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Expression of myogenic factors in skeletal muscle and electric organ of *Torpedo californica*

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Fish electric organ is a skeletal muscle homolog in which many muscle-specific genes are inhibited while acetylcholine receptor is expressed at high levels. The molecular mechanisms underlying this discoordinate regulation have not yet been explored. We have obtained partial sequences for MyoD, myogenin, and myf5 from *Torpedo californica* and have measured their mRNAs in several organs, using ribonuclease protection. We have found that MyoD and myf5 are expressed at comparable levels in muscle and electric organ, whereas myogenin transcripts could not be detected in either tissue. Acetylcholine receptor α subunit mRNA, on the other hand, is two orders of magnitude more abundant in electric tissue. We conclude that neither the loss of contractile proteins from, nor the enhanced expression of acetylcholine receptor genes in, the differentiating electrocyte is a simple consequence of the abundance of myogenic factor messages.

Myogenic factor; Electric organ; Skeletal muscle; Regulation; Acetylcholine receptor; *Torpedo californica*

1. INTRODUCTION

An analysis of the control of acetylcholine receptor (AChR) gene expression is important for an understanding of how myogenic cells differentiate and how they respond to neural signals. Like many genes that are specifically expressed in muscle, all AChR subunit genes analysed to date have been found to contain CANNTG elements (E boxes; MEF1 sites) which are recognized by the members of the MyoD family of regulatory proteins [1–7]. That AChR promoters should share *cis* elements with other muscle-specific genes is perhaps not surprising, since many of these genes are coordinately upregulated during differentiation. However, AChR expression does not always parallel expression of other muscle genes. For example, genes coding for the extrajunctional or embryonic receptor ($\alpha_2\beta\gamma\delta$) are strongly activated following denervation of mature muscle [8] when expression of other muscle genes is little changed. An extreme case of the discoordinate control of the expression of AChR and of a large fraction of muscle-specific proteins is the electric tissue of electric fish. The electric organ is a skeletal muscle homolog that forms during embryonic development. This process has been studied in detail in *Torpedo marmorata* [9–11]. Cells that give rise to the organ initially appear as myoblasts that fuse to form elongated myotubes with a cross-striated contractile apparatus, before they lose their myofibrils and flatten into mature electrocytes. These electrocytes are highly specialized cells that retain the apparatus for chemical excitability and certain proteins serving ancillary functions such as electrogenic ion pumps. However, they have ceased to express components of the myofibrils which in striated muscle comprise the bulk of the cellular protein. How the genes coding for these proteins are turned off while those coding for AChR subunits maintain or even increase their activity is an intriguing problem in muscle gene control. An investigation of this transition may shed light on mechanisms of receptor regulation and more generally on the control of the muscle phenotype.

As a first step in an analysis of electrocyte gene control we have prepared probes for myogenic factors from *Torpedo californica* (a close relative of *Torpedo marmorata*) and measured factor transcript levels in skeletal muscle and electric tissue.

Abbreviations: AChR, acetylcholine receptor; PCR, polymerase chain reaction.

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2. MATERIALS AND METHODS

2.1. *Probes for* Torpedo californica *mRNAs*

Two degenerate oligonucleotides, ccggatccTGCCTII(C/T)ITGGGCITGCAA(A/G)IIITGCAA(A/G)(A/C)GIAA and ccggatccAT(A/G)TAI(C/T)(G/T)IATIGC(A/G)I(A/T)IC(G/T)IAGIAT(C/T)TCIAC(C/T)TT, corresponding to regions shared by the four known myogenic factors, were synthesized (Oligos Inc., Guilford, CT). The upstream oligonucleotide was designed to encode CLPWACKXCKRK, a stretch of twelve amino acids just upstream of the basic DNA binding region, of which ten are conserved in all myogenic genes from Drosophila to Homo. The downstream primer encodes KVEILR(N/S)AIRY, a portion of helix II of the dimerization domain. One μg of *Torpedo californica* genomic DNA was amplified by the polymerase

chain reaction (PCR) under the following conditions: 5 cycles – 95°C/1 min, 37°C/1 min, ramp/2 min, 72°C/30 s; 25 cycles – 94°C/1 min, 55°C/1 min, 72°C/1 s. The flanking *Bam*HI sites generated by the primers in the resulting 210-bp product were cleaved, and the fragments inserted into the M13 vector PhageScript SK (Stratagene, La Jolla, CA). Individual plaques were grown up and sequenced, using Sequenase 2.0, following the manufacturer's recommendations (U.S. Biochemical, Cleveland, OH), to ascertain their identities. Inserts corresponding to MyoD, myogenin, and myf5 were PCR-amplified with the flanking universal Reverse and −20 primers, and transcribed, using the appropriate phage RNA polymerase to generate ^{32}P-labelled antisense riboprobe. A template for the synthesis of *Torpedo californica* AChR α-subunit riboprobe was constructed by purifying a 105-bp *Sau*3AI-*Eco*RV fragment from the plasmid DOLα [12] and subcloning it in the Bluescript plasmid pKSII– (Stratagene, La Jolla, CA).

