The E Protein CTF4 and Acetylcholine Receptor Expression in Development and Denervation Supersensitivity*

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Craig M. Neville‡, Yoon-Hyuk Choe§, Ying-Shuan Lee¶, Daryl Spinner¶, Huey-Jen Tsay†, and Jakob Schmidt***

From the §Department of Biochemistry and Cell Biology, SUNY at Stony Brook, Stony Brook, New York 11794-5215, the ¶Cardiovascular Research Center, Massachusetts General Hospital, Charlestown, Massachusetts 02129-2060, the ‡Department of Biochemistry and Cell Biology, SUNY at Stony Brook, Stony Brook, New York 11794-5215, and the †Samsung Biomedical Research Institute, Seoul, Korea 135-230, and the ¶Institute of Neuroscience, Yang-Ming Medical University, Taipei, Republic of China

Motor activity blocks the extrasynaptic expression of many genes in skeletal muscle, including those encoding ion channels, receptors, and adhesion molecules. Denervation reinduces transcription throughout the multinucleated myofiber, restoring the developmental pattern of expression, especially of the genes coding for the acetylcholine receptor. A screen for trans-acting factors binding to the enhancer region of the α-subunit gene of the acetylcholine receptor identified CTF4, a ubiquitously expressed and alternatively spliced chicken homologue of the human E protein transcription factor HTF4/HEB. Expression of the CTF4 locus closely parallels that of myogenin and acetylcholine receptor during development and maturation of skeletal muscle, but transcription is not similarly regulated by neuronal cues. Alternative splicing within the region encoding the transactivation domain generates two CTF4 isoforms with different tissue distributions, but similar binding affinities for the acetylcholine receptor α-subunit enhancer and similar transcriptional potential when complexed to myogenin. Direct injection of a myogenin, but not a MyoD, antisense expression vector into denervated skeletal muscle caused a significant decrease in the transcriptional activation of a depolarization-sensitive reporter gene. Similarly, injection of a CTF4, but less so of an E12, antisense expression vector impaired the denervation response, further implicating the involvement of a myogenin/CTF4 heterodimer in the expression of AChR genes in vivo.

Vertebrate skeletal muscle development proceeds by fusion of undifferentiated mononucleated myoblasts to form multinucleated myofibers, with a concomitant activation of muscle-specific genes. Although much is known about the cis-elements and the trans-acting factors that confer muscle-specific gene expression in an in vitro cell culture system, the mechanisms responsible for the modulation of the more complex patterns of gene expression present within the myofibers found in muscle have remained elusive. Many of the activated muscle genes, especially those coding for the contractile apparatus and metabolic pathways, are expressed in all nuclei of every myofiber, whereas others are activated in only a subset of fibers or even a fraction of the nuclei within a single cell, or are restricted to a particular developmental period. Upon innervation and maturation of the myofiber, expression of proteins required for the formation of the neuromuscular junction and the myotendinous junction becomes restricted; thus, subsynaptic nuclei are stimulated by a trophic factor released from the nerve terminal to express acetylcholine receptor (AChR) genes, counteracting the ongoing electromechanical activity which eliminates the AChR from extrajunctional regions (1, 2).

Like many skeletal muscle-specific genes, the genes coding for the subunits of the AChR contain E boxes in their regulatory regions that myogenic factors recognize and activate in heterologous expression systems. E boxes are cis-regulatory elements that contain the sequence CANNTG and are found in the enhancers of many developmentally regulated genes from a diverse array of tissues (3). E boxes have been implicated in the dramatic stimulation of receptor gene transcription that is seen during myogenesis in vivo and in vitro and may also play a role in denervation supersensitivity, i.e. receptor gene activation resulting from denervation of the adult muscle fiber (4–7). Similarly, they have been found in the promoters of human (8), mouse (9), and chicken (10) myogenin which are likewise responsive to electrical membrane activity. Denervation does not trigger up-regulation of other genes such as the muscle creatine kinase and myosin light chains 1/3, both of which clearly contain E boxes as crucial control elements in their regulatory regions (11, 12). The context of an E box, i.e. its flanking regions and the trans-acting factors bound to them, may govern whether a particular E box-containing gene will respond to denervation.

The helix-loop-helix (HLH) motif in the eponymous superfamily of transcription factors is a conserved structure that mediates homo- and hetero-dimerization (13); members of this protein family play an important role in differentiation. In basic-helix-loop-helix (bHLH) proteins the HLH motif is immediately preceded by a region rich in basic amino acids that is responsible for site-specific DNA binding to E boxes. The E proteins form one important family of bHLH transcription factors that bind such cis-regulatory elements. Three vertebrate loci have been reported: E2A/PAN/SEF2 (13–15), E2–2/TF2 (16) and HTF4/CTF4/HEB (17–19). E proteins are widely expressed throughout the organism and are involved in the differentiation of many tissues, including the central nervous system, skin, heart, and skeletal muscle; their dimerization

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** To whom correspondence should be addressed: Dept. of Biochemistry and Cellular Biology, SUNY at Stony Brook, Stony Brook, NY 11794-5215; Tel.: 516-632-8561; Fax: 516-632-8575; E-mail: jschmidt@life.bio.sunysb.edu.

