AML1 Is Expressed in Skeletal Muscle and 
Is Regulated by Innervation

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Although most skeletal muscle genes are expressed at similar levels in electrically active, innervated muscle and in electrically inactive, denervated muscle, a small number of genes, including those encoding the acetylcholine receptor, N-CAM, and myogenin, are expressed at significantly higher levels in denervated than in innervated muscle. The mechanisms that mediate electrical activity-dependent gene regulation are not understood, but these mechanisms are likely to be responsible, at least in part, for the changes in muscle structure and function that accompany a decrease in myofiber electrical activity. To understand how muscle activity regulates muscle structure and function, we used a subtractive-hybridization and cloning strategy to identify and isolate genes that are expressed preferentially in innervated or denervated muscle. One of the genes which we found to be regulated by electrical activity is the recently discovered acute myeloid leukemia 1 (AML1) gene. Disruption and translocation of the human AML1 gene are responsible for a form of acute myeloid leukemia. AML1 is a DNA-binding protein, but its normal function is not known and its expression and regulation in skeletal muscle were not previously appreciated. Because of its potential role as a transcriptional mediator of electrical activity, we characterized expression of the AML1 gene in innervated, denervated, and developing skeletal muscle. We show that AML1 is expressed at low levels in innervated skeletal muscle and at 50- to 100-fold-higher levels in denervated muscle. Four AML1 transcripts are expressed in denervated muscle, and the abundance of each transcript increases after denervation. We transfected C2 muscle cells with an expression vector encoding AML1, tagged with an epitope from hemagglutinin, and we show that AML1 is a nuclear protein in muscle. AML1 dimerizes with core-binding factor β (CBF β), and we show that CBF β is expressed at high levels in both innervated and denervated skeletal muscle. PEBP2α, which is structurally related to AML1 and which also dimerizes with CBF β, is expressed at low levels in skeletal muscle and is up-regulated only weakly by denervation. These results are consistent with the idea that AML1 may have a role in regulating gene expression in skeletal muscle.

Vertebrate neuromuscular synapses arise as a result of complex interactions between motor neurons and developing skeletal muscle cells (20). One of the more striking specializations of the neuromuscular synapse is the remarkable concentration of acetylcholine receptors (AChRs) in the postsynaptic muscle membrane (20, 25). Experiments designed to determine how AChR expression is regulated have revealed that a combination of three separate mechanisms mediates the clustering of AChRs at synaptic sites. First, motor neurons synthesize and deposit agrin in the synaptic basal lamina, where it induces a redistribution of non-synaptic AChRs to the newly formed synapse (1, 31, 38). Second, an unknown factor, which like agrin is deposited into the synaptic basal lamina and which is presumably synthesized by motor neurons, activates a signalling pathway leading to transcription of AChR genes selectively in nuclei near the synaptic site (8, 34, 47). Finally, nerve-induced muscle electrical activity represses AChR transcription throughout the muscle fiber, decreasing the number of AChRs expressed in the non-synaptic region (20, 25).

The molecular mechanisms that mediate electrical activity-dependent regulation of AChR genes are unknown, but a clearer view of the electrical activity-dependent signalling pathway is beginning to emerge (23, 29, 51). Recent studies demonstrate that a binding site (E box) for myogenic basic helix-loop-helix (bHLH) proteins in the AChR 8-subunit gene is critical for activity-dependent gene regulation, and these results suggest that a E box-binding protein(s) is directly involved in electrical activity-dependent regulation of the 8-subunit gene (51). Because the level of mRNAs encoding myogenic bHLH proteins rises substantially following denervation (14, 15, 39, 52, 58), these proteins may directly mediate the increase in AChR expression in denervated muscle by binding to the AChR regulatory region (25). Nevertheless, electrical activity-dependent regulation of the AChR subunit genes may be controlled by transcription factors in addition to E box-binding proteins, and expression of other genes that are regulated by electrical activity may be controlled by other pathways and transcription factors. Thus, it remains unclear whether E box-binding proteins are the only transcriptional mediators of electrical activity-dependent regulation.

