-1 binding to the CATR site of the suppressor region. In line with this study, the gel shift pattern in Fig. 5*B* shows that the *in vitro* translated YY-1 protein bound to the 105-bp DNA and yielded a single complex. Complex formation was inhibited by excess wild-type CATR DNA, but not by mutant CATR DNA. Interestingly, hJKTBP1 binding to the negative enhancer region was also competed by the wild-type CATR DNA, whereas the mutant CATR DNA did not
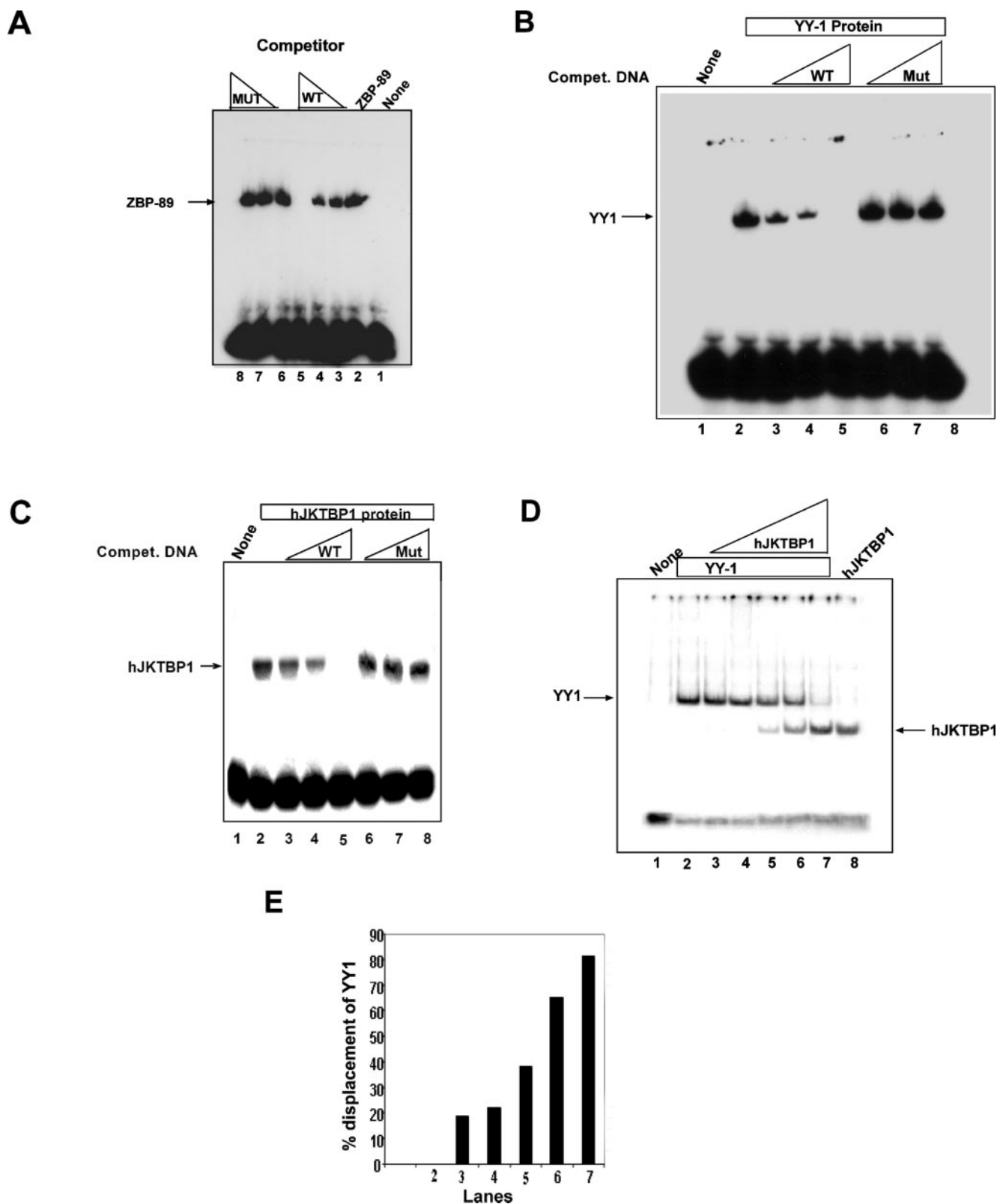
FIG. 5. **Overlapping DNA binding specificities of the YY-1 and hJKTBP1 proteins.** The ZBP-89, hJKTBP1, and YY-1 proteins translated *in vitro* in rabbit reticulocyte lysate (10 μl each) were used for binding to 0.4 ng of ^{32}P-labeled 105-bp DNA for each reaction in *A–C* or to 0.05 ng of CATR DNA in *D* as described in the legend to Fig. 4. *A*, binding specificity of the ZBP-89 protein. Increasing amounts (5, 10, and 20 M excess) of wild-type DNA (*WT*; *lanes 3–5*) or mutant DNA (*Mut*; *lanes 6–8*) were used for competition. *B*, binding specificity of the YY-1 protein. Increasing amounts (5, 10, and 20 M excess) of wild-type CATR DNA (*lanes 3–5*, respectively) or mutant CATR DNA (*lanes 6–8*, respectively) were used for competition (*Compet.*). *C*, binding specificity of the hJKTBP1 protein. Increasing amounts (5, 10, and 20 M excess) of wild-type CATR DNA (*lanes 3–5*, respectively) or mutant CATR DNA (*lanes 6–8*, respectively) were used for competition. *D*, binding of the YY-1 and hJKTBP1 proteins to CATR DNA exclusive of each other. In *lanes 2* and *8*, 10 μl of YY-1 or hJKTBP1 was used. In *lanes 3–7*, a constant amount of YY-1 (10 μl) and increasing amounts of hJKTBP1 (5–15 μl) were used for binding reactions. The YY-1- and hJKTBP1-specific complexes are marked appropriately. *E*, displacement of YY-1 bound to the 105-bp DNA by hJKTBP1. Gels in *D* were quantified by imaging with a Molecular Dynamics STORM system. The extent of YY-1-bound complex in *lane 2* was considered as 100% for calculating the percent displaced YY-1 by increasing amounts of hJKTBP1.

