Spatial restriction of AChR gene expression to subsynaptic nuclei

ALEXANDER M. SIMON, PETER HOPPE[1] and STEVEN J. BURDEN

Biology Department, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA
[1]Jackson Laboratories, Bar Harbor, Maine 04609, USA

Summary

Acetylcholine receptors (AChRs) and the mRNAs encoding the four AChR subunits are highly concentrated in the synaptic region of skeletal myofibers. The initial localization of AChRs to synaptic sites is triggered by the nerve and is caused, in part, by post-translational mechanisms that involve a redistribution of AChR protein in the myotube membrane. We have used transgenic mice that harbor a gene fusion between the murine AChR delta subunit gene and the human growth hormone gene to show that innervation also activates two independent transcriptional pathways that are important for establishing and maintaining this non-uniform distribution of AChR mRNA and protein. One pathway is triggered by signal(s) that are associated with myofiber depolarization, and these signals act to repress delta subunit gene expression in nuclei throughout the myofiber. Denervation of muscle removes this repression and causes activation of delta subunit gene expression in nuclei in non-synaptic regions of the myofiber. A second pathway is triggered by an unknown signal that is associated with the synaptic site, and this signal acts locally to activate delta subunit gene expression only in nuclei within the synaptic region. Synapse-specific expression, however, does not depend upon the continuous presence of the nerve, since transcriptional activation of the delta subunit gene in subsynaptic nuclei persists after denervation. Thus, the nuclei in the synaptic region of multinucleated skeletal myofibers are transcriptionally distinct from nuclei elsewhere in the myofiber, and this spatially restricted transcription pattern is presumably imposed initially by the nerve.

Key words: neuromuscular synapse, human growth hormone, transgenic mice, synaptic development.

Introduction

In adult muscle fibers, acetylcholine receptors (AChRs) are virtually confined to the small patch of membrane directly beneath the presynaptic nerve terminal (Salpeter, 1987). This arrangement is established during development in a series of steps that begin when the developing motor nerve interacts with embryonic myotubes and causes a redistribution of a fraction of AChRs that were already present on the myotube membrane (Anderson and Cohen, 1977; Ziskind-Conhaim et al., 1984; Role et al., 1985). Thereafter, AChRs are excluded from non-synaptic regions of the myofiber membrane and they remain concentrated at the synaptic site (Burden, 1977).

The maintenance of a high concentration of AChRs at the synapse requires replacement of AChRs at synaptic sites (Fambrough, 1979), and there is evidence that this replacement of synaptic AChRs is accomplished by local insertion of AChRs into the postsynaptic membrane (Role et al., 1985). In addition, there is evidence that AChR polypeptides are synthesized locally in the synaptic region, since the mRNAs encoding the different subunits of the AChR are highly concentrated at synaptic sites in adult myofibers (Merlie and Sanes, 1985; Fontaine and

Changeux, 1989; Goldman and Staple, 1989; Brenner et al., 1990).

The mechanisms responsible for accumulation of AChR mRNAs at synaptic sites are unclear, but could involve selective stabilization of AChR mRNAs in the synaptic region, transport of AChR mRNAs to the synaptic region, or selective transcription of AChR genes by the subset of nuclei that are within the synaptic region.

We constructed transgenic mice that contain a gene fusion between 1.8 kbp of 5' flanking DNA from the murine AChR delta subunit gene and the human growth hormone (hGH) gene, to determine whether cis-acting elements in the AChR gene could restrict expression of hGH to the synaptic region of skeletal myofibers. Because hGH is processed in intracellular organelles prior to secretion, and because these organelles are closely associated with nuclei, we could infer the nuclear source of hGH transcription by studying the spatial distribution of intracellular hGH by immunocytochemistry. We show here that hGH in these transgenic mice is restricted to the perinuclear region of nuclei within the synaptic region of the myofiber, and we conclude that transcription of the endogenous AChR delta subunit gene is confined to nuclei that are situated at the synaptic site. These data indicate that

synaptic nuclei are transcriptionally distinct from nuclei elsewhere in the multinucleated skeletal myofiber and that the synaptic site provides a signal that acts locally to activate transcription of certain genes in nuclei that are positioned close to the synaptic site.

Materials and methods

Construction of transgenic mice

C57BL/6J females were mated with LT/Sv males, and zygotic male pronuclei were injected with a gene fusion between the murine AChR delta subunit gene ($-1,823/+24$) (Baldwin and Burden, 1988) and the hGH gene (Selden et al., 1986). The embryos were transferred to B6/SJL F1 pseudopregnant females (Wagner et al., 1981). Five founder mice (δGH1-5), which had integrated the transgene into their germ line, were the source of five different transgenic lines. All integrations are autosomal, and they range from a few copies to more than ten copies of the transgene. Mice from each transgenic line express hGH in skeletal muscle, although the absolute level of hGH expression and the degree of muscle-specific hGH expression differs among the five lines (Table 1). An additional founder mouse was apparently sterile and another founder mouse contained the transgene but did not express hGH in any tissue; we did not establish lines from these mice. hGH (20-100 ng/ml) is detectable by radioimmunoassay in the serum of newborn transgenic mice.

Histology

Immunofluorescence

Adult mice were fixed by perfusion (4% formaldehyde in 0.9% sodium chloride with 0.2 M sucrose), and dissected muscles were fixed further by immersion in fixative for 1 hr. The tissue was rinsed with 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.3 (PBS), washed with 20 mM Tris, 150 mM sodium chloride, pH 7.3, permeabilized with PBS-NP40 (0.5% NP40) and incubated with antibodies to hGH (DAKO, Carpinteria, CA) (1/500 in PBS-NP40) for 3-6 hr. at room temperature. The tissue was washed with PBS for 0.5 hr., incubated with fluorescein-labelled goat anti-rabbit IgG

Table 1. *hGH mRNA levels in muscle and non-muscle tissue*

Line #	Den. muscle	Heart	Liver	Spleen
δGH1	100	30.0	<0.5	10
δGH2	100	<0.2	<0.2	1
δGH3	100	0.4	0.3	20
δGH4	100	40.0	<6.0	10
δGH5	100	2.0	90.0	10

