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# Chromatin modifications that support acetylcholine receptor gene activation are established during muscle cell determination and differentiation

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## Abstract

Localization of acetylcholine receptors (AChRs) to the postsynaptic region of muscle is mediated in part by transcriptional mechanisms. An important way of regulating transcription is through targeting histone modifications on chromatin to distinct gene loci. Using chromatin immunoprecipitation, we examined the developmental regulation of certain histone modifications at the AChR epsilon subunit locus, including methylations at lysine residues K4 and K27 and acetylations at K9 and K14. We modeled various stages of muscle development in cell culture, including pre-determined cells, committed but undifferentiated myoblasts, and differentiated myotubes, and modeled synaptic myotube nuclei by stimulating myotubes with neuregulin (NRG) 1. We found that a pattern of histone modifications associated with transcriptional activation is targeted to the AChR epsilon subunit locus in myotubes prior to stimulation with NRG1 and does not change upon addition of NRG1. Instead, we found that during muscle cell determination and differentiation, specific histone modifications are targeted to the AChR epsilon subunit locus. Within the gene, at K4, dimethylation is induced during muscle cell determination, while trimethylation is induced during differentiation. At K27, loss of trimethylation and appearance of monomethylation occurs during determination and differentiation. In addition, in a region upstream of the gene, K4 di- and trimethylation, and K9/14 acetylation are induced in a distinct developmental pattern, which may reflect a functional regulatory element. These results suggest synaptic signaling does not directly target histone modifications but rather the histone modification pattern necessary for transcriptional activation is previously established in a series of steps during muscle development.

### Keywords

Acetylcholine receptor; Chromatin; Histone modifications; Muscle differentiation; Neuromuscular junction

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## Introduction

Formation of the neuromuscular synapse involves the coordinated development of a highly differentiated presynaptic nerve terminal and a highly specialized postsynaptic apparatus [1]. One of the important actions within muscle cells for postsynaptic specialization is selective transcription of genes that encode postsynaptic proteins within the few muscle nuclei that are situated at synaptic sites [2]. Of the synaptic proteins, the mechanisms used to localize acetylcholine receptors (AChRs), which involve transcriptional and other processes, have been the most extensively studied and have served as a basis for understanding in general how synaptic proteins are localized.

Because localization of AChRs to synapses occurs in part by a transcriptional process, the transcriptional regulation of the genes encoding AChRs constitutes an important part of how they are localized. For the AChR $\varepsilon$ subunit gene, which has been particularly well characterized, transcriptional regulation occurs in response to multiple conditions [3-6]. Transcription is regulated by cell type, occurring only in skeletal muscle cells that have become fully differentiated. Within muscle cells, transcription is confined to synaptic nuclei, presumably due to them receiving a localized signal. In addition, transcription is temporally regulated, with maximal levels occurring following 1–2 weeks of postnatal development.

According to various cell culture and in vivo studies, muscle-specific and synapse-specific aspects of AChR $\varepsilon$ subunit gene regulation are facilitated by distinct transcriptional regulatory controls, which presumably act in conjunction with each other to confine transcription to muscle synaptic nuclei [4,7-9]. Synapse-specific transcription has been studied in vivo, including by muscle injection or with transgenic mice, and using cell culture in which myotubes are stimulated with a synaptic signal. Muscle-specific transcription has been studied in cell culture, in which transcriptional induction accompanies muscle differentiation. In these various studies, a binding site for Ets proteins has been implicated in synapse-specific transcription, while other elements, which might include an E-box that binds myogenic transcription factors, have been implicated in myotube specific transcription.

To study synapse-specific transcription in cell culture, a synaptic signal that has been used is neuregulin (NRG) 1. NRG1, which is a secreted signaling protein, is localized at the neuromuscular synapse and can induce transcription of synaptic genes including the AChR $\varepsilon$ subunit gene in cultured muscle cells [10]. However, according to mouse genetic approaches in which NRG1 signaling was disrupted in muscle, NRG1 signaling was found to not be required for synapse-specific transcription, although disruption of NRG1 signaling modestly reduced the amounts of AChRs and AChR mRNA at synapses [11,12]. Instead, signaling by agrin, a motor neuron-derived ligand, and muscle-specific kinase (MuSK), a receptor tyrosine kinase, is involved in synapse-specific transcription in vivo [13-15]. For unknown reasons, however, stimulation of the agrin-MuSK pathway does not promote synaptic transcription in cultured muscle cells [7]. Because the same transcription factor binding sites were found to have a critical role in regulating synaptic transcription that is induced by NRG1 in cultured muscle cells and at synaptic nuclei in vivo, the same transcriptional mechanisms appear to mediate NRG1-induced transcription in cultured cells and synapse-specific transcription [8,9,16,17]. Thus, despite the uncertainty about the role of NRG1 signaling at synapses, NRG1-stimulated cultured muscle cells are likely to be a valid model system for studying the mechanisms of synapse-specific transcription.

An important way in which transcription can be regulated, presumably including for the AChR $\varepsilon$ subunit gene, is through local modifications of chromatin, and a large part of how chromatin is modified is through post-translational modifications of histones [18].