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The abbreviations used are: AChR, acetylcholine receptor; bHLH, basic helix-loop-helix; CAT, chloramphenicol acetyltransferase; HLH, helix-loop-helix; LH, loop-helix; MDF, myogenic determination factor; bp, base pair(s); kb, kilobase pair(s).
with members of another bHLH family, the myogenic determination factors (MDFs), results in the transactivation of a wide array of muscle-specific genes. Each of the primary transcripts arising from the three E protein loci is alternatively spliced to give rise to multiple isoforms with distinct differences in DNA binding and dimerization domains, nuclear localization signals, and consensus sites for phosphorylation. This also holds for CTF4. As in the case of its rat homologue REB (20/SCBP (21), alternative splicing of the primary transcript produces two gene products, one with 24 amino acids inserted in the middle of the LH domain, a well conserved transcription-activation motif unique to E proteins (22). The transcripts encoding the two proteins exhibit characteristic spatial and temporal distribution patterns as well as subtle differences in their gene activation capabilities.

CTF4 was originally identified by its ability to recognize a potent enhancer in the AChR α-subunit promoter, prompting us to investigate its potential role in AChR gene regulation, both during muscle cell differentiation and the subsequent innervation-induced restriction of receptor expression to the neuromuscular junction. Myogenin, one of the four MDFs, is itself regulated by innervation and the innervation-dependent electrical activity of the sarcolemma (reviewed in Ref. 23), and therefore a potential participant in the regulation of AChR subunit genes. Whether myogenin exclusively or preferentially interacts with a specific E protein partner when activating AChR gene expression is not known.

The differences in depolarization sensitivity of E boxes in muscle-specific promoters may be a result of specific target preferences among bHLH proteins; individual factors and factor combinations have been shown to select specific sequences (3, 24, 25). To investigate the possible participation of CTF4 in the up-regulation of AChR genes in denervated muscle, we combined an antisense strategy with the technique of in-muscle plasmid injection. Here we present in vivo antisense suppression results, and show that myogenin and to a lesser extent CTF4 contribute to the denervation-triggered activation of AChR expression in skeletal muscle.

**EXPERIMENTAL PROCEDURES**

**Animal Experiments**—White Leghorn cockerels (Hall's Brothers Hatchery, North Brookfield, MA), were anesthetized 3–4 days after hatching with an intraperitoneal injection of ketamine (50–100 mg/kg), and their crural musculature was excised. The nerve was perfused unilaterally as described previously (26). At the desired time after denervation, animals were sacrificed; the shank muscles of the operated and control legs were isolated and rinsed with phosphate-buffered saline, then processed for the preparation of muscle extracts; nuclear extracts were prepared by the method of Dignam et al. (27). The effects of electrical stimulation were measured 6–7 days after denervation; the denervated leg musculature was stimulated in 100-Hz trains, 2-s duration, applied once every min. Plasmid injection was carried out as described by Wolff et al. (28). The AChR α-subunit regulatory region drives expression of reporter enzymes following AChR induction by nerve section; thus, the denervation-induced expression of p20kbLuc resembles that of the endogenous AChR subunit genes. Reporter plasmids were injected 24 h prior to denervation. Three days after denervation, when luciferase is maximally expressed, animals were sacrificed, and muscle tissue frozen quickly in liquid nitrogen and pulverized with pestle and mortar. Powdered specimens were resuspended in lysis buffer (100 mM potassium phosphate, pH 7.8, 1 mM Mg-dithiothreitol, 1% Triton X-100) and incubated on ice for 1 h with frequent vortexing. After centrifugation at 14,000 rpm for 15 min, the supernatant was assayed for enzyme activity. All animal experimentation followed protocols approved by the Stony Brook Institutional Animal Care and Use Committee. For chicken embryos, fertilized White Leghorn eggs were obtained from local hatcheries and incubated at 38 °C in a humidified incubator.

**Measurement of mRNA**—Total RNA was extracted by the guanidinium isothiocyanate procedure of Chirgwin et al. (29). RNA was quantified spectrophotometrically and checked for integrity by electrophoresis. Messenger RNA levels were measured by ribonuclease protection assays essentially as described previously (30).