In all five lines the highest level of hGH mRNA is seen in denervated muscle, but only δGH2 shows nearly complete muscle specificity. Because there is no consistent pattern of tissue expression among these lines, it seems likely that the site of transgene integration and cryptic elements in the hGH gene (Low et al., 1989) determine the level of hGH expression in non-skeletal muscle tissue. The levels of hGH mRNA in heart, liver, spleen and denervated skeletal muscle were determined by RNase protection (Materials and methods). The amount of hGH mRNA/total RNA in denervated muscle from each line was assigned 100%, and the values of hGH mRNA/total RNA in other tissues are expressed relative to denervated muscle.
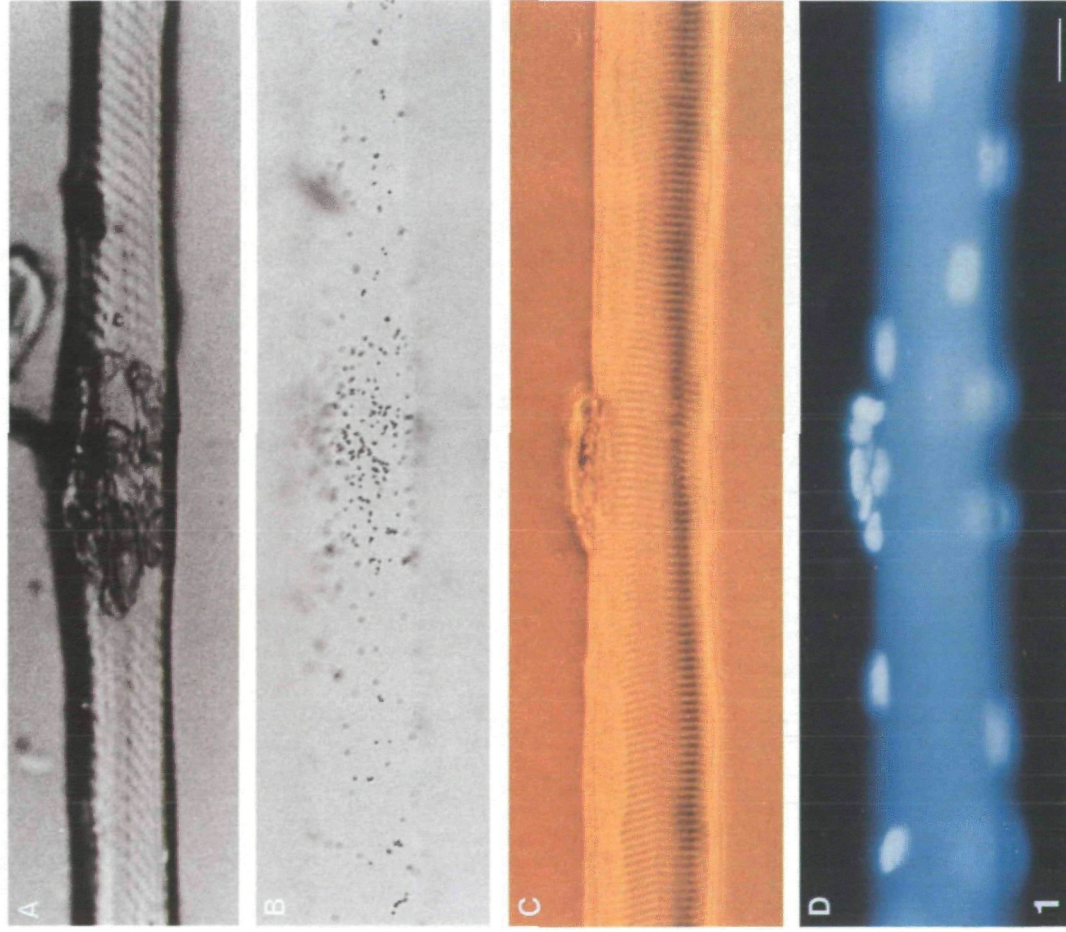
(Cappel, Malvern, PA) and tetramethylrhodamine-coupled alpha-bungarotoxin (TMR-α-BGT) in PBS-NP40 for 3-6 hr. at room temperature, washed with PBS for 0.5 hr. and fixed in 100% methanol at $-20°C$. Muscles, or individual dissected muscle fibers, were mounted whole (in 20% glycerol, 80 mM sodium bicarbonate, pH 9, with 10 μg/ml p-phenylenediamine) and were viewed with filters selective for either fluorescein or rhodamine (Burden, 1982). Our attempts to detect hGH with enzyme-coupled antibodies were unsuccessful; lack of hGH staining was probably due to poor penetration of these reagents into muscle tissue. Most experiments were performed on diaphragm, intercostal, extensor digitorum longus and soleus muscles; the pattern of hGH staining was indistinguishable among the different muscles. Staining for hGH in muscle that was fixed only by immersion, and not by perfusion, was capricious; lack of hGH staining under these conditions was probably due to secretion of hGH during the time required for dissection of deeper muscles prior to their fixation. Because of the low level of hGH expression in lines δGH4 and δGH5 and the reduced fertility of line δGH3, we restricted the immunocytochemical analysis to lines δGH1 and δGH2.

In situ hybridization

Muscles were fixed in 4% formaldehyde in PBS for 1 hr., stained for cholinesterase and dehydrated. Single muscle fibers were dissected in a drop of water on silated microscope slides, allowed to dry overnight at 40°C and processed for in situ hybridization (Kintner and Melton, 1987). The ^{35}S-labelled anti-sense delta subunit RNA probe (1.9 kbp long) was synthesized with SP6 polymerase from SP65-delta cDNA (LaPolla et al., 1984). Exposures were for 4-5 days.

RNA analysis

RNA was isolated from tissue with guanidinium thiocyanate as described (Chomczynski and Sacchi, 1987). The levels of delta subunit, skeletal muscle actin and hGH mRNAs were measured by RNase protection (Baldwin and Burden, 1988), were quantitated with a phosphorimager (Molecular Dynamics, Sunnyvale, CA), and were corrected for non-specific hybridization by subtracting the value for protection with tRNA. The ^{32}P-labelled RNA probes extended from nucleotide 1 to 582 in the hGH gene (Selden et al., 1986), nucleotide 234 to 730 in the delta subunit gene (Baldwin and Burden, 1988), and nucleotide 1258 to 1661 in the actin gene (Hu et al., 1986).

Results

AChR delta subunit mRNA is highly concentrated at synaptic sites

mRNAs encoding the four subunits of the AChR are enriched at synaptic sites (Fontaine and Changeux, 1989; Goldman and Staple, 1989; Brenner et al., 1990). The distribution of delta subunit mRNA in a single isolated myofiber is illustrated in Fig. 1, which shows that delta subunit mRNA is concentrated at the synaptic site, whereas nuclei are present throughout the myofiber.

Fig. 1. AChR delta subunit mRNA is concentrated at synaptic sites. Mouse diaphragm muscle was stained for acetylcholinesterase, and individual muscle fibers were dissected and processed for in situ hybridization using a ³⁵S-labelled-anti-sense delta RNA probe (Materials and methods). (A) The synaptic site on the myofiber is identified by cholinesterase staining. (B) The autoradiographic grains are concentrated at the synaptic site. (C) The single synaptic site on another myofiber is identified by the nerve terminal and associated Schwann cells, which are seen clearly on the myofiber surface. (D) The nuclei of both the myofiber and Schwann cells contribute to a cluster of nuclei at the synaptic site. Three to six myofiber nuclei are present at each synaptic site, and the density of myofiber nuclei in non-synaptic regions is 2.5 to 3-fold less. Myofiber nuclei are located at the periphery of the cell and are found throughout the myofiber (D). Bar=7.5 μm for (A,B) and 15 μm for (C,D).
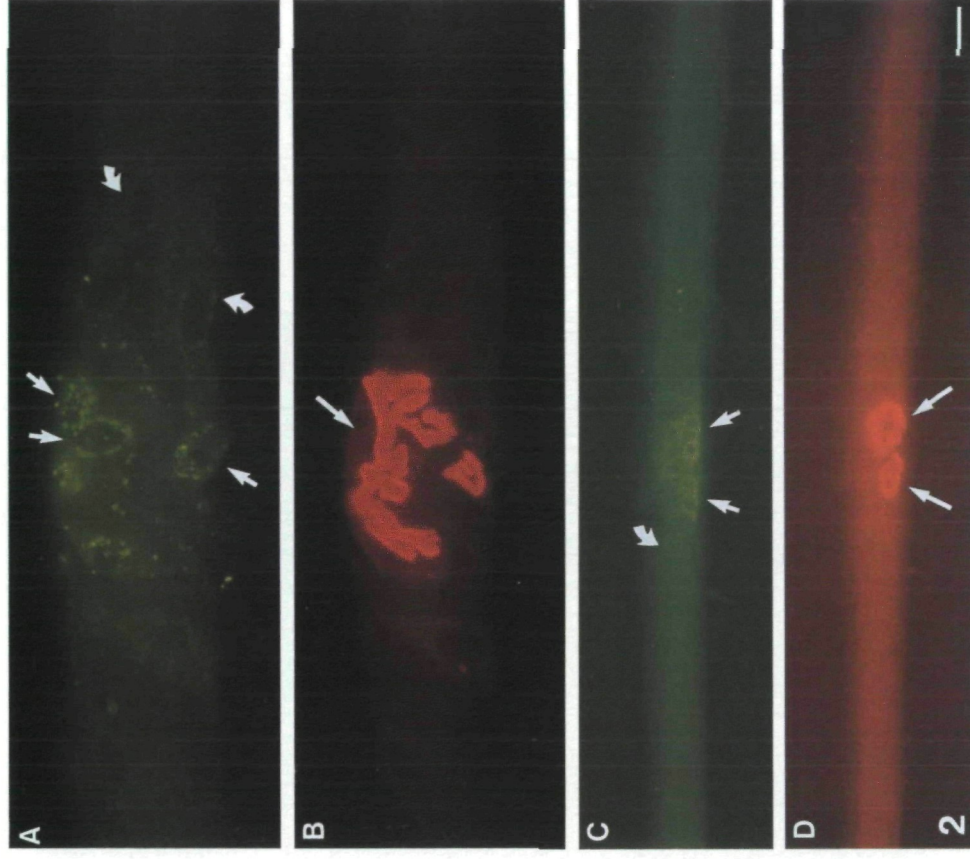