TABLE I
List of wild-type and mutant oligonucleotides

Only the sense strand sequences of synthetic double-stranded DNAs are shown. Underlined nucleotides in mutant sequences indicate substitutions. WT, wild-type; Mut, mutant.

WT NFE1′	CAGAGTGGAGGAGGGAA
Mut NFE1′	CGGAGAGGAGGGGGAG
WT GTG	GTGGGGGGGGGGGTGTACTG
Mut GTG	CTCTAAGGGTTTGGCTCTAC
WT CATR	GGCCATTGCAACAATTTGGTA
Mut CATR	GGCCGTTGCAACGGTCCGGTA
WT ψCArG	TAACAAACACAACAGG
Mut ψCArG	TAACGGACGCGACAGA

interfere with complex formation (Fig. 5*C*). These results suggest the possibility that the YY-1 and JKTBP1 proteins bind to the same region of the negative enhancer.

We next investigated whether YY-1 and JKTBP1 bind to the 105-bp DNA exclusive or inclusive of each other under *in vitro* conditions. As shown in Fig. 5*D*, YY-1 alone formed a relatively slowly migrating complex (*lane 2*), whereas hJKTBP1 alone formed a faster migrating complex (*lane 8*). Under conditions when >80% of the probe was occupied by YY-1 increasing levels of added hJKTBP1 caused a steady decline in the level of the YY-1-specific complex (*lanes 3–7* and Fig. 5*E*). At the same time, there was a corresponding increase in the level of the hJKTBP1-specific complex (Fig. 5*D*), suggesting that the two proteins compete for the same binding site. Furthermore, at all protein concentrations used, no binary complexes indicative of dual protein occupancy on the DNA template was seen. These results suggest that the two factors bind to the same region exclusive of each other.

Transcription Activation and Repression of the COX Vb Promoter by ZBP-89, JKTBP1, and YY-1—The role of the three protein factors in the transcription activity of the COX Vb promoter was studied by cotransfection of promoter constructs with ZBP-89, YY-1, and hJKTBP1 cDNAs. Fig. 6*A* shows that in undifferentiated C2C12 myoblasts, overexpression of ZBP-89 and hJKTBP1 yielded 2.7–3-fold higher activity, suggesting that both of these proteins function as transcription activators. On the other hand, transfection with YY-1 cDNA with or without ZBP-89 cDNA caused ~80–90% inhibition of activity compared with activity in control cells, suggesting transcription repression by this factor. Interestingly, the repressor activity of YY-1 was nearly completely reversed by coexpression with hJKTBP1 cDNA, further suggesting divergent roles of these two latter factors in the transcription activity of the COX Vb promoter. Expression of ZBP-89 and hJK-TBP1 together yielded ~5-fold higher activity, indicating additive effects of the two proteins on transcription activation. Essentially a similar pattern of activity was obtained in differentiated myotubes, although, as expected, the relative activity was generally 6–7-fold higher (Fig. 6*B*).

The role of the GTG sequence (GTGGGGGGGGGGGTG) in YY-1-mediated transcription repression and hJKTBP1-mediated activation was investigated by mutations directed to individual motifs of the promoter. As shown in Fig. 6*A*, mutations targeted to the GTG element vastly reduced the transcription activity, which was not reversed by transfection with ZBP-89, hJKTBP1, or YY-1 cDNA in undifferentiated myoblasts. On the other hand, mutations targeted to the CATR motif (GGCCAT-TGCAACAATTTGGTA) caused a near 2-fold increase in the activity of the promoter in undifferentiated C2C12 cells, which was not altered by transfection with any of the three cDNAs (Fig. 6*A*). Mutations of the GTG site had a similar inhibitory effect in differentiated myotubes, which was not reversible by transfection with any of the three cDNAs (Fig. 6*B*). Interestingly, unlike what was observed in myoblasts, mutations of the
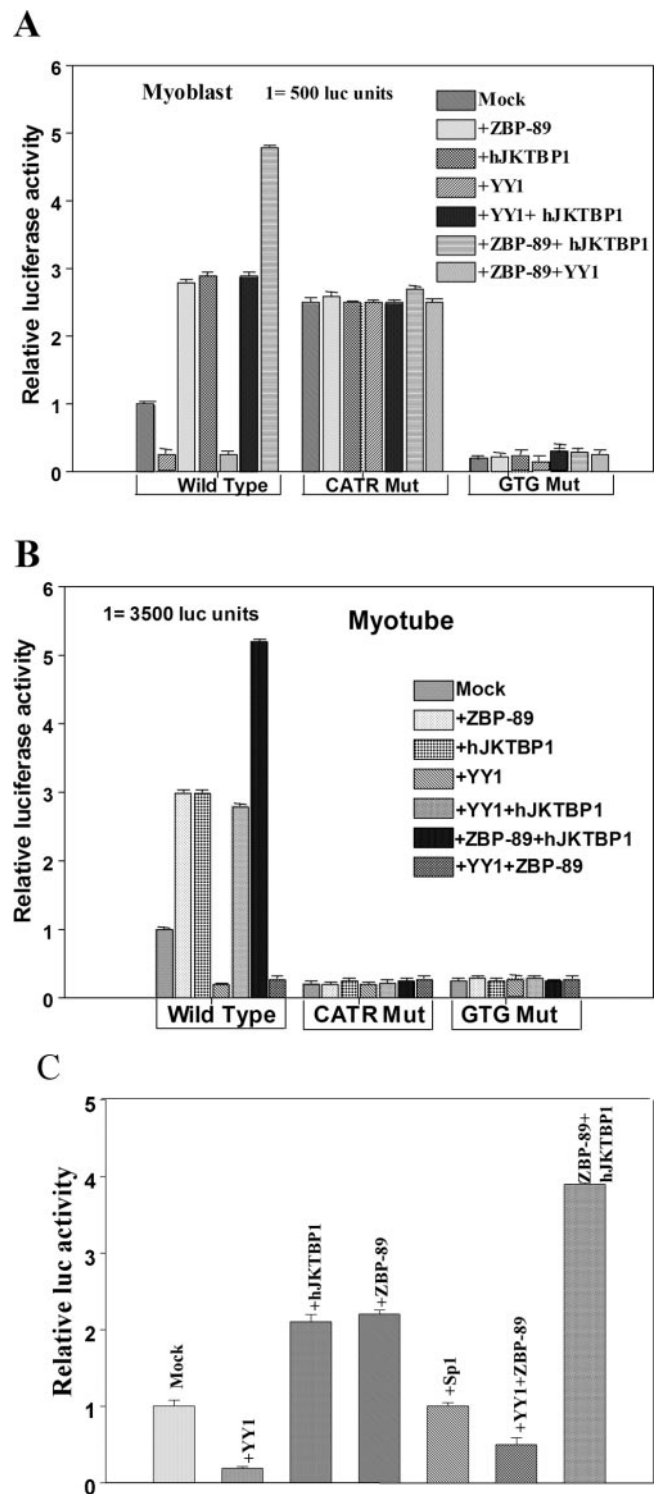

