Part 1

Differentiation and Maturation of Tongue Striated Muscles

Results

Expression of desmin, muscle creatine kinase, fast and slow troponin C genes during myogenesis in tongue and hind limb muscles

Figure 2 shows developmentally related changes in desmin gene expression in mouse tongue and hind limb muscles from E11 to newborn stages. Desmin mRNA in tongue muscle was detected at E11. Its quantity increased by 239% (p <0.0001) from E11 to E15 and then plateaued after E15. On the other hand, small quantity of desmin mRNA in hind limb muscle was observed at E11. Its quantity increased by 723% (p <0.0001) from E11 to E17 but, did not change significantly between E17 and newborn stages.

Muscle creatine kinase mRNA in both tongue and hind limb muscles was not detected at E11 but was initially detected at E13 (Figure 3). The quantity of muscle creatine kinase mRNA in tongue muscle increased by 288% between E13 and 15, and plateaued after E15. In the case of hind limb muscle, the quantity of muscle creatine kinase mRNA increased by 644% (p <0.0001) from E13 to E17 and then decreased by 27% (p <0.0001) between E17 and newborn stages.

As shown in Figure 4, fast troponin C mRNA in both tongue and hind limb muscles was not detected at E11 but was initially detected at E13. Their quantities increased by 62% in tongue muscle (p <0.0001) and by 65% in hind limb muscle (p <0.0001) between E13 and E15. The quantity of tongue fast troponin C mRNA did not change significantly after E15, whereas the quantity of fast troponin C mRNA in hind limb continued to increase up to E17 and then declined to 84% of E17 value

at newborn stage (p <0.01)

Figure 5 shows developmentally related changes in slow troponin C gene expression in tongue and hind limb muscles. Slow troponin C mRNA in tongue muscle was expressed at E11. Its quantity increased markedly (about 12-fold, p <0.0001) between E11 and E13 and plateaued between E13 and E15, and then declined to less than 2% of the E15 value at newborn stage (p <0.0001). On the other hand, slow troponin C mRNA in hind limb muscle was also expressed at E11 and increased in its quantity markedly (about 8-fold, p <0.0001) up to E15 but did not change significantly after E15.

Expression of myoD family gene expression during myogenesis in tongue and hind limb muscles

Figures 6 ~ 9 show developmentally related changes in myoD family expression in the mouse tongue and hind limb muscles.

Myf5 mRNA was expressed through all developmental stages in both tongue and hind limb muscles (Figure 6). Myf5 mRNA in tongue muscle was highly expressed at E11 and E13, and then decreased in its quantity to 36% of the E13 amount at newborn stage (p <0.0001), while the expression of hind limb myf5 mRNA was relatively constant throughout all developmental stages. No significant difference in the quantity of hind limb muscle myf5 mRNA was found between any two developmental groups.

Figures 7 and 8 show developmentally related changes in myoD and myogenin mRNA expression, respectively, in both tongue and hind limb muscles.

The quantities of both myoD and myogenin mRNA in tongue muscle increased by 46% ($p<0.001$) and 55% ($p<0.05$), respectively, between E11 and E13. Their quantities began to decrease at E13 and, at newborn stage, became 28% (myoD, $p<0.0001$) and 21% (myogenin, $p<0.0001$) of E13 value. In the case of hind limb muscle, the stage when both the quantities of myoD and myogenin mRNAs began to decrease was two days later than that in tongue muscle. The quantities of both myoD and myogenin mRNA increased by 50% ($p<0.001$) and 146% ($p<0.0001$), respectively, between E11 and E15 and began to decrease at E15. Their quantities decreased by 49% (myoD, $p<0.0001$) and 46% (myogenin, $p<0.0001$) between E15 and newborn stages.

MRF4 mRNA was initially detected at E13 in tongue muscle but at E15 in hind limb muscle (Figure. 9). The quantity in tongue muscle increased markedly (about 17-fold, $p<0.0001$) from E13 to E17 and then plateaued after E17. In the case of hind limb muscle, the quantity of MRF4 mRNA increased quite linearly from E15 to newborn stages.

Discussion

In Part 1, I defined the stages in which the determination of myoblasts, formation of myotubes, and maturation of myofibers occurred during myogenesis in tongue and hind limb muscles. Furthermore, I analyzed the expression profiles of myoD family genes during the development of tongue and hind limb muscles. I correlated the expression profiles of myoD family genes with myoblast determination, myotube formation and myofiber maturation during the development of tongue and hind limb muscles.

Desmin is known to be a marker for the initial step of skeletal myogenesis such as determination (Babai *et al.*, 1990; Mayo *et al.*, 1992). Therefore, the present finding that desmin mRNA was detected in the mouse tongue and hind limb even at E11 (Figure 2) indicated that tongue and hind limb precursor cells were determined to become myoblasts in somites before E11, which is in accordance with the previous immunohistochemical data (Babai *et al.*, 1990; Mayo *et al.*, 1992). The data that desmin positive cells are observed in E9 mouse somites (Schaart *et al.*, 1989; Mayo *et al.*, 1992) supports our assertion.

