Specific Activation of the Acetylcholine Receptor Subunit Genes by MyoD Family Proteins*

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Whether the myogenic regulatory factors (MRFs) of the MyoD family can discriminate among the muscle gene targets for the proper and reproducible formation of skeletal muscle is a recurrent question. We have previously shown that, in Xenopus laevis, myogenin specifically transactivated muscle structural genes in vivo. In the present study, we used the Xenopus model to examine the role of XMMyoD, XMMyf5, and XMRF4 for the transactivation of the (nicotinic acetylcholine receptor) nAChR genes in vivo. During early Xenopus development, the expression patterns of nAChR subunit genes proved to be correlated with the expression patterns of the MRFs. We show that XMMyf5 specifically induced the expression of the δ-subunit gene in cap animal assays and in endoderm cells of Xenopus embryos but was unable to activate the expression of the γ-subunit gene. In embryos, overexpression of a dominant-negative XMMyf5 variant led to the repression of δ- but not γ-subunit gene expression. Conversely, XMMyoD and XMRF4 activated γ-subunit gene expression but were unable to activate δ-subunit gene expression. Finally, all MRFs induced expression of the α-subunit gene. These findings strengthen the concept that one MRF can specifically control a subset of muscle genes that cannot be activated by the other MRFs.

Myogenesis is regulated by four transcription factors, MyoD, Myf5, MRF4, and myogenin, known as the muscle regulatory factors (MRFs) of the MyoD family of basic helix-loop-helix proteins (1). From genetic studies, distinct roles have been proposed for each MRF in muscle differentiation. MyoD and Myf5 are essential in establishing the muscle lineage, whereas myogenin and MRF4 control the terminal differentiation of myofibers (2, 3). The four MRFs form heterodimers with E-protein family members and bind to a consensus DNA sequence, CANNTG, also called the E-box, to activate transcription of muscle-specific genes (4). A largely unexplored question is whether active MRFs can discriminate among the different muscle gene targets or activate transcription at all E-box-containing promoters. Many molecular details have been worked out for individual MRFs, but no global study has been done with all four factors. A recent study by Bergstrom et al. (5) focused on MyoD, which serves as a paradigm for the MRF mode of function.

Regarding the question whether a single gene or an array of genes is under the transcriptional control of an individual MRF, using the Xenopus model, we have recently shown that, among the MRFs, myogenin played a unique role in the transactivation of several structural genes of the contractile apparatus, e.g. β-tropomyosin and myosin heavy chain (6). However, whether such specificity applies to other MRFs still remains controversial. Xenopus laevis muscle development provides an attractive model to address this question since, in this system, primary myogenesis occurs in the absence of myoblast fusion and is devoid of myogenin expression (7), thus allowing comparison of the role of MyoD, Myf5, and MRF4 in the control of early gene expression.

Myogenesis is characterized by the sequential expression of specific gene families, including those that mediate communication between motor neurons and muscle. Synaptic transmission at the neuromuscular junction is mediated through the nicotinic acetylcholine receptor (nAChR), a pentameric complex of four homologous subunits with a molar stoichiometry of α2, β, δ, and γ or ε (8). Expression of the nAChR subunits and the distribution of the receptors among muscular fibers is developmentally regulated. nAChR mRNA levels are highest during myogenic differentiation. Motor innervation inhibits nAChR expression by repressing transcription of the nAChR subunit genes, whereas denervation results in reaccumulation (9). The nAChR γ- to ε-subunit switch allows the expression of two types of channel, namely an embryonic channel composed of α-, β-, δ-, and γ-subunits, distributed throughout the fiber, and an adult channel containing α-, β-, δ-, and ε-subunits, exclusively expressed at the motor endplate (10, 11).

