Int. J. Dev. Biol. 43: 027-037 (1999)

Original Article

Murine tongue muscle displays a distinct developmental profile of MRF and contractile gene expression

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ABSTRACT Few studies have addressed the molecular differences that exist between muscles of the body and those of the craniofacial apparatus. In this study, we characterize the molecular events associated with determination and differentiation of the tongue musculature. We assess the expression of myogenic regulatory factors as well as the developmentally regulated myosin heavy chain, (MHC), genes which serve as markers of differentiation. These results suggest that tongue and limb muscle form by distinct molecular pathways. The myoblasts that contribute to the formation of the tongue preferentially express *Myf-5* during myoblast determination rather than *MyoD*. Subsequently, isolated regions of myogenin expression mark the differentiation of first, the small primary myofibers and later, the larger secondary myofibers. Analysis of differentiation markers demonstrates that the tongue muscle also assumes a unique profile of MHC expression as compared to that of the muscles of the body. Unlike the myoblasts of the developing limb, which express embryonic and neonatal forms of MHC and later express *MHC-slow*, the tongue myoblasts co-express *MHC-embryonic*, *MHC-slow* and *MHC-fast* isoforms from gestational age E12. Proteins for MHC embryonic and MHC fast isoforms are detected almost simultaneously. Interestingly, *MHC-slow* transcripts do not appear to be translated into a detectable MHC slow protein at any developmental stage assayed. These results provide further evidence to suggest that skeletal tongue muscle represents a myoblast lineage that develops differently than the limb.

KEY WORDS: *tongue, myogenesis, MRF, myosin heavy chain*

Introduction

The generation of cell type diversity is illustrated by analyzing the formation of vertebrate skeletal muscle. Individual skeletal muscles have distinct physical requirements that are met by the existence of a number of functionally distinct contractile apparatuses. In order to attain such molecular and physiologic diversity, myogenic cells progress through specific developmental events; determination, differentiation and maturation. While several of these events have been examined in limb and axial muscles in the body, these processes have not been characterized in muscles found in the craniofacial apparatus. The distinct embryonic origins of axial and craniofacial skeletal muscles may be reflective of different processes of muscle development.

The Myogenic Regulatory Factors, (MRFs), have been found to have an important role during skeletal muscle development. Initially the four MRF family members, *MyoD* (Davis *et al.*, 1987), *Myf-5* (Braun *et al.*, 1989), *myogenin* (Edmonson and Olson, 1989; Wright *et al.*, 1989), and *MRF4* (Rhodes and Konieczny, 1989; Miner and Wold, 1990) were defined by their ability to induce the expression of muscle specific genes; such as myosin and *MCK*, in non-myogenic mesodermal cells (Olson, 1990 review). Although each of the four MRFs is expressed in a temporal-spatial specific manner during vertebrate embryogenesis, null mutations in each of these genes has provided the clearest representation of their individual functions *in vivo*. Though functional redundancy was shown to exist between

Abbreviations used in this paper: MCK, muscle creatine kinase; MRFs myogenic regulatory factors; MHC, mycsin heavy chain; RT-PCR, reverse transcription-polymerase chain reaction.

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0214-6282/99/$10.00

Printed in Spain
www.lg.ehu.es/ijdb

individual knock-outs of *MyoD* and *Myf-5*, the removal of both genes resulted in the ablation of muscle cells in all areas of the embryo, demonstrating their crucial role in determination of the skeletal muscle lineage (Braun *et al.*, 1992; Rudnicki *et al.*, 1992, 1993). Removal of the *myogenin* gene produced fetuses possessing muscle beds filled with undifferentiated myoblasts (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993). *MRF4* was shown to play a functional role in the maintenance of maturation (reviewed in Olson *et al.*, 1996).

Embryologically, skeletal muscle is derived from two primary sources. In the body, lateral mesoderm forms compartmentalized structures termed somites. These mesodermal cells either express MRFs and proceed to differentiate *in situ* or, alternatively, they migrate into the limb fields, where they express particular MRFs and differentiate (Ott *et al.*, 1991; Ordahl and Le Dourain, 1992; Smith *et al.*, 1994). In the craniofacial apparatus, the majority of muscles are derived from non-segmented paraxial mesoderm termed somitomeres (Noden, 1991). Within the trunk of the developing embryo, the myotomal portions of the somites have been shown to possess the ability to produce two distinct lineages. Specifically, the lateral somite gave rise to migratory myoblasts that populate the limb, while the medial aspects of the somite gave rise to axial and other muscles (Ordahl and Le Dourain, 1992). Of the somitomeric derived muscles, medial paraxial mesoderm yielded a subset of the ocular muscles, while the lateral paraxial mesoderm of the somitomeres produced most of the non-ocular, craniofacial muscles (Couly *et al.*, 1992, 1993). Whole occipital transplants between chick and quail demonstrated that the majority of tongue muscle was derived from occipital somites 1-4 (Noden, 1991; Couly *et al.*, 1993). To date, similar lineage analysis subdividing medial-lateral and dorsal-ventral domains of occipital somite derived muscles has not been completed.

In addition to the physical distinction between somites and somitomeres found on the rostral-caudal axis, the myoblast's extracellular environment also depends on the site from which they are derived along this axis. For instance, myoblasts from paraxial mesoderm of either somitomeres or occipital somites have been observed to invade locations and become mingled with neural crest derived mesenchyme. Trunk somite myoblasts, on the other hand, have been found to interact with lateral mesoderm derived mesenchyme (Hazelton, 1970; Noden, 1991; Couly *et al.*, 1993).

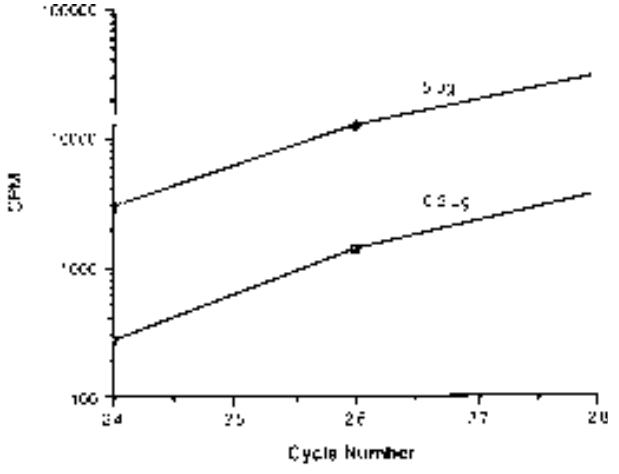

Fig. 1. Template and cycle titration of cDNA. *PCR was carried out with different starting template RNA-cDNA. Shown here are the results using the GAPDH primers with 0.5(g) and 5.0(g) of starting RNA-cDNA. This experiment was repeated using the MRF primers.*

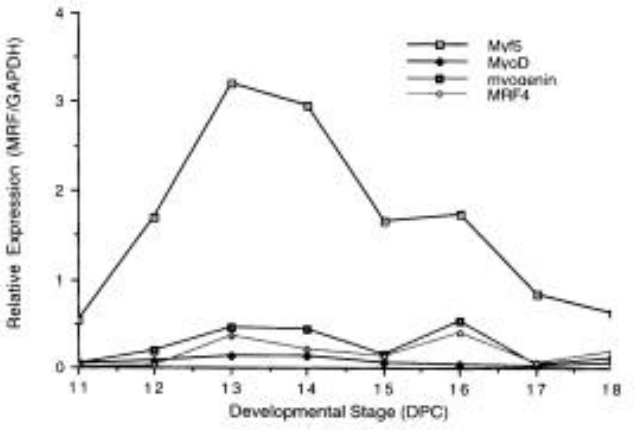

Fig. 2. Temporal expression pattern of the MRF genes during embryonic tongue myogenesis. *The transcript levels were determined by quantitative PCR. Similar results were obtained in two independent experiments. See text for details.*

Although extensive molecular characterizations of limb and axial muscle development have been completed, relatively little information exists on the development of the molecular phenotype of craniofacial muscles, such as the tongue. Several recent studies on the upstream regulatory events specifying myoblast populations provide strong evidence to suggest that some important differences exist between muscles along the rostral-caudal axis (reviewed in Miller, 1992; Patapoutian *et al.*, 1993; Tajbakhsh *et al.*, 1996). For example, a cross of the *Pax3* and *Myf-5* null mutant mice produces offspring that lack axial and limb muscles as well as a portion of the tongue muscle (Bladt *et al.*, 1995; Tajbakhsh *et al.*, 1997). However, the remainder of the tongue as well as the other craniofacial muscles remain unaffected. Null mutation of the *c-met* receptor, a tyrosine kinase gene downstream to *Pax3*, was also shown to produce selective deficiencies in the formation of specific muscles. This mutant lacked muscles derived from migratory populations, namely, the limbs, and interestingly, a portion of the tongue (Bladt *et al.*, 1995; Epstein *et al.*, 1996). Our studies provide further support for the existence of heterogenous myoblast populations. Namely, the craniofacial tongue muscle expresses the molecular markers of commitment and differentiation in a spatially and temporally distinct pattern.