FIG. 6. **Variable functional role of the CATR motif in undifferentiated and differentiated C2C12 myocytes and 3T3 fibroblasts.** The effects of cotransfection with various cDNAs on the transcription activities of wild-type, mutant (*Mut*) CATR, and mutant GTG promoter constructs in undifferentiated (*A*) and differentiated (*B*) C2C12 myocytes and 3T3 fibroblasts (*C*) were assayed as described under "Materials and Methods." 0.5 μg of cDNA was used in each transfection. The only exception was with the YY-1 and hJKTBP1 combination, when 1 μg of the latter was used for reversing the repressive effects of YY-1. Values represent means ± S.D. of three to five individual assays. *luc*, luciferase.

CATR site had an inhibitory effect on the activity of the promoter in differentiated myotubes (Fig. 6*B*). These results show that the GTG motif with its bound ZBP-89 functions as an

activator in both myoblasts and myotubes. On the other hand, the CATR motif appears to mediate the repressor activity in undifferentiated myoblasts, but has an activator function in differentiated myotubes. The divergent functional role of this motif may be related to its ability to bind to YY-1 and hJKTBP1 exclusive of each other. The results also show that an intact CATR motif is required for mediating the repressor and activator functions of YY-1 and hJKTBP1, respectively.

In contrast to the putative transcription activator role of ZBP-89 for the COX Vb promoter, several reports show that the factor represses transcription of a number of other promoters (21, 43). To exclude the possibility that the observed transcription activation of the COX Vb promoter by ZBP-89 is cell-specific, we tested its effect in 3T3 fibroblasts. As shown in Fig. 6C, the promoter activity was inhibited by cotransfection with YY-1, but was induced by ~2-fold with hJKTBP1 and also ZBP-89. Cotransfection with YY-1 markedly reduced the transcription activation by ZBP-89. A combination of ZBP-89 and hJKTBP1 showed a nearly 4-fold higher activity, confirming the additive effects of these factors as seen in C2C12 myocytes. Consistent with the protein binding patterns (data not shown), transfection with Sp1 cDNA did not affect the transcription activity (Fig. 6C). These results essentially confirm that both ZBP-89 and hJKTBP1 function as transcription activators, whereas YY-1 functions as a repressor of COX Vb promoter activity.

Physical Interaction of ZBP-89 with CATR Motif-bound YY-1 and JKTBP1—The need for an intact GTG motif for the YY-1- and JKTBP1-mediated transcription repression and activation suggested a possible physical interaction of the proteins involved or the possible involvement of a bridging factor that brings about functional synergy. This possibility was initially investigated by gel shift analysis using various combinations of *in vitro* translated proteins. As shown in Fig. 7A, YY-1 alone and ZBP-89 alone formed complexes with the 105-bp DNA that exhibited nearly comparable migration rates. Both of these complexes were supershifted by their specific antibodies. A combination of YY-1 and ZBP-89 formed an additional slowly migrating complex, suggesting the formation of a higher order complex. The latter was supershifted to a similar level by both anti-YY-1 and anti-ZBP-89 antibodies, further supporting the possibility of a physical association between the two proteins. Fig. 7B shows a similar gel shift analysis with the ZBP-89 and hJKTBP1 proteins. These two proteins formed differently migrating complexes with the 105-bp DNA that were supershifted to different levels by their respective antibodies. hJKTBP1 and ZBP-89 together formed three complexes, of which the two faster migrating complexes comigrated with the hJKTBP1-specific and ZBP-89-specific complexes (*lane 6*). The slowest migrating complex appears to be a higher order complex of the two proteins since it was supershifted by both the anti-hJK-TBP1 and anti-ZBP-89 antibodies. These results suggest that JKTBP1 and YY-1 form higher order complexes with adjacently bound ZBP-89 protein.

