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Cloning and Characterization of Muscle-Specific Kinase in Chicken

Fanny C. F. Ip,* David G. Glass,† David R. Gies,†
Janet Cheung,* Kwok-On Lai,* Amy K. Y. Fu,*
George D. Yancopoulos,† and Nancy Y. Ip*

**Department of Biochemistry and Biotechnology Research Institute, Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong, China; and †Muscle Molecular Biology, Regeneron Pharmaceuticals, 777 Old Saw Mill River Road, Tarrytown, New York 10591-6707*

Muscle-specific kinase (MuSK) is part of the receptor complex that is involved in the agrin-induced formation of the neuromuscular junction. In the rodent, prominent mRNA expression of MuSK is restricted to skeletal muscle while the expression of agrin can also be detected in brain and certain nonneuronal tissues. The recent identification of *Xenopus* MuSK reveals that MuSK can be detected in tissues other than skeletal muscle, such as the neural tube, eye vesicles, and spleen. In this study, we describe the cloning and characterization of the chicken ortholog of MuSK and demonstrate that the regulation of MuSK expression in muscle is conserved from avian to rodent. Abundant mRNA expression of MuSK can be detected in early embryonic chick muscle and is up-regulated after nerve injury. More importantly, we also demonstrate that, in the chicken, MuSK mRNA is expressed during development in brain and liver, suggesting possible novel functions for MuSK. Furthermore, the regulatory profile of MuSK expression in chick muscle closely parallels that observed for acetylcholine receptor, in terms of both mRNA expression and protein localization. Finally, studies with paralyzed chicken muscle as well as with cultured chick myotubes demonstrate the dependence of MuSK on both electrical activity and trophic factors.

INTRODUCTION

The formation of the neuromuscular junction (NMJ) involves the interactions of anterograde and retrograde signals (Daniels, 1997; Meier and Wallace, 1998; Sanes and Lichtman, 1999). Several nerve-derived factors have been shown to regulate the pre- and postsynaptic differentiation during the development as well as the maintenance of mature NMJ (Fischbach and Rosen, 1997; Ruegg and Bixby, 1998). For example, the redistribution of acetylcholine receptors (AChRs) into tight clusters beneath the motor nerve terminal is induced by agrin during NMJ formation (Nitkin *et al.*, 1987; Tsim *et al.*, 1992). Although there are several cell-surface proteins capable of binding agrin, a receptor tyrosine kinase called muscle-specific kinase (MuSK) is the key mediator of agrin's actions (Glass *et al.*, 1996).

MuSK was cloned from a rat denervated muscle cDNA library (Valenzuela *et al.*, 1995); MuSK orthologs have thus far been cloned in human, mouse (Ganju *et al.*, 1995), and *Xenopus* (Fu *et al.*, 1999a). MuSK possesses all the domains characteristic of the traditional receptor tyrosine kinases, including a signal peptide, an extracellular (EC) domain, a transmembrane region, and an intracellular (TK) domain that contains intrinsic kinase activity. In rodents, the mRNA expression pattern of MuSK seems to be tightly restricted to muscle (Valenzuela *et al.*, 1995). MuSK is prominently expressed during embryogenesis in developing myotomes; mRNA levels stay high in muscle until birth, at which point the expression decreases. Once the muscle is innervated, MuSK protein becomes localized to the NMJ, and its expression is restricted to synaptic nuclei (Valenzuela *et al.*, 1995). Immunohistochemical analysis reveals that MuSK protein is colocalized with AChR in rat muscle since the early embryonic stages (Bowen *et al.*, 1998). If the muscle is denervated or immobilized, MuSK is dramatically up-regulated, and its expression is derepressed in nonsynaptic nuclei (Valenzuela *et al.*,

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1995). MuSK activation represents a critical step in the agrin signaling pathway by mediating the agrin-induced aggregation of AChRs in cultured myotubes (Glass *et al.*, 1996; Glass and Yancopoulos, 1997). Mice lacking functional MuSK do not form NMJ, and similar defects are seen in mice deficient in agrin (DeChiara *et al.*, 1996; Gautam *et al.*, 1996).

There are alternatively spliced forms of agrin, differing by the inclusion of small exons at three sites in the molecule, X, Y, and Z (Rupp *et al.*, 1991; Tsim *et al.*, 1992). Only the isoforms which contain inserts at the Z site are active on MuSK at physiological concentrations (Glass *et al.*, 1996), although there is a report that agrin containing only the Y insert, if expressed in COS cells which directly contact muscle, can cause AChR aggregation (Ferns *et al.*, 1993). In the mouse, agrin mRNA transcripts which contain only a Y insert have been detected throughout the brain, as well as in nonneuronal tissues, including muscle and vascular tissues. However, forms which contain the Z insert, and which are most active on MuSK, are found only in spinal and brain-stem motor neurons (Stone and Nikolics, 1995). In other rodent studies, agrin mRNA has been identified in cerebellar granule cells and somatosensory cortical neurons (So *et al.*, 1996; Li *et al.*, 1997).

It has long been known that the expression of AChR is regulated by both trophic factors and electrical activity (Klarsfeld *et al.*, 1989; Witzemann *et al.*, 1991). A nerve-derived factor, neuregulin (NRG), induces the transcription of AChR and voltage-gated sodium channel (Falls *et al.*, 1990; Corfas and Fischbach, 1993; reviewed in Fischbach and Rosen, 1997) while calcitonin gene-related peptide (CGRP) has also been demonstrated to up-regulate the expression of AChR (Fontaine *et al.*, 1987). Extensive studies have also reported the activity dependence of the AChR gene regulation (Klarsfeld *et al.*, 1989; Kues *et al.*, 1995). In contrast, very little is known about the controlling factors that regulate the expression of MuSK.

The studies on MuSK expression reported thus far are on rodent and amphibian tissues; studies with the avian system during development and after nerve injury would contribute toward our understanding of the functions of MuSK. In this report, we describe the isolation and characterization of chicken MuSK. We find that the mRNA expression and regulation of MuSK in muscle is conserved from avian to rodent. Interestingly, expression of chicken MuSK mRNA can also be detected in brain and other tissues, suggesting the possibility that MuSK has additional roles that are distinct from that of NMJ organization. Furthermore, studies with paralyzed chicken muscle as well as with cultured chick myotubes demonstrate the dependence of MuSK on both electrical activity and trophic factors.