Results

Myogenic regulatory factor (MRF) expression during embryonic and fetal development of tongue muscle

The homogeneously fast-twitch phenotype in mature tongue muscle led to the examination of the MRF expression profile during embryonic and fetal tongue development (Prigozy *et al.*,

1997). Accumulation and distribution patterns of MRF expression were determined by semi-quantitative RT-PCR, Western Blot analysis and immunohistochemistry. MRF expression levels and distribution patterns were determined between gestational days E11 and E17. These time points sample both embryonic (primary, E12-14 approximately) and fetal (secondary, E15-17 approximately) waves of myogenesis (reviewed in Cossu and Molinaro, 1987). Additionally, our *in vitro* analysis of tongue myoblasts, initiated at E12 and E15, support these times as being equivalent in their ability to produce characteristic primary and secondary fibers. Transcription levels were determined by a semi-quantitative method of cycle titration RT-PCR. First, the sensitivity and linearity of amplification were demonstrated by plotting the log of the forward primer incorporated (log CPM) versus the cycle number (Fig. 1). This demonstrated that the amount of PCR product generated at different cycle numbers was proportional to the amount of RNA input (Fig. 1). This methodology was then applied to the quantitation of MRF transcript levels during fetal tongue myogenesis.

Embryonic day 11 (E11) of gestation showed relatively little MRF transcription products while on E12, the first of the MRFs, *Myf-5*, was upregulated to 50% maximal levels (Fig. 2). *Myf-5* continued to be upregulated until its expression peaked on day 13, a time corresponding to the primary wave of myogenesis. The primary wave of myogenesis is characterized by populations of small myoblasts or 2-3 nuclei myotubes (Hauschka, 1974). This peak expression was maintained for another day (E14) and then declined to approximately 70% maximum expression on E15 and E16. These stages are thought to roughly correspond to the second genesis of myofibers, a phase where large, syncytial myotubes are observed morphologically (Hauschka, 1974). Low expression levels of *Myf-5* were maintained for the remainder of gestation. Of the other MRFs, only *myogenin* demonstrated a small rise in expression at E16 (Fig. 2). These levels were roughly half the expression level of *Myf-5* at the equivalent time point and occurred at the developmental stage corresponding to the secondary wave of myogenesis. Both *MRF4* and *MyoD* were detectable by this methodology though only *MRF4* to a level measurable by protein analyses.

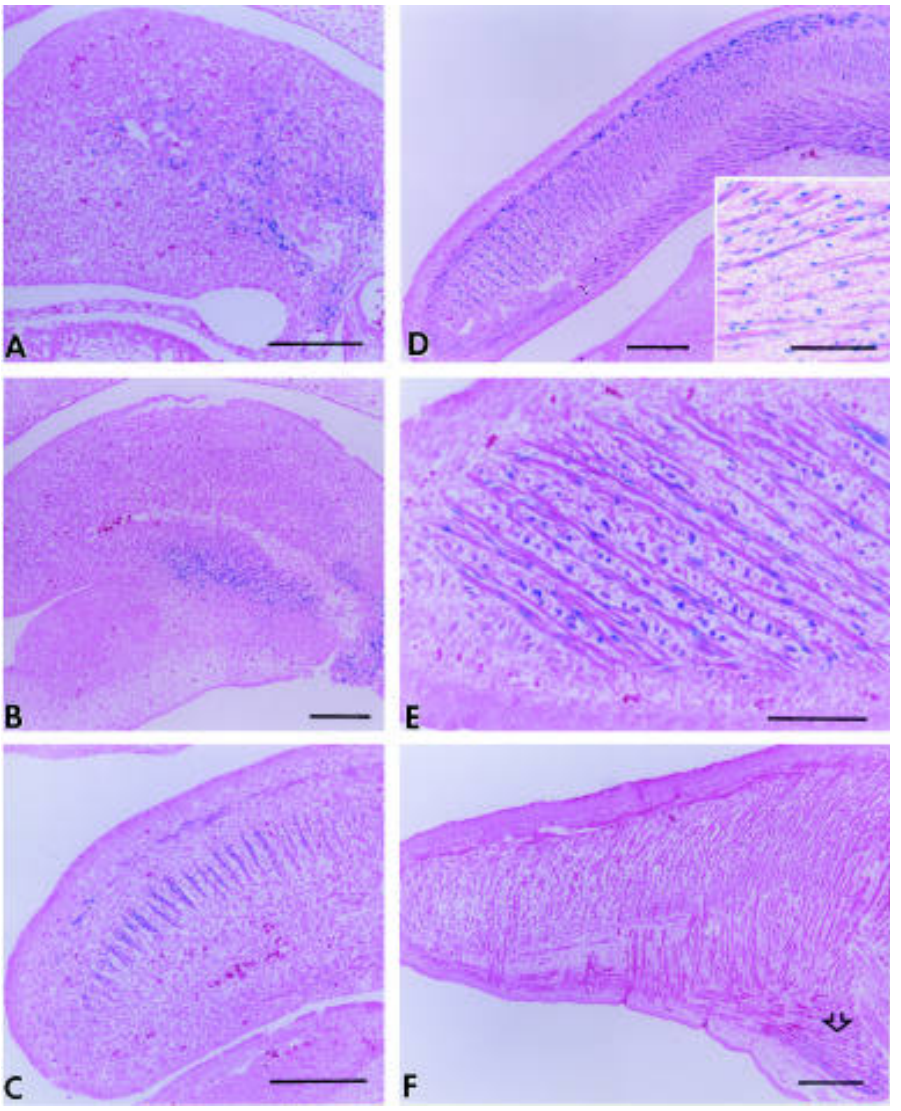

Fig. 3. Saggital sections through various gestational stages of fetal murine tongues transgenic for Myf-5-lacZ and stained for immunogalactosidase. (A) *E11.5 tongue bud,* **(B)** *E12.5 tongue,* **(C)** *anterior E13.5 tongue,* **(D)** *E14.5 tongue; E14.5 posterior tongue insert,* **(E)** *anterior E15.5 tongue,* **(F)** *E17.5 tongue, arrow points to Myf-5 positive cells in the ventral, posterior portion of the tongue. Photos (A) through (F) depict from left to right, an anterior to posterior orientation. Bar in A,B,C,D,F, 200 μm; in D insert and E, 100 μm.*

MRF protein distribution was examined by immunohistochemistry and in the case of *Myf-5*, by nuclearly localized β-galactosidase activity, between gestational days as early as E10.5 until birth. Of the polyclonal antibodies tested, Myf-5, Myogenin and MRF4 epitopes were recognized. Greater emphasis was placed on monitoring the markers of determination (*MyoD* and *Myf-5*) and the marker of differentiation, *myogenin*. Myf-5 expression was determined by close analysis of a developmental series of *Myf-5-lacZ* transgenic embryos and fetuses. Myf-5 positive cells, noted by their blue nuclei, were first detected at E10.5 in relatively small numbers in the posterior regions of the first branchial arch. At E11.5 and E12.5, an increased number of β-gal positive cells were identified in the posterior regions of the developing lateral lingual processes of the tongue (Fig. 3A,B). At E13.5, anterior regions of the tongue contained Myf-5 positive cells most notably in alternating rows of interior forming fibers (Fig. 3C). By E14.5, cells expressing Myf-5 could be found throughout the tongue (Fig. 3D, insert). Myf-5 expression continued through day 17.5, though by E15.5, the Myf-5 positive cells localized primarily to the anterior