The possible physical association of these protein pairs, *viz.* ZBP-89/YY-1 and ZBP-89/JKTBP1, was further tested by co-immunoprecipitation. Initially, we tested ^{35}S-labeled *in vitro* translation products programmed in rabbit reticulocyte lysate. As shown in Fig. 8A, immunoprecipitation of a mixture of ZBP-89 and hJKTBP1 proteins with their respective antibodies resulted in the pull down of the other protein as well. However, the preimmune IgG fraction failed to immunoprecipitate either of these proteins. A similar co-immunoprecipitation of ZBP-89 and YY-1 is also shown in Fig. 8B. However, hJKTBP1 did not co-immunoprecipitate with YY-1 either with anti-hJKTBP1 or anti-YY-1 antibody (data not shown), suggesting no physical
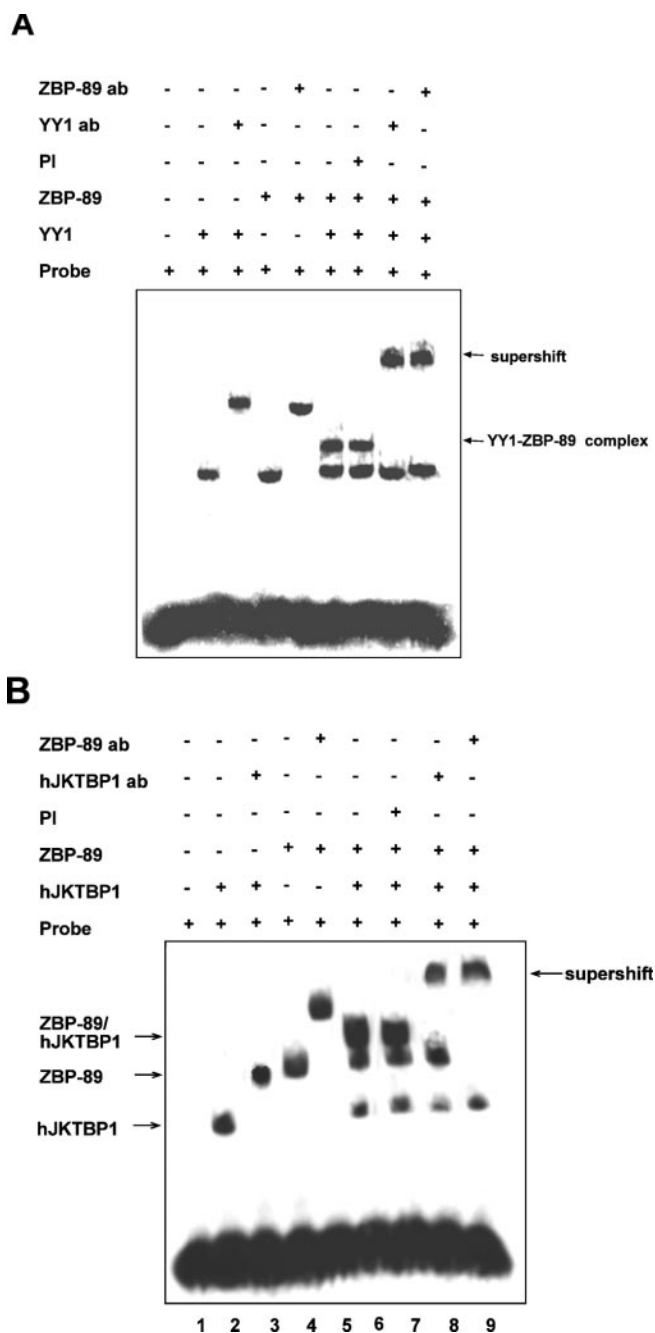

FIG. 7. **Formation of a higher order ZBP-89 complex with both the YY-1 and hJKTBP1 proteins.** Gel mobility shift assays were carried out with ^{32}P-labeled 105-bp negative enhancer probe using *in vitro* translated ZBP-89 and YY-1 (*A*) or ZBP-89 and hJKTBP1 (*B*) proteins (10 µl each). Antibody (*ab*) supershift was carried out using the indicated antibodies as described in the legend to Fig. 5 and under "Materials and Methods." *PI*, preimmune IgG.