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Modifications to histones can regulate transcription of specific genes by being targeted to regions of chromatin where these genes are located. Among the histone modifications that can occur, the best characterized are certain acetylation and methylation events, which have important roles in activating or silencing transcription. Various histone-modifying proteins that add or reverse these modifications often are recruited to specific regions of chromatin by forming complexes with particular DNA-binding proteins, thereby targeting particular histone modifications to these locations.

For the AChR *ε* subunit gene, in which expression is detected only in synaptic nuclei of differentiated skeletal muscle, presumably some set of histone modifications occurs that is involved in turning on AChR transcription, but it is not known the developmental stages in which these occur. Because distinct transcriptional regulatory elements appear to control the cell-type and synapse-specific aspects of transcriptional regulation, the particular histone modifications that occur at each developmental stage presumably depend in part on whatever histone-modifying proteins are being recruited to specific regulatory elements that are functional at that stage. Modifications that ultimately participate in transcriptional activation could potentially be induced during myogenic cell fate determination, muscle fiber differentiation, or in response to a synaptic signal, or during some combination of these processes.

In the current studies, we examined the developmental regulation of certain histone modifications at the AChR *ε* subunit locus using cultured cells that modeled various stages of muscle development. We found that a pattern of histone modifications associated with transcriptional activation was targeted to the AChR *ε* subunit locus in myotubes prior to stimulation with the synaptic signal NRG1 and did not change upon addition of NRG1. We found instead that stepwise changes to histone modifications, which are consistent with transcriptional activation, occurred during earlier developmental stages. These results suggest that synaptic signaling does not directly target histone modifications but rather the histone modification pattern necessary for transcriptional activation is previously established during muscle development.

## Materials and methods

To obtain myoblasts, C2C12 myoblasts were grown in DMEM with 20% bovine growth serum (hyclone) to 40–50% confluency. To obtain myotubes, myoblasts were grown to confluence and then placed in differentiation medium (DMEM with 4% horse serum) for 4 days. For myotubes treated with NRG1, NRG1 (R&D Systems) at 100 ng/ml was added to myotubes that had been in differentiation medium for 3 days, and myotubes were maintained for one additional day. NIH 3T3 fibroblasts were grown in DMEM with 10% bovine calf serum to 80–90% confluency.

Cells that had been grown on 15 cm dishes (two dishes for fibroblasts or myotubes, four dishes for myoblasts) were scraped in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4$, 1.4 mM $KH_2PO_4$) containing protease and acetylase inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 10 mM sodium butyrate), pelleted at 2500×*g*, and resuspended in ~5X the packed cell volume (PCV) of nuclear extract buffer (10 mM Tris–HCl, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, with protease and acetylase inhibitors). Cells were again pelleted at 2500×*g* and resuspened in nuclear extract buffer to give final volume of ~3X of original PCV and incubated 10 min on ice. Nuclei were released using ten strokes on a Dounce homogenizer with type B pestle, pelleted at 3500×*g* for 5 min, and resuspended in 600 μl nuclear extract buffer with 1 mM $CaCl_2$. The absorbance at 260 nm was measured using a 5 μl sample that was diluted 1:100 in 5 M urea, 2 M NaCl. Nuclei were again

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pelleted at 3500×*g* for 5 min and resuspended in nuclear extract buffer with 1 mM $CaCl_2$, 0.4% NP-40 to give an absorbance at 260 nm of 50.

Nuclei were digested with micrococcal nuclease (Worthington) at 0.06 units/μl for 10 min at 37°C. Reactions were stopped by adding EDTA to 10 mM and centrifuged at 3500×*g* for 5 min. The supernatant was supplemented with NaCl to 150 mM, incubated at 4°C for 20 min, and centrifuged at 8200×*g* for 15 min, and the resulting supernatant (designated S1) was saved. The pellet (from the centrifugation immediately following digestion) was resuspended in 300 μl 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 5 mM EDTA, with protease and acetylase inhibitors, incubated at 4°C for 1 h with rocking, passed through a 20 gauge needle 4 times and a 25 gauge needle 4 times, and centrifuged at 8200×*g* for 10 min, and the supernatant (designated S2) was saved. The combined supernatants (S1 and S2) were diluted with an equal volume of ChIP buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, with protease and acetylase inhibitors).