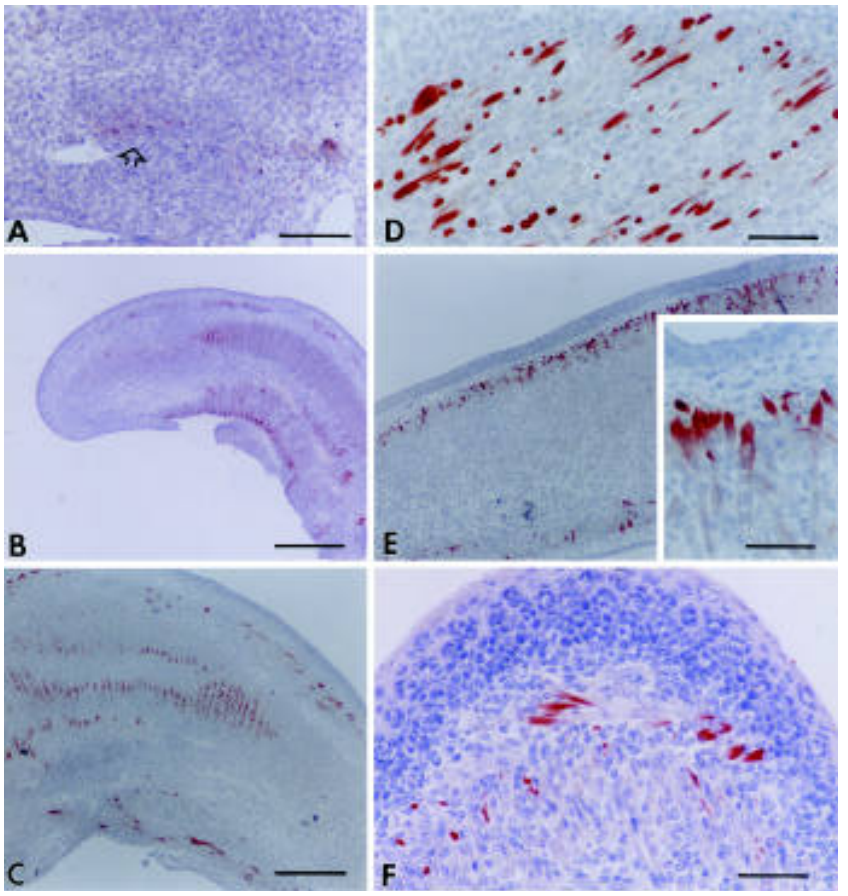

Fig. 4. Saggital sections through the developing murine tongue muscle immunostained with myogenin antibody demonstrating localized populations of positive myoblasts. **(A)** *E11 posterior tongue bud,* **(B)** *E13*, **(C)** *E14*, **(D)** *anterior portion of the E15 tongue,* **(E)** *E16 anterior; E16 insert,* **(F)** *anterior E17. Photos (A), (B), (D) and (E) depict from left to right, an anterior to posterior orientation, while photo (C) is oriented with the posterior on the left. Photo (F) depicts the tip of the tongue muscle. Bar in A, 100 μm; in B,C and E, 200 μm and in D,E insert and F, 50 μm.*

portion of the tongue (Fig. 3E) and less so to cells in the dorsal and ventral longitudinal myofibers. By E17.5 β-galactosidase positive cells were found in small populations of cells only in the ventral, posterior region of the tongue (Fig. 3F). MyoD positive cells were not localized to the developing murine tongue by immunohistochemistry, though immunoreactive cells were located in various myotomal compartments of E11 whole-embryo, control sections.

Myogenin epitopes were noted as early as E11 in a small number of cells found posteriorly, in the developing lateral lingual processes of the tongue (Fig. 4A, arrow). In the E13 tongue, increased numbers of cells, primarily in the posterior portions of the tongue muscle demonstrated myogenin expression (Fig. 4B). The most posterior portions contained positive cells chiefly in the myotubes of the body of the tongue. The distribution of myogenin protein at E14 was relatively similar to day E13 (Fig. 4C). Myogenin immunopositive cells were also observed in the dorsal and ventral longitudinal fibers, only their distribution reached slightly further anteriorly as compared to E13 samples. By E15, myogenin positive cells were seen to extend even further anteriorly (Fig. 4D), while the ventral, longitudinal myotubes had become negative. At E16, myogenin expression was observed along the length of the tongue in vertical myofibers interdigitating with longitudinal myofibers, while the fibers located within the body of the muscle appeared to be negative for myogenin protein (Fig. 4E). In addition, myogenin protein appeared to be expressed predominantly in the terminal nuclei of the vertical fibers (Fig. 4E, insert). This observation has also been noted repeatedly in primary tongue and limb myoblast cultures. And finally at E17, the myogenin positive cells appear to have declined dramatically being found primarily in fibers of the anterior portion of the tongue. Other positive cells can be noted in random locations throughout the rest of the E17 tongue and presumably, could be satellite cells (Fig. 4F).

Representative sections of *Myf-5-lacZ* tissue with β-gal stain were concomitantly immunostained with the myogenin antibody to determine whether Myf-5 and myogenin colocalize spatially and temporally. At the time when Myf-5 expression appeared most ubiquitous in the transgenic animal, E14.5, myogenin protein was detected in a much more restricted subset of cells near the posterior regions of the tongue (Fig. 5A). One day later at E15.5, when Myf-5 expression had become restricted to the anterior most regions as well as ventral posterior regions of the tongue, myogenin expression had expanded into the body of the muscle as well as to smaller populations of cells in the more anterior ventral and dorsal aspects of the tongue (Fig. 5B). At higher magnifications, it was apparent that not all myogenin positive cells were coexpressing Myf-5 protein (Fig. 5C, arrow). It is not known whether this observation reflects groups of myoblasts that had once expressed Myf-5 and were competent to express myogenin or if these are separate populations of myoblasts that were determined by some other factor than Myf-5.

Western Blots were completed to determine expression levels of Myf-5, MyoD and myogenin in the murine tongue muscle at the selected gestational times of E14, E15, E18, as well as in the adult. These time points represent primary, early secondary, secondary and the adult lineage of myoblasts as defined by previously noted morphological criteria. These blots demonstrated that Myf-5 expression was high at E14 and E15 but diminished appreciably by E18. By adulthood, the expression of Myf-5 was undetectable (Fig. 6). Myogenin expression was detectable at much lower levels but was detectable during all prenatal time points surveyed. Myogenin expression was also undetectable by adulthood (Fig. 6). Expression of MyoD was not detected by immunoblotting.

Myosin heavy chain (MHC) genes are differentially regulated during tongue myogenesis

After assessing the expression of MRFs, the expression patterns of downstream, contractile genes were also ana-