Fig. 2. Delta subunit gene is transcribed preferentially in synaptic nuclei. hGH is concentrated at synaptic sites of muscle from transgenic mice that harbor a gene fusion between the AChR delta subunit gene and the hGH gene. Two muscle fibers are shown, one in A and B and the other, at lower magnification, in C and D. Single muscle fibers were double-labelled with antibodies against hGH (A,C) and with TMR-α-BGT to stain AChRs (B,D), mounted whole, and viewed with optics selective for either fluorescein (A and C) or rhodamine (B and D). Most myofibers have a single synaptic site (B, long arrow), and the presence of two adjacent synaptic sites is uncommon (D, long arrows). hGH is concentrated at the perinuclear region of nuclei immediately beneath the postsynaptic membrane (A,C, short arrows). Lower levels of hGH are detectable in the perisynaptic region (10-50 μm from the edge of the synaptic site; A,C, curved arrows), whereas hGH is not detectable in non-synaptic regions. Bar=12 μm for (A,B) and 30 μm for (C,D).
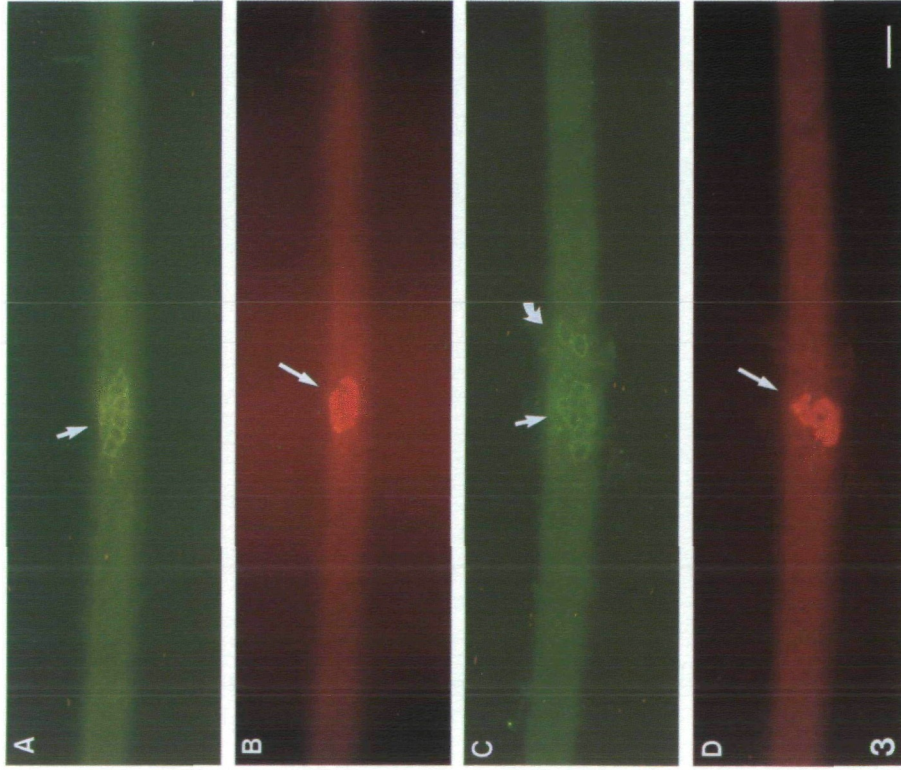
Fig. 5. Non-synaptic nuclei in denervated muscle are heterogeneous, and nuclei that express the delta subunit gene at an enhanced rate are positioned close to non-synaptic AChR clusters. Three muscle fibers are shown, one in A and B another in C and D, and a third in E and F. The muscle fibers were double-labelled with antibodies against hGH (A,C,E) and with TMR-α-BGT to stain AChRs (B,D,F). Non-synaptic AChR clusters were detected with TMR-α-BGT on approximately one-third of the denervated myofibers (B,D,F, long arrows), and hGH was observed in the region of each AChR cluster (A,C,E, short arrows). Non-synaptic AChR clusters are usually found further than 500 μm from synaptic sites (B,D), although they can occur adjacent to the denervated synaptic site (F, curved arrow). In some cases the hGH-positive nuclei are in the vicinity of AChR clusters (C,D), whereas in other cases, hGH-positive nuclei are found precisely underlying AChR clusters (A,B and E,F). The denervated synaptic site is readily distinguished from non-synaptic AChR clusters by its characteristic shape and size, and was identified on each myofiber that had non-synaptic AChR clusters. Bar=12 μm for (A,B,C,D) and 30 μm for (E,F).

Fig. 3. Preferential transcription of the delta subunit gene in synaptic nuclei does not require the continuous presence of the nerve. The extensor digitorum longus muscle was denervated by cutting the sciatic nerve, and single muscle fibers, which were denervated for 4 days, were labelled with antibodies against hGH (A,C) and with TMR-α-BGT (B,D) and mounted whole. Two muscle fibers are shown, one in A and B and the other in C and D. The denervated synaptic site is marked by TMR-α-BGT (B,D, long arrow). hGH is concentrated at the perinuclear regions of nuclei immediately beneath the postsynaptic membrane of denervated muscle (A,C, short arrow). hGH is readily detectable in the perisynaptic region of denervated muscle (C, curved arrow). hGH staining is more intense at synaptic sites and at perisynaptic regions in denervated muscle than in innervated muscle. Bar=30 μm.
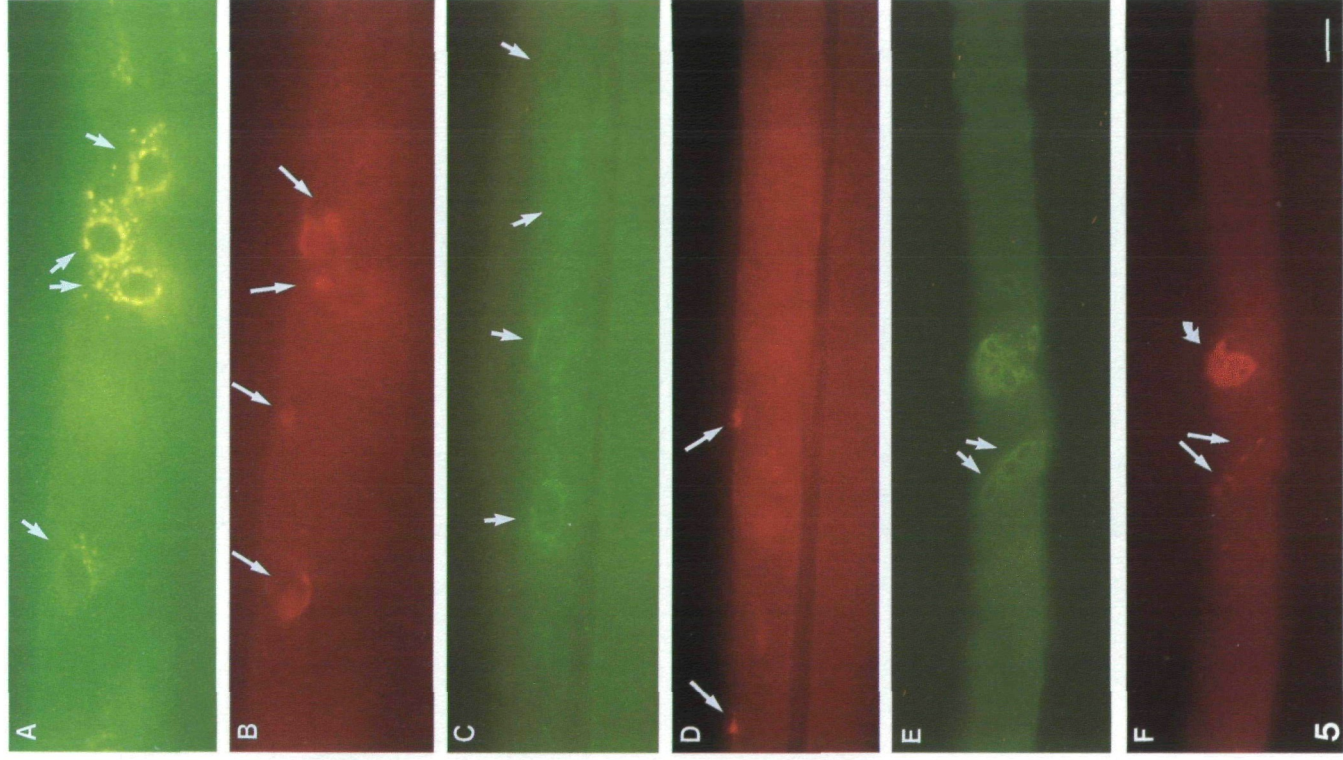
AChR delta subunit gene is transcribed selectively in subsynaptic nuclei

Accumulation of delta subunit mRNA at synaptic sites could be caused by transcriptional and/or post-transcriptional mechanisms. In order to determine whether cis-acting elements in the delta subunit gene restrict expression to the synaptic region of skeletal myofibers, we constructed transgenic mice that contain a gene fusion between 1.8 kbp of 5' flanking DNA from the delta subunit gene and the hGH gene (Materials and methods).