RESULTS

Molecular Cloning of Chicken MuSK

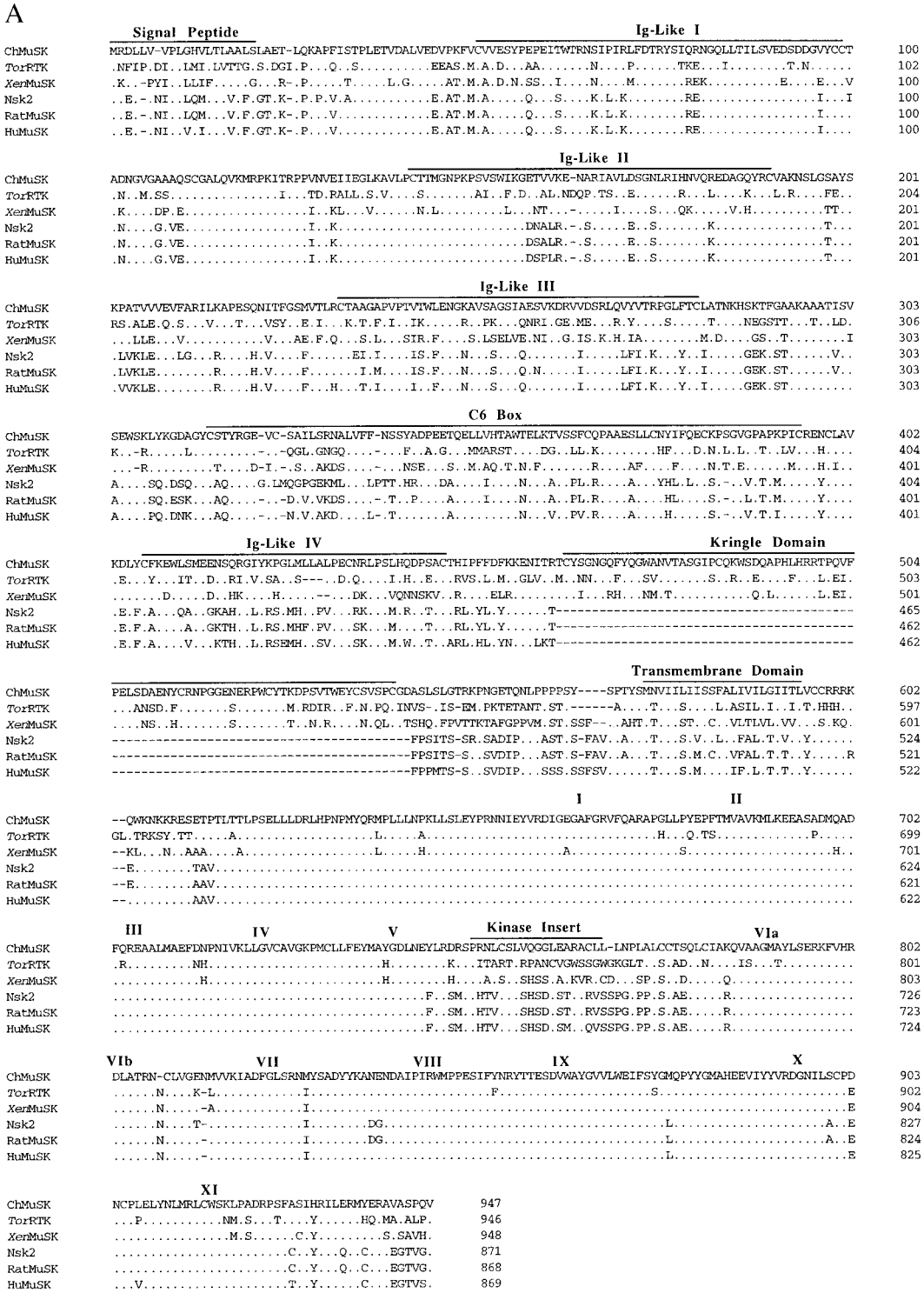
Using the rat MuSK tyrosine kinase domain as a probe, a small cDNA fragment encoding a portion of the chicken MuSK kinase domain was isolated during a low-stringency screen of an E10 chick phage library. The cDNA fragment was sequenced, and oligo primers were prepared so that PCR-based approaches could be performed on a chick myotube cDNA plasmid library, resulting in the isolation of a full-length chicken MuSK cDNA (see Experimental Methods).

The deduced chicken MuSK protein is 947 amino acids long, including the secretion signal peptide (Fig. 1A), and has most of the key structural domains defined in mammalian MuSK. A major structural difference between mammalian MuSK and the orthologs found in the lower species, such as *Torpedo* and *Xenopus*, is the absence of a Kringle domain in the extracellular region of rat, mouse, and human MuSK. The chicken MuSK reported in this study has a Kringle domain and therefore represents the third ortholog of this family which contains a Kringle domain in the same location as that found in *Torpedo* and *Xenopus* (Fig. 1A). Amino acid sequence comparison between chicken MuSK and other orthologs reveals an overall identity of ~70% (Fig. 1B). The most variable region of MuSK between species is located before the transmembrane domain. Chicken MuSK is 74% identical to *Xenopus* MuSK (70% ectodomain; 86% cytodomain), 66% identical to *Torpedo* MuSK (62% ectodomain; 80% cytodomain), ~75% identical to mammalian MuSK (68–72% ectodomain; 87–88% cytodomain). The recently identified functional tyrosine Y533 in rat MuSK is also conserved in chicken MuSK with the presence of the conserved sequence NPXY (Herbst and Burden, 2000).

Developmental Expression Profile of Chicken MuSK and Rat MuSK

To compare the temporal and spatial expression of MuSK and AChR in rat and chicken tissues, Northern blot analysis using an EC domain probe (as described under Experimental Methods) was performed on RNA samples prepared from various rat and chicken tissues at different developmental stages, i.e., E14 to adult in rat and E7 to adult in chicken. A major transcript of

A



B

	EC domain (%)	TK domain (%)	Kringle domain (%)	Overall identity (%)
*Tor*RTK	61.8	79.4	64.5	65.9
*Xen*MuSK	69.5	85.9	73.4	73.8
Nsk2	68.4	87	---	74.4
RatMuSK	72.5	87	---	76
HuMuSK	72.4	88.4	---	77.2