interaction between these two proteins. The *in vivo* interaction of ZBP-89 with endogenous YY-1 and JKTBP1 was tested by immunoprecipitation of nuclear extracts from C2C12 myoblasts and myotubes with anti-YY-1 antibody, anti-hJKTBP1 antibody, or preimmune IgG. The resulting immunoprecipitates were probed with anti-ZBP-89 antibody by immunoblot analysis. As shown in Fig. 8C, the input nuclear extracts from C2C12 myotubes and myoblasts were probed with various antibodies to determine the steady-state levels of different proteins. Both myotube and myoblast extracts contained comparable levels of ZBP-89 and the nuclear marker protein p97. However, the myotube extract contained relatively lower levels
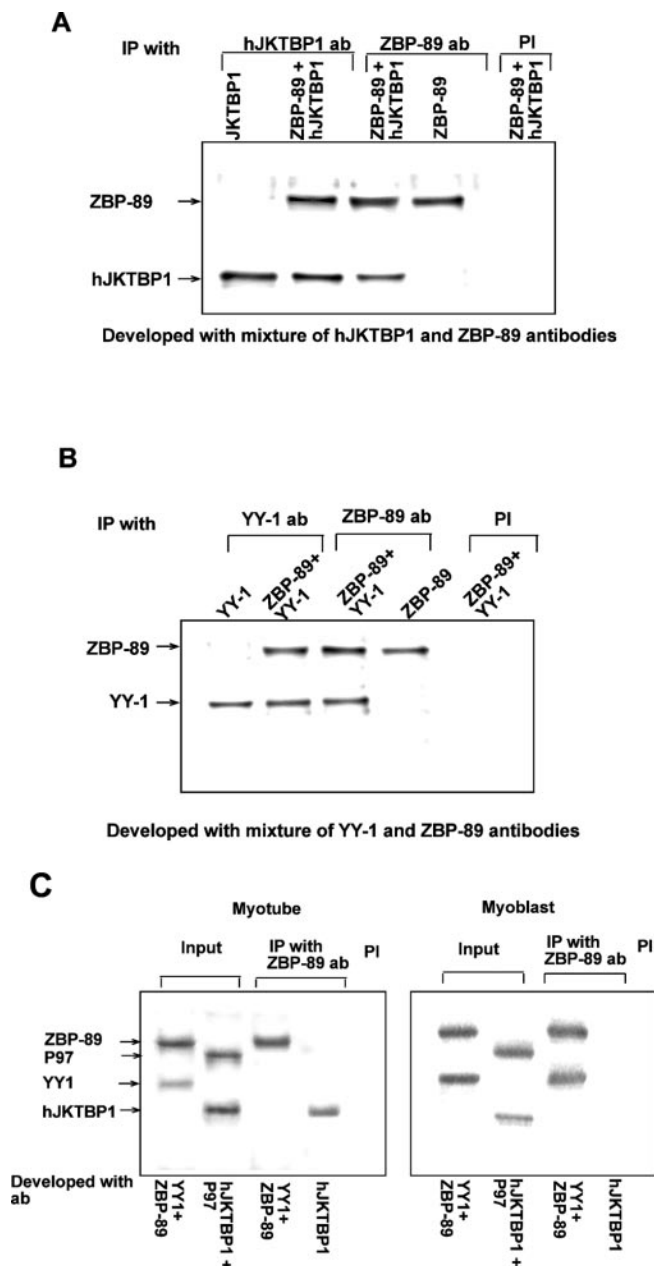
FIG. 8. **Physical interaction of ZBP-89 with YY-1 and JKTBP1 under *in vitro* and *in vivo* conditions.** In *A* and *B*, *in vitro* translated proteins (10 μl each) were mixed, preincubated for 20 min at 30 °C, and subjected to co-immunoprecipitation assays. In *C*, nuclear extracts from undifferentiated C2C12 myoblasts and differentiated myotubes (100 μg of protein each) were subjected to immunoblot analysis with the indicated antibodies before (*Input*) or after immunoprecipitation with the specified antibodies. *A*, *in vitro* translated hJKTBP1 and ZBF-89 were immunoprecipitated (*IP*) individually or together with the indicated antibodies (*ab*). As a control, preimmune IgG (*PI*) was used for immunoprecipitation. Immunoprecipitates were subjected to immunoblot analysis using a mixture of anti-hJKTBP1 and anti-ZBP-89 antibodies. *B*, same as in *A*, except that the YY-1 protein and its specific antibody were used in place of hJKTBP1 and its specific antibody. *C*, nuclear extracts from myoblasts (*right panel*) or myotubes (*left panel*) (100 μg each) were subjected to gel electrophoresis before (*Input*) or after immunoprecipitation with anti-ZBP-89 antibody or preimmune IgG as indicated. Blots corresponding to individual lanes were probed with the antibodies indicated at the bottom. The level of p97 was used as a loading control.

of YY-1, but higher levels of JKTBP1 compared with the myoblast nuclear extract. With the myotube nuclear extract, anti-ZBP-89 antibody efficiently pulled down the JKTBP1 protein, but not detectable YY-1 protein. On the other hand, with the

myoblast extract, anti-ZBP-89 antibody efficiently pulled down the YY-1 protein, but not significant JKTBP1 protein. These results provide direct evidence for *in vivo* physical association of ZBP-89 with the YY-1 and JKTBP1 proteins. The results also provide confirmatory evidence for the vastly variable levels of YY-1 and JKTBP1 in undifferentiated myoblasts and differentiated myotubes.

YY-1 Domains Needed for Interaction with ZBP-89 and Repressor Function—Fig. 9*A* shows a schematic of various structural and functional domains of YY-1 and mutations targeted to different zinc fingers. As shown previously (22), YY-1 contains an acidic helix, an acidic stretch, and a His stretch close to the N terminus as part of the transcription activation domain. At the C terminus of the protein, there are four zinc fingers, of which the first three have been shown to be important for binding to the YY-1 site from the adenovirus P_{5+1} promoter (44). The zinc finger domain is also required for transcription repression. Since zinc finger domains are generally implicated in both DNA binding and protein-protein interaction, we tested zinc finger domain mutants for binding to the CATR DNA motif and also for interaction with ZBP-89. The Gal4-YY-1-(1–414) construct contained all four zinc fingers intact. The Gal4-YY-1-(1–397) construct had part of the fourth finger deleted. The Gal4-YY-1-(1–370) construct had part of the third finger and the entire fourth finger deleted. The Gal4-YY-1-(1–331) construct contained only the intact first finger, whereas the Gal4-YY-1-(1–200) construct had all four fingers deleted. Point mutations C298S, C327S, and C360S disrupted the first, second, and third zinc fingers, respectively.

Gel shift analysis with CATR DNA (Fig. 9*B*) showed that Gal4-YY-1-(1–414) and Gal4-YY-1-(1–397) bound to the DNA. The binding of Gal4-YY-1-(1–414) was competed by a 40 M excess of unlabeled CATR DNA. Gal4-YY-1-(1–370), Gal4-YY-1-(1–331), and Gal4-YY-1-(1–200) did not bind to the CATR DNA. Similarly, point mutations C298S, C327S, and C360S failed to bind to the CATR DNA. In support of studies by Galvin and Shi (44), these results show that intact first, second, and third zinc fingers are required for DNA binding.

