Separate pathways for synapse-specific and electrical activity-dependent gene expression in skeletal muscle

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SUMMARY
Signaling between nerve and muscle is mediated by multiple mechanisms, including two transcriptional pathways. Signals provided by the nerve terminal activate transcription of acetylcholine receptor (AChR) genes in myofiber nuclei near the synaptic site, and signals associated with myofiber electrical activity inactivate AChR gene expression throughout the myofiber. These opposing effects of innervation are conferred by 1.8 kb of 5′ flanking DNA from the AChR δ subunit gene. These results raise the possibility that synapse-specific and electrical activity-dependent gene expression are mediated by the same DNA sequence and that activation and repression are determined by differential regulation of the same DNA binding protein. We produced transgenic mice carrying AChR δ subunit-hGH gene fusions, and we show here that a binding site (E-box) for myogenic basic helix-loop-helix proteins is required for electrical activity-dependent but not for synapse-specific gene expression of the δ subunit gene. These results indicate that a change in the expression or activity of an E-box binding protein(s) mediates electrical activity-dependent gene regulation and that synapse-specific and electrical activity-dependent gene expression require different DNA sequences. Moreover, we show here that the cis-acting elements for both aspects of innervation-dependent gene regulation are contained in 181 bp of 5′ flanking DNA from the AChR δ subunit gene.

Key words: Acetylcholine receptor, neuromuscular synapse, transgenic mice, myogenin, MyoD

INTRODUCTION
Neuromuscular synapses form as a consequence of inductive interactions between nerve and muscle. Following contact between nerve and muscle during development, the nerve terminal and the postsynaptic region of the muscle fiber undergo a complex differentiation program and become specialized for synaptic transmission. One of the more striking and well-studied aspects of postsynaptic differentiation is the accumulation of acetylcholine receptors (AChRs) at synaptic sites, and the regulation of this process has served as a model for understanding mechanisms of synaptic differentiation (Jennings and Burden, 1993; Hall and Sanes, 1993).

Both post-translational and transcriptional mechanisms have an important role in accumulating AChRs at synaptic sites and excluding AChRs from non-synaptic regions of muscle. The post-translational process is controlled by agrin, a protein that is synthesized by motor neurons and deposited into the synaptic basal lamina, where it triggers a redistribution of AChRs from non-synaptic membrane to synaptic sites (McMahan, 1990; Nastuk and Fallon, 1993). The number and distribution of AChRs in skeletal muscle is also regulated by two transcriptional processes. One transcriptional pathway activates AChR gene expression in myofiber nuclei near the synaptic site, leading to an accumulation of AChR mRNA and protein at synaptic sites (Klarsfeld et al., 1991; Simon et al., 1992; Sanes et al., 1991; Burden, 1993). The signal for synapse-specific gene expression is not known, but like agrin, it is located in the synaptic basal lamina (Jo and Burden, 1992; Brenner et al., 1992; Goldman et al., 1991). Myofiber electrical activity controls a second transcriptional pathway that inactivates AChR gene expression in nuclei throughout the myofiber (Tsay and Schmidt, 1989; Merlie and Kornhauser, 1989; Simon et al., 1992). Because electrical activity regulates the expression of genes encoding adhesion molecules, transcription factors and voltage-gated channels (Covault et al., 1986; Hahn and Covault, 1992; Witzemann and Sakmann, 1991; Eftimie et al., 1991; Neville et al., 1992; Yang et al., 1991), knowledge of the electrical activity-dependent signalling pathways will be an important step in understanding how myofiber electrical activity controls muscle physiology.

Cell culture studies have shown that the cis-acting sequences for electrical activity-dependent expression of the AChR δ subunit gene are contained in less than 200 bp of 5′ flanking DNA (Chahine et al., 1992; Dutton et al., 1993). These results raise the possibility that synapse-specific and electrical activity-dependent gene expression are mediated by the same DNA sequence and that activation and repression are determined by differential regulation of the same DNA binding protein. We produced transgenic mice carrying AChR δ subunit-hGH gene fusions, and we show here that a binding site (E-box) for myogenic basic helix-loop-helix proteins is required for electrical activity-dependent but not for synapse-specific gene expression of the δ subunit gene. These results indicate that a change in the expression or activity of an E-box binding protein(s) mediates electrical activity-dependent gene regulation and that synapse-specific and electrical activity-dependent gene expression require different DNA sequences. Moreover, we show here that the cis-acting elements for both aspects of innervation-dependent gene regulation are contained in 181 bp of 5′ flanking DNA from the AChR δ subunit gene.