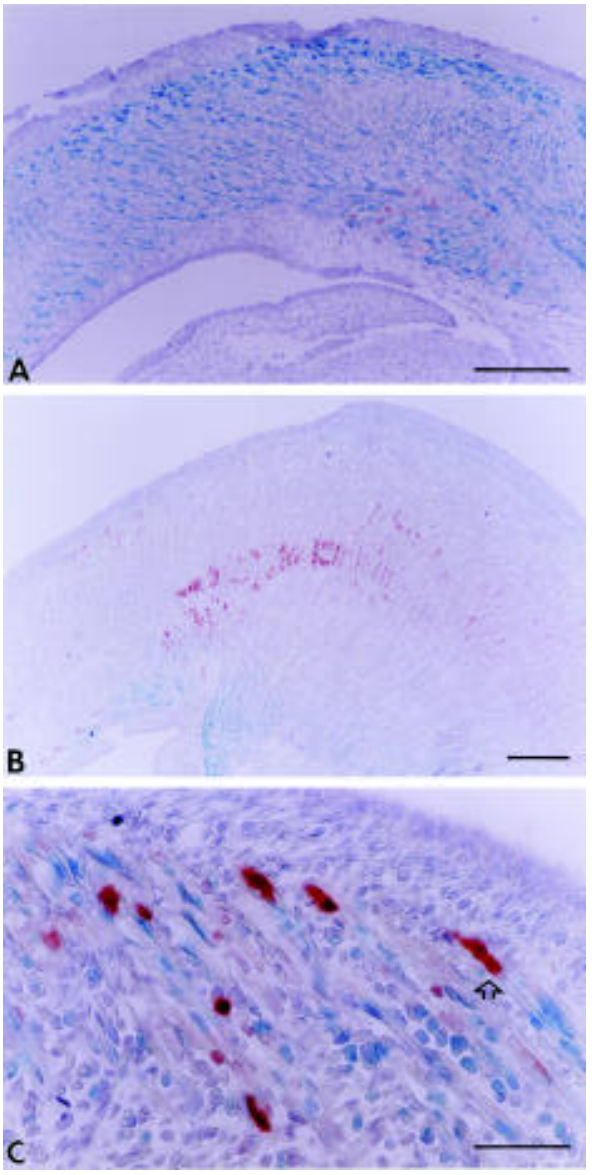

Fig. 5. Saggital sections of *Myf-5-lacZ* fetal mouse tongues concomitantly immunostained with myogenin antisera. *Myf-5 positive cells appear turquoise in color while cells immunopositive for myogenin appear red.* **(A)** *Middle to posterior E14.5 tongue; arrow points to myogenin positive cells in the posterior regions of the tongue.* **(B)** *Middle to posterior E15.5 tongue.* **(C)** *Higher magnification of an E15.5 tongue demonstrating Myf-5 and myogenin positive cells in close proximity to one another but not always expressing both proteins (arrow). Bar in A and B, 200 μm; in C, 50 μm.*

lyzed. Previous studies have shown that while redundancy exists between *Myf-5* and *MyoD*, coordinated expression between pairs of myogenic regulatory factors and downstream contractile genes is often muscle specific (Ontell *et al.*, 1993). The assessment of contractile gene expression by immunoblotting and immunohistochemical localization served to determine whether the occipital somite derived tongue muscle possessed a unique pattern of coordinated expression associated with the acquisition of a homogeneously fast-twitch phenotype.

Previously, RT-PCR analysis of *MHC-fast* and *MHC-slow* isoforms was completed for the gestational series E12 through E18 as well as in newborn and adult tongue (Prigozy *et al.*, 1997). To extend these findings, we have analyzed mRNA expression of the *MHC-embryonic* isoform for the same developmental period. *MHC-embryonic* expression was detected at E12, one day earlier than that of *MHC-fast* and *MHC-slow*. The expression of *MHC-embryonic* continued through development but was undetectable in adult tongue.

Immunohistochemical analysis of MHC isoforms provided a spatial localization that complemented transcription data and demonstrated a disparity between transcription and translation products. In this study, a series of previously characterized, developmentally specific antibodies to the various MHC isoforms were utilized. The appearance of the MHC-fast isoform commenced between late E12 and early E13 (Fig. 7A,B). As was observed with the protein localization of particular MRFs, MHC isoforms were expressed in temporally and spatially distinct patterns. At E13, MHC-fast positive cells were found in the posterior regions of the developing body of the tongue (Fig. 7B). Additionally, myotubes in posterior, ventral regions of the future longitudinal muscles were also immunoreactive to MHC-fast. Posterior-dorsal regions were immunopositive but in what appear to be mononuclear myoblasts or very small myotubes. These likely correspond to the development of the small, primary myotubes also observed in a multitude of other skeletal muscles (Hauschka, 1974; Ontell *et al.,* 1993). At E14, MHC-fast protein appears to have extended its expression domain to include more anterior regions of the centrally located myotubes (Fig. 7C). Dorsal regions contained MHC-fast positive cells that were further developed in size and appearance. Interestingly, a clear pattern of alternating rows of horizontally oriented myotubes became apparent. The E15 tongue showed minor differences of MHC-fast distribution in comparison to E14 with

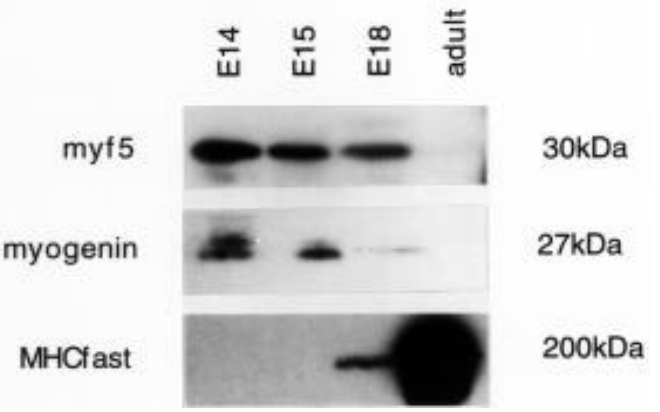

Fig. 6. Western blot analysis of Myf-5, myogenin, and MHC-fast expression at selected gestational points of murine tongue myogenesis. *A total of 50 μg of protein was run for each gestational time point. The same blot was probed with the various antibodies with only Myf-5, myogenin, and MHC-fast showing immunoreactivity. Both MyoD and MHC-slow were undetectable during these time points.*

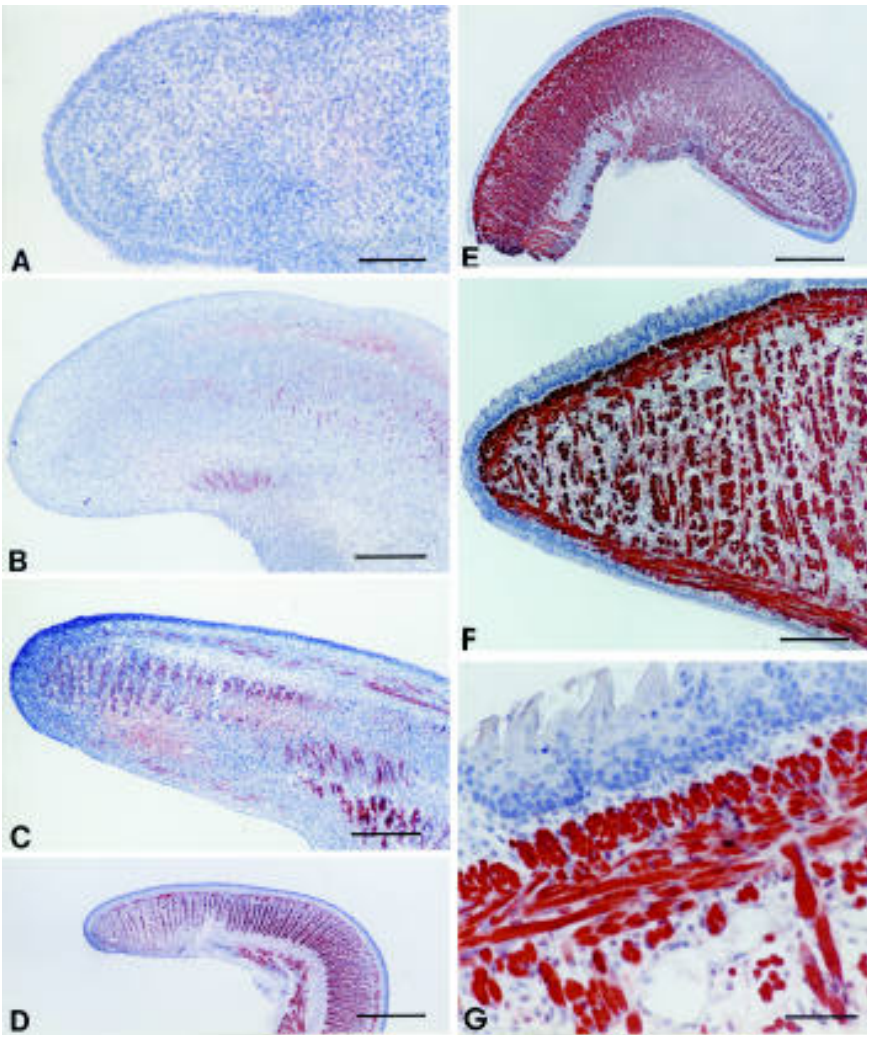

Fig. 7. Immunolocalization of MHC fast isoform expression in the developing murine tongue. (A) *E12 tongue bud,* **(B)** *E13*, **(C)** *E14*, **(D)** *E15,* **(E)** *E17*, **(F)** *newborn, low magnification,* **(G)** *newborn, high magnification. Photos (A) through (D), (F) and (G) depict from left to right, an anterior to posterior orientation, while photo (E) is oriented with the posterior on the left. Bar in A 100 µm; in B,C and F, 200 µm; in D and E, 500 µm and in G, 50 µm.*

the most obvious difference being the increased distinctiveness of the alternating rows of MHC-fast positive, MHC-fast negative horizontal myotubes (Fig. 7D). This unique banding pattern disappeared at E16 in an apparently posterior to anterior manner with the appearance of fibers in the formerly MHC-fast negative bands. E17 and neonatal tongues displayed a homogeneously fast MHC phenotype with dense, striated, organized fibers that were oriented in all three dimensions (Fig. 7E-G).