The accumulation to a high density of one type of nAChR at the neuromuscular junction is the result of transcriptional activation of nAChR subunit genes in subsynaptic muscle nuclei. Members of the MyoD gene family are likely to play a central role in the regulation of nAChR subunit expression. All the genes coding for nAChR subunits contain one or more E-boxes in their regulatory regions, which the MRFs indifferently recognized and bound (12). Each MRF is capable of increasing specifically nAChR subunit promoter activity in transient co-transfection assays (13, 14). However, MRF4 preferentially activates the expression of the ε-subunit gene (15).

To date, no in vivo study has directly addressed the individual role of each MRF in patterning nAChR subunit expression. In the present study, we compared the expression patterns of MRFs and nAChR subunit genes during early Xenopus development, showing that these expression patterns are temporally
correlated. We then compared the in vitro abilities of MyoD, Myf5, and MRFP4 to trigger the expression of each nAChR subunit gene. Among the MRFPs, only MyoD specifically induced expression of the β-subunit gene in cap animal assays and in endoderm cells of Xenopus embryos, whereas it was unable to induce expression of the γ-subunit gene. In addition, overexpression of a dominant-negative MyoD variant led to reduced expression of the γ-subunit gene expression in embryos. The β- and γ-subunit genes also differentiated among MRFPs for their transactivation in contrast to the α-subunit gene, whose expression was induced by all MRFPs tested.

**EXPERIMENTAL PROCEDURES**

**Animals**

Adult *X. laevis* were maintained at 22 °C in tap water and fed once a week.

**Plasmid Constructions**

For expression in *Xenopus* embryos, the clones pSP64T-XMyoD, pSP64T-XMyf5, and pSP64T-XMRFP4 have already been described (16–18). MRFP-FLAG constructs were obtained by a variant of the QuikChange site-directed mutagenesis method (Stratagene) using forward primers, 5'-CAGGACCTCTATCAGCTTATATTATAAAAGATGAGCGATGAAACC-3', and 5'-TATACAGGACGGTGAGAAATGGAGGGAAGAAGG-3'. The fidelity of the sequence amplified by the polymerase chain reaction was verified by DNA sequencing.

The dominant-negative XMyf5-DN was constructed as described previously (19). Briefly, the 5′-bp fragment coding for amino acid residues 2–295 of the *Drosophila* repressor engrailed domain was inserted between the last coding codon and the stop codon by directed cloning (20), using a PCR method also developed in our laboratory. The primers used were 5′-TGGAGAAGTATTATAAAGATGACGATGACAAG-3′ and 5′-TGAGGTGTTAAGTTGTAACCTTGTTACTGTATTTATAC-3′. The fidelity of the sequence amplified by PCR was verified by DNA sequencing.

**Western Blot Analysis**

A FLAG epitope tag introduced at the C terminus of the MRF constructs was used as a control of the translation efficiency of the synthetic mRNAs. Five MRFP-FLAG-injected embryos were homogenized at stage 8 in 100 μl of buffer containing 25% glycerol, 50 mM KCl, 50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 2 mM dithiothreitol, 2 mM MgCl2. Proteins were extracted after two rounds of centrifugation at 13,500 rpm for 10 min at 4 °C. Proteins were resolved by electrophoresis on an 8% polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS-PAGE), transferred to a polyvinylidene difluoride membrane, and reacted with an anti-FLAG monoclonal antibody M2 (Sigma). Briefly, the membranes were blocked with TBS-T (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 0.1% Tween 20) containing 5% dried milk for 1 h at room temperature; incubated with a 1/20000 dilution of 5 μg/ml M2 in TBS-T with 5% dried milk for 1 h at room temperature; washed and finally incubated with a 1/40000 dilution of anti-mouse horseradish peroxidase-conjugated antibody (Transduction Laboratories) in TBS-T with 5% dried milk. Antibody binding was revealed using ECL according to the manufacturer's instructions (Amersham Biosciences).

