

## Synaptic basal lamina contains a signal for synapse-specific transcription

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### Summary

**Nuclei in the synaptic region of multinucleated skeletal myofibers are transcriptionally distinct, since acetylcholine receptor genes are transcribed at a high rate by these nuclei, but not by nuclei elsewhere in the myofiber. Although this spatially restricted transcription pattern is presumably imposed by the motor nerve, the continuous presence of the nerve is not required, since synapse-specific transcription persists after denervation. These results suggest either that a transcriptional signal persists at synaptic sites after nerve terminals have degenerated, or that a transcriptional pattern in the myofiber, once established, is stable in the absence of a nerve-derived signal. To distinguish between these possibilities, we**

**denervated muscle and damaged the myofibers and specialized cells located near synaptic sites, and then studied transcription of an acetylcholine receptor gene in myofibers that regenerated in their original basal lamina sheaths, but remained denervated. We show that synapse-specific transcription is re-induced in these regenerated myofibers, and we conclude that a signal for synapse-specific transcription is stably maintained in the synaptic basal lamina.**

Key words: extracellular matrix, neuromuscular synapse, acetylcholine receptor, gene expression, transgenic mice.

### Introduction

Acetylcholine receptors (AChRs) are highly concentrated in the postsynaptic membrane of skeletal muscle fibers (Salpeter, 1987). Two different signalling pathways lead to the localization of AChR protein at synaptic sites. The signal for one pathway has been identified as agrin (Nitkin et al., 1987), a protein which is synthesized by motor neurons (Magill-Solc and McMahan, 1988) and which is secreted into the basal lamina at synaptic sites (Reist et al., 1987, 1991), where it stimulates a redistribution of AChR protein from non-synaptic membrane to the synaptic site (Godfrey et al., 1984). The second signalling pathway activates transcription of AChR genes selectively in those myofiber nuclei that are positioned close to the synaptic site (Klarsfeld et al., 1991; Sanes et al., 1991; Simon et al., 1992). The signal for this pathway is not known. The experiments presented here were designed to determine the location of the signal for this pathway as a first step to the identification of the signal.

We showed previously that the AChR subunit gene is transcribed specifically in the synaptic nuclei of skeletal myofibers (Simon et al., 1992). We also showed that the continuous presence of the nerve is not required to maintain transcription in synaptic nuclei, since synapse-specific transcription is maintained for at least four days after denervation. We reasoned that a signal could persist after denervation if it was associated with the synaptic basal lamina (Burden et al., 1979; McMahan and Slater, 1984), or if it was provided by Schwann cells (Birks et al., 1960) or specialized fibroblast-like cells (Connor and McMahan, 1987; Gatchalian et al.,

1989; Weis et al., 1991), both of which are located near synaptic sites. Alternatively, the effect of a signal might persist after the nerve terminal is removed if a transient signal imprinted a pattern of transcription in the myofiber that was subsequently stable in the absence of the signal, as others have suggested (Witzemann et al., 1991). The present study was designed to determine whether the signal for synapse-specific transcription is associated with the synaptic basal lamina.

Following damage to nerve and muscle, both nerve terminals and myofibers degenerate, but the myofiber's basal lamina sheath remains intact (Marshall et al., 1977; for reviews, see Sanes, 1983 and Burden, 1987). Original synaptic sites in the damaged muscle can be recognized in the absence of nerve terminals and myofibers, because cholinesterase activity persists in the basal lamina at synaptic sites (McMahan et al., 1978). Subsequently, new myofibers regenerate within the original myofibers' basal lamina sheaths, and axons reinnervate the regenerated myofibers. If axon regeneration is prevented or delayed, myofibers will regenerate in the absence of nerve terminals, but AChRs will still re-accumulate at original synaptic sites (Burden et al., 1979; Bader, 1981). This re-accumulation of AChRs also occurs when myofibers regenerate in the absence of nerve terminals, Schwann cells and other presynaptic cells (McMahan and Slater, 1984), indicating that the signal responsible for AChR clustering is associated with the synaptic basal lamina. This signal was subsequently identified as agrin (Nitkin et al., 1987). Agrin causes clustering of AChR protein on cultured myotubes (Godfrey et al., 1984; Campanelli

et al., 1991), but does not alter the rate of AChR synthesis (Godfrey et al., 1984). Thus, agrin is not a likely candidate for the signal that induces synapse-specific transcription; the identity and source of this signal remain unclear.