**Enzyme Assays**—Chloramphenicol acetyltransferase (CAT) and luciferase assays followed established procedures (31).

**Plasmids**—Expression vectors were constructed by cloning cDNAs for chicken MyoD (32), myogenin (33), and the two isoforms of CTF4 (Promega) into a pEMSV vector (Promega, Madison, WI). A CTF4 plasmid cDNA insert was ligated into the vector pBKCMV (Stratagene, La Jolla, CA). To assess tissue and stage specificity of expression of CTF4, a riboprobe synthesized from a 198-base template corresponding to the 3′- untranslated region was used for mRNA analysis. In order to determine the relative abundance of the two CTF4 transcripts in the same assay, another riboprobe was generated from the 5′ splice junction, was generated by cloning 50 bp of annealed synthetic oligonucleotides, containing the 36-bp α-subunit enhancer with its 2 E boxes (GGCGGGCCATCAAGTGTGTATGGCCTGAAACAGGTGGTG) and flanking BamHI linkers, into the Smal site of Bluescript SK + (Stratagene). Labeled probes, either 49 or 97 bp long, were generated by excision with BamHI or a combination of HincIII and XbaI, respectively, followed by Klenow fill-in. An 83-bp fragment of the chick AChR β upstream sequence (307 to 125) which contained a single E box was cloned into the HincIII and XhoI sites of pBR322. po2kbCAT and po2kbLuc contain 2 kb of AChR α-subunit upstream inserted upstream inserted into the pCAT-Basic and pGLO2-Basic vectors (Promega, Madison, WI), respectively. Plasmids for in vitro expression: pChMyoG/SK− contains 1.1 kb of full-length chicken myogenin cDNA and pCTF4a10/SK−, 4.2 kb of CTF4 cDNA (clone no. 10), both inserted into the EcoRI site of pBluescript SK− (Stratagene). pCMV-E12 encoding the chick E12 protein was obtained from Bruce Paterson.

**Immunohistochemistry**—The crural musculature from an animal that had been denervated 3 days previously was removed from both ipsi- and contralateral limbs and fixed in 4% paraformaldehyde in phosphate-buffered saline. Immunohistochemistry was performed on paraffin-embedded sections, which were incubated with the CTF4-specific antibody 251, a biotinylated anti-rabbit antibody, alkaline phosphatase-conjugated avidin, and finally the enzyme substrate Fast Red (Sigma).

**Cell Culture and Transfections**—NIH/3T3 cells were maintained in growth medium (Dulbecco's modified Eagle's medium containing 10% supplemented calf serum) before transfection. Cells (1.5–3×10^5) were plated 2 days prior to transfection on 100-mm dishes and reseeded with growth medium 2 h prior to transfection. Transient transfections (10 μg of reporter plasmids, po2kbCAT or po2kbLuc, 10 μg of effector plasmids, and 2 μg of internal control plasmids, pRSVcat or pRSVLuc) were performed by the calcium phosphate precipitation method as described previously (35) except that medium was changed 12 h after introduction of DNA without gelatin sandwich. Twenty-four h after transfection, the medium was changed to serum-deficient medium (Dulbec-co modified Eagle's medium containing 2% heat-inactivated horse serum). Three days after transfection, the cells were rinsed twice with 5 ml of phosphate-buffered saline and harvested in 1 ml of 40 mM Tris-HCl, pH 7.8, 1 mM EDTA, and 150 mM NaCl. The cell suspension was centrifuged for 7 s at 14,000 rpm, and the pellet resuspended in 100 μl of lysis buffer. After a 1-h incubation on ice, the cell lysis mixture was spun again, and the supernatant assayed or stored at −70 °C.

**In Vitro Transcription and Translation**—In vitro translation was performed using the TNT® reticulocyte lysate system (Promega) in 50 μl of buffer mixture containing 1 μg of DNA template, 25 μg of rabbit reticulocyte lysate, 1 μm amino acid mixture minus methionine, 4 μl of [35S]methionine at 10 mCi/ml, 5 units of RNA polymerase, and 40 units of RNase inhibitor (Boehringer Mannheim) at 30 °C for 1 h. The [35S] labeled proteins were resolved on 10% SDS-polyacrylamide gel.

**Bacterial Expression of Fusion Proteins**—Chicken myogenin and CTF4 DNA fragments were cloned into the pGEX vector (Pharmacia Biotech Inc.) encoding glutathione S-transferase. The induced fusion proteins were affinity-purified on glutathione-agarose beads and used in binding studies and for the generation of polyclonal antibodies.