The sequence domains of YY-1 required for interaction with the ZBP-89 protein were determined by co-immunoprecipitation of *in vitro* translated wild-type and mutant Gal4-YY-1 proteins and ZBP-89. The protein pairs were immunoprecipitated with antibody to ZBP-89, and the immunoprecipitates were then probed with a mixture of anti-ZBP-89 and anti-YY-1 antibodies. Fig. 9*C* shows that the intact Gal4-YY-1-(1–414) protein interacted with ZBP-89 with the highest efficiency, while Gal4-YY-1-(1–397) and mutations C327S, C298S, and C360S bound at 30–50% reduced efficiency. Gal4-YY-1-(1–370), Gal4-YY-1-(1–331), and Gal4-YY-1-(1–200) failed to interact with the ZBP-89 protein. Essentially similar results were obtained when the protein pairs were first immunoprecipitated with anti-YY-1 antibody (data not shown).

The results of transcription analysis of the COX Vb promoter in Fig. 9*D* show that Gal4-YY-(11–414) and Gal4-YY-(1–397), which were capable of binding to CATR DNA and interaction with the ZBP-89 protein, were effective in transcription repression in C2C12 myotubes. Deletion mutants Gal4-YY-(1–370), Gal4-YY-(1–331), and Gal4-YY-(1–200) and point mutations C298S, C327S, and C360S, which did not show DNA binding activity, failed to show repressor activity. These results therefore suggest that protein-protein interaction between ZBP-89 and YY-1 alone is not sufficient for transcription repression of the COX Vb promoter, but that YY-1 binding to promoter DNA is also critical for this activity.
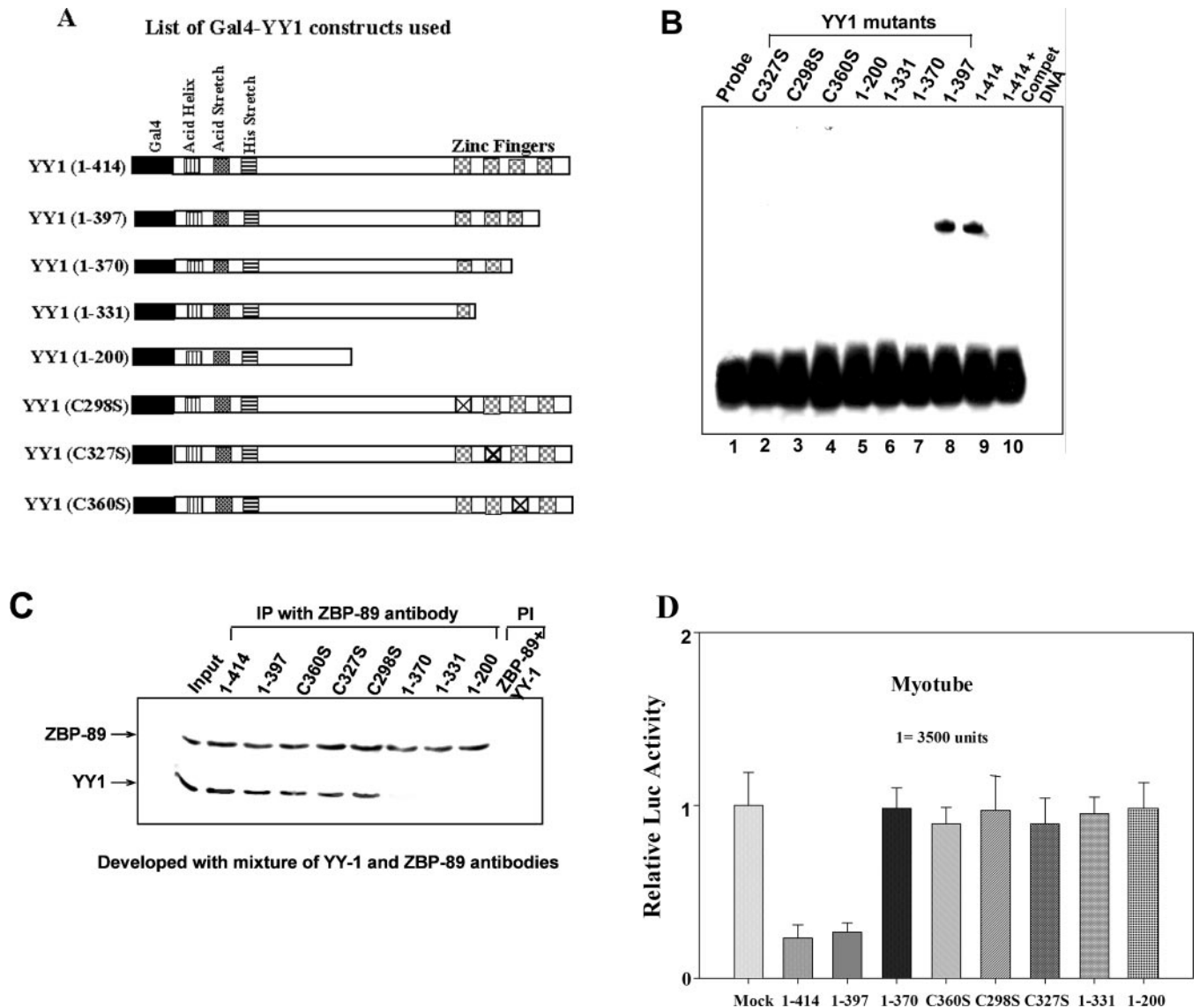
Regulation of COX Vb Gene Expression during Myogenesis