Developmental isoforms of MHC were expressed in a temporally and spatially specific fashion as well. While expression of MHC-embryonic at E12 was similar to the distribution of MHC-fast, at E13, MHC-embryonic positive cells in the central pre-muscle region reached much further anteriorly (Fig. 8A,B). Alternating rows of MHC-embryonic immunopositive cells were evident one day prior to those observed with the MHC-fast antibody. At day 14, myoblasts positive for MHC-embryonic were detected in the most anterior regions of the tongue (Fig. 8C). And by day 17 of gestation, the MHC-embryonic isoform could be found in nearly all muscle cells of the fetal tongue (Fig. 8D). Developmental isoforms of MHC-embryonic myofibers decreased significantly in numbers by the time of birth, two days later. With the exception of a group of MHC-embryonic positive fibers in the anterior, ventral portion and in the anterior-most fibers, newborn tongue muscle was entirely absent of MHC-embryonic immunoreactive cells (Fig. 8E, insert and F).

Neonatal isoforms of MHC were not found until day 15 of gestation (Fig. 9A-C). At this time, myotubes positive for MHC-perinatal appeared in a posterior to anterior pattern. Expression of MHC-perinatal was found uniformly in the developing tongue by day 17 and continued in this manner through the first few days of neonatal life (Fig. 9D-F).

Analysis of *MHC-slow* expression demonstrates a sharp contrast between transcription product and translation product data. While transcripts of *MHC-slow* isoforms are clearly evident from late E12 until late in gestation, immunolocalization of MHC-slow, although present, is weak and indistinct in pattern (Fig. 10). Low level immunoreactivity was noted in dorsal and ventral areas from E12 through E15 (Fig. 10A-D). By birth, MHC-slow is clearly absent in the murine tongue (Fig. 10F). Positive controls were performed with this antibody and demonstrated that antigen recognition when present, was strong, localized intracellularly and was easily identified (Fig. 10E)

Immunoblotting was also completed to determine general levels of MHC-slow and MHC-fast protein and to demonstrate that the detection of transcription products did not predict the production of a translated protein. Selected time points during gestation were used to survey various developmental stages. Detection of MHC fast was negligible at E14 and E15, while at E18 MHC fast protein was readily detectable (Fig. 6). By adulthood, abundant MHC-fast was observed as well as a number of lower molecular weight isoforms. And although the MHC slow antibody was easily able to detect the MHC slow protein in positive controls, it was undetectable at all sampled tongue developmental stages.

Discussion

While previous characterizations detailing the temporal and spatial expression of both muscle regulatory molecules as well as the ensuing complement of developmentally

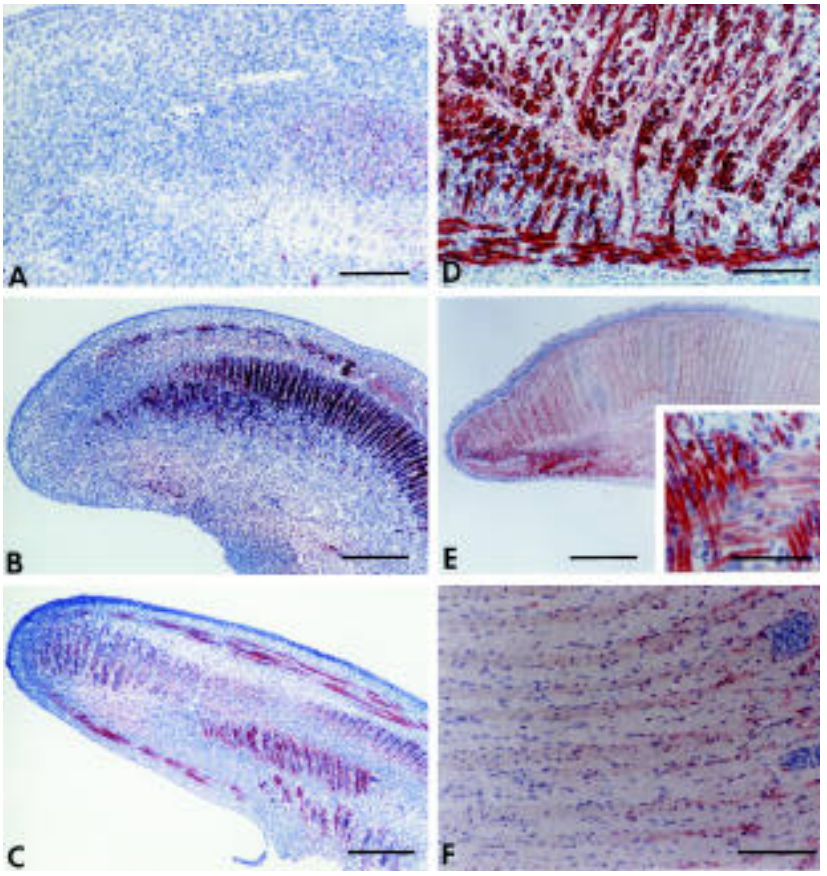

Fig. 8. MHC developmental isoform localization in: (A) *E12 tongue bud,* **(B)** *E13*, **(C)** *E14*, **(D)** *E17*, **(E)** *newborn; insert,* **(F)** *newborn. Photos (A) through (F) depict from left to right, an anterior to posterior orientation. Bar in A,D and F, 100µm; in B,C and E, 200 µm and in E insert, 50 µm.*

specific contractile proteins have been completed in a number of muscles, complete studies in craniofacial muscles have not been undertaken. Our study demonstrated that the tongue muscle has distinct features of molecular differentiation when compared to limb and axial muscles.

MRF transcription analysis of murine tongue muscle myoblasts identified *Myf-5* expression at levels significantly higher than other myogenic regulatory factors for the duration of the developmental period analyzed (E10.5-E17.5). Protein analysis, both immunoblots and immunolocalizations, as well as analysis of *Myf-5-lacZ* transgenic mice supported the dominant role of *Myf-5* as a mediator of tongue muscle determination. Research done on limb muscles has indicated that both *MyoD* and *Myf-5* were expressed at high and comparable levels from days E11 through E13 in the front limb and at E12.5 through E14.5 in the hind limb (Hannon *et al.*, 1992, reviewed in Yun and Wold, 1996). In the epaxial component of the somites that gives rise to the deep muscles of the back, *Myf-5* is expressed first, and later in the development of these muscles, both *Myf-5* and *myogenin* are expressed simultaneously. This pattern of MRF expression is most similar to what we have observed in the tongue.

Although mutational analysis of the various MRFs has concluded that functional redundancy exists between certain members of this family, primarily between *MyoD* and *Myf-5* in myoblast determination, other studies have shown the temporal and spatial specific patterns of expression likely represent the existence of several myogenic lineages. The present results during tongue muscle determination and commitment would support that hypothesis. As further support, analysis of the upstream regulatory elements of the *Myf-5* gene has identified specific regions able to direct both branchial arch expression as well as expression in the hypoglossal cord (Patapoutian *et al.*, 1993; Tajbakhsh *et al.*, 1996). Only one other group of skeletal muscles appears to be programmed to predominantly express *Myf-5* during development and those are the paraspinal and intercostal muscles derived from the dorsomedial epaxial region of the somite (reviewed in Yun and Wold, 1996; Kablar *et al.*, 1997). However, despite the pattern similarities in *Myf-5* expression observed in the epaxially derived muscles and the tongue musculature, only a portion of the tongue is affected in both the *c-met* and the *Pax3-Myf-5* mutants. Epaxial muscles, on the other hand, develop normally in c-met mutants. Both the *c-met* and the *Pax3-Myf-5* mutants bear defects in myoblast lineages thought to be migration dependent (Bladt *et al.*, 1995; Epstein *et al.*, 1996; reviewed in Yun and Wold, 1996; Tajbakhsh *et al.*, 1997). These previous studies of axial and appendicular muscles have outlined important distinctions not only between muscles derived from migratory populations and non-migratory populations of cells but also between muscles of the head and muscles of the body. Thus, it appears that the formation of the tongue may require more than one specified lineage of myoblasts. Future lineage analysis will determine what types of genetic pathways determine the myogenic populations that give rise to the tongue or whether the tongue represents a hybrid muscle, one that is developmentally similar to both the muscles of the head and to those of the body.