In the present study, we damaged the synaptic region of the sternomastoid muscle, thus destroying myofibers, nerve terminals and synapse-associated cells (McMahan and Slater, 1984), and then we asked whether synapse-specific transcription was re-induced in new myofibers that regenerated within the original myofiber basal lamina sheaths in the absence of nerve terminals and perisynaptic cells. We show that AChR transcription is re-induced at original synaptic sites, and we conclude that the signal for synapse-specific transcription is associated with the synaptic basal lamina.

## Materials and methods

The sternomastoid muscle from transgenic line GH1 (Simon et al., 1992) was denervated by resecting ~2 mm of the muscle nerve, and reinnervation was prevented for at least two weeks by suturing the proximal end of the cut nerve to a nearby muscle. The central, innervated portion of the muscle was frozen by applying the flat surface of a brass block, which had been chilled in liquid nitrogen, to the muscle for thirty seconds; after the muscle had thawed, the process was repeated twice (McMahan and Slater, 1984).

The time course of myofiber degeneration and regeneration was determined by light microscopic histological analysis of muscle cross-sections. We found that muscle degeneration was nearly complete by 2-3 days after damage and that myofibers regenerated by 1-2 weeks after damage (data not presented). A few myofibers (5 to 10%), which were opposite the exposed, frozen side of the muscle, did not degenerate completely, and we avoided this region of the muscle in our analysis.

Synaptic sites in normal and regenerated muscle (9-10 days after damage) were identified by their characteristic size and shape as visualized by staining with tetramethylrhodamine  $\beta$ -bungarotoxin (TMR- $\beta$ BGT). Original synaptic sites in degenerated muscle (three days after damage) were identified by staining for cholinesterase (Karnovsky, 1964; Burden et al., 1979). Human growth hormone (hGH) expression was detected with antibodies against hGH and fluorescein-coupled secondary antibodies. Myofibers were mounted whole, and viewed with optics selective for rhodamine or fluorescein (Simon et al., 1992).

Non-synaptic hGH expression was calculated by examining 750  $\mu$ m of the non-synaptic region from each myofiber, beginning 300  $\mu$ m from the original synaptic site, and measuring the length of any hGH-expressing region. 16% of the non-synaptic region in denervated, regenerated muscle contains detectable hGH; this hGH expression generally consisted of one to five patches. The majority of myofiber nuclei are not associated with such patches, since the 750  $\mu$ m region contains ~60 nuclei, which are distributed uniformly along the length of the myofiber.

## Results

We showed previously that the skeletal muscle AChR  $\beta$  subunit gene is transcribed at an enhanced rate in the synaptic nuclei of multinucleated myofibers (Simon et al., 1992). We constructed transgenic mice that contain a gene fusion between the murine AChR  $\beta$  subunit gene (-1,823/+24) and the human growth hormone (hGH) gene, and we showed that hGH is present at high levels in the Golgi apparatus around

synaptic nuclei, but not around nuclei in the remainder of the myofiber. These experiments indicated that a signal associated with the synaptic site acts locally to activate transcription of the AChR  $\beta$  subunit gene in myofiber nuclei that are positioned near the synaptic site. We also showed that synaptic nuclei remain transcriptionally distinct in the absence of the nerve, since synapse-specific transcription persists for at least four days after denervation (Simon et al., 1992). The experiments described here were designed to determine whether the signal for synapse-specific transcription is maintained in the myofiber or is provided by structures that are external to the myofiber.