In addition to its role in repressing AChR gene expression, nerve-induced muscle activity has been implicated as a regulator of other steps in the formation and maturation of synapses. For example, motor neuron survival, motor neuron growth, and editing of initial synaptic connections are affected by nerve-induced muscle activity (20). To understand how muscle activity regulates muscle structure and function, we used a subtractive-hybridization and cloning strategy to identify and isolate genes that are expressed preferentially in innervated or denervated muscle. We identified several genes that are expressed preferentially in denervated muscle and one gene that is expressed preferentially in innervated muscle. One of the genes that we found to be regulated by electrical activity

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is the recently discovered acute myeloid leukemia 1 (AML1) gene (37).

Disruption and translocation of the human AML1 gene are responsible for a form of acute myeloid leukemia (16, 37). AML1 is a DNA-binding protein, containing a DNA-binding region homologous to the Drosophila runt protein (5, 12, 16, 27). The normal function of AML1, however, is not known, and its expression and regulation in skeletal muscle were not previously appreciated. AML1 is thought to dimerize with core-binding factor β (CBF β) (40, 41, 54), and translocations in the β-subunit gene likewise cause acute myeloid leukemia (30). The AML1/CBF β heterodimer was purified independently on the basis of its ability to bind core sequences in murine leukemia virus enhancers (49, 53), which are important for viral pathogenesis (21, 50). Because of its potential role as a transcriptional mediator of electrical activity, we characterized expression of the AML1 gene in innervated, denervated, and developing skeletal muscle.

MATERIALS AND METHODS

Subtractive-hybridization and cloning. cDNA was synthesized as described elsewhere (55), except that only 2 μg of poly(A)⁺ RNA was included in the first strand synthesis reaction. Linkers were added to the blunt-ended cDNA, and cDNA in the range of 150 bp to 1 kb was gel purified and pooled. PCR amplification, photobiotinylation of driver DNA, hybridization, and removal of biotinylated driver DNA were done as described elsewhere (55), except that only 2 μg of target DNA and 40 μg of driver DNA were included in the hybridization reaction mixtures. Four rounds each of long and short hybridizations were carried out before the subtracted DNA was cloned into the EcoRI site of pBluescript IISK+ to generate the subtracted libraries. The libraries were screened with random-primed ³²P-labelled subtracted probe, and positive colonies were then subjected to further analysis. DNA was sequenced with Sequenase, according to the manufacturer’s (U.S. Biochemicals) instructions.

We initially screened 20 colonies from the denervated subtracted library; 13 colonies contained cDNA encoding B-crystallin, and 7 colonies contained cDNA encoding either N-CAM, filamin, or AML1. We prepared a second subtracted library by subtracting crystallin sequences from the original library, and we screened 19 colonies from this denervated subtracted library. Eighteen colonies contained cDNAs encoding mRNAs that are expressed at higher levels in denervated than in innervated skeletal muscle; these included cDNAs encoding AChR subunits, N-CAM, filamin, DEN1, and DEN2.

RNA analysis. RNase protection assays were performed as described previously (54). Briefly, antisense RNA was hybridized with total RNA (10) in hybridization buffer overnight at 42°C. Nonhybridized RNA was digested with RNases (A and T₁) for 1 h at 30°C. Following protease K digestion and phenol extraction, the protected RNA was precipitated by ethanol and fractionated in 5% denaturing polyacrylamide gels.

mRNA was purified by oligo(dT) cellulose chromatography (3), electrophoresed in a 1% formaldehyde agarose gel, and transferred to a GeneScreen (Du Pont) membrane. DNA probes were labelled by random-hexamer priming. The membranes were hybridized overnight in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA [pH 7.7]), 5× Denhardt’s solution, 0.1% sodium dodecyl sulfate (SDS), and 100 μg of salmon sperm DNA per ml at 68°C. The filters were washed in 0.1% SDS–0.1× SSPE for 1 h at 68°C and exposed for 2 days with an intensifying screen. Northern (RNA) blots and RNase protection assays were quantitated with a PhosphorImager.