Growth hormone is normally expressed and secreted by somatotroph cells of the anterior pituitary (Herlant, 1964), but the hormone can be expressed and secreted by a variety of non-pituitary cell types in transgenic mice (Palmiter et al., 1983; Trahair et al., 1989). Cells that have a regulated secretory pathway contain the hormone in secretory granules and in the golgi apparatus, whereas cells that have only a constitutive secretory pathway contain the hormone largely in the golgi apparatus (Trahair et al., 1989; Roth et al., 1990). Since myofibers are not regarded as secretory cells and are thought to possess a constitutive but not a regulated, secretory pathway (Kelly, 1985; Burgess and Kelly, 1987), we expected intracellular hGH to be found in the golgi apparatus of myofibers. Further, because the golgi apparatus is closely associated with nuclei, and because mRNAs do not diffuse over long distances in myotubes (Ralston and Hall, 1989a,b; Pavlath et al., 1989; Rotundo, 1990), we reasoned that the nuclear source of hGH transcription could be inferred by studying the spatial distribution of golgi that contain hGH.

We examined the intracellular distribution of hGH in skeletal myofibers from two lines of transgenic mice containing the gene fusion between the delta subunit and hGH genes using antibodies against hGH and immunofluorescence. Fig. 2 shows that hGH expression is restricted to the synaptic region of myofibers, and that intracellular hGH is perinuclear and, by light microscopy, appears to be associated with the golgi apparatus.

The perinuclear region of nuclei immediately beneath the postsynaptic membrane are labelled intensely, and labelling is diminished in the perisynaptic region (10-50 μm from the synaptic site). hGH is not detectable in non-synaptic regions of normal muscle, although nuclei are found throughout the myofiber (Fig. 1). These results show that cis-acting sequences in the transgene are sufficient to confer synapse-specific expression of hGH in innervated skeletal muscle.

Our data indicate that the transgene is transcribed at an enhanced rate in subsynaptic nuclei. Although the transgene encodes 24 nucleotides of mRNA from the 5' untranslated region of the delta subunit gene, this sequence is neither conserved between delta subunits genes from other species (chicken δ, Wang et al., 1990; *Xenopus* δ, Burden, unpublished observation), nor between genes encoding other AChR subunits (human α, Noda et al., 1983; chicken α, Klarsfeld et al., 1987; mouse γ, Crowder and Merlie, 1988); therefore, these 24 nucleotides are not likely to be critical for localizing delta subunit and hGH mRNAs to synaptic sites. Thus, our data indicate that myofiber nuclei that are positioned close to the synaptic site transcribe the endogenous delta subunit gene at a higher rate than nuclei elsewhere in the myofiber.

Synaptic activation persists in the absence of the nerve

Synaptic specializations, including accumulations of AChR and mRNAs encoding AChR subunits, persist at synaptic sites after denervation (Fambrough, 1979; Fontaine and Changeux, 1989; Goldman and Staple, 1989; Witzemann et al., 1991). In order to determine whether synapse-specific transcription of the delta subunit gene was maintained after denervation, we examined the distribution of hGH in denervated muscle. Fig. 3 shows that hGH is concentrated at synaptic sites in muscle that was denervated for 4 days. Like hGH at normal synaptic sites, hGH at denervated synaptic sites is perinuclear and is apparently associated with the golgi apparatus. Because hGH mRNA has a half-life of 2-20 hours (Diamond and Goodman, 1985), most of the hGH that is detected at denervated synaptic sites was probably transcribed and translated after denervation. Thus, enhanced transcription of the delta subunit gene at synaptic sites does not require the continuous presence of the nerve.

Denervation causes an increase in the turnover of synaptic AChRs and an increase in the level of AChR protein and AChR mRNA in perisynaptic regions (Salpeter, 1987; Goldman and Staple, 1989), and these changes could be caused by an increase in the rate of AChR transcription. Consistent with this idea, the level of hGH in synaptic and perisynaptic regions appears greater in denervated than in innervated muscle (Figs 2,3). These results are consistent with the idea that electrical activity suppresses transcription of the delta subunit gene throughout the myofiber and that enhanced transcription at synaptic sites in denervated muscle is caused by the removal of electrical activity dependent repression and the persistence of synapse-specific activation.

Myofiber electrical activity has an important role in repressing AChR expression, and denervation of skeletal muscle causes an increase in the abundance of AChRs and mRNAs encoding AChR subunits in non-synaptic regions (Fambrough, 1979; Evans et al., 1987). Delta subunit mRNA levels increase 10- to 20-fold after denervation (Fig. 4), and this increase can be prevented, or reversed, by direct electrical stimulation of denervated muscle (Goldman et al., 1988; Witzemann et al., 1991). We measured delta subunit and hGH mRNA levels in innervated and denervated muscle to determine whether electrical activity regulates the delta subunit gene by transcriptional mechanisms. Fig. 4 shows that denervation of muscle causes a 10- to 20-fold increase in endogenous delta subunit mRNA levels and a similar increase in hGH mRNA levels. Thus, this 1.8 kbp from the delta subunit gene contains the cis-acting regulatory elements that confer electrical activity dependent gene regulation.
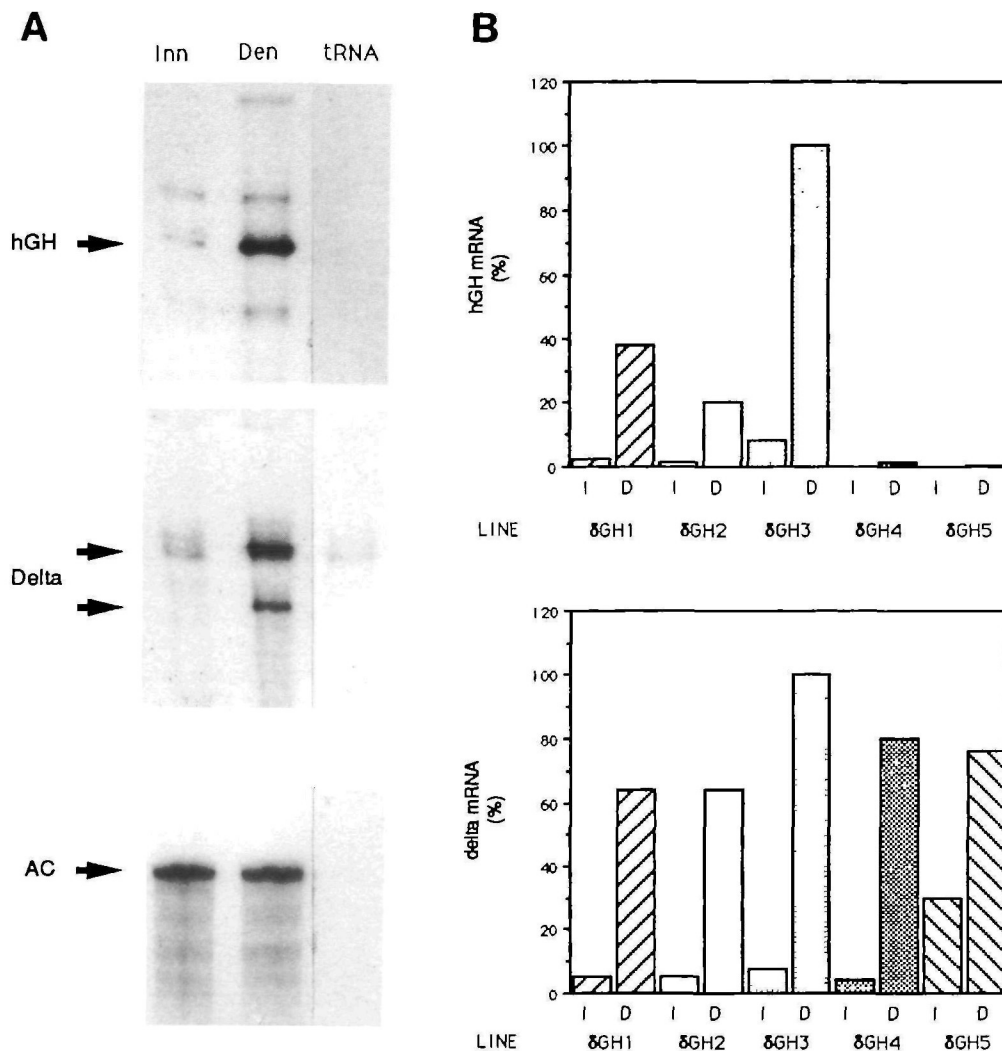
A