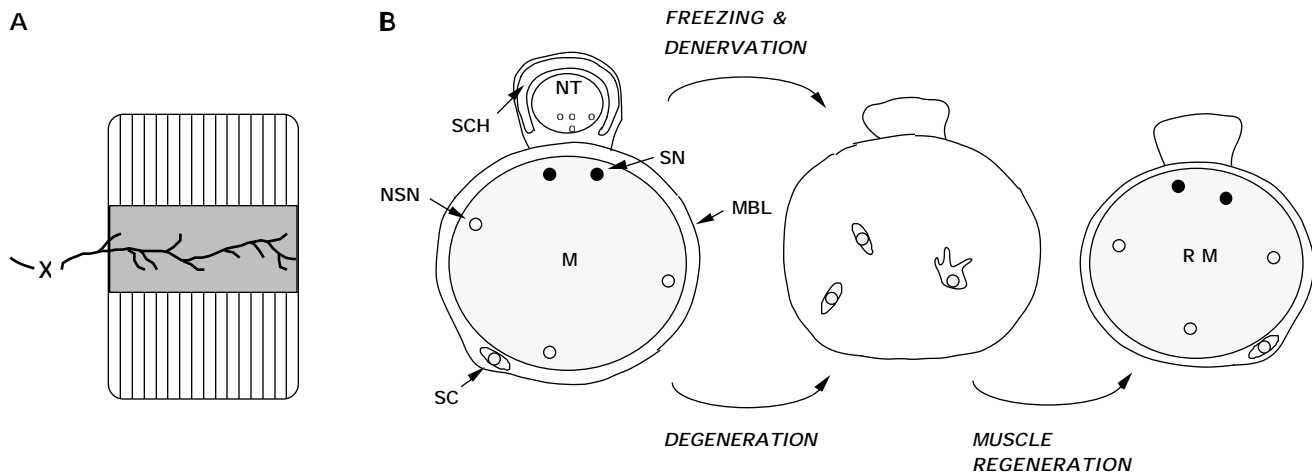
### *Degeneration and regeneration of myofibers in the absence of presynaptic cells*

The sternomastoid muscles of these transgenic mice were denervated by cutting the nerve to the muscle, and the central, innervated region of the muscles were damaged by freezing and thawing (Fig. 1; McMahan and Slater, 1984). These procedures result in degeneration of the myofibers, nerve terminals, and other cells that are present at synaptic sites, including Schwann cells and specialized fibroblast-like cells, within 2-3 days after damage (see Materials and methods); the myofiber basal lamina sheaths, however, remain intact (Vracko, 1974; Marshall et al., 1977; Burden et al., 1979; McMahan and Slater, 1984). Muscle satellite cells, which survive in the undamaged regions of the muscle, proliferate in response to damage of original myofibers, and these cells fuse to form regenerated myofibers within 1-2 weeks (Fig. 1; Vracko, 1974; Marshall et al., 1977).

### *Induction of synaptic gene expression in denervated, regenerated myofibers*

The distribution of intracellular hGH in normal and regenerated myofibers was determined by immunofluorescence (Simon et al., 1992). Synaptic sites, identified by their characteristic size and shape, are readily located in normal muscle by staining for AChRs with TMR- $\beta$ BGT. Original synaptic sites in denervated, regenerated muscle are also readily located, because AChRs are known to re-accumulate at original synaptic sites in denervated, regenerated myofibers by post-translational mechanisms (Burden et al., 1979; McMahan and Slater, 1984; Godfrey et al., 1984), and these AChR accumulations have the same characteristic size and shape as those at normal synaptic sites (Burden et al., 1979; McMahan and Slater, 1984; Rich and Lichtman, 1989).

In normal muscle, the AChR transgene is transcribed at an enhanced rate in synaptic nuclei, and so hGH is present at high levels in the Golgi apparatus associated with nuclei in the synaptic region (Fig. 2; Simon et al., 1992). 2-3 days after damage, the original myofibers have degenerated, and hGH is not detectable in the damaged region (Fig. 3). One week later, myofibers have regenerated in the absence of the nerve and perisynaptic cells, and these new myofibers express hGH in the Golgi apparatus at original synaptic sites (Fig. 2). hGH is re-expressed at all original synaptic sites in denervated, regenerated myofibers (Table 1), and the level of hGH expression at these sites is similar to that found at normal