200 μl portions were each incubated overnight at 4°C with a modification-specific histone H3 subunit antibody: 7 μl Millipore 07-448 (1MeK27), 7 μl Millipore 07-449 (3MeK27), 9.5 μl Millipore 07-030 (2MeK4), 5 μl Abcam 8580 (3MeK4), 7 μl Upstate 06-599 (AcK9/14), 17.5 μl Santa Cruz 2027 (IgG control). A portion was retained to use as input control. Samples containing antibodies were incubated with 25 μl protein A agarose (50% slurry), which had been pre-washed with ChIP buffer and pre-absorbed with 1.9 μg sonicated single stranded herring sperm DNA, at 4°C for 1 h with rotation. Samples were loaded onto empty micro-bio spin columns (Bio-Rad), centrifuged 30 s at 700×*g*, washed 5X, with each wash consisting of 5 min incubation in 400 μl ChIP buffer followed by centrifugation for 30 s at 700×*g*. Columns were eluted using 400 μl per elution of ChIP buffer without inhibitors, with an initial elution containing 1.5% SDS and a second elution containing 0.5% SDS, and each elution consisting of a 15 min incubation followed by centrifugation for 30 s at 700×*g*. Combined elutions or input that had SDS added to 1% were digested with 1 μl 20 mg/ml proteinase K at 65°C for 1 h, extracted with phenol/chloroform, supplemented with 10 μg glycogen and NaCl to 0.2 M, precipitated using 2.5 volumes ethanol, washed with 70% ethanol, and resuspended in 280 μl TE. 5 μl of input material was analyzed by agarose gel electrophoresis to confirm that 140 bp mononucleosome-sized fragments had been isolated.

Quantitative PCR (qPCR) with real-time fluorescence detection was performed, as described previously [19], using 5 μl of immunoprecipitated material per reaction. Chromatin corresponding to the acetylcholine receptor ε subunit gene was analyzed with different sets of PCR primers, in which the number assignment of each primer set refers to the position of the 5′ end of the forward primer relative to the transcription start site, and the amplicons generated range in size from 69 to 86 bp. For measurements with each PCR primer set, the cycle threshold ($C_t$) values from immunoprecipitation samples were normalized to the $C_t$ values from the input sample to obtain a level of enrichment.

Analysis of acetylcholine receptor ε subunit gene expression by qRT-PCR was performed as described previously [19].

## Results

In our studies on developmental regulation of histone modifications at the AChR ε subunit gene, we have focused on several modifications within the H3 subunit, based on their well characterized functions and their potential for being developmentally regulated: two different methylation levels of lysine 4 (K4), di-methylation (2MeK4) and tri-methylation (3MeK4); two different methylation levels of lysine 27 (K27), mono-methylation (1MeK27)

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and tri-methylation (3MeK27); and acetylation at lysine 9 and 14 (AcK9/14) [18,20-22]. 3MeK4 and acetylations, including on K9 and K14, are associated with transcriptionally active genes, being targeted around transcriptional start sites and in the case of 1MeK27 also extending into gene body regions. 2MeK4 is associated with both transcriptionally active genes and with non-expressed genes that can become active upon further stimulation, leading to the idea that 2MeK4 functions to maintain genes in a transcriptionally permissive or poised state [21,23,24]. 3MeK27 is associated with repressed genes, being targeted throughout the gene body and upstream regulatory regions. Because proteins that add and reverse these modifications to histones can be recruited to specific genes by forming complexes with transcription factors [18,22], developmental regulation of these histone modifications in muscle could occur through interactions of histone modifying proteins with transcription factors functioning at particular developmental stages.

Because AChR transcription can be induced by synaptic signals, we determined if histone modifications are involved in transcriptional induction of the AChR $\varepsilon$ subunit gene by NRG1. We used ChIP to examine histone modifications and whether they are affected by NRG1 signaling. We prepared mononucleosome-sized chromatin fragments, using micrococcal nuclease digestion, from C2C12 myotubes that had either been untreated or stimulated with NRG1. We used antibodies specific for modified histones to immunoprecipitate chromatin, such that each antibody was able to pull down a collection of chromatin fragments that contain histones with a particular modification. We quantified immunoprecipitated chromatin fragments that were derived from different regions around the AChR $\varepsilon$ subunit gene by performing real-time PCR. Our PCR analysis spanned throughout the genomic region containing the AChR $\varepsilon$ subunit gene, which is transcribed from +1 to +4312, including regions that are upstream of the transcription start site (−1.4 and −0.5 kb), near the start site (+42), and in the gene body (+1.7 kb). By analyzing these regions, we should be able to determine where histone modifications are being targeted and compare their levels to surrounding basal regions. As shown in Fig. 1, histone modifications associated with transcriptional activation are elevated within the AChR $\varepsilon$ subunit locus in myotubes prior to stimulation with NRG1. 2MeK4, 3MeK4, and AcK9/14 all showed peak levels near the transcription start site. Elevated levels of 2MeK4 and to a lesser extent 3MeK4 were also seen in the proximal upstream region (−0.5 kb). Only low levels of these modifications were seen in more distal upstream (−1.4 kb) and gene body regions (+1.7 kb), which presumably represents background levels because these regions had similarly low levels in the various cell types examined. For 1MeK27, high levels were seen extending from the upstream region (−1.4 kb) throughout the gene body, with low, background levels in the more distal upstream region. In contrast, 3MeK27, which is associated with transcriptional repression, was not detected within the AChR $\varepsilon$ subunit locus in myotubes (Fig. 3), indicating that this repressive influence is already turned off prior to NRG1 stimulation.