**Electrophoretic Mobility Shift Assay**—Appropriate amounts of bacterial extracts, in vitro translated reporter protein, were incubated with typically 10,000 cpm of the 32P-labeled α-subunit enhancer probe in a total volume of 20 μl. The binding reactions contained 20 mM Hepes (pH 7.9), 100 mM potassium chloride, 0.2 mM ethylene diamine tetraacetic acid, 20% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol, 3 μg of poly(dA-dT) or poly(dI-dC), and 0.5 μg of yeast tRNA, and were incubated at 37 °C for 15 min. The
protein-DNA complexes were resolved by electrophoresis through 3.5–5% (w/v) nondenaturing polyacrylamide gels at 100 V for 1.5 h at room temperature. The gel and electrophoresis buffer contained 106 mM Tris, 89 mM boric acid, and 0.1% Nonidet P-40. For supershifts, 0.3 μl of anti-CTF4 or preimmune serum was added to these mixtures.

RESULTS

CTF4 Contains Alternative Transactivation Domains—The core enhancer sequence of the chicken AChR α-subunit gene contains two E boxes that are the primary elements responsible for mediating transcriptional control both during development and upon denervation (2). A double-stranded oligonucleotide containing four copies of the enhancer was used as a probe in an affinity screening protocol to isolate factors involved in neuron-regulated muscle gene transcription. Of the four DNAs isolated from an E10 whole chicken embryo λgt11 cDNA library, three encoded the E protein CTF4 (18), a homologue of the human gene HTF4 (17/HEB) (19) (Fig. 1). All three CTF4 cDNAs contained a 72-bp region encoding a 24-residue peptide which is absent from the human protein. A search for additional CTFE protein isoforms was performed with degenerate oligonucleotide primers targeting regions conserved in all known vertebrate E proteins, in a polymerase chain reaction utilizing reverse transcription-polymerase chain reaction (data not shown). The longer fragment corresponds to the cDNA isolated by the affinity screening protocol above, while the shorter fragment is derived from an alternatively spliced mRNA encoding an otherwise identical polypeptide except for lacking the 24 amino acids found in the original CTF4 clones, but absent in HTF4/HEB (Fig. 1). As a similar splicing pattern has been seen in rat (see above), we have adopted the nomenclature proposed for REB proteins (20) and designated cDNAs encoding the shorter and longer proteins CTF4α and CTF4β, respectively. The 24-residue insertion interrupts the loop-helix (LH) motif transactivation domain contained by all other E proteins (22).

Expression Is Especially Abundant in Brain and Skeletal Muscle of the Early Fetus—The transcriptional regulation of the CTF4 gene was examined by ribonuclease protection assay, utilizing a probe that partly spans the alternatively spliced exon located within the transactivation domain (Fig. 1). During the development of the chicken embryo, CTF4 is continuously transcribed at high levels, but steady-state transcript levels drop considerably after embryonic day 12 (Fig. 2). The two splice variants are present at significant levels in the early developing embryo from the first time point examined, E5.5, with little overall change in the ratio of the two mature transcripts throughout development. The expression of the two CTF4 transcripts was then examined in several different tissues of E10 embryos. Both transcripts were found in all tissues assayed, but their concentration ratios vary by over an order of magnitude. Absolute levels of the CTF4 transcripts vary widely also, with high expressing tissues such as brain and muscle containing about ten times more than liver. The processed transcript lacking the alternatively spliced exon predominates in the majority of tissues, including liver, skeletal muscle, bone marrow, gizzard, and in particular lung. Only in the brain is the long form more abundant; expression levels in the developing brain are higher than in any other embryonic tissue examined and remain moderately high, though fluctuating, throughout development. CTF4 transcripts are most abundant in the telencephalon and cerebellum of the neonate animal, but rare in the brain stem and optic lobe. The CNS also contains a minor band of about 180 nucleotides; this size is consistent with a CTF4 isoform containing the three amino-terminal residues encoded by the alternatively spliced exon. Although this region contains a splice-donor consensus sequence, we were unable to demonstrate the existence of such a cDNA utilizing reverse transcription-polymerase chain reaction (data not shown).

Developmental Regulation of Expression in Skeletal Muscle—The AChR is expressed along the entire length of the muscle fiber surface during early stages of development. As the muscle matures, transcription of the genes encoding the AChR receptor becomes restricted to the newly formed neuromuscular junctions as a result of repression of gene activity in extrajunctional muscle nuclei (reviewed in Ref. 36). In the crural muscles, restricted expression occurs during early fetal development (E13–15), resulting in significantly fewer receptor transcripts. Temporal expression patterns of the myogenic factors and CTF4 were measured in developing muscle. The down-regulation of the myogenic factors MyoD, myogenin, and myf5 (but not of herculin/MRF4) shortly after the shift to the fetal

Fig. 1. CTF4 aligned to other members of the E protein family. The nucleotide sequence encoding the CTF4 transactivation domain with the alternative exon and flanking regions is shown. The segment used for synthesis of the isoform-discriminating riboprobe is underlined; it protects 171 (CTF4α) and 203 nucleotides (CTFβ), respectively. The deduced polypeptide sequence of CTF4 (GenBank™ locus MMU16322). Conserved residues of the transactivation domain are shown in bold.