**Fig. 1.** Diagram illustrating normal, degenerated, and regenerated myofibers. (A) The sternomastoid muscle was denervated (X) and the central, innervated zone of the muscle (hatched) was damaged by freezing (Materials and methods). (B) A cross-section of a neuromuscular synapse shows a single normal myofiber (M), the nerve terminal (NT) with associated Schwann cell (SCH), and the myofiber basal lamina (MBL), which ensheathes the myofiber and is specialized in the synaptic cleft. The AChR subunit gene is expressed at a greater rate in synaptic nuclei (SN) than in non-synaptic nuclei (NSN). Muscle satellite cells (SC) are distributed throughout normal muscle, where they are found between the myofiber basal lamina and the myofiber plasma membrane. Following freezing and thawing of the central, innervated zone of the sternomastoid muscle, all cells in the damaged portion of the muscle, including Schwann cells, specialized fibroblast-like cells, and the central portion of the original myofiber, degenerate within two days, while the basal lamina sheath of the myofiber remains intact (McMahan and Slater, 1984). Muscle satellite cells from outside the damaged region proliferate following damage of the original myofiber and fuse to form regenerated myofibers (RM) within original myofiber basal lamina sheaths.

synaptic sites (Fig. 2). These data indicate that the synaptic basal lamina contains a signal that activates transcription of the AChR subunit gene in normal and regenerated myofibers. Although we do not know whether the signalling molecule in the basal lamina is synthesized by nerve or muscle, the localization of this molecule must ultimately be controlled by the motor neuron.

**Table 1.** *hGH expression at synaptic sites and in non-synaptic regions*

Muscle	Synaptic sites expressing hGH (%)	Length of non-synaptic region expressing hGH (%)
Normal	100 ( $n > 100$ )	0 ( $n > 100$ )
Degenerated	7 ( $n = 42$ )	ND
Regenerated	100 ( $n = 59$ )	16 ( $n = 59$ )

hGH expression is detectable at all synaptic sites in normal myofibers and at all original synaptic sites in denervated, regenerated myofibers. In normal and regenerated muscle, hGH is perinuclear and is apparently associated with the Golgi apparatus. hGH expression was observed infrequently at original synaptic sites in degenerating muscle; in these cases, the staining appeared disorganized and was not perinuclear.

hGH expression is not detectable in the non-synaptic region of normal muscle. In denervated, regenerated muscle, hGH expression is found in a portion of the non-synaptic region (see Materials and methods). Because hGH expression is found rarely in the non-synaptic region of denervated myofibers (Simon et al., 1992), it seems likely that some hGH expression in regenerated myofibers is attributable to their immaturity. Similarly, AChR subunit mRNA is initially expressed throughout denervated, regenerated myofibers, and only later becomes restricted to synaptic sites (Goldman et al., 1991). Small clusters of AChRs are found in the non-synaptic region of denervated myofibers (Ko et al., 1977), and all non-synaptic AChR clusters in denervated, regenerated muscle are in proximity to nuclei that express hGH (see Simon et al., 1992); however, most expressing nuclei in the non-synaptic region are not associated with AChR clusters. The number ( $n$ ) of muscle fibers that were examined, in at least two muscles, is indicated.