As shown in Fig. 1, upon addition of NRG1 to myotubes, the levels of histone modifications at the AChR $\varepsilon$ subunit locus associated with transcriptional activation did not markedly change. Within the peak region of each histone modification, NRG1 did not change the extent by which the modification was elevated. Also, the pattern within the gene of peak and low regions for all these histone modifications, with the exception of 3MeK4, was not altered by NRG1 treatment. For 3MeK4, while the location of peak levels in both untreated and NRG1-stimulated myotubes was positioned near the transcription start site, in NRG1-stimulated myotubes, elevated levels of 3MeK4 extended further downstream within the gene, although to a diminished degree. Whether this extended region of 3MeK4 in NRG1-stimulated myotubes has any functional significance is not clear, as the level of expression of genes generally correlates with the amount of 3MeK4 that is located around their start site. To confirm that NRG1 was inducing AChR transcription in myotubes in which histone

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modifications were being studied, real-time RT-PCR was used to measure mRNA levels for the AChR $\varepsilon$ subunit gene. As shown in Fig. 1, NRG1 robustly induced AChR expression at the mRNA level, presumably through increased transcription, consistent with previous reports. Thus, the mechanism by which NRG1 signaling induces transcription of AChR $\varepsilon$ subunit gene does not involve induction of particular histone modifications associated with active genes.

Transcription of the AChR $\varepsilon$ subunit gene, in addition to being responsive to synaptic influences, which we modeled by adding NRG1, also appears to be subject to separate regulatory influences as part of the developmental program of muscle cells. Histone modifications involved in transcriptional activation of this gene could be regulated during steps of muscle development, in particular during muscle cell determination or differentiation. To determine how histone modifications were regulated during these developmental stages, we evaluated histone modifications by ChIP using different cell types which served as models for these different developmental stages. Fibroblast cell lines, including NIH 3T3 cells, which we used in these studies, model pre-determined cells because these cells are not part of the muscle lineage yet can be converted to myoblasts by expression of the muscle determination factor MyoD or related proteins [25]. Muscle cell lines, including C2C12 cells, serve as a model for muscle cells that are either committed or differentiated, because under different culture conditions, these cells can either propagate as myoblasts, which are committed but undifferentiated muscle cells, or can terminally differentiate into myotubes.

We performed ChIP with fibroblasts, myoblasts, and myotubes to determine if histone modifications at the AChR $\varepsilon$ subunit locus are regulated during muscle cell determination and differentiation. As shown in Fig. 2, methylation at K4 showed stepwise changes during muscle cell determination and differentiation. In pre-determined fibroblasts, low, near background levels of 2MeK4 and 3MeK4 were seen throughout the gene. In committed but undifferentiated myoblasts, 2MeK4 was substantially induced while 3MeK4 remained at low, background levels. 2MeK4 in myoblasts showed peak levels near the transcription start site. Elevated levels were also seen in the proximal upstream region (−0.5 kb), while only low, background, levels were seen in more distal upstream and gene body regions. When myoblasts were differentiated into myotubes, 3MeK4 was substantially induced, primarily near the transcription start site. Even though 3MeK4 was induced, 2MeK4 still remained elevated, and to a modestly greater extent than in myoblasts, indicating that 3MeK4 induced during differentiation was not replacing 2MeK4 but occurring in addition to it.

For AcK9/14, as shown in Fig. 2, pre-determined fibroblasts exhibited somewhat elevated levels near the transcription start site relative to upstream and downstream regions of the gene. Induction of AcK9/14 was seen during progression through subsequent stages, with stepwise increases occurring, although to a fairly modest degree, both in myoblasts and upon differentiation to myotubes. Induction of AcK9/14 at these stages was confined to the region around the transcription start site.

As shown in Fig. 3, methylation at K27 also showed stepwise changes at the AChR $\varepsilon$ subunit gene during muscle cell determination and differentiation. In pre-determined fibroblasts, 3MeK27, which is associated with transcriptional repression, was elevated in all regions that we examined, extending from −3.1 kb throughout the gene body, although its levels partially tapered off toward the 3′ end of the gene body. In contrast, for 1MeK27, which is associated with transcriptional activation, low, near background levels were seen in fibroblasts within the regions being examined. In committed myoblasts, 3MeK27 was lost, with no detectable levels within the AChR $\varepsilon$ subunit gene, and remained at undetectable levels upon differentiation to myotubes. Loss of 3MeK27 in myoblasts and myotubes was

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accompanied by an increase in 1MeK27, increasing both in myoblasts and upon differentiation to myotubes. The elevated region of 1MeK27 in these cells, which extended from −1.4 kb throughout the gene body, encompassed most of the region exhibiting 3MeK27 in fibroblasts, consistent with 3MeK27 marks being converted to 1MeK27 during muscle cell determination and differentiation.