Fig. 2. CTF4 expression during development. Ribonuclease protection assays were used to determine levels of the two CTF4 transcripts in whole chicken embryos (top panel); in the developing brain (center left; P5, 5 days after hatching); and various brain regions and tissues of an E10 embryo (center right; bottom). Upper bands reflect expression of CTFβ, lower bands CTFα.
stage (E13) is accompanied by a drop in mRNAs encoding AChR α and δ subunits, and a decline in CTF4 transcripts (Fig. 3A). Both the short and long CTF4 transcripts were present throughout the embryonic and early fetal period of muscle development. To determine if changes in transcript levels are accompanied by changes in protein concentrations, we performed supershift analysis. As is shown in Fig. 3C, CTF4-like immunoreactivity is abundant in the early embryo leg, but disappears toward the second week in ovo, accompanying a general decline in E box binding activity as measured with a probe derived from the AChR δ-subunit enhancer (Fig. 3B). These changes parallel, and possibly precede, the long known and pronounced reductions in AChR protein levels in the embryonic hind limb musculature (36).

Response in Skeletal Muscle to Neuronal Signals—As CTF4 is developmentally expressed in the hind limb muscle in a pattern similar to the AChR α-subunit and the myogenic factors MyoD, myogenin, and myf5, we investigated whether these genes are all under similar mechanistic control. The myogenin gene in particular responds to the establishment and disruption of neuromuscular transmission (33, 38, 39). The response of the CTF4 gene to denervation was therefore determined in the crural muscles of neonate chickens (Fig. 4, top panel). Whereas AChR α-subunit message levels increase 80-fold by day 3 postdenervation (37) and myogenin mRNA rises by more than 200-fold (33), CTF4 transcript levels remain fairly constant (~2-fold increase), and CTF4 protein, as measured by supershift analysis, also increases only modestly over a 3-day period (data not shown), indicating that the motor neuron affects CTF4 gene expression relatively little. Similarly, whereas electrical stimulation of chronically denervated skeletal muscle causes an immediate decline in transcript levels for AChR α subunit and myogenin (33), the CTF4 gene does not respond to the depolarization signal under these conditions (Fig. 4, bottom panel). This could result from an mRNA half-life that significantly exceeds the duration of the experiment or may indicate independence from neuronal signals.

E Box Binding of CTF4 in Homo- and Heterodimeric Form—We next tested the ability of the two CTF4 isoforms to bind to the α50 probe which encompasses the α-subunit enhancer and its two E boxes (35). Both CTF4α and CTF4β are capable of binding the probe; for the longer isoform this result was expected, as it had originally been identified by a protocol selecting for single gene products. Two other E proteins, the E2A products E12 and E47, and the MDF myogenin were also examined for their binding activity. While E47 alone displayed binding activity, E12 failed to efficiently bind to α50 as a homodimer, presumably because of the inhibitory domain at the N-terminal which is absent in other E proteins (41). Similarly, myogenin displays low affinity and binds the probe only at high concentrations. As seen in Fig. 5, combination of an E protein with myogenin (in amounts that by themselves do not generate band shifts) potentiated formation of complexes that migrate to a position intermediate between that seen with either bHLH protein alone, indicating the formation of heterodimers; this was confirmed by antibody disruption experiments (not shown). No significant difference between CTF4α and CTF4β heterodimers in the amounts and patterns of shifted bands were observed, suggesting that the additional 24 amino acids at the LH domain do not affect DNA binding. These experiments show that the CTF4 proteins, like E12, efficiently form heterodimers with myogenin.

Transactivation of the AChR α-Subunit Promoter—To determine the activation potential of CTF4, expression plasmids

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H.-J. Tsay and Y.-S. Lee, unpublished results.
line was chosen as the transfection recipient as it contains low levels of endogenous E proteins. Results of these experiments are shown in Fig. 6. Only CTF4α was capable of efficiently transactivating pα2kbCAT without additional cofactors. Enzymatic activity due to transactivation of the reporter construct by CTF4β was detectable only with extended incubation of the assay reaction. Since gel shift analysis of the two isoforms revealed little difference in their DNA binding abilities, it is likely that the additional 24 residues of CTF4β modulate the transactivating function. The further addition of myogenin to fibroblasts increased reporter gene activity. Synergistic effects were particularly apparent when limiting amounts of effector plasmids were used. Under such conditions, whereas either myogenin or CTF4 by themselves failed to activate the reporter gene, their combination resulted in activation. Transactivation is more pronounced with the short isoform, suggesting that an intact transactivation domain is required for optimal activity.