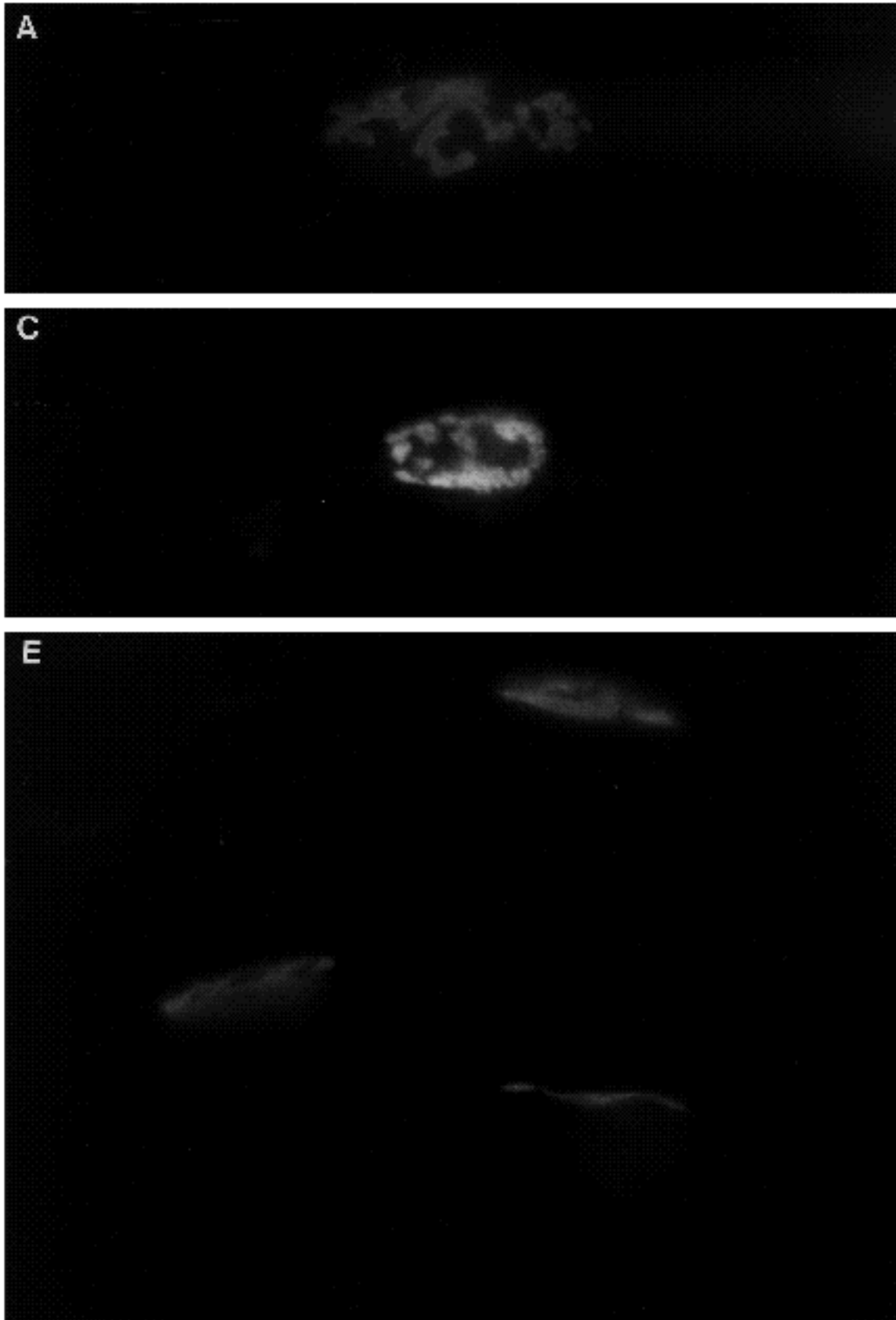
#### *Non-synaptic expression in denervated, regenerated myofibers*

hGH expression is not detectable in the non-synaptic region of normal muscle (Table 1), and it is only rarely detected by immunofluorescence in the non-synaptic region of denervated muscle (Simon et al., 1992). hGH is detectable, however, in a portion of the non-synaptic region in denervated, regenerated muscle (Table 1). The lack of electrical activity in these denervated myofibers is presumably responsible for some of this non-synaptic expression (Fambrough, 1979); however, it seems likely that some of the non-synaptic expression is attributable to the immaturity of the regenerated myofibers. Consistent with this idea, we observe high levels of hGH expression in cultured embryonic myotubes derived from our transgenic lines.