**Embryo Injection and Animal Cap Assays**

Capped mRNAs of XmyoD, Xmyf5, XMRFP4, XEF1-a, and XMRFP6-DN (dominant-negative XMyf5) were synthesized *in vitro* from linearized plasmids (*XbaI* restriction enzyme, New England BioLabs) using the SP6 Message Machine kit (Ambion). Injections were performed using a oil-based microinjector (nanoject, Drummond). *Xenopus* embryos were placed in Modified Marc 15 medium in 100 μl (19). Briefly, the 891-bp fragment coding for amino acid residues 11–148 of XMyoD, XMyf5, and XMRF4 was used as a control of the translation efficiency of the synthetic mRNAs. Five MRFP-FLAG-injected embryos were homogenized at stage 8 in 100 μl of buffer containing 25% glycerol, 50 mM KCl, 50 mM Tris-HCl, pH 7.4, 0.1% Tween 20) containing 5% dried milk for 1 h at room temperature; incubated with a 1/2000 dilution of 5 μg/ml M2 in TBS-T with 5% dried milk for 1 h at room temperature; washed and finally incubated with a 1/40000 dilution of anti-mouse horseradish peroxidase-conjugated antibody (Transduction Laboratories) in TBS-T with 5% dried milk. Antibody binding was revealed using ECL according to the manufacturer's instructions (Amersham Biosciences).

**RESULTS**

**In Situ Hybridization**

**RNA Isolation and RT-PCR Analysis**

Total RNA was isolated from *Xenopus* embryos as described by Chomczynski and Sacchi (21). Embryos and larvae were staged according to Nieuwkoop and Faber (22). First-strand cDNAs were synthesized from 1 μg of total RNAs with oligo(dT) priming using Superscript reverse transcriptase (Invitrogen) at 37 °C for 1 h. PCR assays were performed as described elsewhere. 1 μCi of [3P]dCTP was added to each PCR product, and the PCR products were resolved electrophoretically on a 20% polyacrylamide gel. The expression of ornithine decarboxylase (ODC) was first examined as a control for equal amplification of cDNAs. The primers used for RT-PCR were: 5′-GTCATAGTGGAATGTTGATGCTA-3′ and 5′-GGTCTCAGGAGACGAAAGGA-3′ (OCD), 5′-TCCTCCTGTTAGATCCTGGA-3′ and 5′-ACCTGCCCTAAGGTGCTCA-3′ (AChR α3), 5′-TGTTGCTCAAGCTTGGCAGACG-3′ (AChR γ3), 5′-AGCCTTGCTATACGACGCTA-3′ and 5′-TGATGCTGGAGAAAGGG-3′ (AChR α1), 5′-ATGACCTGCGTATCCACAGC-3′ (AChR α2). The fidelity of the sequence amplified by PCR was verified by DNA sequencing.

**Densitometry**

The relative density of silver grains/mm² was determined in the somites of five unilaterally injected embryos. Relative density was measured on an average of 10 sections for each embryo and 20% Steinberg (20). The relative density was determined by following the ability of MRFs to activate *nAChR* subunit gene expression in embryos. In addition, *nAChR* subunit gene expression was never investigated together in the same samples from developmentally staged embryos (Fig. 1). The expression of *nAChR* subunit gene was determined by *in situ* hybridization. The ability of MRFPs to activate expression of *nAChR* subunit genes in nonsonic cells, 32-cell stage embryos were co-injected with 2 ng of MRFP mRNA and 2.5 ng of *nAChR* subunit gene RNA into the D1 blastomere. To determine in situ the effect of XmyoD-DN, two-cell stage embryos were unilaterally injected with 2 ng of XmyoD-DN and 2 ng of capped green fluorescent protein mRNA as a cell tracer. Following injection, embryos were incubated in 20% Steinberg's solution until the appropriate stage was reached.