Epitope tagging of AML1. The hemagglutinin (HA) epitope tag was added to the carboxyl terminus of AML1 by PCR. The HA-tagged AML1 cDNA was inserted into a myosin light-chain vector (MDAF, kindly provided by J. P. Merlie). Mouse C2 cells were grown and transfected as described previously (48). Pooled stably transfected cells were stained with a monoclonal antibody (12CA5) against the HA epitope (57).

Site-directed mutagenesis. The AChR δ-subunit regulatory sequence, nucleotides −840 to +25, was mutagenized by site-directed mutagenesis (48). The mutations were confirmed by sequencing, and the mutated regulatory sequence was subcloned into pφhGH (46). AChR δ-hGH and pSV2-CAT plasmids were cotransfected into C2 myoblasts, the amount of human growth hormone (hGH) secreted from myotubes was determined by a radioimmunoassay 4 or 5 days after transfection, and the amount of secreted hGH was normalized to the level of chloramphenicol acetyltransferase (CAT) activity (48). Duplicate dishes were included in each experiment; expression from duplicate dishes varied by less than 20%.

Electrophoretic mobility shift assay. AML1 binding to wild-type and mutated sequences was assayed by an electrophoretic mobility shift assay (54). Oligonucleotides were end labelled and purified with a Biospin-3 column (Bio-Rad), and AML1/CBF β proteins were translated in reticulocyte lysates (Promega).

Nucleotide sequence accession number. The GenBank accession number for the rat AML1 sequence is L35271.

RESULTS

Identification of mRNAs that are regulated by innervation. We used a PCR-mediated subtractive-hybridization and cloning method (55) to identify genes which are expressed in skeletal muscle and which are regulated by innervation. Approximately one dozen genes are known to be expressed at higher levels (greater than threefold) in denervated than in innervated muscle, including the genes encoding AChR (33), N-CAM (11), sodium channels (59), N-cadherin (19), and myogenin (14, 15, 39, 52, 58). We screened a denervated-minus-innervated library with a subtracted probe (see Materials and Methods) and identified four of the approximately one dozen genes that are known to be up-regulated following denervation (Table 1). We also identified three cDNAs that encode known proteins (filamin, MAP1a, and AML1) whose expression was not known to be regulated by innervation and two cDNAs that encode novel proteins whose expression increases substantially following denervation. To the best of our knowledge, only the GLUT-4 gene is known to be expressed at lower levels (greater than twofold) in denervated than in innervated muscle (9). To determine whether innervation causes an increase in expression of additional genes, we screened an innervated-minus-denervated library with a subtracted probe (see Materials and Methods), and we identified one cDNA that encodes a novel protein whose expression is ~10-fold greater in innervated than in denervated muscle (Table 1).

One of the genes, whose expression increases following denervation, encodes the recently discovered AML1 protein. In this study, we characterize expression of AML1 during skeletal muscle differentiation and following denervation.

AML1 expression increases following denervation. We isolated several cDNA fragments encoding the rat homolog of human AML1 from our denervated-minus-innervated muscle library, and we used the cDNA fragment to isolate a cDNA
encoding full-length AML1 protein from a rat skeletal muscle library. The 1.8-kbp cDNA contains an open reading frame of 1.35 kbp that encodes a 48.5-kDa protein which is highly homologous to human (99%) and mouse (99%) AML1 (5) and less homologous (82%) to the related mouse protein, PEBP2α (41). The rat cDNA encodes an AML1 protein that is identical in length to the mouse AML1 protein but longer than the human AML1 protein.

To confirm that AML1 expression is regulated by innervation, we measured the levels of AML1 mRNA in innervated and denervated muscle by an RNase protection assay. Figure 1 shows that AML1 is expressed in rat skeletal muscle and that AML1 expression increases substantially (50- to 100-fold) following denervation (Table 1).

Because Schwann cells and other perisynaptic cells respond to denervation (7, 18, 43), we considered the possibility that perisynaptic cells, rather than muscle cells, accounted for the increased AML1 expression in denervated muscle. Muscle can be conveniently dissected into a synaptic region, containing perisynaptic cells, and a nonsynaptic region, lacking perisynaptic cells (34). We dissected the synaptic and nonsynaptic regions of innervated and denervated skeletal muscle and measured the level of AML1 expression in each region.