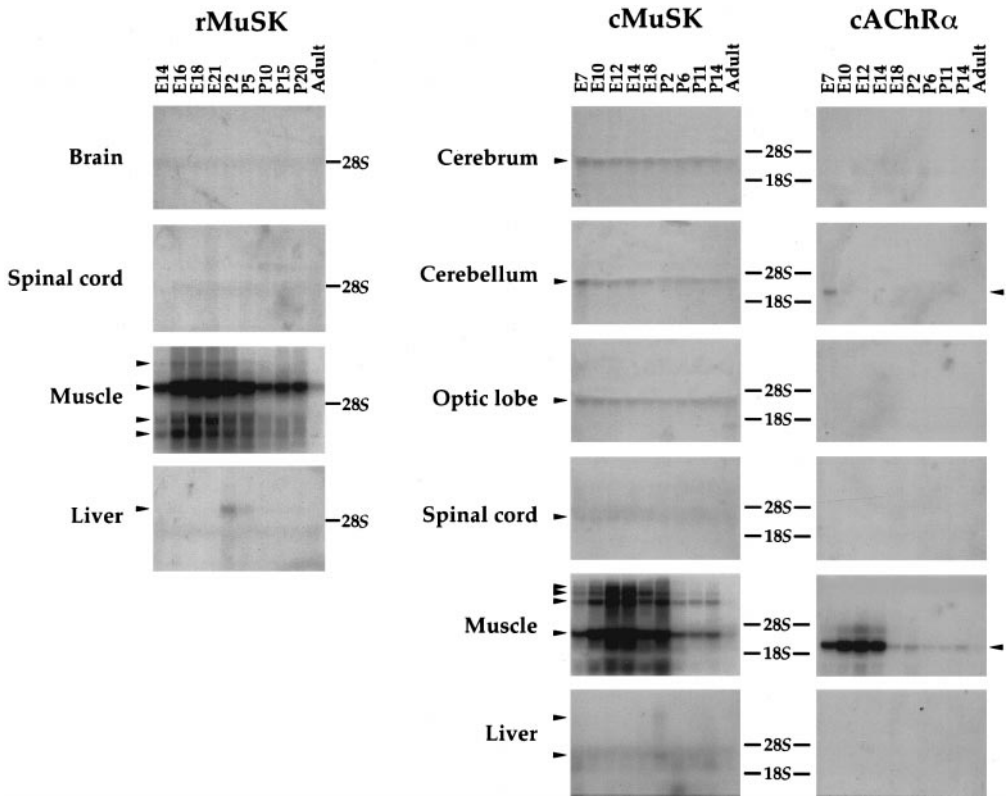


FIG. 2. Developmental mRNA expression profiles of MuSK in rat and chick tissues. Rat tissues were dissected at different stages of development: embryonic day 14 (E14) to E21, postnatal day 2 (P2) to P20, and adult. Chicken tissues were dissected from E7 to E18, P2 to P14, and adult. Expression of MuSK was examined in rat tissues (rMuSK, left) and chicken tissues (cMuSK, middle) and compared to the expression profile of AChRα (cAChRα, right). Ribosomal RNA bands (18S and 28S) are indicated, and the tissues examined are shown on the left of the blots. Arrowheads indicate the detectable transcripts of MuSK and AChRα. For MuSK, an extracellular domain probe was used as described under Experimental Methods while the entire coding region of AChRα was used as a probe.

~7.4 kb and two smaller transcripts were detected for MuSK in rat skeletal muscle (Fig. 2, left). The level of rat MuSK transcript increased significantly from E14 to E16 (approx threefold) and remained at a high level during the embryonic stages followed by a gradual decrease to low levels in adult (Fig. 2, left). A previous developmental study performed by *in situ* hybridization did not allow for the discrimination of the multiple transcripts demonstrated here (Bowen *et al.*, 1998). Similar to the findings by Valenzuela *et al.* (1995), MuSK was not detected in rat brain and spinal cord. Unexpectedly, a low but detectable level of MuSK expression was observed in rat liver from P2 and was not detectable at P20 upon overexposure (Fig. 2; data not shown), perhaps suggesting expression of MuSK by a hematopoietic compartment which is in liver during this stage of development.

Similar to MuSK expression in rat muscle, MuSK transcripts in chick muscle were also increased from E7 to E10, stayed high until P2 stage, and declined slowly to adult (Fig. 2, middle). Multiple transcripts were also detected in chicken muscle, i.e., two major (~11 and

FIG. 1. Amino acid sequence comparison of chicken MuSK to its counterparts in other species. (A) Comparison of chicken MuSK (ChMuSK) amino acid sequence to its counterparts, *Xenopus* MuSK (*Xen*MuSK), *Torpedo californica* RTK (*Tor*RTK), human MuSK (HuMuSK), and mouse MuSK (Nsk2). Only differences from the consensus sequence are indicated, and hyphens denote missing residues. The signal peptide, Ig-like domains, C6 box, Kringle domain, transmembrane domain, and conserved domains of the kinase region are indicated. (B) Percentage identity of amino acid sequence of chicken MuSK to other orthologs in terms of EC domain, TK domain, Kringle domain, and overall identity.

~4.0 kb) and two minor (~13 and ~15 kb). These MuSK transcripts were prominent in developing chick skeletal muscle of stage E7 to P2. From P6 to adult, only the two major transcripts were detected (Fig. 2). In contrast to rat MuSK expression, low levels of chick MuSK mRNA were detected in the nervous system, including cerebrum, cerebellum, optic lobe (Fig. 2, middle), and even spinal cord after overexposure (data not shown). While the expression of MuSK transcripts in chick cerebrum and optic lobe remained detectable at low levels throughout development, it was expressed at a higher level in E7 cerebellum. Interestingly, expression of MuSK mRNA was also found in P2 chicken liver, similar to that observed for rat (Fig. 2).

Since MuSK mediates aggregation of the AChR, a comparison of MuSK and AChRα expression was performed in chicken tissues. The expression of AChRα transcripts (~3.2 kb) increased in E7 to E10 chick skeletal muscle but dramatically declined after E14 (Fig. 2, right), which is consistent with the previous findings reported by Moss *et al.* (1989) and parallels that of chicken MuSK (Fig. 2, middle). Intriguingly, a low level of AChRα transcript was detected in E7 chick cerebellum, and disappeared by E10, coincident with the profile of MuSK expression (Fig. 2, right).

To compare the subcellular localization of MuSK in chicken muscle during development, double immunostaining was performed using a MuSK-specific antibody (Fu *et al.*, 1999a) and rhodamine-conjugated α-bungarotoxin (Fig. 3). While the expression of AChR clusters at the developing neuromuscular synapse was already visible during early embryonic development (as early as E10), MuSK-specific immunostaining did not appear to be confined to the synapse and was diffusely distributed on the muscle fiber (Fig. 3). Colocalization of MuSK and AChR staining was observed at E19 (Fig. 3, indicated by arrowheads) and the extent of colocalization increased throughout the postnatal stages. In adult skeletal muscle, MuSK and AChR proteins were precisely colocalized at the mature NMJ, in a manner similar to that observed for rat (Fig. 3).

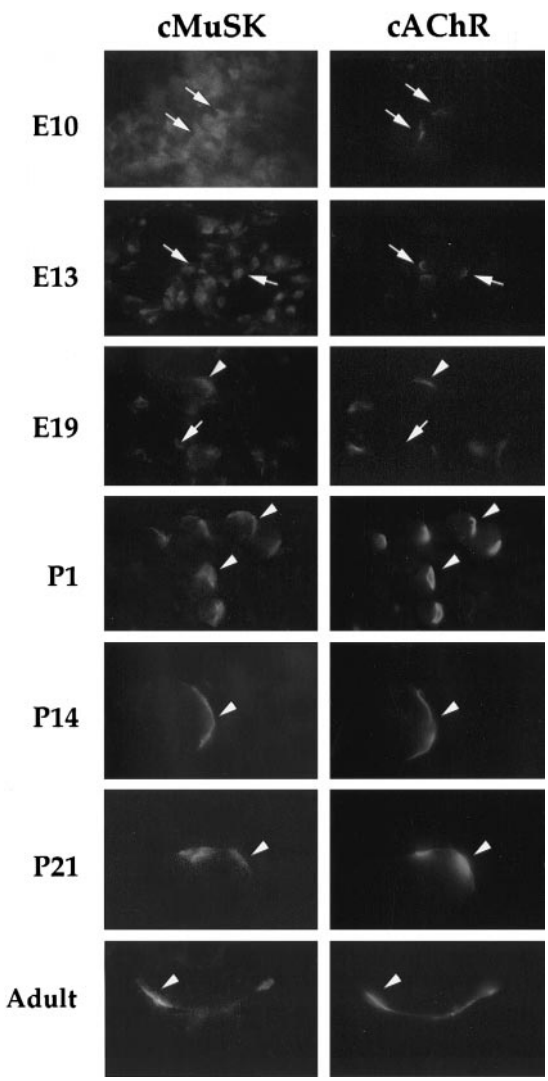


FIG. 3. Localization of MuSK in chicken skeletal muscle during development. Chicken muscle sections were prepared from various stages of development (E10 to E19, P1 to P21, and adult). MuSK was detected by MuSK-specific antibody followed by FITC-conjugated secondary antibody (left). AChR was stained with rhodamine-conjugated α-bungarotoxin (right). Arrows indicate the noncongruent clusters of MuSK and AChR while arrowheads show the colocalization of MuSK and AChR. Original magnification, 100×.