B

Fig. 4. Denervation causes an increase in the rate of transcription from the delta subunit gene. (A) The level of hGH, endogenous delta subunit and actin mRNAs in innervated (Inn) and denervated (Den) muscles from line δGH2 were determined by RNase protection (Materials and methods). The position of the protected bands are indicated with arrows. (B) In four lines (δGH1-4), the level of hGH and delta subunit mRNAs, normalized to the level of actin mRNA, is 10 to 20-fold greater in denervated than in innervated muscle. The level of hGH expression in lines δGH4 and δGH5 is about 40-fold lower than in line δGH3. Lower left leg muscles were denervated by cutting the sciatic nerve, and innervated and denervated muscles were assayed 4 days later. The value for denervated muscle from line δGH3 was assigned 100%, and all other values are expressed relative to δGH3 denervated muscle. The abundance of actin mRNA was 20% lower in denervated than in innervated muscle.

Non-synaptic nuclei in denervated muscle are heterogeneous, and expressing nuclei are colocalized with AChR clusters

Although denervation causes an increase in the level of AChRs, most AChRs in non-synaptic regions of denervated myofibers are distributed diffusely and are not detectable with TMR-α-BGT (Fambrough, 1979). However, a small fraction of these non-synaptic AChRs aggregate, and these AChR clusters can be detected with TMR-α-BGT (Ko et al., 1977). We do not detect hGH by immunofluorescence in most non-synaptic areas of denervated muscle; we suspect that it is present, but that immunofluorescence is insufficiently sensitive to detect this low level of expression. However, hGH is detectable at non-synaptic regions where AChR clusters are found (Fig. 5). The intensity of hGH staining at these non-synaptic AChR clusters is similar to that observed at synaptic sites, and non-synaptic hGH, like synaptic hGH, is perinuclear and is apparently associated with the golgi apparatus. These results indicate that non-synaptic nuclei in denervated myofibers are transcriptionally heterogeneous, since the delta subunit gene is transcribed at different rates in

different non-synaptic nuclei (see also Fontaine and Changeux, 1987; Berman et al., 1990). Further, these results indicate that the processes of clustering AChRs and enhancing AChR transcription are linked, and that colocalization of AChR transcription and AChR clusters does not require a signal from the nerve.

Signal and pathway appear shortly after synapse formation

Accumulation of AChRs at developing synapses is an early event in synapse formation and occurs several days before birth in rodents (Frank and Fischbach, 1979; Dennis, 1981; Slater, 1982). However, synaptic structure and function continue to be modified during the next few weeks, and the full complement of adult synaptic properties are not acquired until about one month after birth (Dennis, 1981; Slater, 1982; Schuetze and Role, 1987). In order to determine at what point synaptic nuclei become distinct, we examined the distribution of hGH in muscle from newborn mice. Fig. 6 shows that hGH is concentrated at synaptic sites in newborn muscle. Like synaptic staining in adult muscle, synaptic hGH staining in developing muscle is perinu-
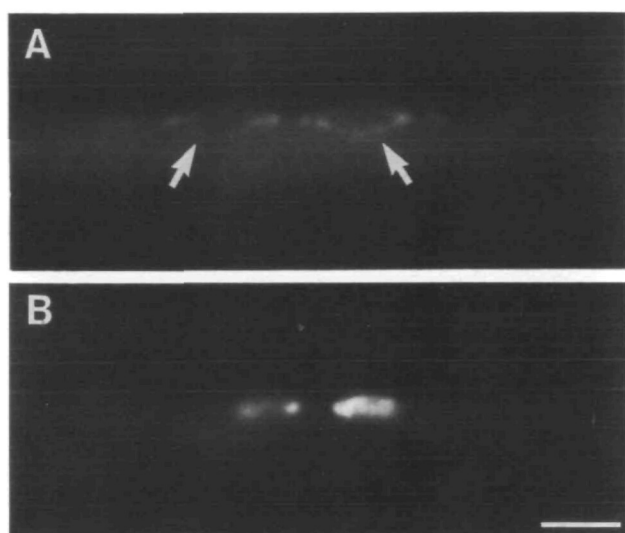
Fig. 6. Delta subunit gene is transcribed preferentially in synaptic nuclei in newborn muscle. hGH is concentrated at synaptic sites in muscle from newborn transgenic mice. An individual muscle fiber from the diaphragm muscle, which was double-labelled with antibodies against hGH (A) and with TMR-α-BGT (B), was mounted whole and viewed with optics selective for fluorescein (A) and rhodamine (B). hGH is concentrated at the perinuclear region of nuclei immediately beneath the postsynaptic membrane (arrows); there are less nuclei at synaptic sites in newborn muscle than in adult muscle. Bar=10 μm.

clear. However, synaptic staining in newborn muscle appears less intense than in adult muscle. Thus, synaptic nuclei become transcriptionally distinct early during synapse formation and before postjunctional folds have developed, polyneuronal innervation has been eliminated, or AChR channels with short open-times have appeared (Schuetze and Role, 1987).

Myogenin is not expressed specifically in subsynaptic nuclei

Myogenic basic-helix-loop-helix (bHLH) proteins are important regulators of muscle gene expression during myogenesis, and their targets include the genes encoding AChR subunits (Piette et al., 1990). Previous studies have shown that the levels of myogenin and MyoD1 mRNA are high in muscle from newborn mice and decrease during the following two weeks (Duclert et al., 1991; Eftimie et al., 1991). Further, the levels of myogenin and MyoD1 mRNA, like AChR mRNA, are regulated by innervation, since they increase following denervation (Duclert et al., 1991; Eftimie et al., 1991). We sought to determine whether myogenic bHLH proteins might be involved in synapse-specific expression of the AChR delta subunit gene, and we used an antibody against myogenin to determine whether myogenin is concentrated in subsynaptic nuclei. We restricted our analysis to myogenin, because antibodies against MRF4 and myf-5 are not available, and we could not detect staining in newborn or adult muscle with available antibodies to MyoD1 (Tapscott et al.,
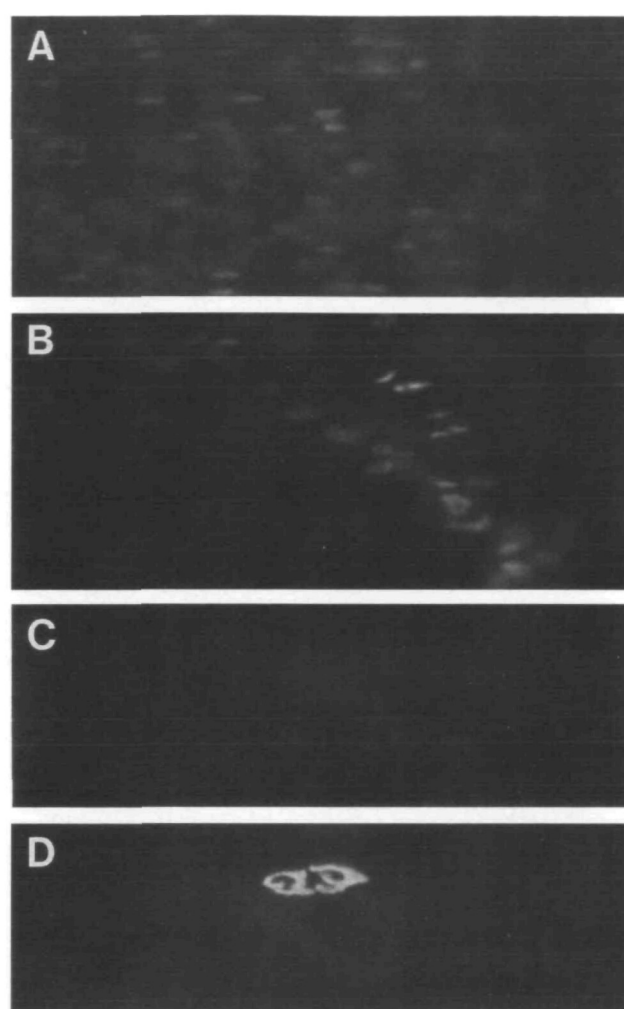