The finding that myogenin is expressed throughout the examined period coincides with myogenin's hypothesized role in differentiation of myoblasts. In particular, the apparent increase of expression at E16 likely coincides to the onset of differentiation of secondary fibers (Nabeshima *et al.,* 1993). It is noteworthy that, unlike the ubiquitous expression of both *Myf-5* transcripts and Myf-5 positive cells during tongue development, immunolocalization data of myogenin demonstrates that differentiation of tongue myoblasts occurs in waves. These waves appear to progress grossly from the posterior to the anterior of the tongue. In addition, the seemingly stable levels of myogenin by immunoblotting may demonstrate that while groups of myoblasts may appear to express myogenin quite strongly, only sub-populations are doing so at any one time.

Another interesting observation is that throughout gestation, myogenin positive fibers were stained in either a

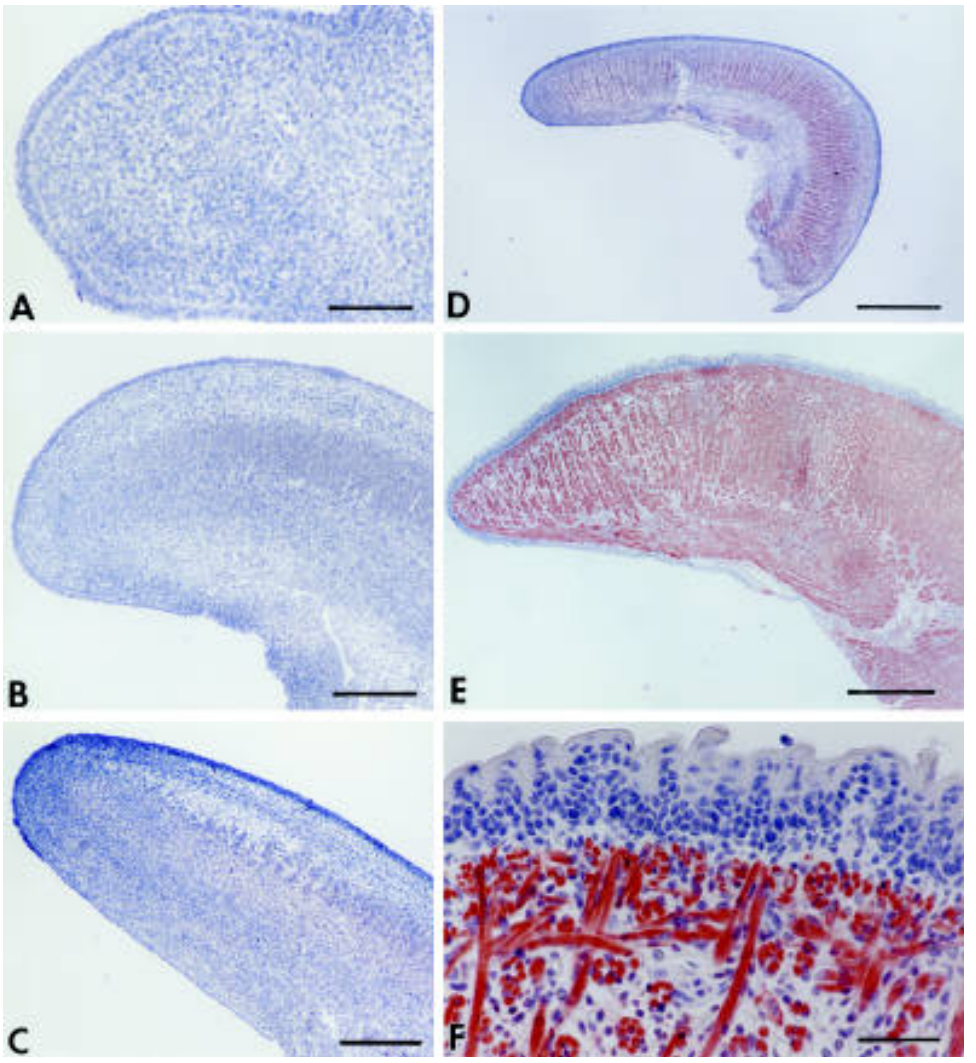

Fig. 9. Immunolocalization of the MHC neonatal isoform in: (A) *E12 tongue bud,* **(B)** *E13*, **(C)** *E14*, **(D)** *E17*, **(E)** *newborn; insert,* **(F)** *newborn. Photos (A) through (F) depict from left to right, an anterior to posterior orientation. Bar in A, 100 μm; in B,C and E, 200 μm; in D, 500 μm and in F, 50 μm.*

nuclear or a perinuclear manner. In select fibers, it is evident that this staining often occurs at the terminal nucleus. Interestingly, during the developmental series a pattern of myogenin positive expression appears in a roughly posterior to anterior fashion as well as in a plane specific manner, (i.e., groups of longitudinal fibers alternating with groups of transverse fibers).

The onset of the developmentally programmed expression of the MHC family also appears to differ in tongue muscle from other skeletal muscles of the body. In the limb, embryonic and neonatal MHC isoforms are transcribed at E11.5 and E12 respectively, followed by the *MHC-slow* isoform at E13. Within the myotome, a slightly different profile presents, *MHC-embryonic*, *MHC-fast* and *MHC-slow* transcription are all initiated at E9.5, with *MHC-neonatal* being observed at E10.5 (Buckingham *et al.*, 1992; Ontell *et al.*, 1993). Notably, the tongue expresses embryonic, fast and slow isoforms of MHC at a developmental stage later than that of the myotome and limb. Proteins of embryonic and fast MHC isoforms were concomitantly detected at late E12. MHC neonatal protein was detected at E17 and maintained into the perinatal period. While the fast isoform of MHC is transcribed into adulthood, the embryonic and slow isoforms of MHC were absent by the time of birth. Of special interest is the observation that although *MHC-slow* is apparently transcribed throughout development, no protein was detected by either immunoblotting or immunohistochemistry. Future research should consider this disparity when assaying the acquisition of contractile phenotypes by either transcription or protein analysis methods alone.

Our study suggests that the relatively simple MRF expression profile, as well as the pre-natally determined, homogenous contractile phenotype of the tongue may provide an interesting avenue in which to address the regulation of myogenic determination and contractile gene expression in the head.

Materials and Methods

Tissue collection

Swiss-Webster mice were bred on site. Day 0, hour 0 of gestation was established by the presence of a vaginal plug. Pregnant mice were sacrificed daily from day 11 through day 17 of gestation. Embryonic, fetal, newborn and adult tongues were obtained and quickly frozen for RNA and protein isolation procedures or fixed in Carnoy's fixative for immunostaining procedures.

Nucleic acid isolation

Total RNA was isolated from mouse tongue muscles by homogenization on ice in 4M guanidinium thiocyanate, 0.3M sodium acetate, and 1% β-mercaptoethanol. Homogenates were laid onto a 5.7M caesium chloride, 0.3M sodium acetate cushion and spun at 80K rpm for 3 h at 20.0°C. RNA pellets were rinsed in 70% alcohol and resuspended in DEPC-treated water containing 0.1 U/μL RNase inhibitor (Boehringer Mannheim). Optical densities were used to measure total RNA concentration as well as the absence of protein contamination.

Competitive PCR by cycle titration

One μg aliquots of total RNA were reverse transcribed by incubation in 50 μM oligo (dT)15 (Gibco), 1X RT buffer, 10 mM dNTPs (Boehringer Mannheim), 0.1 mM DTT, and 200U of MMLV-

reverse transcriptase (GIBCO) for one hour at 42°C. Successful reverse transcription was determined by the positive amplification of GAPDH by PCR.