MuSK Transcripts with Tyrosine Kinase Domain

As part of an effort to gain insight into the multiple transcripts of chicken MuSK, two MuSK cDNA inserts (TK1 and TK2, as described under Experimental Methods) flanking the different regions of tyrosine kinase domain were used to perform Northern blot analysis. Interestingly, all the multiple transcripts in chicken skeletal muscle as well as the single transcript in E7 cerebellum could be detected by both TK probes of MuSK (Fig. 4). The expression profile of chicken MuSK in skeletal muscle using TK probes (Fig. 4) was similar to that observed for EC probe (Fig. 2, middle). Surprisingly, two smaller transcripts of chicken MuSK were detected in adult optic lobe and developing liver using the two TK probes but not the EC probe. The significance of these smaller transcripts found in chicken tissues remains to be determined.

The expression of MuSK in embryonic chick brain

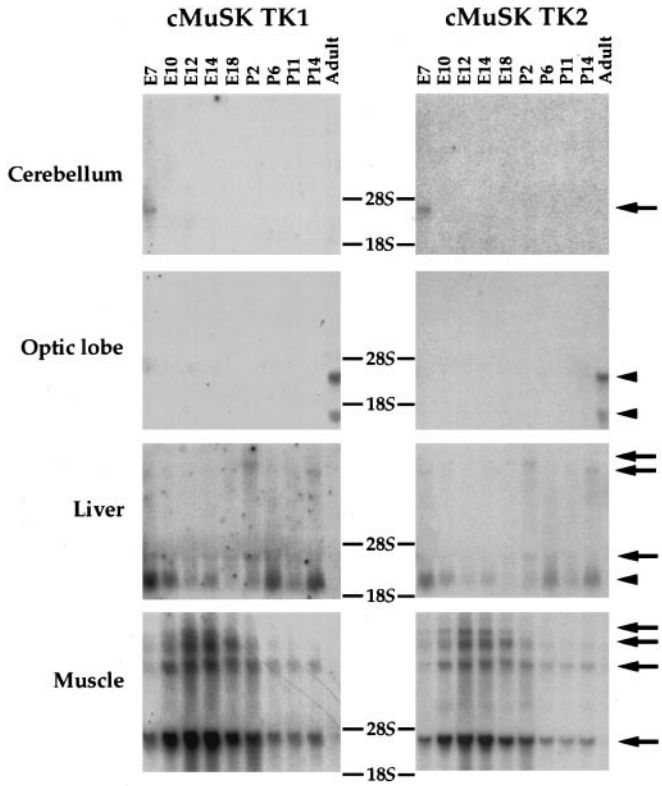


FIG. 4. Developmental expression of MuSK transcripts with kinase domain in chicken tissues. RNA blots similar to those depicted in Fig. 2 were hybridized with two TK probes (as described under Experimental Methods) flanking different regions of the tyrosine kinase domain of chicken MuSK (cMuSK). Ribosomal RNA bands (18S and 28S) are indicated between the blots and the tissues examined are indicated on the left. Arrows indicate the transcripts in muscle, while arrowheads indicate the MuSK transcripts detected in tissues other than skeletal muscle.

and postnatal liver raised the possibility that MuSK may also exist in other tissues. RT-PCR followed by Southern blot analysis was performed to detect the expression of MuSK in various tissues of P29 chick. Primers flanking the EC and TK domains were used to amplify MuSK cDNA fragments containing the kinase domain (Fig 5, top). As expected, abundant level of MuSK expression was detected in P29 chicken muscle. A detectable level of MuSK transcript was also observed in kidney, gizzard, intestine, and testis. Low level of MuSK expression was found in all other tissues examined, except bursa of Fabricius (Fig. 5, top). In addition to the predicted size of the amplified PCR fragments (arrowheads, similar to the form isolated from muscle), multiple bands were also observed, perhaps suggesting the existence of isoforms of chicken MuSK. Amplified products were not obtained in both negative controls (i.e., no RNA and no RT). Chicken GAPDH was also amplified as a control (Fig. 5, bottom). Our finding indicates that MuSK expression can also be detected in chicken tissues in addition to skeletal muscle.

Expression of MuSK in Denervated Skeletal Muscle

Since the developmental expression of chicken MuSK is similar to that of rat MuSK, it is possible that the expression of chicken MuSK is regulated in a similar manner after nerve injury. The expression of MuSK transcripts in denervated skeletal muscle was compared in chicken and rat, both of P18. Nerve transection resulted in a rapid increase of MuSK transcript in both rat and chicken gastrocnemius muscle, albeit to a different extent (Fig. 6A). While rat MuSK expression increased more than 10-fold in denervated muscle by day 1 (Fig. 6A, left), only an ~3-fold increase was found in chicken muscle (Fig. 6A, right). The two smaller transcripts of rat MuSK that were evident in embryonic muscle reappeared in denervated muscle 1 day postoperation (Fig. 6A, left, indicated by arrows). Furthermore, the up-regulation of MuSK expression in both rat

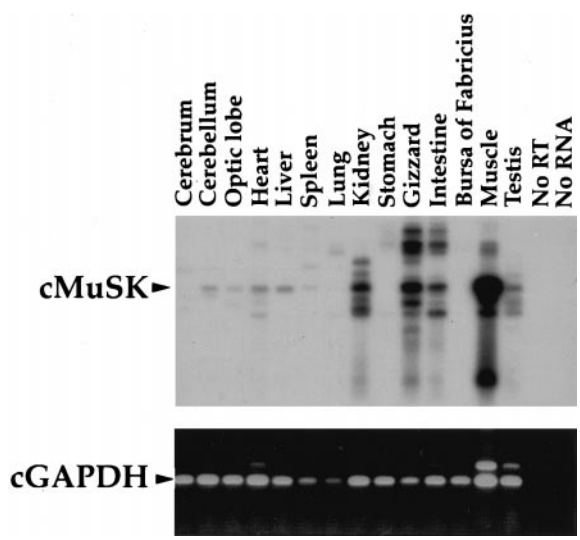


FIG. 5. mRNA expression of MuSK in adult chicken tissues by RT-PCR analysis. Expression of MuSK was examined in different chicken tissues (P29) using a pair of primers flanking the EC domain and the TK domain (top). Low level of MuSK expression in liver, spleen, and lung was revealed by longer exposure (data not shown). GADPH was amplified as control (bottom). Arrowheads indicate the identified form of MuSK and GAPDH. Amplified products were not obtained for the "no RT" or "no RNA" controls.