Our analysis of histone modifications at the AChR *ε* subunit locus revealed that in a particular part of the upstream region, residing near −3 kb, 2MeK4, 3MeK4, and AcK9/14 were being regulated in a way that was distinct from what was seen closer to the transcription start site, which may have functional consequences. As shown in Fig. 4, induction in this region did not occur in predetermined fibroblasts, where 2MeK4, 3MeK4, and AcK9/ 14 were seen at relatively low levels that were fairly uniform with surrounding upstream and downstream regions. In committed but undifferentiated myoblasts, 2MeK4, 3MeK4, and AcK9/14 were all substantially induced in a distinct part of the upstream region that peaked around −3 kb, to levels substantially higher than that seen near the transcription start site. Low, background levels were seen more distally (−4 kb) and proximally (−1.4 kb), separating the upstream peak from the elevated region near the transcription start site. When myoblasts were differentiated into myotubes, 2MeK4, 3MeK4, and AcK9/14 remained elevated around −3 kb, although to a somewhat lesser degree than in myoblasts. Treatment of myotubes with NRG1 did not alter the pattern or levels of histone modifications in this region (Fig. 4). Distinct regions nearby a gene but outside the transcription start site in which 2MeK4, 3MeK4, and AcK9/14 are elevated can be indicative of certain functional elements, including enhancers and insulators. Because these histone modifications are induced in myoblasts and myotubes, but not in pre-determined fibroblasts, a functional element might be present in this region that is regulated by muscle cell determination.

## Discussion

In these studies, we addressed how histone modifications at synapse-specific genes are developmentally regulated by examining histone modifications at the AChR *ε* subunit locus using a cell culture model. Histone modifications associated with transcriptional activation were present in myotubes in the absence of NRG1, and the extent by which these histone modifications occurred was not altered by NRG1, indicating that the induction of AChR transcription by NRG1 signaling does not directly target histone modifications. Instead, during muscle cell determination and differentiation, histone modifications were targeted to the AChR *ε* subunit locus in a series of distinct steps to achieve a histone modification pattern that could promote transcriptional activation.

The ways in which we found that histone modifications were targeted suggests how different histone modifying enzymes that alter methyl or acetyl marks might function to regulate expression of the AChR *ε* subunit gene. Histone methyltransferases and histone demethylases are generally specific for a single residue, so presumably distinct enzymes act at K4 and K27 residues on histones at the AChR *ε* subunit gene locus. K4 methyltransferases presumably act in committed but undifferentiated myoblasts to target K4 methylation to the transcription start site region (2MeK4) and to the distal upstream region (2MeK4 and 3MeK4), and further act in differentiated myotubes to target 3MeK4 to the transcription start site region. K27 methyltransferases presumably function in pre-determined cells to target 3MeK27 throughout the AChR *ε* subunit gene. In committed but undifferentiated myoblasts, histone demethylases, which can remove di- and tri-methyl marks on K27, might then act to remove 3MeK27. Histone acetyltransferases, which typically have a broad specificity for various lysine residues on histones, presumably act to target AcK9/14 to the transcription start site region, most prominently in differentiated

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myotubes but to a lesser degree at earlier developmental stages, and to the distal upstream region in myoblasts and myotubes.

In order for histone modifying enzymes to regulate the AChR $\varepsilon$ subunit gene, these enzymes would need to be recruited to the region of chromatin containing this gene. Recruitment of histone modifying enzymes can occur by their interactions with specific transcription factors. In previous studies with certain muscle-specific genes, interactions between transcription factors and histone modifying enzymes have been found, which might have a role in regulating the AChR $\varepsilon$ subunit gene. Myogenic transcription factors, including MyoD and MEF2, recruit histone acetyltransferases, including p300 and PCAF, to activate genes in differentiated muscle cells [26]. The transcription factor YY1 recruits the K27 methyltransferase Ezh2 to silence muscle-specific genes in undifferentiated muscle and non-muscle cells [27]. Also, various examples have been reported for the recruitment of K4 methyltransferases and K27 demethylases to certain loci by their interactions with transcription factors [22,28]. Additional experiments will be required to determine particular complexes of transcription factors and histone modifying enzymes that are being recruited to the AChR $\varepsilon$ subunit gene at different developmental stages.

For methylation at K4, in which 2MeK4 near the transcription start site is induced in committed but undifferentiated myoblasts and 3MeK4 is induced upon differentiation to myotubes, the different degrees of methylation could be regulated differently and have different functions. It has been suggested that 2MeK4 occurring distinct from 3MeK4 functions to maintain genes in a transcriptionally permissive or poised state. Genes in yeast that are in a repressed state and that have the ability to become active in response to particular signals are marked by 2MeK4 but not by 3MeK4, and upon activation, these genes exhibit both 2MeK4 and 3MeK4 [23]. Some studies suggest a similar role for 2MeK4 in higher eukaryotes. For example, in the chicken $\beta$-globin locus, where genes exhibit developmentally regulated gene expression, the relatively high levels in erythrocytes of 2MeK4 at developmental stages where particular genes are not expressed suggest 2MeK4 has a role for making these genes poised for expression [24]. Our results are consistent with 2MeK4 functioning in myoblasts, where transcription of the AChR $\varepsilon$ subunit gene in not detectable, to maintain this gene in a transcriptionally permissive state, ready to become activated. The different levels of K4 methylation at the transcription start site region of the AChR $\varepsilon$ subunit gene, 2MeK4 in myoblasts, and 3MeK4 in myotubes, suggest that different methylase components are functioning at this gene at these two stages. For histone K4 methylases, a variety of catalytic subumits each function similarly as part of a conserved core complex consisting of ASH2L, RbBP5, and WDR5. While these methylase complexes perform both 2MeK4 and 3MeK4, lack ASH2L in the complex results in reduced 3MeK4 of target histones without effecting 2MeK4 [29]. Thus, differences in ASH2L occupancy could be one possible way of specifying different methylation patterns in myoblasts and myotubes.