**Comparison of the Expression Patterns of nAChR Subunits and MRFPs during Early Xenopus Development**—Although a number of studies have already described the expression patterns of either the nAChR subunits or the MRFPs during the development of several species including *Xenopus* (23–25), these expression patterns were never investigated together in the same set of experiments. To compare the expression patterns of the genes for the nAChR subunits and MRFPs during early *Xenopus* development, we performed a time course analysis with RNA samples from developmentally staged embryos (Fig. 1). The onset of appearance of the nAChR subunit mRNAs followed the time course of the MRFP mRNAs. All subunit genes, except the e-subunit, were expressed as early as stages 12–15, i.e. starting 8 h after the appearance of XMyoD and XMyf6 transcripts,
suggesting a direct gene activation (5). Comparison of MRF and nAChR expression at later stages of development proved to be more complex. mRNA expression decreased from stage 19 for XMyoD and XMyf5 and from stage 22 for XMRF4, and MRF mRNAs became undetectable at stage 28. The decrease in the expression of the α-, δ-, γ-subunit genes followed the decreasing expression of the MRF genes, albeit at different rates. Indeed, δ-subunit gene expression became undetectable after stage 24, whereas α- and γ-subunit expression persisted until stage 30. Interestingly, the γ-subunit gene displayed a biphasic expression pattern with peaks of expression at stage 17 and stage 22. Finally, β- and ε-subunit gene expression persisted throughout early development.

Selective nAChR Subunit Gene Activation by MRFs—To determine whether the pattern of transcription of each nAChR subunit gene during development can be attributed to differences in transactivation by MRFs, we compared the specificity of each MRF for transactivation of nAChR subunit genes, using the Xenopus animal cap assay. In this assay, two-cell stage embryos were bilaterally injected at the animal pole with 2 ng of synthetic XMyoD, XMyf5, or XMRF4 RNA. The same FLAG epitope tag was inserted at the C terminus of each MRF coding sequence to permit evaluation of the relative amount of each MRF injected (Fig. 2C). Animal poles were dissected at the blastula stage and cultured until the late gastrula stage for molecular analysis. The animal caps were analyzed by RT-PCR for the expression of nAChR subunit genes. Since cultured animal pole explants normally form epidermis, any expression of a muscle gene in these cells solely reflects the activity of the transcription factor gene injected. As shown in Fig. 2, mRNAs for the nAChR subunits were detected only in the MRF-RNA-injected samples, and no signal was obtained following injection of control XEF1a mRNA. A specific activation of δ-subunit mRNA was observed after XMyf5 injection (Fig. 2A). No signal was detected for δ-subunit gene expression with either XMyoD or XMRF4. β-subunit transcription was activated by XMyf5 or XMyoD, but not by XMRF4, whereas γ-subunit transcription was activated by XMyoD and XMRF4.
but not by XMyf5. α-subunit mRNA was found to be activated in all animal caps except in the control XEF1α. In contrast, expression of the ε-subunit gene was not induced by any of the MRFs injected.

To detect a possible dose effect for the gene activation specificity, we examined the expression of the nAChR δ-subunit genes in embryos injected with 6 ng of synthetic MRF RNAs. Although the differences between the MRF transactivation potentials were less marked, we fully confirmed the data obtained with the 2 ng-injected embryos. In particular, γ-subunit gene expression was activated by each MRF (Fig. 2B). In addition, the preferential role of XMyf5 for transactivation of the δ-subunit gene, as well as of XMyf5 and XMyoD for transactivation of the β-subunit gene, were confirmed in these conditions. Finally, expression of the ε-subunit gene was not detected at any dose of MRF injected.

In Vivo δ-Subunit Gene Activation by XMyf5—To confirm that MRFs could discriminate between the nAChR subunit targets in vivo, XMyf5 was overexpressed in a predetermined blastomere. In the 32-cell stage embryo, the cells derived from the D1 blastomere mainly give rise to endodermal structures (26). XMyf5 and nβ1-galactosidase RNAs or XMyoD and nβ1-galactosidase RNAs were injected into the D1 blastomere of 32-cell stage embryos. At stage 35, the injected embryos were assessed for the presence of δ- or γ-subunits of the nAChR mRNAs, respectively activated by XMyf5 and by XMyoD, in endodermal tissue. In situ hybridization analysis revealed a discrete but significant expression of δ-subunit transcripts in some of the endoderm cells derived from the XMyf5-injected blastomere (Fig. 3). No hybridization signal was detected in endoderm cells with the nAChR-γ probe, neither in the embryos injected with XMyoD nor in the embryos injected with XMyoD. In contrast, the myotomal tissue of all the injected embryos displayed high levels of hybridization with the nAChR-γ and -δ probes (data not shown).