Key words: Acetylcholine receptor, neuromuscular synapse, transgenic mice, myogenin, MyoD
et al., 1991; Neville et al., 1992), myogenin could mediate the increase in AChR gene expression that follows loss of myofiber electrical activity.

In a previous study we used transgenic mice to show that the cis-acting sequences for both synapse-specific and electrical activity-dependent expression of the AChR δ subunit gene are contained in 1.8 kb of 5′ flanking DNA (Simon et al., 1992). These results raise the possibility that the same cis-acting element(s) confers both aspects of innervation-dependent expression. Here, we show that the critical cis-acting sequences for both aspects of innervation-dependent expression are not distributed throughout the δ subunit regulatory region but are contained in 181 bp of the δ subunit promoter. Moreover, we show that an E-box near the transcription start site of the δ subunit gene is required for electrical activity-dependent but not for synapse-specific gene expression. These results indicate that a change in the expression or activity of an E-box binding protein(s) mediates electrical activity-dependent gene regulation and that synapse-specific and electrical activity-dependent gene expression require different DNA sequences.

MATERIALS AND METHODS

Founder mice carrying the δ(−1,823/mut. E1/+25)-hGH transgene were produced using methods similar to those described previously (Simon et al., 1992) and were the source of thirteen different lines. We prepared and stained extensor digitorum longus and soleus muscles for hGH expression as described previously (Simon et al., 1992). Synaptic hGH staining was observed in three lines, and the pattern of hGH staining in each line is indistinguishable from that in lines carrying a δ(−1,823/+25)-hGH transgene. hGH expression is evident at all synaptic sites in muscle fibers from mice carrying a δ(−1,823/+25)-hGH transgene (Simon et al., 1992) but only at 8-35% of synaptic sites in muscle fibers from mice carrying an E1-mutated transgene. We measured hGH mRNA in the three lines that have synaptic staining and in five of the ten lines that lack synaptic staining. Each of the three lines that have synaptic staining express hGH mRNA (Table 1), whereas four of the five lines that lack synaptic staining do not express detectable hGH mRNA. One line lacks synaptic staining, but expresses very low levels of hGH mRNA.

We produced six lines carrying the δ(−181/+25)-hGH transgene. Synaptic hGH staining was observed in three lines carrying the δ(−181/+25)-hGH transgene, and the pattern of hGH staining is indistinguishable from that in mice carrying a δ(−1,823/+25)-hGH transgene. We measured hGH mRNA in the three lines that had synaptic staining and in one of the three lines that lacked synaptic staining; we found that only the lines with synaptic staining express detectable hGH mRNA. We produced one line carrying the CK(−1,200/+54)-hGH transgene (Dutton et al., 1993).

The sequence of E1 (CACCCTG) was mutated (CACCAG) by site-directed mutagenesis, causing a >60-fold decrease in affinity for recombinant MyoD or myogenin (Simon and Burden, unpublished results) and a 20-fold reduction in δ subunit expression in cultured myotubes (Simon and Burden, 1993). Leg muscles were denervated for 4-5 days, and mRNA levels were measured by RNase protection and quantitated by using a Phosphor-Imager as described previously (Simon et al., 1992).

RESULTS

AChR mRNA in adult myofibers is concentrated at synaptic sites (Merlie and Sanes, 1985; Fontaine et al., 1988; Goldman and Staple, 1989; Brenner et al., 1990). In a previous study we used transgenic mice to study the mechanisms responsible for localizing AChR mRNAs to synaptic sites (Simon et al., 1992). These mice carry a gene fusion between 1.8 kb of 5′ flanking DNA from the murine AChR δ subunit gene and the human growth hormone (hGH) gene. Because hGH is processed in intracellular organelles prior to secretion, and because these organelles are closely associated with nuclei, we could infer the nuclear source of hGH transcription by studying the spatial distribution of intracellular hGH by immunocytochemistry. We showed that hGH is restricted to the synaptic region of the myofiber, and we concluded that transcription of the endogenous AChR δ subunit gene is confined to nuclei situated at the synaptic site (Simon et al., 1992; see also Klarsfeld et al., 1991; Sanes et al., 1991; Duclert et al., 1993).

This 1.8 kb of 5′ flanking DNA contains three binding sites (E-boxes) for myogenic basic helix-loop-helix (bHLH) proteins. The E-box nearest to the transcription start site, E1, unlike the other two E-boxes, is important for muscle-specific gene expression (Simon and Burden, 1993). To determine whether E1 is necessary for synapse-specific gene expression, we produced mice carrying a transgene with a mutation in E1. Fig. 1 shows that this transgene, δ(−1,823/mut. E1/+25)-hGH, is expressed selectively at synaptic sites. These results demonstrate that E1 is not required for synapse-specific expression.