Variations in the amounts of MRF transcripts at different stages of tongue muscle development were quantitated by PCR cycle titration essentially as described by Hannon *et al.* (1992) with some modifications. The forward primers were end-labeled with P^{32} by polynucleotide kinase (GIBCO BRL) to a specific activity of 6x105 cpm/pmole. The standardized PCR reaction mix consisted of PCR buffer (Perkin-Elmer), 200 mM dNTPs, 0.25 mM forward primer, 0.25 mM reverse primer and 0.05 U/µL of Taq polymerase (Perkin-Elmer). Quantitative PCR reactions were initiated by adding 80 µL of the PCR reaction mix to 20 µL of cDNA on ice. Temperature cycling following the previously described thermal profile was completed in a Perkin-Elmer DNA thermal cycler.

First, the sensitivity and the linearity of amplification were established by carrying out PCR template and cycle titrations with GAPDH primers. These primers were used to amplify PCR products from 0.5 and 5 µg of total embryonic day 13 tongue RNA. Each sample of RNA was reverse transcribed and one-tenth of each resultant cDNA was used in a separate PCR amplification. The amount of PCR product that was generated at different cycle numbers was determined by removing samples after the completion of specific cycle numbers. The PCR products were electrophoresed on 6% polyacrylamide gels and the ethidium bromide stained bands were excised from the gel for scintillation counting. The amount of radioactive forward primer that was incorporated was then determined.

In order to simultaneously utilize each of the MRF primer sets, the annealing time was extended to 1 min, 20 sec. Representative PCR reaction tubes were removed from the instrument following the completion of cycles 24, 26, 28 and 30 and samples were electrophoretically separated on 6% polyacrylamide gels. Subsequently, the gels were stained with ethidium bromide and the PCR products cut from the undried gel with a razor blade and immersed in Bio-Safe scintillation fluid for counting in a Beckman LS 5000 CE liquid scintillation counter. At least two determinations were made for each transcript level at all developmental stages. Primer sequences and annealing temperatures are provided in Hannon *et al.* (1992).

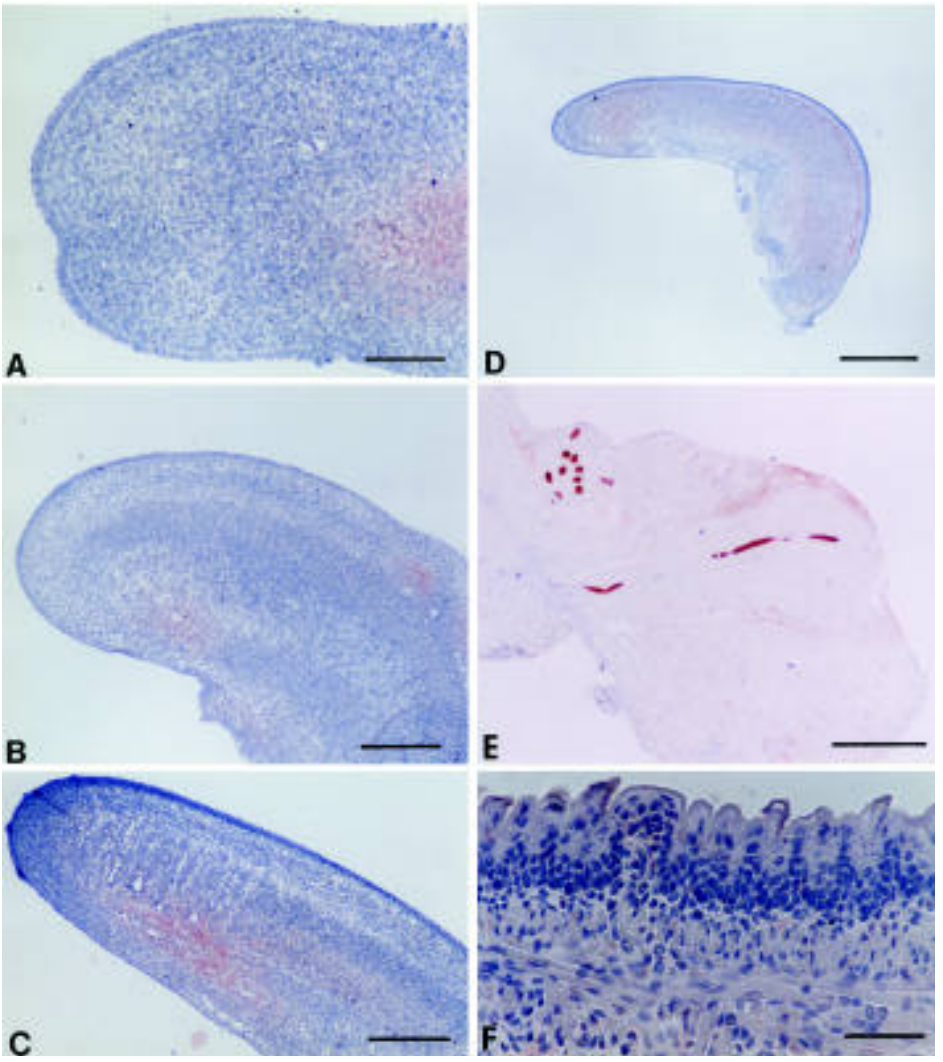

Fig. 10. MHC slow localization in the developing tongue. (A) *E12 tongue bud,* **(B)** *E13*, **(C)** *E14*, **(D)** *E17*, **(E)** *newborn; MHC slow positive cells in the muscles located anterior to the tongue,* **(F)** *newborn. Photos (A) through (F) depict from left to right, an anterior to posterior orientation. Bar in A, 100 µm; in B and C, 200 µm; in D, 500 µm; in E, 300 µm and in F, 50 µm.*

Immunohistochemistry

Carnoy's fixed tissues were dehydrated, penetrated with xylenes and paraffin and oriented sagitally in blocks and stored at 4°C. Paraffin blocks were then sectioned and placed onto Histostik coated slides (Sigma). Sections to be immediately immunostained were warmed, deparaffinized, and rehydrated through graded alcohols. Antigen exposure was enhanced by mild trypsinization (for MHCs only) and microwave treatment (5 min at 50% power) in a citrate buffer (2 mM citric acid, 8 mM sodium citrate, pH 6). Slides were also quenched for peroxidase activity in a fresh mixture of 10% hydrogen peroxide, 90% methanol for 10 min. After tissue preparation, slides were blocked with 10% goat serum for 10-20 min at RT. The sections were incubated with the appropriate primary antibody for one hour at RT (MHC monoclonal antibodies from Novocastra, Newcastle, England; MRF polyclonal antibodies, Santa Cruz Biologicals, Santa Cruz, CA). Subsequently, the slides were incubated with goat-anti-mouse IgG (MHC monoclonals) or goat-anti-rabbit IgG (MRF polyclonals) and processed routinely with an immunoperoxidase, secondary detection kit (Zymed).

Collection of Myf-5-lacZ embryos and β-galactosidase staining

The *Myf-5-lacZ* heterozygous females (Tajbakhsh *et al.*, 1996) were bred with wild type male, CD-1 mice and were harvested at gestational points E10.5 through E17.5 for β-galactosidase staining. Whole embryos were used from stages E10.5 and E11.5, whole heads from stages E12.5 and E13.5, and mandibles only from the older fetuses (E14.5-E17.5). Tissues were fixed for 30 min

in 2% paraformaldehyde, 0.1% glutaraldehyde, washed in PBS, and then placed in X-gal staining solution consisting of the following: 0.01% sodium deoxycholate, 0.2% NP-40, 2 mM MgCl2, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 400 ug/mL X-gal. Embryos from each time-point were stained for 1-2 h at 37°C. Older stage embryos (E13.5-E17.5) were maintained in the X-gal staining solution overnight at room temperature. All embryos were rinsed and post-fixed in 4% paraformaldehyde overnight. β-galactosidase positive specimens were then dehydrated and paraffin embedded for sectioning and counter-staining.