Expression of CTF4 Is Required for Maximal Myogenin-dependent Gene Activity in Denervated Muscle—To determine whether CTF4 participates in AChR gene activation in either innervated or denervated muscle we adopted a strategy of CTF4 elimination in muscle fibers through intramuscular injection of plasmid DNA expressing antisense RNA. Since only a small fraction of the muscle fibers of the injected muscle take up the plasmid (41), the effect of an inhibitory transgene on endogenous AChR expression would remain undetectable. The pα2kbLuc reporter, whose activity in denervated muscle mimics and parallels the activity of the endogenous AChR α-subunit gene, was co-injected as a reporter to permit monitoring the effect of antisense RNA expression on the targeted transcription factor. To test the validity of the antisense approach, the effect of suppressing myogenin which is likely to be the major activator of the AChR subunit genes (33) was examined first. Administration of the anti-myogenin expression vector pCMVantiMG reduced denervation-induced activity to as low as 16% of control, depending upon the amount of antisense plasmid used (Fig. 7A). In contrast, exogenous myogenin induced the reporter by about 10-fold in innervated muscle (Fig. 7B). The specificity of the inhibitory effect of the antisense construct was established by the rescue of pα2kbLuc expression with exogenous myogenin (Fig. 7C) and by the relative inefficiency of MyoD suppression (Fig. 7D). The possible involvement of E proteins in the denervation response was examined in the same fashion. Specifically, the effects of antisense-CTF4 and antisense-E12 were analyzed; as shown in Fig. 7, E and F, the targeting of either E protein affects the dener-
CTF4 and Acetylcholine Receptor Expression

Fig. 7. Effect of antisense constructs on reporter gene expression in vivo. The indicated amounts of antisense construct or vector were injected intramuscularly, together with standard amounts of reporter plasmids (40 μg of p2khLuc; 5 μg of pRSV-CAT), as described in the Methods section. Luciferase/CAT ratios are shown as percent of values from denervated muscle not treated with antisense constructs. IN, innervated; DN, denervated muscle; MG, myogenin; MD, MyoD.

DISCUSSION

In this study, we characterize the expression pattern of the gene encoding the chicken E protein CTF4 and describe its ability to transactivate, in concert with myogenin, the gene encoding the α-subunit of the AChR. As the chicken homologue of the human, rat, and mouse proteins HEB, REB, and ME1, CTF4 is a member of the A class of bHLH proteins and ubiquitously expressed. The primary transcript of CTF4 is alternatively spliced, with the longer mRNA containing an additional 72-bp segment encoding 24 amino acids whose presence disrupts a transactivation domain characteristic of the gene products of all three E protein loci. This region, including the flanking residues of the transactivation activation domain, is perfectly conserved at the amino acid level when compared with its homologues in the rat REB (20)/SCBP (21) and a Torpedo californica protein, attesting to the evolutionary antiquity and presumptive significance of this domain. The CNS also contains a minor band of about 180 nucleotides which may arise from alternate splicing at Cga/GTtG (splice junction consensus in capitals) within the intron shown in Fig. 1. Three different transcripts of the homologous gene have also been observed in the rat (21).

Basic-HLH proteins must dimerize before becoming functional transcription factors capable of binding to an E box-containing DNA sequence. As a rule such protein complexes are heterodimers; in fact some bHLH-containing proteins, such as c-myc and in particular the MDFs, do not efficiently form homodimers. This does not hold for CTF4; homodimers of both isoforms of CTF4 were equally capable of binding to the E box-containing α-subunit enhancer. In addition, homodimers of the shorter, but less so of the longer, isoform of CTF4 were able to transactivate a reporter gene under control of the α-subunit promoter in NIH/3T3 fibroblasts, a cell line containing very low levels of endogenous E proteins. As homodimers of each CTF4 isoform are equally able to bind to the E boxes of the α-subunit enhancer, differences in reporter enzyme levels are likely due to the relative effectiveness of the respective transactivation domains. When myogenin was co-expressed in the same experiment to allow for the formation of CTF4 heterodimers, only minimal differences in reporter activity were observed for the two E protein isoforms. The transactivation domain of myogenin apparently obviates the need for the E protein to provide such a domain itself. As has been pointed out previously, the alternative domain is predicted to form an ankyrin motif (20) which is likely to mediate protein-protein interactions. Ankyrin motifs exclude members of the Rel family of transcription factors from the nucleus during particular phases of the cell cycle by sequestering them in an inactive cytoplasmic complex with IκB (42). Although E proteins are abundantly expressed in both proliferating and recently differentiated cells, they possess potent growth-suppressive activity that must be carefully regulated to prevent premature exit from the cell cycle at the G₁ restriction point (43). It is possible that the ankyrin motif might allow for precise regulation of nuclear E protein concentrations during the cell cycle, an aspect that would not be reflected in our assays for gene expression in differentiated myofibers.