A previous study compared histone acetylation levels around the AChR $\varepsilon$ subunit gene locus in myotubues that were either untreated or stimulated with NRG1 [30]. While in these studies, H3 AcK9/14 was detected around the AChR $\varepsilon$ subunit gene locus in untreated myotubes, which is consistent with our results, these studies also found an increase in AcK9/14 in response to NRG1, which conflicts with our results. The chromatin analysis in the previous study had lower resolution than in our experiments, owing to a heterogeneous sized population of chromatin fragments, which averaged approximately 1 kb, being immunoprecipitated and a single set of PCR primers being used in the subsequent analysis. Thus, it could not be determined specifically where around the AChR $\varepsilon$ subunit gene locus the increased AcK9/14 was being detected, including whether it was being targeted to the region around the start site in a manner associated with gene activation. In our studies, chromatin was analyzed at higher resolution, with 140 bp homogenous sized fragments

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being immunoprecipitated and a series of primer sets spanning throughout the gene region being used in the subsequent analysis. Because our analysis using multiple PCR primers (Fig. 1 and additional primers not shown) makes it unlikely that we would have failed to detect any increase in AcK9/14 that might have been occurring, the reason for the differing results between the two studies is not readily apparent. Even though we found that AcK9/14 was unaltered by NRG1, we are confident that we are detecting AcK9/14 at the AChR $\varepsilon$ subunit locus because we found that AcK9/14 was specifically targeted around the transcription start site, which is the pattern thought to be associated with transcriptional activation, and we found that AcK9/14 levels increased in determined and differentiated muscle cells, which would be expected to promote transcriptional activation.

The induction of 2MeK4, 3MeK4, and AcK9/14 in the region around −3 kb in myoblasts and myotubes, which occurs in a distinct way from what is found closer to the transcription start site, might be indicative of a functional element. In particular, regions nearby a gene but distinct from the transcription start site in which these histone modifications are elevated can be indicative either of enhancers that activate transcription at a distance or of insulators, which are DNA elements that separate chromatin domains [20,31,32]. Insulators can function either as barriers that stop spreading of repressive chromatin or as enhancer blockers that block action of enhancers on promoters of neighboring genes. Transgenic mice generated with the upstream flanking sequences of the AChR $\varepsilon$ subunit gene linked to a reporter are consistent with a barrier insulator being present between 1 and 3.5 kb upstream of the start site, based on these sequences conferring position independence to transgene expression, which is a property associated with barrier insulators. In these transgenic mice, 3.5 kb of upstream sequence is sufficient to confer appropriate expression that mimics the endogenous gene consistently in multiple lines of mice, indicative of position-independent expression, while other transgenes containing less than 1 kb of upstream sequence are properly expressed in only small proportion of lines but silent in most lines [4]. Other studies with transcriptional reporter assays in myotubes in culture, in which varying lengths of AChR $\varepsilon$ subunit gene upstream sequence were examined, suggest that within 3.5 kb upstream of the start site, an enhancer is not present [6]. In order to determine directly the function of an element that might be present in the region around −3 kb, specific assays for insulator function and enhancer function will need to be performed.

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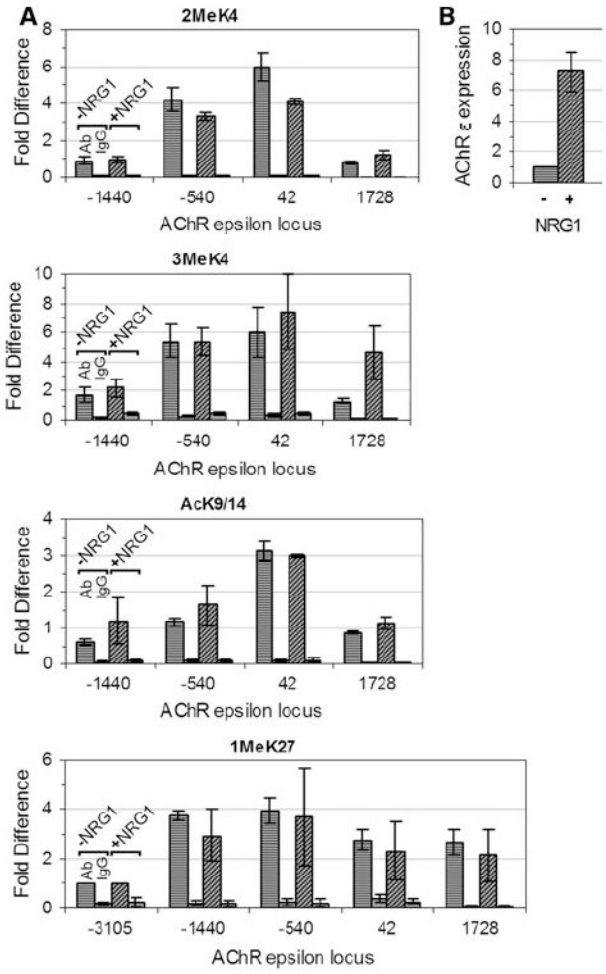