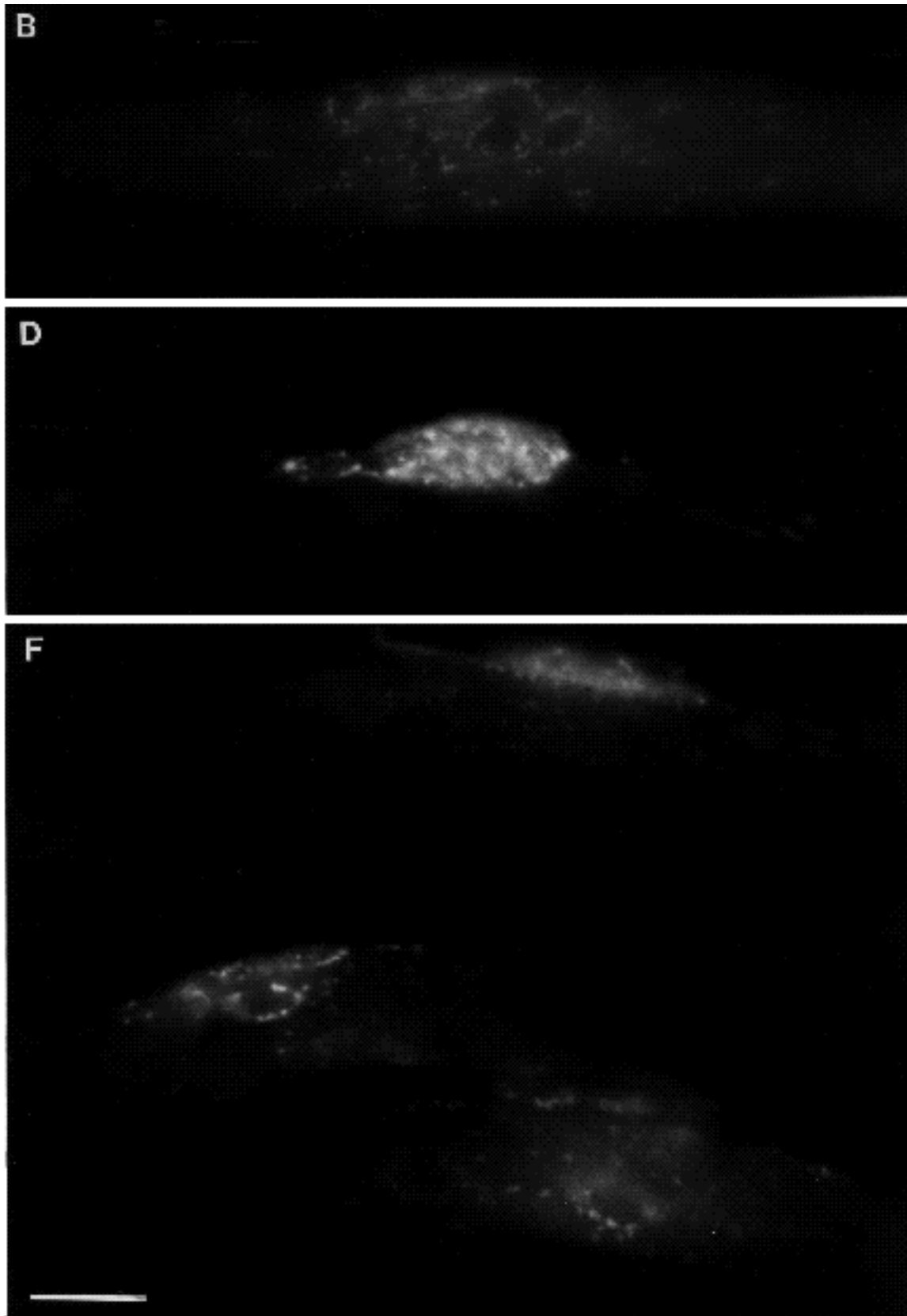
## Discussion

This study demonstrates that a signal for synapse-specific gene expression persists at synaptic sites after the removal of the original myofiber, and that this signal can re-induce expression of the AChR subunit gene at synaptic sites in new, regenerated myofibers. Because these myofibers regenerated in the absence of nerve terminals, Schwann cells and perisynaptic cells, but within the original myofiber's basal lamina sheath, we conclude that the signal for synapse-specific transcription is associated with the synaptic basal lamina (Fig. 4).