Fig. 7. Myogenin is not expressed specifically at synaptic sites. Diaphragm muscles from newborn rats (A,B), or adult rats (C,D) were labelled with a monoclonal antibody (F5D) against myogenin (A,C) and TMR-α-BGT (B,D). A whole mount of newborn muscle is shown in A and B, whereas a single adult myofiber is shown in C and D. Myogenin is expressed in nuclei throughout developing myofibers from newborn rat muscle, but myogenin is not concentrated at synaptic sites (A,B). The whole mount of the innervated zone of newborn diaphragm muscle contains more than one hundred myofibers, but only several dozen of the myofibers and synaptic sites are in focus. Myogenin is not detectable at synaptic sites or non-synaptic regions of innervated, adult muscle (C,D). Bar=30 μm.

1988). Fig. 7 shows that myogenin is expressed in nuclei throughout developing myofibers in newborn muscle, but that myogenin is not detectable in innervated, adult muscle. These results are consistent with the data for myogenin mRNA and support the idea that myogenin could have a role in coupling changes in myofiber electrical activity to changes in AChR gene expression (Duclert et al., 1991; Eftimie et al., 1991). However, because myogenin is not concentrated at synaptic sites, either in newborn or adult muscle (Fig. 7), these data do not support the idea that myogenin has a central role

in activating AChR genes selectively in subsynaptic nuclei.

Discussion

This study demonstrates that transcription of the AChR delta subunit gene in innervated, adult muscle is confined to nuclei that are situated within the the synaptic region. These results indicate that synaptic nuclei are transcriptionally distinct from nuclei elsewhere in the multinucleated skeletal myofiber and that the synaptic site provides a signal that acts locally to activate transcription of a select set of genes in nuclei that are positioned close to the synaptic site.

The restricted spatial distribution of AChR gene expression indicates that a synaptic signal, which is presumably provided by the motor neuron, exerts its effect over a highly circumscribed region of the myofiber. hGH is highly concentrated immediately beneath the postsynaptic membrane, and expression of hGH is less abundant 10-50 μm from the synaptic site and is not detectable further than 50 μm from the synaptic site. These distances are similar to those measured for spread of signalling information in other transduction systems (Lamb et al., 1981; Matthews, 1986), and these data indicate that the skeletal myofiber need not have unique mechanisms to restrict the spatial flow of information. The spatial influence of the synaptic signal may be restricted further by repressive signals provided by electrical activity, and this additional influence may explain why hGH is readily detectable in the perisynaptic region of denervated myofibers. Although our results are consistent with the idea that transcriptional activity decreases exponentially from the synaptic site, our immunofluorescence methods were not quantitative, and diffusion of RNA or rapid translocation of organelles associated with the golgi apparatus could also produce a gradient of intracellular hGH. Nevertheless, our data provide an estimate of the distance over which the synaptic signal exerts its effect.

Spatial restriction of delta subunit gene expression does not depend upon the continuous presence of the nerve, since hGH remains concentrated at synaptic sites following denervation. Thus, either the nerve-derived signal that is responsible for activation of the delta subunit gene in subsynaptic nuclei remains at synaptic sites for at least several days following denervation, or the effect of this signal persists after the nerve is removed. In either case, the transcriptional machinery associated with activation of the delta subunit gene persists in the subsynaptic nuclei of adult muscle and does not require the continuous presence of the nerve.

AChRs can cluster at non-synaptic sites on denervated myofibers and on embryonic myotubes (Ko et al., 1977; Vogel et al., 1972; Fischbach and Cohen, 1973), and the mechanisms that regulate the formation of these non-synaptic clusters have served as a model for formation of AChR clusters at synapses (Schuetze and Role, 1987). We show here that AChR accumulation at non-synaptic sites *in vivo* is associated with regions of increased delta subunit transcription. Thus, there appears to be heterogeneity among the non-synaptic nuclei, since a small number of nuclei in non-synaptic regions of denervated muscle express the delta subunit gene at an enhanced rate, similar to synaptic nuclei. Because our results show that highly expressing nuclei need not be positioned at the synaptic site, colocalization of AChR transcription and AChR clusters does not require a signal from the nerve.

We do not know whether there is a causal relationship between increased density of surface AChRs and increased AChR transcription; however, since the expressing non-synaptic nuclei are not randomly distributed but are associated in small groups, it seems unlikely that random activation of individual nuclei causes the appearance of surface AChR clusters. It is not clear what triggers the formation of these synaptic specializations at non-synaptic sites; however, it is possible that a pathway, which is normally activated by the nerve at synaptic sites, can be activated independently of the nerve and lead to all specializations normally found at the synaptic site, including both surface clustering of AChRs and nuclear activation of AChR genes.

At present we have little information regarding the nature of the signal from the nerve that activates AChR gene expression at synapses. The signal could be released by the nerve and stably maintained in the synaptic basal lamina, like agrin, the extracellular matrix molecule that causes AChRs to cluster at synaptic sites (Nitkin et al., 1987). Nevertheless, agrin itself does not appear to be a likely candidate for the transcriptional signal, since agrin causes a redistribution of surface AChRs that is not accompanied by an increase in AChR synthesis (Godfrey et al., 1984). Alternatively, the signal may be released from the nerve and be required only transiently to activate a pathway or to assemble a structure, and the signal may not be provided constitutively (Brenner et al., 1990). Identification of the synaptic signal and its receptor will be important steps in understanding how nerve-muscle signalling is mediated, and in understanding how transcription is activated.

Myogenin is a transcription factor that has an important role in initiating muscle differentiation (Wright et al., 1989), and the abundance of myogenin mRNA is regulated by innervation (Duclert et al., 1991; Eftimie et al., 1991). Myogenin is not detectable at synaptic sites in adult muscle, but is present in nuclei throughout newborn muscle. These data do not support the idea that myogenin has a central role in activating AChR genes selectively in subsynaptic nuclei. However, these data are consistent with the idea that myogenin has a role in initiatiating AChR gene expression during myogenesis and that myogenin could be involved in coupling changes in electrical activity to changes in AChR gene expression (Piette et al., 1990; Duclert et al., 1991; Eftimie et al., 1991). It will be important to determine whether any of the other myogenic bHLH proteins are concentrated in subsy-

naptic nuclei. In any case, the identification of the transcription factors that activate gene expression selectively in subsynaptic nuclei will be facilitated by analysis of additional transgenic lines and identification of the important *cis*-acting regulatory elements in the delta subunit gene that confer synapse-specific expression.

mRNAs encoding the alpha, beta and epsilon subunits of the AChR are also highly concentrated at synaptic sites in adult myofibers (Merlie and Sanes, 1985; Fontaine and Changeux, 1989; Goldman and Staple, 1989; Brenner et al., 1990), and it would seem likely that the genes encoding these subunits are also transcriptionally activated in the subsynaptic nuclei of adult muscle. A previous report has shown that a transgenic mouse line that harbors a gene fusion between the chicken AChR alpha subunit 5' flanking region and a β-galactosidase reporter gene, expresses β-galactosidase in newborn muscle (Klarsfeld et al., 1991). Expression is enriched in the central region of the muscle, where most synapses are located; however, staining extends far beyond synaptic sites in newborn muscle (Klarsfeld et al., 1991). Therefore, it is unclear whether the expression pattern in newborn muscle reflects preferential expression of the transgene by synaptic nuclei as proposed (Klarsfeld et al., 1991), or whether the pattern reflects the developmental history of myotubes, in which myoblast fusion begins in the central region of the muscle (Kitiyakara and Angevine, 1963; Bennett and Pettigrew, 1974; Braithwaite and Harris, 1979). Since expression of β-galactosidase is not detectable after postnatal day 4, the authors were not able to evaluate this latter possibility. The absence of expression in adult muscle could be due to silencing of the transgene, but it is also possible that the transgene, while it contains the elements for muscle-specific and electrical activity-dependent regulation (Merlie and Kornhauser, 1989), lacks an element necessary for synapse-specific expression.