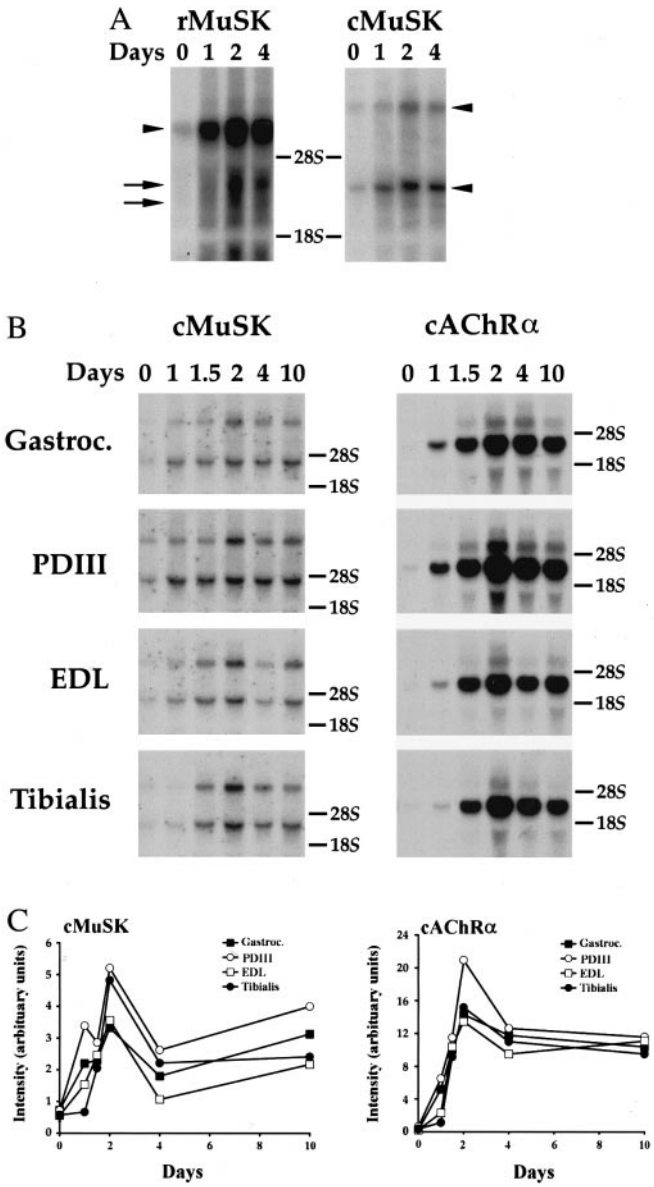


FIG. 6. mRNA expression of MuSK in denervated skeletal muscles of rat and chick. (A) MuSK and AChRα transcripts in denervated skeletal muscle of the P18 rat (rMuSK; left) and chicken (cMuSK; right) were examined at different times (0–4 days) after nerve transection. Arrowheads indicate the major transcripts of MuSK in rat and chicken, while arrows indicate the minor transcripts of rat MuSK. Ribosomal RNA bands (18S and 28S) are indicated between the blots. (B) cMuSK and cAChRα transcripts were examined in different types of chicken leg muscles, including gastrocnemius (Gastroc.), flexor perforans et perforatus digitii III (PDIII), extensor digitorum longus (EDL), and tibialis. Ribosomal RNA bands (18S and 28S) are indicated on the right. (C) Relative profiles of the mRNA expression of cMuSK and cAChRα in different types of chicken leg muscles after denervation. Quantitation of the cMuSK (lower band) and cAChRα transcripts was performed using a scanning digitizer and expressed as arbitrary units.

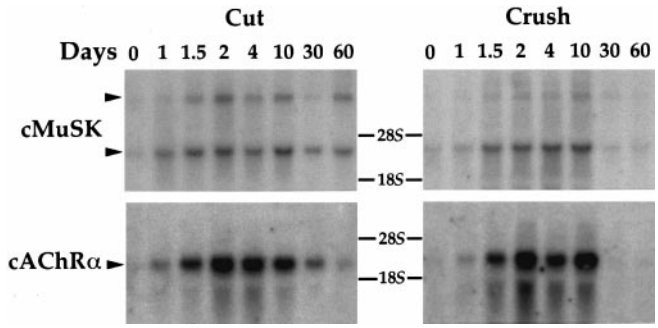


FIG. 7. Comparative expression of MuSK and AChRα mRNA in P18 chicken skeletal muscle after nerve transection and crush. cMuSK (top) and cAChRα (bottom) mRNA expressions were examined in gastrocnemius muscle at different times (0–60 days) following nerve cut (left) and crush (right). Positions of the two ribosomal RNA bands (18S and 28S) are indicated between blots.

and chicken peaked at day 2 after denervation (Fig. 6A), which is consistent with the temporal changes in MuSK protein expression observed in denervated rat muscle.

The up-regulation of chicken MuSK mRNA after nerve injury was examined in other leg muscles (including flexor perforans et perforatus digiti III (PDIII), extensor digitorum longus (EDL), and tibialis) of P18 chicken (Fig. 6B, left). The level of MuSK transcript was increased at day 1 after denervation in all muscles examined, with some variations in the extent (Fig. 6B, left). The temporal changes in MuSK transcript, however, differed for various muscles examined, i.e., denervated tibialis was the slowest in response to injury (Figs. 6B, left, and 6C). The efficacy of nerve transection was confirmed by the dramatic up-regulation of AChRα mRNA in denervated muscles (Figs. 6B, right, and 6C).

Expression of Chicken MuSK in Muscle Following Nerve Cut and Crush

The expression of chicken MuSK transcripts in skeletal muscles was compared following nerve cut and crush for up to 60 days (Fig. 7). While the expression of MuSK transcript was increased in chicken gastrocnemius muscle at day 1, and the level stayed high up to 60 days after nerve cut, the increase of MuSK transcript after nerve crush returned to basal levels by day 30 (Fig. 7, top). Two major MuSK transcripts were detected in muscle after nerve injury (Fig 7, top), but the smaller of these two transcripts was predominantly up-regulated in muscle after nerve crush (Fig. 7, top). Similar to the expression profile of chicken MuSK, the level of AChRα

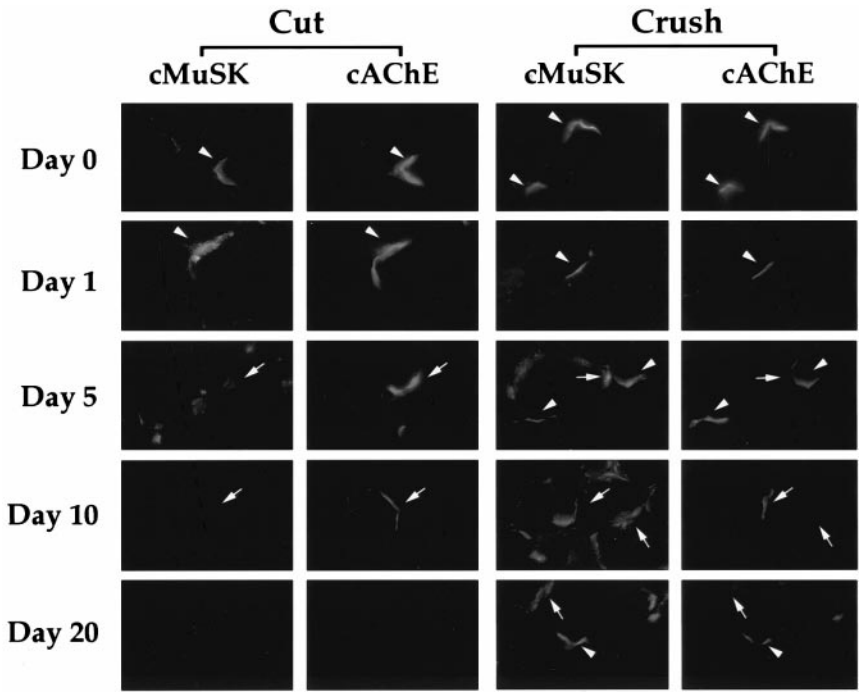


FIG. 8. Localization of MuSK in chicken skeletal muscle after denervation. Muscle sections were prepared at different times after sciatic nerve cut (left) or crush (right). MuSK was detected by MuSK-specific antibody followed by FITC-conjugated secondary antibody. AChE was detected with AChE antibody followed by rhodamine-conjugated secondary antibody. Arrows indicate the noncongruent clusters of MuSK and AChE while arrowheads show the colocalization of MuSK and AChE. Original magnification, 63×.