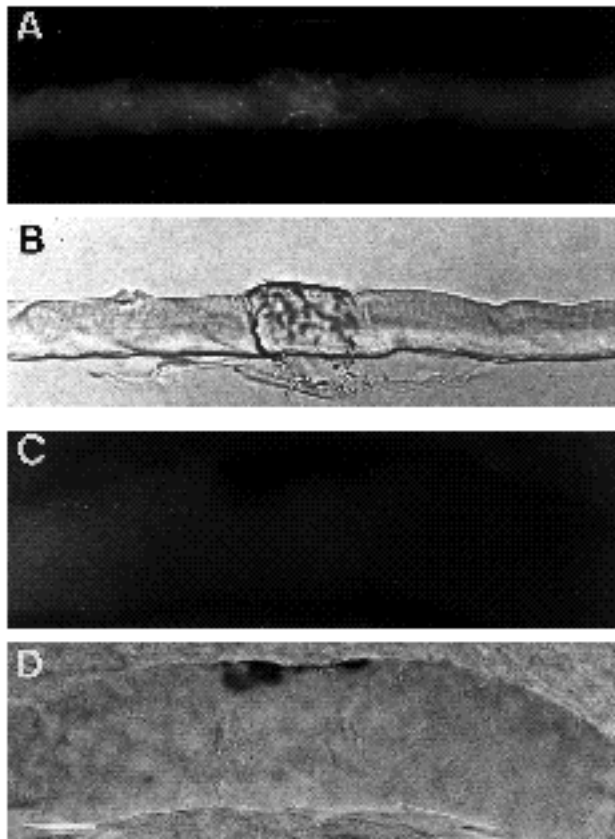
Previous studies have shown that the synaptic basal lamina has a key role in triggering presynaptic (Sanes et al., 1978) and postsynaptic differentiation (Burden et al., 1979; McMahan and Slater, 1984), and these studies led to the



**Fig. 2.** The  $\alpha$ -subunit gene is transcribed preferentially by myofiber nuclei at original synaptic sites in denervated, regenerated muscle. A single myofiber from a normal muscle is shown in A and B, a single denervated, regenerated myofiber from a muscle that was denervated and damaged ten days previously is shown in C and D, and a group of denervated, regenerated myofibers is shown in E and F. Myofibers

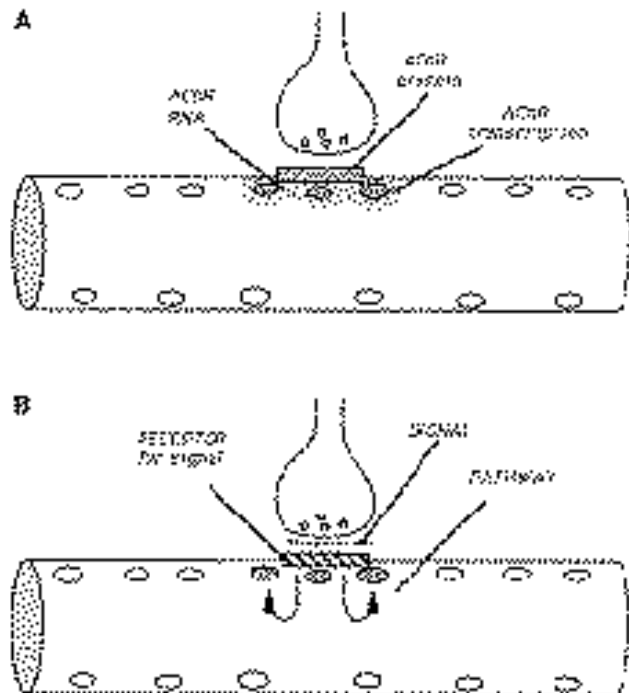


were double-labelled with TMR- $\alpha$ -BGT (A,C,E) to stain AChRs and antibodies against hGH (B,D,F), mounted whole, and viewed with optics selective for rhodamine (A,C,E) or fluorescein (B,D,F) (Simon et al., 1992). hGH is concentrated at the perinuclear region of nuclei near synaptic sites both in normal and denervated, regenerated muscle. Bar, 20  $\mu$ m.



**Fig. 3.** Degenerating muscle does not express hGH. A single myofiber from a normal muscle is shown in A and B and the original synaptic region of denervated, damaged muscle, which was frozen three days previously, is shown in C and D. Myofibers were labelled with antibodies against hGH (A,C) and stained for cholinesterase (B,D) (Karnovsky, 1964) to mark original synaptic sites. hGH is expressed at synaptic sites in normal muscle, but hGH is not detectable in degenerated muscle. Striations are readily visible in normal myofibers (B), whereas they are absent from degenerating myofibers (D). Because the cholinesterase reaction product can obscure the immunofluorescence signal, we restricted our analysis to myofibers in which the cholinesterase stain was situated underneath or on the side of the myofiber. Bar, 20  $\mu$ m.