AChR gene expression increases following denervation, causing an increase in the abundance of AChR mRNAs (Jennings and Burden, 1993; Hall and Sanes, 1993). We showed that 1.8 kb of 5′ flanking DNA from the δ subunit gene confers electrical activity-dependent gene regulation, because hGH mRNA levels are 10- to 20-fold higher in denervated than in innervated muscle of mice carrying a δ(−1,823/+25)-hGH transgene (Simon et al., 1992; see also Merlie and Kornhauser, 1989). To determine whether E1 is required for such regulation, we denervated muscle from mice carrying the E1-mutated transgene and measured the level of hGH mRNA in innervated and denervated muscle. Fig. 2 shows that mutation of E1 blocks electrical activity-dependent regulation: muscle dere

<table>
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<th>Transgene</th>
<th>Expression in Den./Inn. muscle</th>
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<tr>
<td>δ(−1,823/+25)-hGH</td>
<td></td>
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<tr>
<td>line 5GH1</td>
<td>27±1.3</td>
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<td>δ(−1,823/mut. E1/+25)-hGH</td>
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<td>line 450</td>
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<td>line 456</td>
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<td>line 460</td>
<td>2.3±0.72</td>
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<tr>
<td>δ(−181/+25)-hGH</td>
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<tr>
<td>line 4</td>
<td>50±20</td>
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<tr>
<td>line 11</td>
<td>38±12</td>
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<td>line 468</td>
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Duplicate measurements of hGH mRNA expression were made from each muscle. The level of hGH expression was normalized to the level of actin expression, and the ratio of expression in denervated/innervated muscle was determined. The mean ± s.e.m. from several (n) animals are shown. Because the abundance of actin mRNA is ~20% lower in denervated than in innervated muscle (Simon et al., 1992), the values shown would be reduced by 1.25-fold if hGH expression was normalized to the level of total RNA. E1 is necessary for maximal expression of the transgene in innervated muscle, since hGH mRNA levels are ~7-fold greater in innervated muscle of mice expressing the δ(−1,823/+25)-hGH transgene (Simon et al., 1992) than in innervated muscle of mice expressing the E1-mutated transgene. These results indicate that E1 is required for establishing the level but not the pattern of δ subunit gene expression in innervated muscle.
Innervation-dependent gene regulation causes a 27-fold increase in hGH expression in mice carrying the δ(-1,823/+25)-hGH transgene (C,D). This transgene contains E1 but lacks E2 and E3. This regulatory region confers electrical activity-dependent expression in spontaneously active muscle cells in cell culture (Dutton et al., 1993), and Fig. 2 shows that innervation-dependent gene expression, we constructed mice carrying a transgene containing only 181 bp of 5' flanking DNA from the δ subunit gene. This transgene contains E1 but lacks E2 and E3. This regulatory region confers electrical activity-dependent expression in spontaneously active muscle cells in cell culture (Dutton et al., 1993), and Fig. 2 shows that...

**Fig. 1.** hGH expression is restricted to synaptic sites in muscle fibers from mice carrying either an δ(-1,823/+25)-hGH (A,B) or an δ(-1,823/mut. E1/+25)-hGH transgene (C,D). Intracellular hGH is contained within organelles associated with secretion, including the endoplasmic reticulum, the Golgi apparatus and secretory vesicles (Simon et al., 1992). Synaptic sites (A,C) were identified by staining with tetramethylrhodamine α-bungarotoxin (TMR-α-BGT), and hGH (B,D) was detected by indirect immunofluorescence (Simon et al., 1992). Bar, 10 µm.

Innervation causes a 27-fold increase in hGH expression in mice carrying the δ(-1,823/+25)-hGH transgene but only a 1.6-fold increase in mice carrying the E1-mutated transgene (Table 1). These results demonstrate that E1 is required for electrical activity-dependent gene regulation.

To delineate more precisely the cis-acting sequences for innervation-dependent gene expression, we constructed mice carrying a transgene containing only 181 bp of 5' flanking DNA from the δ subunit gene. This transgene contains E1 but lacks E2 and E3. This regulatory region confers electrical activity-dependent expression in spontaneously active muscle cells in cell culture (Dutton et al., 1993), and Fig. 2 shows that...
this sequence confers electrical activity-dependent regulation in transgenic mice. Fig. 2 shows that the δ(-1,823/+25)-hGH transgene, like the δ(-1,823/+25)-hGH transgene, is selectively expressed at synaptic sites. Thus, the cis-acting sequences for both aspects of innervation-dependent expression are located near the proximal promoter.