transcript was increased dramatically in gastrocnemius muscle at 1.5 days and recovered to control level at 30 days after nerve crush (Fig. 7, bottom). Similar results were obtained with other leg muscles after nerve crush (data not shown).

To determine the subcellular localization of MuSK and AChR in denervated skeletal muscle, their distributions were compared with acetylcholine esterase (AChE) by immunohistochemical analysis (Fig. 8). Chicken AChE has been shown to remain localized at NMJ for at least 2 weeks after nerve transection (Massoulié *et al.*, 1993). Frozen sections of chicken muscle after nerve cut and crush were double-immunostained by MuSK and AChE (Fig. 8). MuSK and AChE proteins were colocalized at the NMJ at day 0 and day 1 after nerve cut and crush. At day 5 and day 10 after nerve cut, while AChE staining was still confined to the NMJ, MuSK clusters became dispersed throughout the muscle fibers. MuSK- or AChE-specific staining could no longer be observed at day 20 after denervation. However, in nerve-crushed muscle at day 5, clusters of MuSK staining, some noncongruent with AChE at the synaptic sites, could be detected. At day 10 after nerve crush, MuSK clusters were observed to be reconfined to NMJ and colocalized with AChE at day 20 (Fig. 8).

Expression of MuSK Transcripts in TTX-Paralyzed Skeletal Muscle

In both the rat and the chicken muscles, the expression of MuSK is up-regulated after nerve injury and recovered to normal level coincident with the period of nerve regeneration. To further examine the role of synaptic activity in the regulation of MuSK expression *in vivo*, the leg muscles of the animals were paralyzed by TTX, which was delivered by an implanted auto-osmotic pump. Pumps containing Hanks' solution served as controls. The expression of MuSK and AChRα was examined in the TTX-paralyzed and control leg muscles of the operated side in rat and chicken (Fig. 9). In TTX-paralyzed muscles, MuSK transcripts were up-regulated as was observed in denervated muscles. The induction of MuSK and AChRα mRNA after 2 days of TTX treatment was comparable to that in muscle 2 days after denervation.

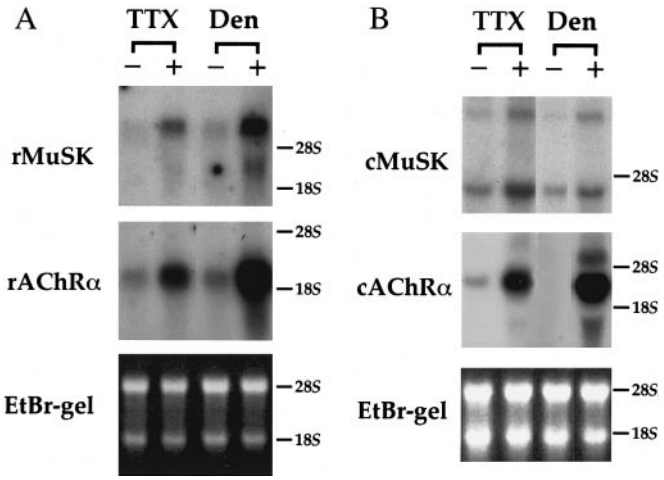


FIG. 9. Expression of MuSK in TTX-paralyzed skeletal muscle. The mRNA expression of MuSK and AChRα was examined in rat (A) and chicken (B) muscle after TTX paralysis or nerve cut for 2 days. Total RNA was extracted from gastrocnemius muscle of TTX-paralyzed (TTX+), Hanks' solution control (TTX−), denervated (Den+), or sham-operated control (Den−) 2 days after the operation. The Northern blots were hybridized with cMuSK (top) and cAChRα (middle). Ribosomal RNA bands (18S and 28S) are indicated on the right. Equal loading of RNA is shown by the ethidium bromide-stained gel at the bottom.

Regulation of MuSK by Pharmacological Agents and Trophic Factors in Primary Chick Muscle Culture

As a first step to examine the mechanisms that are involved in the regulation of MuSK expression, primary chick myotubes were treated with a variety of chemicals or trophic factors for 2 days. Since extensive studies have been reported on the regulation of AChRα mRNA in cultured chick myotubes treated with pharmacological agents (Klarsfeld and Changeux, 1985), the mRNA expression of AChRα was simultaneously examined as control. Treatment with TTX (1 μM), a blocker of the voltage-dependent Na^+ channel, resulted in a rapid and complete cessation of spontaneous contractions of myotubes. While the expression of MuSK transcripts was up-regulated by ~2-fold in TTX-treated cultures, an ~10-fold increase of chicken AChRα mRNA was detected (Fig. 10, left). Treatment with dantrolene, which blocks the calcium efflux from the sarcoplasmic reticulum, or verapamil, a calcium channel blocker, also caused an increase in MuSK expression. In the presence of the Ca^{2+} ionophore A23187, the effect of TTX on MuSK expression was completely blocked (Fig. 10, left). The regulation of MuSK expression by the agents observed in this study closely parallels the profile observed for AChRα (Klarsfeld and Changeux, 1985).

The expression of AChRα can be induced by the treatment with trophic factors such as NRG and CGRP. The ability of these trophic factors to increase the expression of MuSK in chick myotube cultures was examined. Similar to the expression of AChRα, MuSK transcript was up-regulated by NRG and CGRP by ~3- and ~1.5-fold, respectively (Fig. 10, right). CNTF treatment was used as control; significant regulation of MuSK expression was not observed in cultured myotubes treated with CNTF.