FIG. 9. **Sequence domains of YY-1 required for interaction with ZBP-89 and for transcription repression of the COX Vb promoter.** *A*, deletion and point mutations of Gal4-YY-1 (Gal4 protein fused to the N terminus of YY-1). Point mutations C360S, C298S, and C327S selectively disrupted the third, first, and second zinc fingers from the N terminus, respectively. *B*, DNA binding abilities of mutant proteins. Gel mobility shift assays were carried out using ^{32}P-labeled CATR probe and *in vitro* translated Gal4-YY-1 proteins as described in the legend to Fig. 7. Gal4-YY-1-(1–331), Gal4-YY-1-(1–200), C298S, and C327S exhibited no significant binding. *Compet*, competitor. *C*, co-immunoprecipitation of wild-type and mutant Gal4-YY-1 proteins with ZBP-89. *In vitro* translation products (YY-1 and ZBP-89 proteins) were mixed, incubated at 30 °C for 20 min, and immunoprecipitated (*IP*) with anti-ZBP-89 antibody. Preimmune IgG (*PI*) was used as a control. The immunoprecipitates were subjected to immunoblot analysis using a mixture of anti-ZBP-89 and anti-YY-1 antibodies. *D*, transcription repression of COX Vb promoter constructs in differentiated C2C12 myotubes by Gal4-YY-1 constructs. Transfection of cells with promoter constructs (2 μg) and Gal4-YY-1 constructs (0.5 μg) was carried out as described in the legend to Fig. 6 and under "Materials and Methods." Dual luciferase (*Luc*) assays were carried out also as described under "Materials and Methods."

DISCUSSION

A previous study from our laboratory showed that the transcription activity of the mouse COX Vb promoter is induced severalfold during myogenesis, thus providing a rational basis for the high steady-state mRNA and protein levels detected in skeletal and cardiac muscle tissues (6). Additionally, sequence −481 to −320 of the promoter, which contains four putative protein-binding motifs, functions as a transcription suppressor in undifferentiated myoblasts, probably contributing to the low level of expression of the endogenous COX Vb gene. We also proposed that attenuation of transcription suppressor activity by an unknown mechanism was responsible for increased expression of the gene in fully differentiated C2C12 skeletal myotubes. In this work, we have demonstrated that an orchestrated interplay between three different ubiquitously expressed factors, ZBP-89, YY-1, and JKTBP1, which bind to two

adjacent sequence motifs of the promoter, modulates transcription rates during myogenic differentiation. Specifically, our results show that in undifferentiated myoblasts, CATR motif-bound YY-1 physically interacted with GTG motif-bound ZBP-89 to execute its transcription repressor activity. In differentiated myotubes, however, a muscle predominant factor, JKTBP1, bound to the CATR sequence in place of YY-1 and interacted with ZBP-89 to promote transcription activation. This study therefore describes a novel factor-switching mechanism in which two differentiation stage predominant factors that have opposing effects on transcription rates reciprocally occupy the same promoter site and promote transcription activation or repression.

ZBP-89 is a Krüppel-like zinc finger transcription factor that binds to a GT-rich sequence motif (GTGG(G/C)GGGGGGGGTG) in a sequence-specific manner. The human homolog of this

factor is implicated in the transcription activation of T-cell receptor genes (34). In conjunction with the muscle-specific myocyte enhancer factor-2, ZBP-89 has been implicated in the induced expression of β-enolase and other muscle-specific genes during induced myogenesis of C2C12 myocytes (21). ZBP-89 also plays a critical role in the developmentally regulated expression of the gastrin gene in embryonic pancreas and adult stomach (33). The protein factor contains a transcription activation domain at its C terminus and a repressor domain at its N terminus (21), although the factor is more commonly known as a repressor (45). It is known to stabilize the binding of p53 to some critical target genes, resulting in arrest of cell growth (46). In fact, many of the known target genes of ZBP-89 are regulated by mitogens or developmental signals (47, 48). The consensus binding site for ZBP-89 is also found in the promoters of the genes encoding PU1 (49) and CAAT/enhancer-binding protein-α, which play important roles in myeloid differentiation. Thus, ZBP-89 appears to be involved in the regulation of cellular proliferation as well as differentiation. The ability of the factor to mediate transcription activation and repression and to mediate epidermal growth factor-dependent regulation of gene expression is believed to be important in the expression of the gastrin gene at the embryonic stage and also silencing its expression in adult pancreas. Our results with cDNA transfection (Fig. 6, *A–C*) show that ZBP-89 functions as a transcription activator of COX Vb gene expression in both C2C12 skeletal myocytes and 3T3 fibroblast cells. More specifically, interaction of ZBP-89 with CATR box-bound YY-1 or JKTBP1 helps mediate transcription repression or activation of gene expression at different stages of myogenesis.

JKTBP1 (also referred to as hnRNP-DL) belongs to a large family of hnRNPs that have important roles in nuclear RNA processing, transport, stability, and translation regulation of mRNAs in the cytoplasm (50–56). Many members of hnRNP family of proteins have also been shown to bind to double-stranded DNA in a sequence-specific manner and to induce gene-specific transcription repression or activation (37–39, 57, 58–59). Some members of this family of proteins modulate the expression of muscle-specific actin and other genes by binding to CArG motifs at the promoter sites (38). One member of this family designated CCAAT-binding factor A (hnRNP-A/B) exhibits multimodal effects on transcription regulation in that it represses the expression of the immediate-early genes c-*fos* and *zif268* and activates transcription of the rat Ha-*ras* and SP6 κ promoters (57). On the other hand, human JKTBP1 (hnRNP-DL) has been shown to stimulate transcription in a promoter-specific manner (39). Although not fully investigated, it is believed that the RGF domains of hnRNP-associated proteins implicated in RNA binding are also involved in binding to double-stranded CArG DNA (38). Also, the Gln/Gly-rich region of the protein might function as a transcription activation domain. The repressor domains of CCAAT-binding factor A (hnRNP-A/B) and JKTBP1 have not yet been mapped. In the context of the COX Vb promoter, JKTBP1 binds to a novel CATR sequence motif and functions as a transcription activator in differentiated myotubes, a stage at which the nuclear level of JKTBP1 is increased nearly 10-fold. Mutations at this site abolished both ZBP-89- and JKTBP1-dependent transactivation in myotubes, suggesting that functional interaction between these two factors is necessary for realizing the activation potential of both of these factors. In support of this possibility, JKTBP1 indeed physically associates with ZBP-89, as shown by the antibody supershift and antibody pull-down assays. These latter results are consistent with reports showing physical association of members of the hnRNP family (CArG binding factor A) with *ets* family factors PU1 and Elf-1 in the
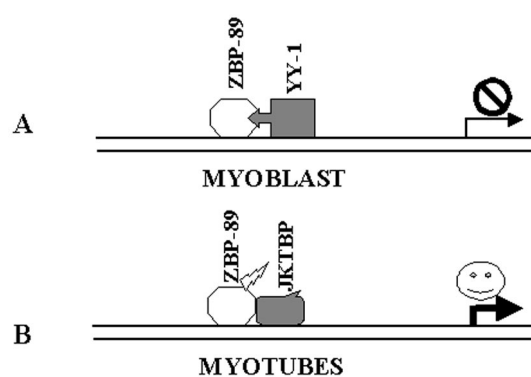