2.2. *Preparation and analysis of mRNA*

Torpedo californica organs, obtained from an adult fish and immediately frozen in liquid nitrogen, were purchased from Marinus, Inc. (Long Beach, CA). Total RNA was extracted by a protocol adapted from the guanidinium isothiocyanate method of Chirgwin et al. [13]. mRNA levels were measured by ribonuclease protection assay essentially as described [14]. The protected bands in the gels were visualized by autoradiography and quantified with a Betascope 603 blot analyzer (Betagen, Waltham, MA).

2.3. *Synthesis of cDNA*

Five μg of total RNA were reverse-transcribed in 50 μl of buffer containing 50 mM Tris-HCl pH 8.3, 75 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM DTT, 4 mM Na pyrophosphate, 1 mM dNTPs, 50 units RNasin (Promega, Madison, WI), 2.5 μg random hexamer, and 50 units AMV reverse transcriptase. The reaction was allowed to proceed at 42°C for one hour, after which the enzyme was heat-inactivated. PCR amplifications were subsequently done on 1-μl aliquots.

2.4. *Isolation of genomic DNA*

Frozen electric tissue was pulverized with a mortar and pestle in the presence of liquid nitrogen. The powder was resuspended in a solution containing 10 mM Tris-HCl pH 7.4, 10 mM EDTA, 150 mM NaCl, 0.4% SDS, and 1 mg/ml proteinase K. It was then incubated at 65°C for two hours, followed by gentle rocking overnight at 37°C. The solution was extracted twice with phenol/chloroform and once with chloroform. One-twentieth of one volume of 3 M sodium acetate and two volumes of ethanol were added. Genomic DNA was spooled on a glass pipette and rinsed in 70% ethanol followed by 100% ethanol. The DNA was allowed to briefly air dry and was dissolved in 10 mM Tris-HCl pH 7.4, 1 mM EDTA.

3. RESULTS AND DISCUSSION

3.1. *Identification of* Torpedo californica *myogenic factor genes*

To determine which MyoD-related genes *Torpedo californica* might possess we amplified genomic DNA by PCR, using as primers two highly conserved regions in the DNA binding-dimerization motif that have enabled us previously to isolate gene fragments of the four chicken myogenic factors [15]. The forward primer encodes the cysteine-rich sequence (CLPWACKXCKRK) just upstream of the basic DNA-binding region; the reverse primer corresponds to Helix II, the region necessary for dimerization. The former is unique to the myogenic factor family and is the most highly conserved region in mammals, while the latter is moderately conserved through the entire HLH super-family [16].

Genomic DNA which should contain potential target sequences at comparable frequency was chosen as template for PCR instead of cDNA because the extent of expression of the myogenic genes in either muscle or electric tissue was unknown. The primers amplify a 194-bp region that is not interrupted in any mammalian gene for which the genomic structure is known [17], although an intron occurs in the Drosophila gene [18]. Three distinct sequences were identified in the initial pool of amplified fragments. Each occurred with approximately equal frequency. Translation of the nucleotide sequences showed these to correspond to MyoD, myogenin, and myf5; the *Torpedo californica* sequences differ from their mammalian counterparts by very few, conservative substitutions (Fig. 1). A phylogenetic analysis of the human myogenic factors suggests that the gene duplication that gave rise to herculin and myogenin preceded that which generated MyoD and myf5 and that, consequently, herculin should be present in the *Torpedo californica* genome. However, herculin could not be identified by PCR in either genomic or cDNA preperations even when gene-specific primers were used.

3.2. *Tissue expression of* Torpedo californica *myogenic factor mRNA*

In higher vertebrates, the myogenic factors act as muscle-specific transactivators, and their expression is restricted to myogenic cells. They all share the ability to convert fibroblasts to muscle cells, but in vivo are expressed in the myogenic lineage at different times of development and consequently may be assumed to serve specific rather than completely exchangeable functions [19]. To assess their tissue expression in *Torpedo californica*, total RNA was isolated from several different organs of an adult specimen and assayed for specific mRNAs. The MyoD message is expressed in both myogenic tissues in approximately equal amounts (1.6 fmol/mg RNA). Message coding for myf5 is present in muscle at similar levels (1.6 fmol/mg RNA), but is somewhat less abundant in electric tissue (0.8 fmol/mg RNA). No myogenin mRNA was detected in either tissue at this stage. Very low levels could have escaped detection; the limits of sensitivity of the assay are estimated at approximately 0.03 fmol per mg total RNA. Quantification of receptor α-subunit message revealed levels in electric organ (28 fmol/mg RNA) exceeding those in skeletal muscle by approximately 300-fold. No evidence for the expression of myogenic factors (or receptor α subunit) was found in any of the other organs tested, including cardiac muscle (Fig. 2).