FIG. 1. AML1 expression increases 50- to 100-fold following denervation. RNA was extracted from 5-day-denervated (Den) or contralateral innervated (Inn) rat lower-leg muscle, and the levels of AML1 and actin mRNAs were measured by RNase protection.

Table 1. mRNAs identified by subtractive-hybridization cloning

<table>
<thead>
<tr>
<th>cDNA</th>
<th>RNA level (denervated/innervated)</th>
<th>Abundance (%) in innervated muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>AChR α subunit</td>
<td>15</td>
<td>0.001</td>
</tr>
<tr>
<td>AChR β subunit</td>
<td>15</td>
<td>0.001</td>
</tr>
<tr>
<td>N-CAM</td>
<td>40</td>
<td>0.001</td>
</tr>
<tr>
<td>αB-crystallin</td>
<td>6</td>
<td>0.02</td>
</tr>
<tr>
<td>MAP1a</td>
<td>12</td>
<td>0.001</td>
</tr>
<tr>
<td>Filamin</td>
<td>5</td>
<td>0.04</td>
</tr>
<tr>
<td>AML1</td>
<td>100</td>
<td>0.0001</td>
</tr>
<tr>
<td>DEN1 (novel but contains immunoglobulin and FN III repeats that are organized like those in titin [28])</td>
<td>50-100</td>
<td>0.001</td>
</tr>
<tr>
<td>DEN2 (no homology)</td>
<td>10</td>
<td>0.05</td>
</tr>
<tr>
<td>INN1 (23-kDa protein that has homology to NSP [44, 56])</td>
<td>1</td>
<td>0.05</td>
</tr>
</tbody>
</table>

*The following mRNAs are known to be up-regulated (greater than threefold) following denervation: crystallin (2), AChR subunits (17), N-CAM (11), sodium channel (SK2 subtype) (59), myogenin (14, 15, 39, 52, 58), glucose transporter (GLUT-1) (6), N-cadherin (19), and CNTFR (22).”

2 shows that AML1 expression in the nonsynaptic region increases 50- to 100-fold following denervation. Because nonsynaptic regions of muscle lack Schwann cells and other perisynaptic cells, this result indicates that muscle fibers are the predominant source of AML1 RNA in denervated muscle.

Although AML1 is expressed at similar levels in synaptic and nonsynaptic regions of innervated muscle, AML1 is expressed preferentially in the synaptic region of denervated muscle (Fig. 2). We do not know the cellular source of the preferential AML1 expression in the synaptic region of denervated muscle; AML1 expression may be greater in the synaptic than in the nonsynaptic region of denervated muscle fibers and/or AML1 expression may also increase in Schwann cells or other perisynaptic cells following denervation.

The distribution of AML1 expression in different tissues has received little attention. Both B-cell lines and T-cell lines express multiple AML1 transcripts (2.1, 4.3, 5.4, and 8.2 kb), although the rank order of their abundance differs among different cell lines (5, 37). Although little is known about the different transcripts, there is evidence that the different transcripts encode different proteins (4). Four AML1 transcripts, which are similar in size to those in B-cell lines and T-cell lines, are expressed in denervated skeletal muscle, and the abundance of each transcript increases following denervation (Fig. 3). The predominant AML1 transcripts in denervated skeletal muscle are ~7.0 and 6.3 kb long.

We measured the time course of AML1 expression following denervation using an RNase protection assay. A twofold increase in AML1 expression is detectable by 1 day after denervation, and a 50- to 100-fold increase is detectable by 5 days after denervation (Fig. 4). The increases in AChR and N-CAM expression following denervation follow a similar time course (11, 33), whereas the increase in myogenin expression precedes that of the other genes (14, 15, 39, 52, 58).