CTF4 exhibits tissue-specific regulation during development. The tissue distribution of the two isoforms in the 10-day embryo is not unlike the one described for REBα and REBβ in the adult rat (20) colon, a predominance of the long form in the brain; a prevalence of the short form in liver, lung, and skeletal muscle; and comparable amounts in the heart. The transcript is expressed at high levels at the early fetal stage (embryonic day 8/9) in both brain and muscle, two tissues that are late to mature. The brain continues to express high levels in the neonate chicken; however, in hind limb skeletal muscle, CTF4 mRNA undergoes a sharp decline at the transition from early to late fetus (embryonic day 14/15). It is at this period that neuromuscular junctions mature in chicken embryo leg muscle (44), and transcription of MyoD and myogenin is reduced to low levels in all muscle nuclei. Since the genes that encode the extrajunctional form of the AChR and the myogenic factors MyoD and myogenin lose activity at about the time of the down-regulation of CTF4, one might expect similar mechanisms to be at play. However, this does not seem to hold for the adult myofiber where denervation dramatically up-regulates, and membrane activity strongly suppresses, AChR and myogenin genes (33, 40) without similarly affecting CTF4 expression.

3 C. Neville, unpublished results.
AChR expression in skeletal muscle depends on the developmental stage of the tissue. While receptor genes are silent in myoblasts, they become activated during differentiation and myofiber formation; later, innervation of the myofiber and the ensuing electromechanical activity suppress the genes. This responsiveness to neuronal cues can be demonstrated by experimental manipulation: Section of the motor nerve causes cessation of activity and de-repression of AChR genes, while imposition of electrical activity again silences them. Consequently, AChR genes are much more active (~5–10-fold) in denervated than in innervated muscle (45). Since in the chicken the genes for MyoD, myf5, and herculin are little affected by innervation, it is likely that AChR gene expression is mainly dependent on myogenin. Our antisense experiments suggest that this is also true for denervation-triggered stimulation of AChR expression. It has previously been shown by Brunetti and Goldfine (46) that expression of the AChR δ subunit in BC3H-1 cells can be suppressed with antisense oligonucleotides directed against myogenin. The observation of these authors is in agreement with our findings, although it does not rule out a role, in muscle tissue, for other MDFs, especially MyoD (which is absent from BC3H-1 cells).

The parallel expression of myogenic factors and receptor genes during embryonic development and in adult muscle suggests a functional relationship, as the transcription factors are expressed when they might be active in stimulating receptor gene expression. The regulatory role of CTF4 during denervation-triggered receptor expression is not as clear, since the denervation response of the CTF4 gene is less pronounced. Nevertheless, transfection of an antisense expression vector leads to significant reduction in the activation of AChR promoters which otherwise is the hallmark of the denervation response. Perhaps the presence of CTF4 is necessary, but by itself not sufficient to stimulate AChR expression; being expressed constitutively, it may eventually become the limiting factor in denervation-induced AChR up-regulation. A possible explanation of the relatively low effectiveness of antisense-CTF4 (compared with the suppression of myogenin) is that, in the absence of CTF4, other E proteins which are still abundant in the nuclei have an opportunity to dimerize with, and activate, myogenin. A similar effect was observed with antisense-E12, even though the suppression of the reporter gene is a little less pronounced than the effect of antisense-CTF4. Perhaps the two E proteins share the task of dimerizing with myogenin; the importance of combined gene dosage effects for the function of E proteins has been pointed out before (47). A role for CTF4 in receptor expression is further suggested by its ability to associate with the α-subunit enhancer, and to enable myogenin and MyoD to do likewise.