No obvious candidate for transactivator of the receptor α subunit gene emerges from these observations. Both MyoD and myf5 are expressed to a comparable degree in the two tissues and may be involved in the

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MyoD

TTNADRRKAATMRERRRLSKVNEAFETLKRCTSTNPNQRLP   Torpedo
-------------------------------S-------   Human

Myogenin

SVTLDRRRAATLREKRRLKKVNEAFEALKRSTLLNPNQRLP   Torpedo
--SV-----------------------------------   Human

myf5

SSNTDRRKAATMRERRRLKKVNQAFETLKRCTSANPNQRLP   Torpedo
-TTH----------------------------TT-------   Human

<----Basic---><----Helix I---><-Loop-><-Helix II-
```

Fig. 1. Aligned predicted amino acid sequences of *Torpedo californica* myogenic factor bHLH regions. *Torpedo californica* genomic DNA was PCR amplified as described in section 2. The deduced amino acid sequences are shown for the three unique amplified DNA fragments. By alignment with their human counterparts, from which they differ in one, 2, and 5 residues, they are identified as the basic region/helix I/loop segments of MyoD, myogenin, and myf5, respectively.

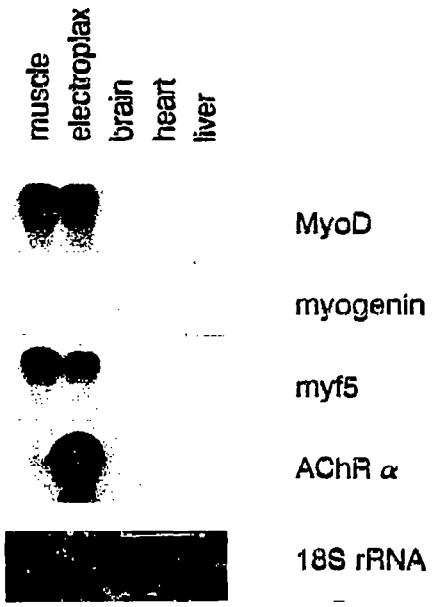

Fig. 2. Expression of myogenic factors in various tissues of adult *Torpedo californica*. Total RNA (30 μg) from each of the indicated tissues was hybridized separately with MyoD, myogenin, myf5, and AChR α subunit riboprobe and subjected to nuclease protection as described in section 2. Relative quantity and integrity of total RNA was determined by electrophoresis of three μg from each tissue in a formaldehyde agarose gel in the presence of ethidium bromide [27]. The 18S rRNA band is shown at the bottom.

transcription of the muscle-specific genes that remain active when myofibers become electrocytes including the genes coding for AChR subunits. MyoD could be involved as it may also regulate the expression of the junctional type of AChR in higher vertebrates where it is present in innervated muscle [20–22] and has been shown in cotransfection experiments to activate the promoters of the chick α [2], and mouse and chick γ [4,7] subunit genes. The large quantitative difference in α-subunit mRNA levels between muscle and electroplax would have to be accounted for by some as yet unknown regulatory feature (e.g. larger amount or higher activity of the factor protein, stabilization of receptor messages, etc.).

The apparent absence of myogenin deserves a comment. In mammals, myogenin is expressed in embryonic muscle, but then virtually disappears during early postnatal development. Upon denervation it is strongly upregulated, about 20- to 40-fold in rat [22] and mouse [20,21], and about 200-fold in the chick where it could play a role in the dramatic proliferation of the extrajunctional or embryonic type of AChR underlying denervation supersensitivity [15]. Perhaps the junctional type of receptor does not require myogenin for expression and instead relies primarily on MyoD which is present in innervated muscle, and is only moderately upregulated upon nerve section, both in rat (~4-fold, [20]) and in chick (~2- to 5-fold [15]). Although the *Torpedo californica* electric tissue AChR comprises a γ subunit, this is a historical quirk of nomenclature rather than an indication of the embryonic nature of the receptor. The *Torpedo californica* γ subunit resembles the mammalian ε subunit in sequence [23], phosphorylation sites [24], and immunoreactivity [25]; the electric organ receptor also has the high conductance and short open time characteristic of an adult endplate receptor in higher vertebrates [26]. Therefore, if the electroplax receptor is of the junctional type which the biochemical and biophysical data seem to suggest, then the two tissues under investigation clearly would not need a factor which in higher vertebrates is linked to the appearance of extrajunctional receptors.

At present we do not know the molecular mechanisms underlying the transdifferentiation of myotubes into electrocytes, and in particular the processes that account for the down-regulation of the myofibrillar proteins and for the enhanced expression of AChR. Our study of the expression of MyoD, myogenin, and myf5 indicates that transcript levels of these myogenic factors and cellular phenotype are not correlated and that other factors (e.g. herculin or proteins outside the MyoD family) and events (e.g. posttranscriptional regulation) must be invoked and remain to be identified in future investigations.

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