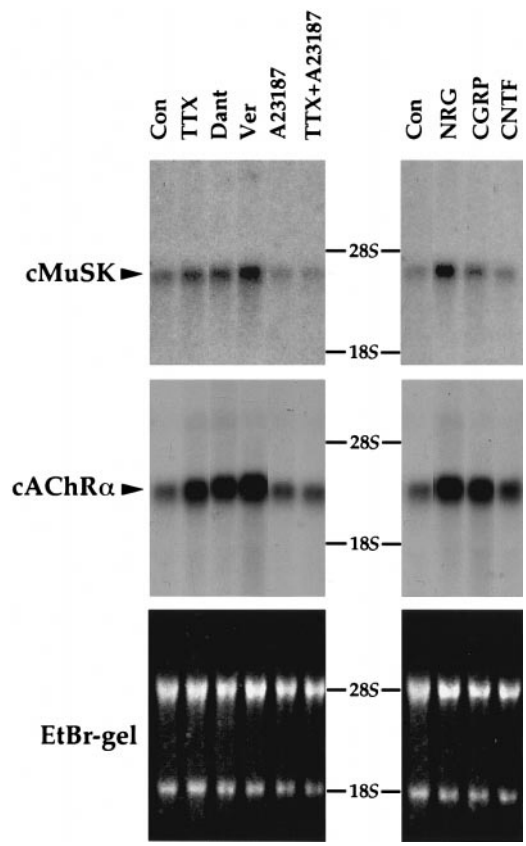


FIG. 10. Expression of chicken MuSK in primary muscle culture. Day 4 chick myotube culture was treated with different chemicals (left) or trophic factors (right) for 2 days as described under Experimental Methods. Pharmacological agents examined included TTX (1 μM), calcium ionophore A23187 (0.25 μM), dantrolene (50 μM), and verapamil (10 μM), while trophic factor treatment included NRG (1 nM), CGRP (100 nM), and CNTF (100 ng/ml). RNA was extracted from treated and control (Con) cultures; the Northern blots were hybridized with cMuSK (top) and cAChRα (middle). Ribosomal RNA bands (18S and 28S) are indicated between blots. Equal loading of RNA is shown by the ethidium bromide-stained gel at the bottom.

DISCUSSION

Rat MuSK has been cloned and characterized to be highly restricted to muscle tissue, inspiring its name as the muscle-specific kinase (Valenzuela *et al.,* 1995). Functional studies identified MuSK as a critical component of the agrin receptor and the mediator of NMJ formation (DeChiara *et al.,* 1996; Glass and Yancopoulos, 1997). While agrin is highly expressed in motor neurons, it is also expressed in brain and nonneuronal tissues, suggesting additional functions distinct from the induction of the NMJ. The lack of an entire complementary expression pattern between rat MuSK and agrin presents something of a paradox. However, the agrin protein is large and complex, and there are several isoforms which do not activate MuSK. Thus, agrin might have additional functions that are independent of MuSK. One possible candidate for an additional receptor for agrin is the ROR tyrosine kinase (Masiakowski and Carroll, 1992), an orphan receptor which shares organizational motifs in its extracellular domain with MuSK (Valenzuela *et al.,* 1995). Studies of protein homologs can help elucidate a protein's function, either by the conservation or loss of functional domains or by comparing activities across various species.

To further assess the function of MuSK, we have cloned its chicken ortholog. We found that chicken MuSK retains the Kringle domain which is present in *Xenopus* MuSK (Fu *et al.,* 1999a) and *Torpedo* RTK (Jennings *et al.,* 1993) but is lacking in mammalian forms of MuSK. This characteristic, together with the finding that chicken MuSK has similar degrees of identity to mammalian MuSK (76%) and to *Torpedo* RTK (66%), suggests that the avian gene is at an evolutionary midpoint between *Torpedo* and rat and provides additional evidence that the *Torpedo* RTK is a MuSK ortholog. The loss of a protein domain in mammalian MuSK, such as the Kringle domain, might hint at some changes in the function of the receptor during evolution.

Analysis of rat and chicken MuSK mRNA expression reveals several transcripts during development, which is also consistent with the observation in *Xenopus* (Fu *et al.,* 1999a). The significance of these different transcripts is not yet clear. Several cDNAs of rat MuSK, representing differentially spliced isoforms, have previously been identified (Valenzuela *et al.,* 1995; Hesser *et al.,* 1999). The very short exon additions or deletions in those isoforms would not account for the change in transcript lengths of chicken MuSK, which is likely due to a variation in the length of untranslated sequence. Efforts are under way in our laboratory to isolate the isoforms of MuSK, such as those in the chicken optic lobe, and identify the potential functions.

The expression profiles of chicken MuSK presented in this study demonstrate that the gene regulation of MuSK in muscle is conserved from avian to rodent: there is a similar peak of expression during myogenesis in the embryo, and there is a parallel up-regulation in rodent and avian MuSK after denervation and paralysis. In rat, MuSK has been reported to be colocalized with AChR as early as E14 and continued to adult NMJ (Bowen *et al.,* 1998). While AChRs already appear as clusters at the chick NMJ at E10 and MuSK is abundantly expressed in chicken muscle during early embryonic stages, colocalization of MuSK and AChR can only be observed at ~E19. The clustering of MuSK proteins at the later stages of synapse formation observed in this study implies potentially different roles for MuSK during chicken development. While it is not clear what accounts for the apparent delay in the clustering of MuSK at the chick NMJ, it is noteworthy that species-dependent differences in the regulation of postsynaptic proteins have previously been reported for AChE and CNTF receptor (Ip *et al.,* 1996).

Our studies on the regulation of MuSK mRNA expression in muscle after nerve injury and in cultured myotubes reveal that the regulatory mechanisms for MuSK parallel AChRα. The up-regulation of MuSK expression in denervated muscle is partly mediated by a loss of electrical activity, which can be mimicked by the addition of Na^+ or Ca^{2+} channel blockers. Interestingly, similar to AChRα, the mRNA expression of MuSK in cultured myotubes can also be regulated by trophic factors such as NRG and CGRP. The mechanism underlying the effects of these factors, however, remains to be elucidated. Further characterization of the regulatory regions in the promoter of MuSK will provide insight into our understanding of the roles of trophic factors, muscle activity, and Ca^{2+} in controlling the MuSK gene expression.

Taken together, the conservation of MuSK regulation in avian and rodent muscle highlights the importance of MuSK as a mediator of synaptogenesis at the NMJ through evolution. Moreover, the restrictive expression pattern of MuSK in mammalian tissues is not observed in lower species, as demonstrated by the detection of MuSK transcripts in several brain tissues of chick (this study) and in nonmuscle tissues of *Xenopus* (Fu *et al.,* 1999a). Such mRNA expression of MuSK, together with the expression of agrin in the brain, suggests a potential role for MuSK in other types of synaptogenesis. The inability to detect MuSK in the rat brain reported in the previous study (Valenzuela *et al.,* 1995) might be due to

the much lower expression levels in nonmuscle tissues. We also report here that both rat and chicken MuSK are expressed at P2 in liver and that this expression disappears by P20. These findings justify further expression studies of MuSK in mammals and anticipate novel roles for MuSK.

EXPERIMENTAL METHODS

cDNA Library and Cloning of Chicken MuSK

An E10 chick λgt11 cDNA library was obtained from Clontech (CA, U.S.A.). Low-stringency hybridization was performed on the E10 library with a *Bgl*II–*Bpu*I 102I probe from the rat MuSK cytoplasmic domain. A clone containing a small insert was obtained and sequenced. Exact oligo primers identical to the putative chicken MuSK cDNA (5′-CATCTGCGACATGCAGGCCGACTTCCAGAG-3′ and its complement and 5′-GCTGACTTTGGCCTCTCAAGGAACATGTATTC-3′ and its complement) together with oligo primers of the vector sequence were used to amplify the full-length chicken MuSK sequence from a chick myotube cDNA plasmid expression library (S. Davis, unpublished data).