δ-Subunit Gene Repression by a Dominant-negative Myf5 Variant—To provide additional evidence for the role of XMyf5 as a preferential regulator of the δ-subunit gene, we tested by in situ hybridization analysis whether a dominant-negative mutant of XMyf5 (XMyf5-DN) could selectively suppress δ-subunit expression in vivo without perturbing γ-subunit expression. Upon unilateral injection into two-cell stage embryos, XMyf5-DN efficiently reduced δ-subunit gene expression (Fig. 4), when this gene is normally activated in the mesoderm. The reduction of δ-subunit gene expression was correlated spatially with the expression of the co-injected lineage tracer green fluorescent protein. In contrast, the level of γ-subunit mRNA was not affected by overexpression of XMyf5-DN. As shown by RT-PCR analysis (Fig. 5), XMyf5-dependent induction of δ-subunit mRNA was significantly reduced by XMyf5-DN, whereas expression of the γ-subunit was unaffected. In these experiments, two-cell stage embryos were bilaterally injected with
A central and recurrent question in the biology of the MyoD family factors is whether each of them has evolved specialized functions or whether they can compensate for each other's function in the precise orchestration of muscle-specific gene expression during myogenesis. Few studies directly addressed this question by comparing the MRF transactivation potentials for specific muscle gene targets. Using the Xenopus model, we have previously shown that Xmyogenin controls a particular set of muscle structural genes, which cannot be activated by the other MRFs (6). Our present study demonstrates that, in vivo, the factors of the MyoD family present in muscle when the neuromuscular junction begins to differentiate, XMMyoD, XMMyf5, and XMRF4, display different specificities for the transactivation of the genes encoding the different subunits of the nAChR. High specificity was observed for activation of the δ-subunit gene by XMMyf5, β- and γ-subunit gene activation was less specific, excluding only one MRF from their activator panel. Finally, activation of the α-subunit gene implies a nonspecific effect of the different MRFs.

Overexpression of any nAChR subunit gene in the cap animal assay is controlled by at least one determination MRF factor, i.e. XMMyoD or XMMyf5. This MRF activation was concomitant with the onset of nAChR transcription, as revealed by comparing the respective expression patterns. Furthermore, expression of the nAChR subunit genes was shown to be dependent on the expression of their corresponding MRF activators since injection of a dominant-negative XMMyf5 altered δ-subunit gene expression. These observations reinforce the role of the MRFs in the initiation of nAChR gene transcription during early development, with the notable exception of the ε-subunit gene. However, no valid prediction can be made on the specific role of XMMyoD, XMMyf5, or the combination of both in promoting the transcription of nAChR genes. Since no difference could be detected in the expression patterns of the nAChR subunits that could account for the number and identity of their relevant MRF activators, it can be speculated that when the two determination factors have an activating capacity, as for the α- and β-subunits, gene activation arises from a competition toward the E-box targets. In this regard, it should be emphasized that the specificity of the initiation of nAChR gene expression by the determination MRFs is dose-dependent, as was shown for the expression of the γ-subunit and to a lesser extent the δ-subunit. Consistent with these data, no difference in the expression of the γ-subunit genes was reported in XMMyoD and Myf5-null mice (27).