Western blot analysis

Frozen tissue was homogenized in an 85°C, 1xSDS gel loading buffer (as per Maniatis), boiled for 10 min, sheared with a 25G needle, and spun for 10 min at 12K. Aliquots were taken for quantitation in a standard Bradford Assay (BioRad Technologies). Fifty µg aliquots were run on 12% SDS-polyacrylamide gels and transferred to PVDF membranes (Boehringer Mannheim) by wet, electrophoretic transfer. Following transfer, membranes were blocked and then probed with primary antibodies (overnight at RT). Detection of antibody binding was performed with a Chemiluminescence Secondary Detection Kit according to manufacturer's instructions (Boehringer Mannheim).

Acknowledgments

We would like to thank Pablo Bringas and Valentino Santos for technical assistance and Los Alamitos high school students; Yoko Tanaka, Stephani Krueger, Vivian Chang and Stacey Nichols, who completed a portion of the immunohistochemistry. Additional thanks go to the labs of Margaret Buckingham and Barbara Wold who developed and provided, respectively, the Myf-5-lacZ transgenics (Tajbakhsh et al., 1996). This work was supported by grant DE09165.

References

BLADT, F., RIETHMACHER, D., ISENMANN, S., AGUZZI, A. and BIRCHMEIER, C. (1995). Essential role for the c-met receptor in the migration of myogenic precursor cell into the limb bud. *Nature 376:* 768-771.

BRAUN, T., BUSCHHAUSEN-DENKER, G., BOBER, E., TANNICH, E. and ARNOLD, H. (1989). A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. *EMBO J. 8:* 701-709.

BRAUN, T., RUDNICKI, M., ARNOLD, H. and JAENISCH, R. (1992). Targeted inactivation of the muscle regulatory gene Myf-5 results in abnormal rib development and perinatal death. *Cell 71:* 369-382.

BUCKINGHAM, M., HOUZELSTEIN, D., LYONS, G., ONTELL, M., OTT, M. and SASSOON, D. (1992). Expression of muscle genes in the mouse embryo. *Soc. Exp. Biol. 46:* 203-217.

COSSU, G. and MOLINARO, M. (1987). Cell heterogeneity in the myogenic lineage. *Curr.Top. Dev. Biol. 23:* 185-208.

COULY, G.F., COLTEY, P.M. and LE DOUARIN, N.M. (1992). The developmental fate of the cephalic mesoderm in quail-chick chimeras. *Development 114:* 1-15.

COULY, G.F., COLTEY, P.M. and LE DOUARIN, N.M. (1993). The triple origin of skull in higher vertebrates: a study in quail-chick chimeras. *Development 117:* 409-429.

DAVIS, R.L., WEINTRAUB, H. and LASSAR, A.B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell 51:* 987-1000.

EDMONSON, D. and OLSON, E. (1989). A gene with homology to the myc similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. *Genes Dev. 3:* 628-640.

EPSTEIN, J., SHAPIRO, D., CHENG, J., LAM., P. and MAAS, L. (1996). Pax-3 modulates expression of the c-Met receptor during limb muscle development. *Proc. Natl. Acad. Sci. USA 93:* 4213-4218.

HANNON, K., SMITH, C., BALES, K. and SANTERRE, R. (1992). Temporal and quantitative analysis of myogenic regulatory and growth factor gene expression in the developing mouse embryo. *Dev. Biol. 151:* 137-144.

HASTY, P., BRADLEY, A., MORRIS, J., EDMONSON, D., VENUTI, J., OLSON, E. and KLEIN, W. (1993). Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature 364:* 501-506.

HAUSCHKA, S. (1974). Clonal analysis of vertebrate myogenesis III. Developmental changes in muscle-colony forming cells of the human fetal limb. *Dev. Biol. 37:* 345-368.

HAZELTON, R. (1970). Radioautographic analysis of the migration and fate of cells derived form the occipital somites in the chick embryo with specific reference to development of the hypoglossal musculature. *J. Embryol. Exp. Morphol. 24:* 455-466.

KABLAR, B., KRASTEL, K., YING, C., ASAKURA, A., TAPSCOTT, S. and RUDNICKI, M. (1997). MyoD and Myf-5 differentially regulate the development of limb versus trunk skeletal muscle. *Development 124:* 4729-4738.

MILLER, J.B. (1992). Myoblast diversity in skeletal myogenesis: how much and to what end. *Cell 69:* 1-3.

MINER, J. and WOLD, B. (1990). Herculin, a fourth member of the MyoD family of myogenic regulatory genes. *Proc. Natl. Acad. Sci. USA 87:* 1089-1093.

NABESHIMA, Y., HANOAKA, K., HAYASAKA, M., ESUMI, E., SHAOWEI, L., NONAKA, I. and NABESHIMA, Y.I. (1993). Myogenin gene disruption results in perinatal lethality because of severe muscle defect. *Nature 364:* 532-535.

NODEN, D. (1991). Cell movements and control of patterned tissue assembly during craniofacial development. *J. Craniofac. Genet. Dev. Biol. 11:* 192-213.

OLSON, E. (1990). MyoD family: a paradigm for development? *Genes Dev. 4:* 1454-1461.

OLSON, E., ARNOLD, H., RIGBY, P. and WOLD, B. (1996). Know your neighbors: three phenotypes in null mutants of the myogenic bHLH gene MRF4. *Cell 85:* 1-4.

ONTELL, M., ONTELL, M.P., SOPPER, M., MALLONGA, R., LYONS, G. and BUCKINGHAM, M. (1993). Contractile protein gene expression in primary myotubes of embryonic mouse hindlimb muscles. *Development 117:* 1435-1444.

ORDAHL, C. and LEDOUARIN, N. (1992). Two myogenic lineages within the developing somite. *Development 114:* 339-353.

OTT, M., BOBER, E., LYONS, G., ARNOLD, H. and BUCKINGHAM, M. (1991). Early expression of the myogenic regulatory gene, Myf-5, in precursor cells of skeletal muscle in the mouse embryo. *Development 111:* 1097-1107.

PATAPOUTIAN, A., MINER, J., LYONS, G. and WOLD, B. (1993). Isolated sequences from the linked myf-5 and MRF4 genes drive distinct patterns of muscle-specific expression in transgenic mice. *Development 118:* 61-69.

PRIGOZY, T., DALRYMPLE, K., SHULER, C. and KEDES, L. (1997). Differential expression of troponin C genes during tongue myogenesis. *Dev. Dynamics 209:* 36-44.

RHODES, S. and KONIECZNY, S. (1989). Identification of MRF4: a new member of the muscle regulatory factor gene family. *Genes Dev. 3:* 2050-2061.

RUDNICKI, M., BRAUN, T., HINUMA, S. and JAENISCH, R. (1992). Inactivation of MyoD in mice leads to upregulation of the myogenic bHLH gene Myf-5 and results in apparently normal muscle development. *Cell 71:* 383-390.

RUDNICKI, M., SCHNEGELSBERG, P., STEAD, R., BRAUN, T., ARNOLD, H. and JEANISCH, R. (1993). MyoD or myf-5 is required for the formation of skeletal muscle. *Cell 75:* 1351-1359.

SMITH, T., KACHINSKY, A. and MILLER, J. (1994). Somite subdomains,

muscle cell origins and the 4 muscle regulatory factor proteins. *J. Cell. Biol. 127:* 95-105.

TAJBAKHSH, S., BOBER, E., BABINET, C., POURNIN, S., ARNOLD, H. and BUCKINGHAM, M. (1996). Gene targeting the Myf-5 locus with nlacZ reveals expression of this myogenic factor in mature skeletal muscle fires as well as early embryonic muscle. *Dev. Dynamics 206:* 291-300.

TAJBAKHSH, S., ROCANCOURT, D., COSSU, G. and BUCKINGHAM., M. (1997). Redefining the genetic hierarchies controlling skeletal myogenesis: Pax-3 and Myf-5 act upstream of MyoD. *Cell 89:* 127-138.

WRIGHT, W., SASSOON, D. and LIN V. (1989). Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. *Cell 56:* 607-617.

YUN, K. and WOLD, B. (1996). Skeletal muscle determination and differentiation: story of a core regulatory network and its context. *Curr. Opin. Cell Biol. 8:* 877-889.

Received: October 1998
Accepted for publication: October 1998