purification of agrin (Nitkin et al., 1987), a basal lamina protein that is made by motor neurons (Magill-Solc and McMahan, 1988) and regulates the distribution of cell surface AChRs by post-translational mechanisms (Godfrey et al., 1984; Campanelli et al., 1991). Identification and purification of agrin rested on the knowledge that AChR clustering activity is a component of the synaptic basal lamina as was shown in studies of regenerating muscle in vivo (Burden et al., 1979; McMahan and Slater, 1984). The results presented here indicate that the synaptic basal lamina regulates the structure of the postsynaptic membrane not only by post-translational mechanisms, but also by regulating transcription of genes encoding synaptic proteins. Because agrin does not appear to increase the rate of AChR synthesis (Godfrey et al., 1984), it would seem unlikely that agrin is the signal for synapse-specific transcription. Many other molecules, however, are also concentrated in the synaptic basal lamina,



**Fig. 4.** A model for synapse-specific transcription. (A) AChR protein, AChR RNA and AChR transcription are localized to the synaptic site (Simon et al., 1992). (B) A signal located in the synaptic basal lamina stimulates a receptor and pathway that activates transcription of the AChR subunit gene in synaptic nuclei. Although the entire myofiber is ensheathed by basal lamina, only the synaptic portion of this basal lamina, which contains the signal, is shown. Although this model proposes that the receptor for this basal lamina signal is localized to synaptic sites, this receptor could also be present in the non-synaptic myofiber membrane.

including acetylcholinesterase (McMahan et al., 1978), s-laminin (Hunter et al., 1989), isoforms of collagen (Sanes et al., 1990), and a heparan sulfate proteoglycan (Swenarchuk et al., 1990), and any of these molecules could be responsible for regulating synapse-specific transcription. In any case, knowledge of the subcellular location of the signal for synapse-specific transcription should facilitate its identification and purification.

Like the AChR subunit mRNA, the mRNAs encoding the  $\alpha$  and  $\beta$  subunits of the AChR are also concentrated at synaptic sites in normal muscle (Merlie and Sanes, 1985; Fontaine and Changeux, 1989; Goldman and Staple 1989; Brenner et al., 1990; Simon et al., 1992), and like the  $\alpha$  subunit gene, the  $\beta$  subunit gene is also transcribed at an enhanced rate in synaptic nuclei of normal muscle (Sanes et al., 1991). The other AChR subunit genes may also be expressed preferentially in synaptic nuclei, although different groups have obtained different results regarding synapse-specific expression of the  $\gamma$  subunit gene (Klarsfeld et al., 1991; Sanes et al., 1991). Thus, it remains unclear whether all AChR subunit genes are preferentially transcribed in synaptic nuclei.

A previous study has shown that the AChR subunit mRNA reaccumulates at original synaptic sites in muscle

that has regenerated in the absence of the nerve (Goldman et al., 1991). Since the methods used in this study do not remove Schwann cells and specialized perisynaptic cells, the authors were not able to determine whether the signal for localizing  $\alpha$  subunit mRNA to original synaptic sites in regenerated muscle is provided by: (1) Schwann cells, which persist at denervated synaptic sites and which secrete acetylcholine and perhaps other signalling molecules (Birks et al., 1960; Burden et al., 1979; Chapron and Koenig, 1989), (2) perisynaptic cells, which synthesize several potentially active molecules (Gatchalian et al., 1989), or (3) the synaptic basal lamina. Because the  $\alpha$  subunit gene is expressed in development later than the  $\beta$  subunit gene (Witzemann et al., 1989; Brenner et al., 1990), these two genes are regulated differently, and the signals that mediate their induction at synapses may be different. Alternatively, the same basal lamina signal may stimulate transcription of both the  $\alpha$  and  $\beta$  subunit genes, and the differences in the timing of expression may be controlled by other mechanisms. Thus, it remains unclear whether the basal lamina signal that activates transcription of the AChR  $\beta$  subunit gene also activates other AChR subunit genes. Our study shows that the signal for synapse-specific expression of the AChR  $\alpha$  subunit gene is contained in the synaptic basal lamina, and this finding will facilitate purification of the signal.

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