Like synaptic nuclei in vertebrate skeletal myofibers, nuclei in the syncytial blastoderm of *Drosophila* are transcriptionally distinct (Nüsslein-Volhard, 1991). For example, expression of the *tailless* gene is restricted to nuclei in the terminal regions (Pignoni et al., 1990), and expression of the *twist* gene is restricted to nuclei in the ventral region of the syncytial blastoderm (Thisse et al., 1988). Polarity is established in the dorsoventral and terminal systems by signals that are synthesized in distinct follicular cells which ensheathe the developing oocyte (Stevens et al., 1990; Stein et al., 1991). Although the dorsoventral and terminal systems use different pathways to interpret the spatial information provided to the oocyte (Anderson et al., 1985; Sprenger et al., 1989; Casanova and Struhl, 1990), in each system polar information is maintained in the egg, and the continuous presence of follicular cells is not required to establish distinct transcriptional activity in subsets of nuclei (Stevens et al., 1990; Stein et al., 1991). The results presented in this study indicate that the presynaptic nerve terminal is the source of a signal which provides spatial information to the myofiber, and

that this signal acts to activate transcription of a select set of genes in nuclei that are situated near the synaptic site. It will be interesting to determine whether similar components and mechanisms are used to establish transcriptionally distinct nuclei in the syncytial blastoderm and in the syncytial myofiber and whether steps involved in pattern formation in *Drosophila* are shared with steps involved in neuromuscular synapse formation.

References

Anderson, K. V., Bokla, L. and Nusslein-Volhard, C. (1985). Establishment of dorsal-ventral polarity in the *Drosophila* embryo: the induction of polarity by the *Toll* gene product. *Cell* 42, 791-798.

Anderson, M. J. and Cohen, M. W. (1977). Nerve induced and spontaneous redistribution of acetylcholine receptors on culture muscle cells. *J. Physiol. (Lond.).* 268, 757-773.

Baldwin, T. J. and Burden, S. J. (1988). Isolation and characterization of the mouse acetylcholine receptor delta subunit gene: identification of a 148-bp *cis*-acting region that confers myotube-specific expression. *J. Cell Biol.* 107, 2271-2279.

Bennett, M. R. and Pettigrew, A. G. (1974). The formation of synapses in striated muscle during development. *J. Physiol. (Lond.).* 241, 515-545.

Berman, S. A., Bursztajn, S., Bowen, B. and Gilbert, W. (1990). Localization of an acetylcholine receptor intron to the nuclear membrane. *Science* 247, 212-214.

Braithwaite, A. W. and Harris, A. J. (1979). Neural influence on acetylcholine receptor clusters in embryonic development of skeletal muscles. *Nature.* 279, 549-551.

Brenner, H.-R., Witzemann, V. and Sakmann, B. (1990). Imprinting of acetylcholine receptor messenger RNA accumulation in mammalian neuromuscular synapses. *Nature* 344, 544-547.

Burden, S. (1977). Development of the neuromuscular junction in the chick embryo: The number, distribution, and stability of acetylcholine receptors. *Dev. Biol.* 57, 317-329.

Burden, S. J. (1982). Identification of an intracellular postsynaptic antigen at the frog neuromuscular junction. *J. Cell Biol.* 94, 521-530.

Burgess, T. L. and Kelly, R. B. (1987). Constitutive and regulated secretion of proteins. *Ann. Rev. Cell Biol.* 3, 243-295.

Casanova, J. and Struhl, G. (1990). Localized surface activity of *torso*, a receptor tyrosine kinase, specifies terminal body patterns in *Drosophila. Genes Dev.* 3, 2025-2038.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analyt. Biochem.* 162, 156-159.

Crowder, C. M. and Merlie, J. P. (1988). Stepwise activation of the mouse acetylcholine receptor δ- and γ-subunit genes in clonal cell lines. *Mol. Cell. Biol.* 8, 5257-5267.

Dennis, M. J. (1981). Development of the neuromuscular junction: inductive interactions between cells. *Ann. Rev. Neurosci.* 4, 43-68.

Diamond, D. J. and Goodman, H. M. (1985). Regulation of growth hormone messenger RNA synthesis by dexamethasone and triiodothyronine. *J. Mol. Biol.* 181, 41-62.

Duclert, A., Piette, J. and Changeux, J. P. (1991). Influence of innervation on myogenic factors and acetylcholine receptor α-subunit mRNAs. *NeuroReport.* 2, 25-28.

Eftimie, R., Brenner, H. R. and Buonanno, A. (1991). Myogenin and

MyoD join a family of skeletal muscle genes regulated by electrical activity. *Proc. Natl. Acad. Sci. USA* **88**, 1349-1353.

Evans, S., Goldman, D., Heinemann, S. and Patrick, J. (1987). Muscle acetylcholine receptor biosynthesis. *J. Biol. Chem.* **262**, 4911-4916.

Fambrough, D. M. (1979). Control of acetylcholine receptors in skeletal muscle. *Physiol. Rev.* **59**, 165-226.

Fischbach, G. D. and Cohen, S. A. (1973). The distribution of acetylcholine sensitivity over uninnervated and innervated muscle fibers grown in cell culture. *Dev. Biol.* **31**, 147-162.

Fontaine, B. and Changeux, J. P. (1989). Localization of nicotinic acetylcholine receptor α-subunit transcripts during myogenesis and motor endplate development in the chick. *J. Cell Biol.* **108**, 1025-1037.

Frank, E. and Fischbach, G. D. (1979). Early events in neuromuscular junction formation *in vitro*. Induction of acetylcholine receptor clusters in the postsynaptic membrane and morphology of newly formed nerve-muscle synapses. *J. Cell Biol.* **83**, 143-158.

Godfrey, E. W., Nitkin, R. M., Wallace, B. G., Rubin, L. L. and McMahan, U. J. (1984). Components of *Torpedo* electric organ and muscle that cause aggregation of acetylcholine receptors on culture muscle cells. *J. Cell Biol.* **99**, 615-627.

Goldman, D., Brenner, H. R. and Heinemann, S. (1988). Acetylcholine receptor α-, β-, γ-, and δ-subunit mRNA levels are regulated by muscle activity. *Neuron.* **1**, 329-333.

Goldman, D. and Staple, J. (1989). Spatial and temporal expression of acetylcholine receptor mRNAs in innervated and denervated rat soleus muscle. *Neuron* **3**, 219-228.

Herlant, M. (1964). The cells of the adenohypophysis and their functional significance. *Int. Rev. Cytol.* **17**, 299-382.

Hu, M. C., Sharp, S. B. and Davidson, N. (1986). The complete sequence of the mouse skeletal α-actin gene reveals conserved and inverted repeat sequences outside of the protein-coding region. *Mol. Cell. Biol.* **6**, 15-25.

Kelly, R. B. (1985). Pathways of protein secretion in eukaryotes. *Science* **230**, 25-32.

Kintner, C. R. and Melton, D. A. (1987). Expression of *Xenopus* N-CAM RNA in ectoderm is an early response to neural induction. *Development.* **99**, 311-325.

Kitlyakara, A. and Angevine, D. M. (1963). A study of the pattern of post-embryonic growth of *M. gracilis* in mice. *Dev. Biol.* **8**, 322-340.

Klarsfeld, A., Bessereau, J.-L., Salmon, A.-M., Triller, A., Babinet, C. and Changeux, J.-P. (1991). An acetylcholine receptor α-subunit promoter conferring preferential synaptic expression in muscle of transgenic mice. *EMBO J.* **10**, 625-632.