Muscle creatine kinase mRNA, a marker for the formation of myotubes (Reporter *et al.*, 1963; Shainberg *et al.*, 1969; Yaffe, 1969), was not detected at E11 but detected at E13 in both tongue and hind limb muscles (Figure 3), indicating that the formation of myotubes in both tongue and hind limb muscles was initiated before E13. Histochemical studies have already shown that myotube formation in mouse tongue and hind limb muscles is initiated between E11 and E13 (Ontell and Kozeka, 1984; Ontell *et al.*, 1988, 1993; Yamane *et al.*, 1997). These data are

consistent with the present data.

The quantity of muscle creatine kinase mRNA did not increase after E15 in tongue muscle but after E17 in hind limb muscle (Figure 3). Muscle creatine kinase is known to increase in parallel with myotube formation (Reporter *et al.*, 1963; Shainberg *et al.*, 1969; Yaffe, 1969). Thus, the present observation indicated that the myotube formation was completed at E15 in tongue muscle but at E17 in hind limb muscle. Electron microscopic studies by Ontell and Kozeka, (1984) and Ontell *et al.*, (1988) have reported that myotube formation is completed at E18 in mouse extensor digitorum longus and at newborn in mouse soleus. These stages are one ~ two days older than the stage estimated by PCR technique in the present study. I assume that the difference is due to time lag between mRNA transcription and formation of myotube structure following protein translation.

I found that fast troponin C mRNA was detected at E13 in both tongue and hind limb skeletal muscles (Figure 4). This data indicates that the maturation to fast-twitch skeletal muscle fiber began at E13 in both tongue and hind limb muscles, because the fast isoform of troponin C is expressed strictly in fast-twitch skeletal muscle fiber (reviewed by Schiaffino and Reggiani, 1996). Fast troponin C mRNA was markedly more abundant than slow troponin C mRNA which was barely detectable at newborn stage in tongue muscle, whereas both fast and slow troponin C mRNAs were still highly expressed at newborn stage (Figures 4 and 5). It has been reported that in both tongue and hind limb muscles fast troponin C increases but slow troponin C decreases gradually with maturation of myofibers, and, finally, fast troponin C becomes the predominant isoform of troponin C in adult tongue and hind limb muscles (Sutherland *et al.*, 1991; Prigozy *et al.*, 1997). the

present observation, therefore, indicates that the maturation to fast twitch muscle fiber is completed in tongue muscle but not completed in hind limb muscle at newborn stage. There is a supportive evidence with respect to tongue muscle that multinucleated myofibers are arranged in a three-dimensional architecture representative of the adult configuration by newborn stage.

In the present study, myf5 mRNA is expressed in tongue and hind limb muscles between E11 and newborn stages. However, studies by using in situ hybridization have reported that the expression of myf5 mRNA disappears by E15 in hind limb muscle (Ott *et al.*, 1991). This discrepancy could be accounted for by the difference in the sensitivity between PCR and in situ hybridization techniques. It appears that myf5 controls myoblast determination (Braun *et al.*, 1992; Rudnicki *et al.*, 1992, 1993). However, since myoblast determination had already been finished before E11 in both tongue and hind limb muscles, a question for the role of myf5 between E11 and newborn stages was raised. Recently it has been reported that myoblasts which express high level of myf5 can not initiate differentiation (myotube formation) (Kitzman *et al.*, 1998). In addition, it is known that adult skeletal muscles contain undifferentiated myogenic stem cells, termed satellite cells, that can regenerate myofibers in response to injury and various kinds of stimulant such as loading, extension, electricity etc (Campion, 1984). Therefore, the present data suggests that myf5 is involved in the maintenance of the satellite cell population in tongue and hind limb muscles after myoblast determination.

I observed that the stage when the quantities of myoD and myogenin mRNAs began to decrease in tongue muscle (E13) were two day earlier than that in hind limb muscles (E15) (Figures 7 and 8). From the expression profiles of muscle

creatine kinase mRNA, I estimated that the stage when myotube formation was completed in tongue muscle (E15) was two day earlier than that in hind limb muscle (E17). It is well known that myogenin is essential for the myotube formation (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993). In addition, it has been recently reported that myoD is involved in the early stages of myotube formation (Kitzman *et al.*, 1998). Therefore, I suggested that the earlier termination of myotube formation in tongue than hind limb muscle was caused by the earlier beginning of decreases in myoD and myogenin mRNA quantities in tongue than hind limb muscles.

In the present study, the initiation of MRF4 mRNA expression (E13) in the tongue muscle preceded that in hind limb muscle (E15) by two days and its quantity increased up to E17 in tongue muscle but up to newborn stage in hind limb muscle (Figure 9). Judging from the expression profiles of fast and slow troponin C (Figures 4 and 5), it was likely that the maturation of tongue myofiber proceeded faster than that of hind limb myofiber. MRF4 is the last one of myoD family to be expressed and involved in the maturation and maintenance of myofibers (Braun and Arnold, 1995; Patapoutian *et al.*, 1995; Block *et al.*, 1996; Zhu and Miller, 1997). Therefore, I suggested that the faster proceeded maturation of myofibers was related to the expression of MRF4 mRNA in both tongue and hind limb muscles.