Although most skeletal muscle genes are expressed at similar levels in fast and slow muscle fibers, a subset of muscle genes are expressed preferentially in certain fiber types (2, 13, 24, 42, 45, 52). To determine whether AML1 is expressed...
Preferentially in fast or slow myofibers, we measured the abundance of AML1 RNA in innervated and denervated rat soleus (slow) and extensor digitorum longus (EDL) (fast) muscles. Figure 5 shows that AML1 is expressed at similar levels in rat soleus and EDL muscles and that denervation causes a 50- to 100-fold increase in AML1 expression in both slow and fast muscles. Thus, the regulation of AML1 expression is similar in slow and fast muscles.

Many genes that are expressed in skeletal muscle are expressed at low levels in myoblasts and at significantly higher levels following myoblast fusion and muscle differentiation. To determine whether AML1 is induced during muscle differentiation, we measured AML1 expression in C2 myoblasts and myotubes. Figure 6 shows that expression of AML1 is not upregulated during muscle differentiation. Indeed, the levels of AML1 expression are similar in myoblasts, myotubes, and denervated adult muscle (Fig. 6).

**CBF β and PEBP2α mRNAs are expressed in skeletal muscle.** AML1 is thought to complex with a second protein, termed CBF β, and the heterodimer, CBF, has a higher affinity for an AML1-binding site (see below) than does AML1 alone (40, 41, 54). Therefore, we determined whether CBF β is expressed in skeletal muscle. Figure 7 shows that CBF β RNA is expressed in skeletal muscle (54). CBF β is more abundant than AML1 in innervated muscle, but CBF β expression increases little following denervation. As a consequence, CBF β and AML1 are expressed at similar levels in denervated muscle.

PEBP2α is structurally related to AML1 and contains a runt homology region (41), but its expression is thought to be restricted to T cells, where it can function as a transcriptional activator (41). To determine whether PEBP2α is expressed in skeletal muscle, we measured the levels of PEBP2α RNA in innervated and denervated muscle. Figure 7 shows that PEBP2α is expressed at low levels in innervated skeletal muscle and at approximately fourfold-greater levels following denervation.

CBF was purified from thymus nuclear extracts on the basis of its ability to bind core sites in viral enhancers (26, 53). To determine whether AML1 is a nuclear protein in skeletal muscle, we transfected an expression vector encoding AML1, tagged with an epitope from HA, into C2 muscle cells and located the epitope-tagged protein by immunofluorescence. Figure 8 shows that AML1 expression is restricted to the nuclei of myoblasts and myotubes. Thus, AML1 is a nuclear protein in muscle.

**References**


**Figure 3**. The level of four AML1 transcripts (7.0, 6.3, 3.9, and 3.4 kb [arrowheads]) increases following denervation. Innervated (Inn) and denervated (Den) rat muscle mRNAs (3 μg) were fractionated by gel electrophoresis and transferred to a GeneScreen membrane. The filter was hybridized with a full-length AML1 cDNA probe and exposed for 2 days with an intensifying screen. The levels of actin mRNA in innervated and denervated muscle were similar (data not presented).

**Figure 4**. Expressions of AML1 and AChR genes increase with similar time courses after denervation. Rat lower-leg muscles were denervated for 1, 2, or 5 days, and the levels of AML1, AChR δ-subunit, myogenin, and actin mRNAs were measured by RNase protection. Inn, innervated muscle.

**Figure 5**. AML1 expression increases in both slow (soleus) and fast (EDL) muscle after denervation. The levels of AML1 and actin mRNAs in innervated (Inn) and denervated (Den) soleus and EDL muscles were quantitated by RNase protection.
The cis-acting region of the AChR δ-subunit gene that confers regulation by electrical activity lacks a functional binding site for AML1/CFB β. A consensus binding site for CFB has been determined by site selection assays (32). The regulatory region of the AChR δ-subunit gene, which confers innervation-dependent regulation (51), lacks this consensus sequence but contains a sequence which deviates from the consensus sequence by a single nucleotide.

We used recombinant AML1/CFB β, synthesized in vitro, to determine whether this variant sequence binds AML1. Figure 9 shows that AML1 and AML1/CFB β bind the variant sequence in the δ-subunit regulatory region but with a 30-fold lower affinity than that of the consensus CBF target sequence.