To control for potential differences in the secretory pathway at synaptic and non-synaptic regions of skeletal muscle that might influence accumulation of hGH, we constructed transgenic mice carrying a gene fusion between hGH and the creatine kinase (CK) regulatory region (Materials and Methods). The CK gene is expressed in skeletal muscle, but is not regulated by innervation (Dutton et al., 1993; Chahine et al., 1992). Fig. 3 shows that hGH in these transgenic mice is not concentrated at synaptic sites but is found throughout the myofiber. Thus, neither sequences in the hGH gene nor mRNA nor potential differences in the secretory pathway in synaptic and non-synaptic regions can account for selective expression of hGH at synaptic sites. Therefore, accumulation of hGH in the synaptic region of myofibers containing δ-hGH transgenes is due to sequences in the δ subunit gene.

DISCUSSION

This study demonstrates that the E-box near the transcription start site of the AChR δ subunit gene is required to confer electrical activity-dependent regulation, and these results indicate that an increase in the expression and/or activity of an E-box binding protein(s) mediates the increase in δ subunit expression following denervation.

Because myogenin expression increases following denervation in all species tested (Witzemann and Sakmann, 1991; Eftimie et al., 1991; Ducrèt et al., 1991; Neville et al., 1992), myogenin is a likely candidate to mediate electrical activity-dependent expression of downstream genes. The steps and mechanisms that mediate regulation of the myogenin gene by electrical activity are not clear, but an E-box in the regulatory region of the myogenin gene is thought to be important for gene expression during myogenesis (Yee and Rigby, 1993). Thus, it is possible that an E-box in the myogenin gene is required for electrical activity-dependent regulation. The regulatory region of the myogenin gene, however, contains additional cis-acting elements, including a MEF-2 binding site, which are important for myogenin gene expression during development (Edmondson et al., 1992; Cheng et al., 1993) and which may have a role in regulating myogenin expression in innervated and denervated muscle.

Multiple mechanisms for modulating the activity of E-box binding proteins have been described (Lassar et al., 1989;
Benezra et al., 1990; Bengal et al., 1992; Li et al., 1992a), including phosphorylation catalyzed by protein kinase C (PKC) (Li et al., 1992b). PKC is a potential negative regulator of myogenic bHLH proteins in innervated muscle, since electrical activity increases PKC activity (Huang et al., 1992), and this increased PKC activity is important for repressing AChR expression in electrically active muscle (Huang et al., 1992; Klarfeld et al., 1989: Laufer and Changeux, 1989). A single threonine residue in the basic region of myogenin is responsible for PKC-mediated inactivation of myogenin activity, and this critical threonine residue is conserved in MyoD, myf-5 and MRF-4 (Li et al., 1992b). Thus, all of the myogenic bHLH proteins are potential substrates for PKC. The data presented here are consistent with the idea that a decrease in myogenin expression and/or a decrease in the activity of myogenic bHLH proteins is responsible for the decrease in AChR expression in electrically active muscle.

E-boxes are clearly not sufficient to confer electrical activity-dependent regulation, since many genes that are expressed in skeletal muscle and that contain E-boxes are not regulated by electrical activity. Moreover, two E-boxes are present in the E1-mutated δ subunit gene, but these E-boxes do not confer regulation by electric activity. Although the mechanisms that distinguish these different E-boxes remain obscure, these results suggest that differences in the core sequence, context and/or position of E-boxes are likely to be important for conferring electrical activity-dependent regulation.

Although our immunocytochemical experiments show that the pattern of hGH expression is not altered by a mutation in E1, the level of expression from synaptic nuclei does appear to be reduced. Because we are unable to detect expression from non-synaptic nuclei in innervated muscle, we do not know whether mutation of E1 causes a similar reduction in expression from non-synaptic nuclei. Thus, we cannot exclude the possibility that E1 contributes to quantitative differences in expression of the δ subunit gene between synaptic and non-synaptic nuclei.

The E-box near the transcription start site of the δ subunit gene is important for muscle-specific and electrical activity-dependent expression. Synapse-specific expression, however, does not require E1. Likewise, E-boxes are dispensable for synaptic expression of the AChR ε subunit gene (Duclert et al., 1993). The cis-acting region for synapse-specific expression contains potential binding sites for several DNA-binding proteins, including AP2, CBF40 and myb (Simon and Burden, 1993), and these proteins, or still others could have a role in synapse-specific gene expression. Knowledge that the regulatory element(s) that confers synapse-specific expression is located within 181 bp of the δ subunit gene should simplify identification of the key cis-acting sequence and facilitate characterization of the proteins that control expression of the δ subunit gene in synaptic nuclei.

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