**Fig. 1.**

Histone modifications associated with transcriptional activation are targeted to the AChR $\varepsilon$ subunit locus in myotubes and are not altered by NRG1. (**a**) ChIP was performed using antibodies (*Ab*) to modified histones or control IgG to immunoprecipitate from C2C12 myotubes that were either untreated or stimulated with NRG1. The enrichment of immunoprecipitated material relative to input material from different regions of the AChR $\varepsilon$ subunit locus was measured by qPCR using the indicated primers. For each region analyzed, the enrichment level, normalized to the level at either +7647 (for 2MeK4, 3MeK4, and AcK9/14) or −3105 (for 1MeK27) is shown. In the absence of NRG1, 2MeK4, 3MeK4, and AcK9/14 were elevated primarily around the transcription start site, while elevated levels of 1MeK27 extended from upstream of the start site throughout the gene body. NRG1 did not change the extent by which these modifications were elevated. (**b**) Even though NRG1 did not alter levels of histone modifications, induction of AChR expression in myotubes by NRG1, which was measured by qRT–PCR, still occurred

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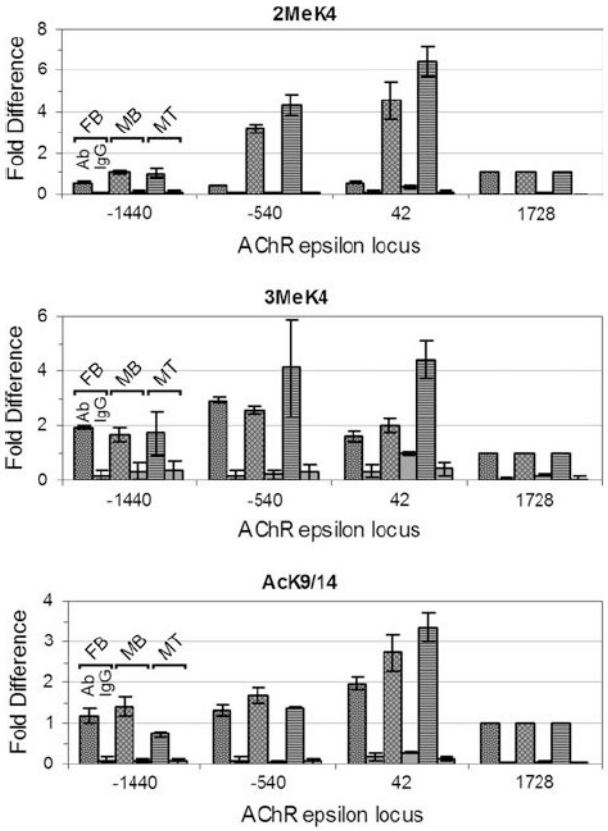


**Fig. 2.**

Histone K4 methylation and K9/14 acetylation are targeted around the transcription start site of the AChR $\varepsilon$ subunit gene during muscle cell determination and differentiation. ChIP was performed using histone antibodies (*Ab*) or control IgG to immunoprecipitate from NIH 3T3 fibroblasts (*FB*), C2C12 myoblasts (*MB*), and C2C12 myotubes (*MT*). qPCR-based measurement of the enrichment level within immunoprecipitated material at different regions, normalized to levels at +1728, is shown. In predetermined fibroblasts, 2MeK4 and 3MeK4 around the transcription start site were largely absent. In committed but undifferentiated myoblasts, 2MeK4 in this region was induced. During differentiation to myotubes, 2MeK4 remained elevated and in addition 3MeK4 was induced. AcK9/14 was targeted around the transcription start site in all cell types examined and was modestly higher in myoblasts and myotubes than in fibroblasts