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REFERENCES
1. Jennings, C. G. B., and Burden, S. J. (1993)Curr. Biol. 3, 75–81
2. Duclet, A., and Changeux, J.-P. (1995)Physiol. Rev. 75, 339–368
3. Weintraub, H., Genetta, T., and Kadesch, T. (1994)Genes Dev. 8, 2203–2211
4. Dutton E. K., Simon, A. M., and Burden S. J. (1993)Proc. Natl. Acad. Sci. U. S. A. 90, 2040–2044
5. Merlie, J. P., Muñoz, J., Cheng, T. C., and Olson, E. N. (1994)J. Biol. Chem. 269, 2461–2467
6. Bessereau, J.-L., Stratford-Perricaudet, L. D., Piette, J., Le Poupo, C., and Changeux, J.-P. (1994)Proc. Natl. Acad. Sci. U. S. A. 91, 1304–1308
7. Walke, W., Xiao, G., and Goldman, D. (1996)J. Neurosci. 16, 3641–3651
8. Salminen, A., Braun, T., Buchberger, A., Jura, S., Winter, B., and Arnold, H. (1991)J. Cell Biol. 115, 905–917
9. Edmondson, D. G., Cheng, T.-C., Cserjesi, P., Chakraborty, T, and Olson, E. N. (1992)Mol. Cell. Biol. 12, 3568–3577
10. Malik, S., Huang, C.-F., and Schmidt, J. (1995)J. Exp. Biol. 230, 88–96
11. Buskin, J. N., and Hausehka, S. D. (1989)Mol. Cell. Biol. 9, 2627–2640
12. Wentworth, B. M., Donoghue, M., Engert, J. C., Berglund, E. B., and Rosenthal, N. (1991)Proc. Natl. Acad. Sci. U. S. A. 88, 1242–1246
13. Murre, C., McCaw, P. S., and Baltimore, D. (1989)Cell 56, 777–783
14. Nelson, C., Shen, L. P., Meister, A., Fodor, E., and Rutter, W. J. (1990)Genes Dev. 4, 1043–1053
15. Cornelissen, B., Thornell, A., Hallberg, B., and Grundstrom, T. (1991)J. Virol. 65, 6084–6093
16. Henthorn, P., Kiledjian, M., and Kadesch, T. (1990)Science 247, 467–70
17. Zhang, Y., Babin, J., Feldhaus, A. J., Singh, H., Sharp, P. A., and Bina M. (1991)Nucleic Acids Res. 19, 4555
18. Tsay, H.-J., Choe, Y.-H., Neville, C. M., and Schmidt, J. (1992)Nucleic Acids Res. 20, 1805; Correction (1992)Nucleic Acids Res. 20, 2624
19. Hu, J. S., Olson, E. N., and Kingston, R. E. (1992)Mol. Cell. Biol. 12, 5031–5042
20. Klein E. S., Simmons, D. M., Swanson, L. W., and Rosenfeld, M. G. (1993)Genes Dev. 7, 55–71
21. Lin, H. H., Li, W.-Y., and Ann, D. K. (1993)J. Biol. Chem. 268, 10214–10220
22. Quong, M. W., Massari, M. E., Zwart, R., and Murre, C. (1993)Mol. Cell. Biol. 13, 792–800
23. Neville, C., and Rosenthal, N. (1996) in Eukaryotic Gene Transcription (Goodbourne, S., ed) pp. 192–233, Oxford University Press, New York
24. Blackwell, T. K., and Weintraub, H. (1990)Science 250, 1104–1110
25. Wright, W. E., Binder, M., and Funk, W. (1991)Mol. Cell. Biol. 11, 4104–4110
26. Shieh, B.-H., Ballivet, M., and Schmidt, J. (1987)J. Cell Biol. 104, 1337–1341
27. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983)Nucleic Acids Res. 11, 1475–1489
28. Wolf, J. A., Malone, R. W., Williams, P., Chong, W., Acsadi, G., Jani, A., and Felgner, P. L. (1990)Science 247, 1465–1468
29. Chirgwin, J. M., Prabhya, E., MacDonald, R. J., and Rutter, W. J. (1979)Biochemistry 18, 5294–5299
30. Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K., and Green, M. R. (1984)Nucleic Acids Res. 12, 7035–7056
31. Ausubel, F. M., Brent, R., Kinston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1995)Current Protocols in Molecular Biology, John Wiley & Sons, New York, pp. 9.7.1 and 9.7.12
32. Lin, Z.-Y., Dechesne, C. A., Eldridge, J., and Paterson, B. M. (1989)Genes Dev. 3, 986–996
33. Neville, C. M., Schmidt, M., and Schmidt, J. (1992)Cell. Mol. Neurobiol. 12, 511–527
34. Lassar, A. B., Buskin, J. N., Lockshon, D., Davis, R. L., Apone, S., Hausehka, S. D., and Weintraub, H. (1989)Cell 56, 823–831
35. Wang, Y., Xu, H.-P., Wang, X.-M., Ballivet, M., and Schmidt, J. (1988)Neuron 1, 527–534
36. Sanes, J., and Hall, Z. W. (1992)Cell/Neuron 7(10), suppl.) 99–121
37. Wang, G., and Schmidt, J. (1976)Brain Res. 114, 524–529
38. Wolf, J. A., Williams, P., Acsadi, G., Jiao, S., Jani, A., and Chong, W. (1991)BioTechniques 11, 474–485
39. Gilmore, T. D. (1996) in Eukaryotic Gene Transcription Goodbourne, S., ed) pp. 102–131, Oxford University Press, New York
40. Pookett, S. (1981)Brain Res. 227, 299–302
41. Tsay, H.-J., and Schmidt, J. (1989)J. Cell Biol. 108, 1523–1526
42. Brunetti, A., and Goldfine, I. D. (1990)J. Biol. Chem. 265, 5860–5869
43. Zhuang, Y., Cheng, T.-C., and Weintraub, H. (1996)Mol. Cell. Biol. 16, 2898–2905