Klarsfeld, A., Daubas, P., Bourachot, B. and Changeux, J. P. (1987). A 5'-flanking region of the chicken acetylcholine receptor α-subunit gene confers tissue specificity and developmental control of expression in transfected cells. *Mol. Cell. Biol.* **7**, 951-955.

Ko, P. K., Anderson, M. J. and Cohen, M. W. (1977). Denervated skeletal muscle fibers develop discrete patches of high acetylcholine receptor density. *Science.* **196**, 540-542.

Lamb, T. D., McNaughton, P. A. and Yau, K.-W. (1981). Spatial spread of activation and background desensitization in toad rod outer segments. *J. Physiol. (Lond.)* **319**, 463-486.

LaPolla, R. J., Mixter-Mayne, K. and Davidson, N. (1984). Isolation and characterization of a cDNA clone for the complete protein coding region of the delta subunit of the mouse acetylcholine receptor. *Proc. Natl. Acad. Sci. USA* **81**, 7970-7974.

Low, M. J., Goodman, R. H. and Ebert, K. M. (1989). Cryptic human growth hormone gene sequences direct gonadotroph-specific expression in transgenic mice. *Molec. Endocrinology.* **3**, 2028-2033.

Matthews, G. (1986). Spread of the light response along the rod outer segment: an estimate from patch-clamp recordings. *Vision Res.* **26**, 535-541.

Merlie, J. P. and Kornhauser, J. M. (1989). Neural regulation of gene expression by an acetylcholine receptor promoter in muscle of transgenic mice. *Neuron.* **2**, 1295-1300.

Merlie, J. P. and Sanes, J. R. (1985). Concentration of acetylcholine receptor mRNA in synaptic regions of adult muscle fibers. *Nature* **317**, 66-68.

Nitkin, R. M., Smith, M. A., Magill, C., Fallon, J. R., Yao, Y. M., Wallace, B. G. and McMahan, U. J. (1987). Identification of agrin, a synaptic organizing protein from *Torpedo* electric organ. *J. Cell Biol.* **105**, 2471-2478.

Noda, M., Furutani, Y., Takahashi, H., Toyosato, M., Tanabe, T., Shimizu, S., Kikyotani, S., Kayano, T., Hirose, T., Inayama, S. and Numa, S. (1983). Cloning and sequence analysis of calf cDNA and human genomic DNA encoding α-subunit precursor of muscle acetylcholine receptor. *Nature* **305**, 818-823.

Nüsslein-Volhard, C. (1991). Determination of the embryonic axes of *Drosophila*. *Development Supplement* **1**, 1-10.

Palmiter, R. D., Norstedt, G., Gelinas, R. E., Hammer, R. E. and Brinster, R. L. (1983). Metallothionein-human GH fusion genes stimulate growth of mice. *Science.* **222**, 809-814.

Pavlath, G. K., Rich, K., Webster, S. G. and Blau, H. M. (1989). Localization of muscle gene products in nuclear domains. *Nature.* **337**, 570-573.

Piette, J., Bessereau, J.-L., Huchet, M. and Changeux, J.-P. (1990). Two adjacent MyoD1-binding sites regulate expression of the acetylcholine receptor α-subunit gene. *Nature.* **345**, 353-355.

Pignoni, F., Baldarelli, R. M., Steingrimsson, E., Diaz, R. J., Patapoutian, A., Merriam, J. R. and Lengyel, J. A. (1990). The *Drosophila* gene *tailless* is expressed at the embryonic termini and is a member of the steroid receptor superfamily. *Cell.* **62**, 151-163.

Ralston, E. and Hall, Z. W. (1989a). Transfer of a protein encoded by a single nucleus to nearby nuclei in multinucleated myotubes. *Science* **244**, 1066-1069.

Ralston, E. and Hall, Z. W. (1989b). Intracellular and surface distribution of a membrane protein (CD8) derived from a single nucleus in multinucleated myotubes. *J. Cell Biol.* **109**, 2345-2352.

Role, L. W., Matossian, V. R., O'Brien, R. J. and Fischbach, G. D. (1985). On the mechanism of acetylcholine receptor accumulation at newly formed synapses on chick myotubes. *J. Neurosci.* **5**, 2197-2204.

Roth, K. A., Hertz, J. M. and Gordon, J. (1990). Mapping enteroendocrine cell populations in transgenic mice reveals an unexpected degree of complexity in cellular differentiation within the gastrointestinal tract. *J. Cell Biol.* **110**, 1791-1801.

Rotundo, R. L. (1990). Nucleus-specific translation and assembly of acetylcholinesterase in multinucleated muscle cells. *J. Cell Biol.* **110**, 715-719.

Salpeter, M. M. (1987). Development and neural control of the neuromuscular junction and of the junctional acetylcholine receptor. In *The Vertebrate Neuromuscular Junction* (ed. M.M. Salpeter) pp. 55-115. New York: Alan R. Liss.

Schuetze, S. M. and Role, L. W. (1987). Developmental regulation of nicotinic acetylcholine receptors. *Ann. Rev. Neurosci.* **10**, 403-457.

Selden, R. F., Howie, K. B., Rowe, M. E., Goodman, H. M. and Moore, D. D. (1986). Human growth hormone as a reporter gene in regulation studies employing transient gene expression. *Mol. Cell. Biol.* **6**, 3173-3179.

Slater, C. R. (1982). Postnatal maturation of nerve-muscle junctions in hindlimb muscles of the mouse. *Dev. Biol.* **94**, 11-22.

Sprenger, F., Stevens, L. M. and Nüsslein-Volhard, C. (1989). The *Drosophila* gene *torso* encodes a putative receptor tyrosine kinase. *Nature* **338**, 478-483.

Stein, D., Roth, S., Vogelsang, E. and Nüsslein-Volhard, C. (1991). The polarity of the dorsoventral axis in the *Drosophila* embryo is defined by an extracellular signal. *Cell* **65**, 725-735.

Stevens, L. M., Frohnhofer, H. G., Klinger, M. and Nüsslein-Volhard, C. (1990). Localized requirement for *torsolike* expression in follicle cells for the development of terminal anlagen of the *Drosophila* embryo. *Nature* **346**, 600-663.

Tapscott, S. J., Davis, R. L., Thayer, M. J., Cheng, P.-F., Weintraub, H. and Lassar, A. B. (1988). MyoD1: A nuclear phosphoprotein requiring a myc homology region to convert fibroblasts to myoblasts. *Science.* **242**, 405-411.

Thisse, B., Stoetzel, C., Gorostiza-Thisse, C. and Perrin-Schmitt, F. (1988). Sequence of the *twist* gene and nuclear localization of its protein in endomesodermal cells of early *Drosophila* embryos. *EMBO J.* **7**, 2175-2183.

Trahair, J. F., Neutra, M. R. and Gordon, J. I. (1989). Use of transgenic mice to study the routing of secretory proteins in intestinal epithelial cells: analysis of human growth hormone compartmentalization as a function of cell type and differentiation. *J. Cell Biol.* **109**, 3231-3242.

Vogel, Z., Sytkowski, A. J. and Nirenberg, M. W. (1972). Acetylcholine receptors of muscle grown *in vitro*. *Proc. Natl. Acad. Sci. USA* **69**, 3180-3184.

Wagner, T. E., Hoppe, P. C., Jollick, J. D., Scholl, D. R., Hodinka, R. L. and Gault, J. B. (1981). Microinjection of a rabbit β-globin gene into zygotes and its subsequent expression in adult mice and their offspring. *Proc. Natl. Acad. Sci. USA* **78**, 6376-6380.

Wang, X.-M., Tsay, H.-J. and Schmidt, J. (1990). Expression of the acetylcholine receptor δ-subunit gene in differentiating chick muscle cells is activated by an element that contains two 16 bp copies of a segment of the α-subunit enhancer. *EMBO J.* **9**, 783-790.

Witzemann, V., Brenner, H.-R. and Sakmann, B. (1991). Neural factors regulate AChR subunit mRNAs at rat neuromuscular synapses. *J. Cell Biol.* **114**, 125-141.

Wright, W. E., Sassoon, D. A. and Lin, V. K. (1989). Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD1. *Cell.* **56**, 607-617.

Ziskind-Conhaim, L., Geffen, I. and Hall, Z. W. (1984). Redistribution of acetylcholine receptors on developing rat myotubes. *J. Neurosci.* **4**, 2346-2349.