RNA Preparation and Northern Blot Analysis

Tissues dissected from rats and chicks of various developmental stages or after denervation were immediately frozen in liquid nitrogen and homogenized. Total RNA was extracted by lithium chloride or guanidium thiocyanate method as previously described (Ip *et al.*, 1995). Total RNA was fractionated on 1% agarose–formaldehyde gel, transferred onto a nylon membrane (MSI, MA, U.S.A.), and cross-linked by UV irradiation. Gels were stained with ethidium bromide to confirm the equal loading of each lane.

cDNA probes used for Northern blot analysis include an EC probe and two TK probes of chicken MuSK (EC: 603-bp cDNA fragment cut by *Apa*I / *Cla*I, corresponding to nt 563–1165. TK1: 594-bp cDNA fragment cut by *Apa*I / *Bgl*II, corresponding to nt 2021–2614. TK2: 256-bp cDNA fragment cut by *Acc*I / *Bgl*II, corresponding to nt 2614–2869). In addition, a 532-bp cDNA fragment (cut by *Ase*I / *Bam*HI, corresponding to nt −6–526), flanking the EC domain of rat MuSK, and an ~1.2-kb full-length cDNA of chicken AChR α-subunit were used. DNA fragments were gel-purified by Qiaex (Qiagen, Germany) and labeled by random priming (Megaprime labeling kit, Amersham). RNA blots were hybridized at 65°C with ^{32}P-labeled probes (1×10^9 cpm) in sodium phosphate buffer (pH 7), 1% bovine serum albumin, 7% SDS, 1 mM EDTA, and 20 μg / ml sonicated salmon sperm DNA. Membranes were washed at 65°C three times in 2× SSC / 0.1% SDS and exposed to X-ray film (XAR-5; Kodak) with intensifying screen at −80°C. Results were confirmed in at least three independent experiments; representative data are presented in this paper.

Immunohistochemical Analysis

Pectoral muscle of developing chicks and gastrocnemius muscle of chicks after denervation or TTX paralysis were put into isopentane and frozen in liquid nitrogen. Tissue sections (10 μm) were cut using a cryostat and thaw-mounted onto gelatin / poly-l-lysine coated slides. Sections were fixed with 2% paraformaldehyde / 5% sucrose in 1× PBS and permeabilized with 0.1% Triton X-100. For the muscle sections collected during various developmental stages, MuSK and AChR were detected by double staining. Muscle sections of denervated and TTX-paralyzed muscles were double-immunostained to detect MuSK and AChE. AChR was detected using rhodamine-conjugated α-bungarotoxin (Molecular Probes, OR, U.S.A.); MuSK (Fu *et al.*, 1999a) and AChE (ACB-1; Randall *et al.*, 1987) were detected by specific primary antibodies and visualized by rhodamine- or fluorescein-conjugated secondary antiserum, respectively. Sections were mounted with Mowiol and analyzed under a Leica fluorescence microscope. Incubation of muscle sections with secondary antiserum alone did not result in any detectable signals.

RT-PCR and Southern Blot Analysis

Synthesis of cDNA was performed using 1 μg total RNA of different tissues from P29 chicken and Superscript II RNase H^- reverse transcriptase (GIBCO, U.S.A.) and amplified by PCR using primers at the EC and TK domains of chicken MuSK. The respective sequences of the 5′ and 3′ primers were 5′-CTCCTGAATCCCAAAACATCACCTTC-3′ and 5′-TGAGCGGTCGCGCAGGTA-3′, giving a 1604-bp PCR product. For GAPDH, which served as a control, the primers used were 5′-TGATGACATCAAGAAGGTGGTGAAG-3′ and 5′-TCCTTGGAGGCCATGTAGTAGGCCAT-3′, giving a 240-bp PCR product. PCR was performed as previously described (Lai *et al.*, 1998). The RT-PCR products were electrophoresed on 1% agarose gel and Southern blot analysis was performed using an 857-bp chicken MuSK probe (cut by *Apa*I, flanking EC to TK domain, corresponding to nt 1165–2021).

Denervation Procedure

Procedures for the denervation were as previously described (Ip *et al.,* 1996). Briefly, P18 chicks and rats were anesthetized by isoflurane inhalation and pentobarbital injection, respectively. The upper thigh of the animal was opened and ~0.5 cm of sciatic nerve was removed using aseptic surgical techniques. For nerve crush experiments, the sciatic nerve was crushed using a pair of forceps chilled on dry ice. Animals were sacrificed at various times postoperation and different denervated leg muscles, including gastrocnemius, PDIII, EDL, and tibialis, were collected for Northern blot analysis. Sham-operated animals served as control.

In Vivo Chronic Paralysis by Tetrodotoxin

The upper thigh of 1-month-old chicken and rat was opened and the sciatic nerve was exposed under isoflurane anesthesia. Surgery procedure was performed as described (Michel *et al.,* 1994) with some modifications. An osmotic minipump (Alzet 2001; Alza Corp., U.S.A.) containing 180 μg/ml TTX, 200 IU penicillin, and 200 μg/ml streptomycin in Hanks' solution was implanted subcutaneously below the rib cage and fixed by 2/O surgical silk. TTX was delivered to the sciatic nerve by the silastic tubing (i.d. 0.078 cm, 10 cm in length) connecting the minipump and positioned by suturing to the surrounding thigh muscle. The filled minipump and tubing were prewarmed in a 37°C incubator for at least 4 h before implantation. The completeness of hindlimb paralysis with the loss of the flexor reflex in response to pinching of the foot toe was checked daily to confirm the effectiveness of the surgery. TTX was delivered at a rate of 4.5 μg/day with a pumping rate of 1 μl/h. This concentration of TTX was found to be optimal in inducing muscle paralysis compared to other concentrations (data not shown). Control experiment was performed using Hanks' solution without TTX and operated as described above. At day 2 postoperation, gastrocnemius muscle was excised and frozen for total RNA extraction and Northern blot analysis.

Primary Muscle Culture

The hindlimb muscles of E11 chicks were dissected in 1× Puck's buffer (0.4% KCl, 2.7 mM NaH_2PO_4, 9.5% NaCl, 6 mM Na_2HPO_4). Muscles were teased, digested with 1× Puck's buffer containing 0.25% trypsin, and terminated by fetal bovine serum. Chick myoblasts were centrifuged, resuspended, and filtered through a 70-μm filter unit. Cells were preplated to reduce the fibroblasts and plated onto rat tail collagen-coated dish at a density of 40 cells/mm^2. Chick muscle culture was maintained in Eagle's minimal essential medium supplemented with 10% (v/v) heat-inactivated horse serum, 2% (v/v) chick embryo extract, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a water-saturated atmosphere of 95% air and 5% carbon dioxide. Myoblasts began to fuse by 3 days after plating and 10 μM cytosine arabinoside was added and treated for 1 day. NRGβ1 (1 nM), CGRP (100 nM), ciliary neurotrophic factor (100 ng/ml), TTX (1 μM), calcium ionophore A23187 (0.25 μM), dantrolene (50 μM), and verapamil (10 μM) were added to the culture for 2 days. Total RNAs were extracted and subjected to Northern blot analysis. All chemicals were obtained from Sigma (MO, U.S.A.) and CNTF from Calbiochem (CA, U.S.A.); recombinant NRGβ1 was prepared as previously described (Fu *et al.,* 1999b).

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