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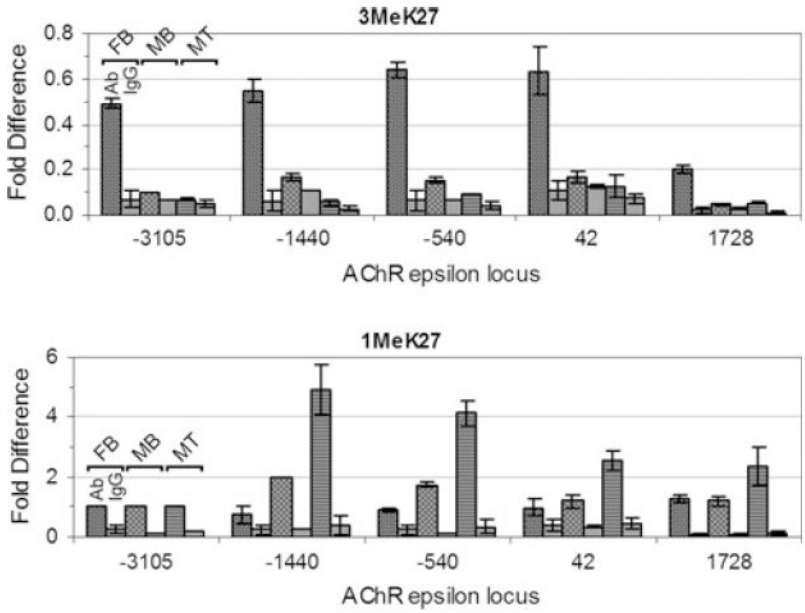


**Fig. 3.**

The histone methylation status at K27 changes during muscle cell determination and differentiation. ChIP was performed using histone antibodies (*Ab*) or control IgG to immunoprecipitate from fibroblasts (*FB*), myoblasts (*MB*), and myotubes (*MT*). qPCR-based measurement of the enrichment level within immunoprecipitated material at different regions, normalized to levels at −3105 (for 1MeK27), is shown. In pre-determined fibroblasts, 3MeK27 throughout the AChR $\varepsilon$ subunit gene locus was elevated. In committed but undifferentiated myoblasts, 3MeK27 that was seen in fibroblasts was absent and was replaced by 1MeK27, which further increased upon differentiation

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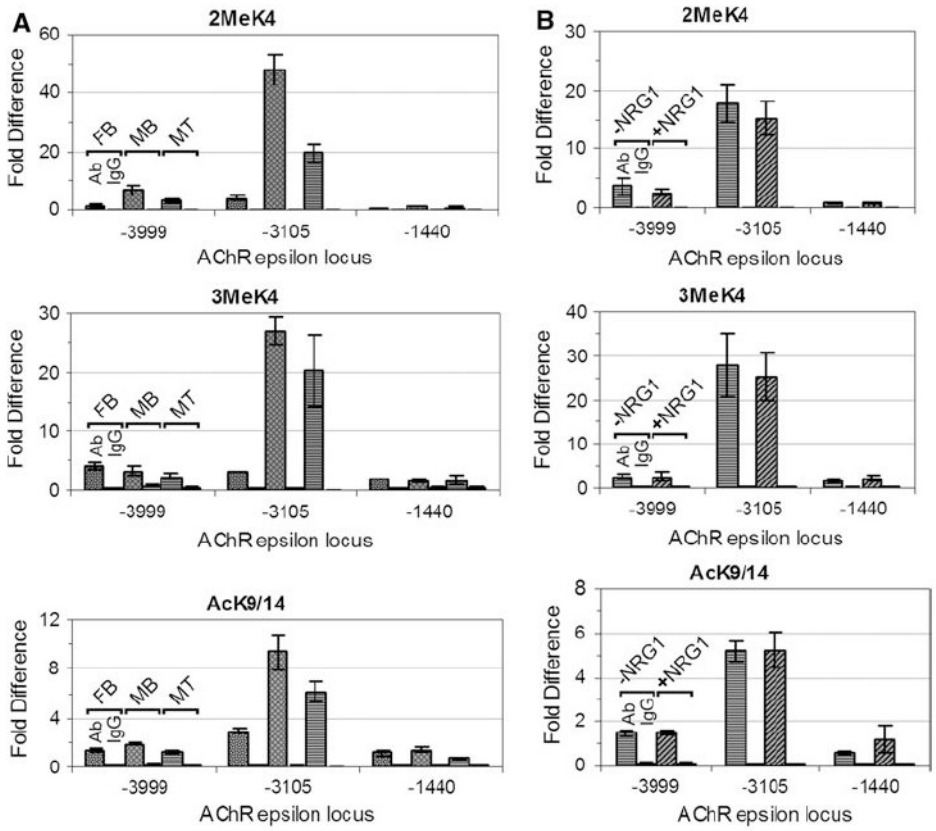


**Fig. 4.**

Histone K4 methylation and K9/14 acetylation are targeted to a part of the upstream region, residing near −3 kb, of the AChR *ε* subunit gene during muscle cell determination and differentiation. (**a**) ChIP was performed using histone antibodies (*Ab*) or control IgG to immunoprecipitate from fibroblasts (*FB*), myoblasts (*MB*), and myotubes (*MT*). qPCR-based measurement of the enrichment level within immunoprecipitated material at different regions, normalized to levels at +1728, is shown. In pre-determined fibroblasts, 2MeK4, 3MeK4, and AcK9/14 in the region around −3 kb were largely absent. In committed but undifferentiated myoblasts, 2MeK4, 3MeK4, and AcK9/14 in this region were induced and remained elevated upon differentiation to myotubes. (**b**) Stimulation of myotubes with NRG1 did not change the extent by which 2MeK4, 3MeK4, and AcK9/14 in the region around −3 kb were elevated