In later developmental steps, each nAChR subunit gene seems to adopt a unique strategy for the maintenance of its transcriptional activation. Activation of the δ-subunit gene during differentiation requires a major activation by XMMyf5, as shown by 1) the results of the cap animal assay, 2) the results of the in vivo injection in 32-cell embryos, and 3) the comparison of the expression patterns during development (Fig. 6A). In vivo, activation of the other subunit genes may require additional factors, which are likely to be MRF family members in the case of the α- and γ-subunits (Fig. 6B) or other factors in the case of the β- and ε-subunits. A functionally significant finding from this study is that XMRF4 most likely has a cooperative function with XMMyoD for the activation of α- and γ-subunit gene expression at subsequent developmental steps. The data from the cap animal assays, showing an activation by XMRF4 only in the case of an activation by XMMyoD, were highly consistent with the relative expression profiles of α- and γ-subunit genes and the expression profiles of XMMyoD and XMRF4. All together, these results suggest that XMRF4 maintains the expression rate of α- and γ-subunit genes, whose activation is initiated by XMMyoD. Thus, XMMyoD and XMRF4 functions may overlap for the transactivation of the nAChR α- and γ-genes. Furthermore, overexpression of XMMyoD in the 32-cell embryos failed to activate γ-subunit expression in endoderm cells in vivo. This might be interpreted as a consequence of an absence of XMRF4. Thus, a working hypothesis, to be tested, is that expression of the γ-subunit gene, effectively triggered by XMMyoD in these conditions, required activation by XMRF4 to display a normal expression level detectable by in situ hybridization. Our results suggest a functional overlap of XMMyoD and XMRF4 and substantiate the results obtained with the knockout mice. In contrast to either a XMMyoD- or a XMRF4-null mutant, which exhibited nearly normal muscle differentiation, XMMyoD-/-/XMRF4-/- mouse embryos have severe muscle defects similar to those seen in myogenin mutants (28). Rather than a critical level of myogenic basic helix-loop-helix proteins insufficient to completely trigger the myogenic program, it can be proposed, in the light of the present study, that the terminal differentiation process relies in part on the correct activation of a particular set of genes, specifically activated by differentiation factors that may act solitarily, such as myogenin, or in tandem, such as XMMyoD and XMRF4. This hypothesis is strengthened by the results reported by Cornelison et al. (29), who examined the genetic requirements for XMMyoD for successful satellite cell myogenesis. These authors have described the dependence of XMRF4 expression on XMMyoD in adult satellite cells. Severe defects in the muscle regenerating process could be observed in the absence of both of these MRFs. In contrast, XMMyoD-/- embryos were phenotypically normal and, interestingly, displayed a significant expression of XMRF4 at a level indistin-
guishable from wild type (30). As a conclusion, differentiation defects likely resulted from a simultaneous deficiency in MyoD and MRF4 activities. Besides, this model accounts for the observation that, in contrast to myogenin, MyoD and MRF4 possess a functionally interchangeable helix III domain implicated in the efficient targeting of endogenous responsive genes (31).

In contrast to the observation by Liu et al. (12) that MRF4 bound the ε-subunit promoter, no MRF was able to trigger ε-subunit gene expression in the cap assay. Thus, these data might reflect a discrepancy between the DNA binding at nAChR promoters and the efficiency of the transcriptional activity. In this regard, studies using heterologous systems have already shown such a discrepancy between MRF binding and transactivation. For instance, several muscle gene promoters, including creatine kinase, myosin light chain I/3, and troponin I, that bound MyoD, myogenin, and MRF4 (4) were not efficiently activated by MRF4 (32). This is also the case for the myogenin promoter to which MRF4 binds but does not activate (33). This feature applied for all the MRFs since Liu et al. (12) demonstrated by chromatin immunoprecipitation assays that the MRFs indifferently bind to the promoters of AChR subunit genes.

Control of nAChR gene transcription is crucial to the development and maintenance of synapses in muscle. The myogenic factor proteins of the MyoD family are essential molecules in regulating the ongoing rates of nAChR gene transcription during early myogenesis in a specific and reproducible manner. In Xenopus, this precise orchestration of nAChR gene activation occurs with a target specificity. Our results further substantiate the concept that an individual MRF can control a subset of muscle genes that cannot be activated by the other MRFs or with dramatically lower efficiency. This concept opens new ways to assign, at the gene level, a particular role to each MRF in myogenesis.

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