FIG. 10. **Proposed model for the switching of protein factors at the CATR motif of the promoter during myogenesis.**

regulation of cell-specific expression of the SP6 κ gene (58).

YY-1, a GL1-Krüppel family zinc finger protein, is a multifunctional transcription factor that can act at promoters, enhancers, and initiator elements (17, 60–63). Here, we have demonstrated that the YY-1 factor functions as a repressor of the COX Vb promoter in murine skeletal myocytes by binding to the CATR motif from within the negative enhancer region. Transfection with YY-1 cDNA caused repression of the COX Vb promoter in myoblasts that was reversed by overexpression of the hJKTBP1 protein (Fig. 6*A*), suggesting that relatively high steady-state levels of YY-1 and relatively low JKTBP1 levels in the nuclear compartment are mostly responsible for *trans*-repression of the gene in undifferentiated myocytes. Chromatin immunoprecipitation analysis indeed confirmed a high level of YY-1 and a very low to negligible level of JKTBP1 bound to the GTG/CATR suppressor region in undifferentiated myoblasts. Mutations at the CATR motif relieved the *trans*-repression in undifferentiated myoblasts, further supporting the possibility that YY-1 is the major contributor for repressor activity at this stage of myogenic differentiation (Figs. 1 and 6). These results also suggest that JKTBP1 and YY-1 have antagonistic functions *in vivo*. Based on these results, we propose a working model for the factor-switching mechanism described in this study. As proposed in Fig. 10, in undifferentiated myoblasts, YY-1 interaction with ZBP-89 may mask the latter's transcription activation domain, thus causing *trans*-repression. Currently, it is not known if the repressor domain of ZBP-89 or that of YY-1 is critical for *trans*-repression. The results of DNA binding and transcription analysis suggest a likely possibility that both YY-1 binding to DNA and its physical interaction with ZBP-89 are critical for this functional outcome. In differentiated myotubes, a relatively low nuclear level of YY-1 but a high level of the JKTBP1 protein may result in preferential binding of the latter factor to the CATR motif, probably due to the mass difference. We hypothesize that association of JKTBP1 with ZBP-89 masks the repressor domain of the latter, causing transactivation. Mutations at the CATR motif abolished both the ZBP-89- and JKTBP1-mediated transactivation, suggesting that intermolecular interaction between these factors is important for mediating activation by ZBP-89 in differentiated myocytes. Since cotransfection with both of these factors has an additive effect on transcription stimulation, it is likely that activator domains of both proteins are involved in the muscle-specific activation of COX Vb gene expression. Further studies are underway to investigate these possibilities.

The CArG motif (CC(A/T)$_6$GG) contains a 5′-ATGG-3′ sequence, which composes the core binding site for YY-1 (61, 62, 64), and such motifs are found in many muscle-specific promoters and enhancers (65, 66). Although initial studies with c-*fos* and α-actin promoters suggested that YY-1 may compete with

serum response factor binding to the serum response element (67), a subsequent study showed that at increasing serum response factor concentrations, YY-1 forms a ternary complex, suggesting that YY-1 may promote the binding of the serum response factor to the serum response element (68). In the present case, both gel shift assays and competition studies did not show the formation of a ternary complex of JKTBP1 and YY-1 with the CATR DNA (Fig. 5*D*). In this respect, the exclusive nature of binding of the two factors to the COX Vb negative enhancer for negotiating transcription activation or repression represents a novel mechanism rarely shown before for differentiation-specific regulation of gene expression in eukaryotes.

Zinc finger domains not only recognize the DNA, but also participate in protein-protein interactions. For instance, the zinc finger of YY-1 interacts with Sp1 (61, 69), and the physical interaction requires YY-1 amino acids 260–331, which correspond to the first zinc finger domain. YY-1 also interacts with c-Myc, but through amino acids 201–343, which encompass the first and part of the second zinc finger (70). On the other hand, E1A interacts with YY-1 through sequence 331–414, which encompasses the second, third, and fourth zinc fingers (71). Our results show that physical association with ZBP-89 requires the region from the third and fourth zinc fingers of YY-1. Furthermore, results with point mutations of individual zinc finger domains suggest that the amino acid sequences in the third and part of the fourth zinc finger, but probably not intact finger structure, are necessary for ZBP-89 binding. Mutational studies also show that mere physical interaction of YY-1 with ZBP-89 is not sufficient for inducing repressor activity (Fig. 9), but its binding to the negative enhancer DNA is critical. These results also preclude the possibility that the repressor function of YY-1 is not merely because of a possible scavenging effect. In summary, our results show that YY-1 and JKTBP1 compete for binding to a novel CATR motif within the negative enhancer region of the COX Vb gene, which forms the basis for vastly varying levels of these two proteins at two different stages of myogenic differentiation.

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