To determine whether the weak AML1-binding sequence is required for δ-subunit gene expression, the AML1-binding site (AACCACC) in the AChR δ-subunit regulatory region (positions −31 to −25) was mutagenized to generate either a nonfunctional (AAGGTCC) or a consensus (AACCACA) binding sequence for AML1 (32). Gene fusions between hGH and wild-type or mutated AChR δ-subunit sequences (nucleotides −840 to +25) were transfected into C2 myoblasts, and hGH expression was measured as described in Materials and Methods. We found that a mutation which prevents AML1/CFB β binding has little or no effect on δ-subunit expression in cultured myotubes. Moreover, mutation of the weak AML1-binding sequence to a consensus AML1-binding sequence has little or no effect on δ-subunit expression. These results indicate that the weak AML1-binding sequence is not a target for AML1-mediated transcriptional activation in myotubes grown in cell culture.

**DISCUSSION**

We used a subtractive-hybridization and cloning strategy to identify genes which are expressed in skeletal muscle and which are regulated by innervation. In our initial screen, we identified approximately one-quarter of the genes that were known to be regulated by electrical activity, indicating that our initial screen was thorough but not exhaustive. We identified several known genes whose expression was not known to be regulated by denervation and several genes encoding novel proteins that are likewise regulated by electrical activity. Further screening of the subtracted libraries and characterization of the identified genes should provide a better understanding of the mechanisms that lead to changes in muscle structure and function following denervation and loss of myofiber electrical activity.

We show that AML1 is expressed in skeletal muscle and is regulated by electrical activity. Although previous studies had shown that AML1 is expressed in 3T3 cells, B-cell lines, and T-cell lines, expression of AML1 in other cell types and tissues had not been examined. Our results demonstrate that AML1 expression is not restricted to the hemopoietic lineage; rather, AML1 is expressed in skeletal muscle, where it is regulated by physiological signals.

Alternative splicing of AML1 RNA results in four transcripts of different sizes. We found that the level of all four AML1 transcripts increases in response to denervation. Although little is known about the different transcripts, the different transcripts encode different proteins which may have different
functions (4, 5, 35, 37). Indeed, others have speculated that certain isoforms of AML1 may bind DNA and activate transcription whereas other isoforms may bind DNA but repress gene expression (35).

Since translocations involving either AML1 or the CBF β-subunit gene lead to leukemia, AML1 might participate in regulation of cell growth or differentiation. Our data, however, do not support the idea that AML regulates proliferation during muscle differentiation, since AML1 is expressed at similar levels in both proliferating myoblasts and differentiated myotubes grown in cell culture. Our studies, however, do not address the possibility that AML1 might be regulated posttranscriptionally during muscle differentiation or that AML1 might regulate muscle growth later in development.

It is not clear how the t(8;21) DNA rearrangement, which results in a fusion between the AML1 and ETO genes, causes leukemia. Since AML1 can bind to target sequences in the T-cell receptor β enhancer and activate transcription (4), it is possible that the gene fusion disrupts the normal transcriptional activity of AML1 and that the function of wild-type AML1 is inhibited by the mutant AML1 protein in a dominant negative manner (4, 36). Alternatively, the gene fusion could result in a novel transcriptional activity, which in turn leads to aberrant gene expression and cell proliferation.

Muscle genes that are regulated by electrical activity, including those that regulate muscle atrophy, motor neuron survival, motor neuron growth, and editing of initial synaptic connections, are potential targets for AML1. Our data, however, demonstrate that the AChR δ-subunit gene is not directly regulated by AML1 in developing muscle, and these results suggest that AML1 does not directly regulate expression of the AChR δ-subunit gene in response to changes in muscle fiber electrical activity. If AML1 has a role in electrical activity-dependent regulation of the AChR δ-subunit gene, then AML1 is likely to act indirectly. The target genes for AML1 and the consequences of increased AML1 expression in muscle may be best examined by increasing AML1 expression in innervated muscle or decreasing AML1 expression in denervated muscle.

REFERENCES