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Technical Article

A somatic gene transfer approach using recombinant fusion proteins to map muscle-motoneuron projections in *Xenopus* spinal cord

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ABSTRACT A combination of somatic gene transfer with fusion protein technology has been developed, thus providing an innovative means of mapping muscle-motoneuronal connections in *Xenopus* tadpole spinal cord. We analyzed whether a neuronal tracer created by the fusion of the *LacZ* gene to the tetanus toxin C fragment (LacZ-TTC) could be produced from plasmid DNA injected into muscle, and whether it could be released and undergo retrograde transport into motoneurons. Plasmids encoding various fusion protein constructions, with or without a signal peptide, were injected into dorsal or caudal muscles of premetamorphic tadpoles. The marker was produced in the muscle at constantly high levels. At one month post-injection, the fusion protein passed the neuromuscular junction and underwent retrograde transport into motoneurons. Transfer into motoneurons was seen for every animal injected, emphasing the high reproducibility and efficiency of the process. No uptake of β-gal protein into motoneurons was observed in the absence of the TTC fragment. Furthermore, no enhancement was obtained by adding a signal peptide. These results provide the first demonstration of the synthesis and transport of a TTC fusion protein produced directly from exogenous DNA in a vertebrate system.

KEY WORDS: *retrograde transport, gene transfer, muscle transfection, tetanus toxin C fragment, motoneuron mapping*

Introduction

Amphibian metamorphosis provides a versatile experimental model for dissecting a number of complex intercellular processes in a freeliving embryo. The model has many advantages, not the least being the fact that the process is orchestrated by a unique signal, thyroid hormone (3,5,3'-triiodothyronine or T3). Thus metamorphosis, and the underlying cellular interactions, can be controlled by modulating the thyroid status of the tadpoles. Of particular interest is the fate of the nervous system as it undergoes remodeling to accommodate the radical changes in target organs as the animal changes from an aquatic to a mainly terrestrial existence. Some of the most remarkable changes include limb growth, eye migration and tail regression. In certain cases new neurons will differentiate in response to T3; this is the case for many neurons implicated in the visual pathways (Hoskins and Grobstein, 1985a,b) and for the neurons innervating the tongue (Grobstein, 1987). In other areas there will be a change of target, such as for the jaw muscles (Alley and Barnes, 1983) whilst others will die, as seems to be the case for motoneurons innervating the caudal but not the dorsal tail muscles (Forehand and Farel, 1982a,b). Indeed, this differential fate of caudal versus dorsal spinal neurons during amphibian tail regression makes the *Xenopus* model of particular interest for analyzing the interdependence of motoneurons and their targets in development, a domain of increasingly active investigation in many vertebrate and invertebrate models (Grieshammer *et al.*, 1998; Chiba, 1999; Kablar and Rudnicki, 1999). Tail regression in *Xenopus* metamorphosis offers the possibility of studying these interactions during post-embryonic development, when all the components are differentiated yet subject to remodeling.

Abbreviations used in this paper: X-Gal, 5-bromo-4-chloro-3-inclolyl β-D-galactoside; β-gal, β-galactosidase; TTC, Tetanus toxin C fragment; IL2, Interleukin-2; CMV, cytomegalo virus.

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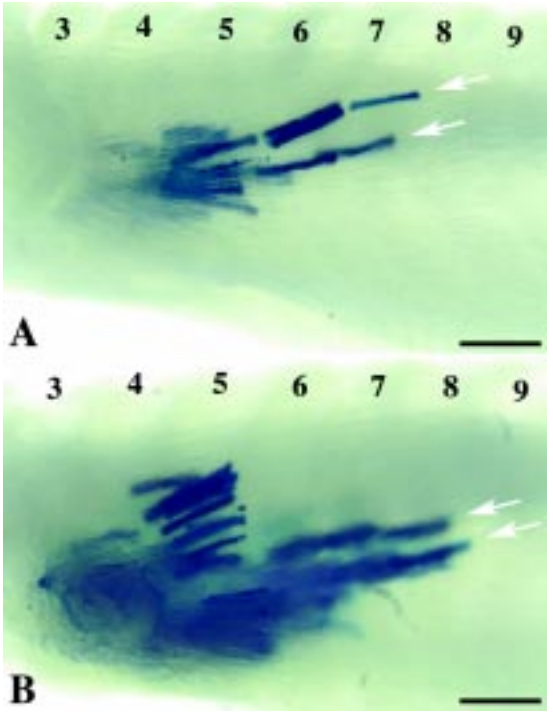

Fig. 1. Protein expression is a function of amount of DNA injected. (A *and* **B)** *In toto X-Gal staining after injection of different concentrations of the control plasmid pCMV-LacZ. For each animal, two injections (1 μl per injection) of 1.5 μg/μl* **(A)** *or 3.5 μg/μl* **(B)** *of plasmid were made into dorsal tail muscle. At 3 days post-injection, a greater number of muscle fibers expressing the β-galactosidase at the higher DNA concentration is seen. White arrows indicate the two injection trajectories and numbers indicate the myomere position from head. Bar, 0.5 mm.*

However, a major restriction to realizing the full potential of this model is the lack of sufficiently refined and precise mapping techniques. Thus, for the most part, the cellular decisions underlying neuronal remodeling in metamorphosis have received limited attention. To date only sparse data is available, which has been obtained principally by applying the horseradish peroxidase (HRP) and the DiI tracer systems (Forehand and Farel, 1982a,b; Roberts and Clarke, 1982; Brenowitz *et al.*, 1983). Despite the elegance of these techniques, they are not applicable for genetic approaches. Neuronal mapping can now be approached with fusion protein gene technology using markers such as Green Fluorescent Protein (GFP) that allows online *in vivo* mapping (Stearns, 1995), or β-galactosidase (Mombaerts *et al.*, 1996) and more recently lectins (Horowitz *et al.*, 1999; Yoshihara *et al.*, 1999). To apply such techniques to the amphibian model, our starting point was the demonstration of the use of tetanus toxin C (TTC) fragment system coupled with a cell marker in mammals (Coen *et al.*, 1997). In this earlier work, a bacteria-based expression system was used to produce a β-gal-TTC fusion protein, which was injected into the tongue of mice and shown to be transported retrogradely to the central nervous system (CNS). The non-toxic recombinant TTC fragment corresponds to the carboxy terminal part of the heavy chain of the tetanus toxin, and is transported by neurons in a similar manner to that of the native toxin (Halpern *et al.*, 1990). This TTC fragment has been used in a variety of approaches including chemical conjugation to enzymes (Fishman and Savitt, 1989; Beaude *et al.*, 1990; Dobrenis *et al.*, 1992) or DNA (Knight *et al.*, 1999) and creation of fusion proteins (Francis *et al.*, 1995; Coen *et al.*, 1997; Figueiredo *et al.*, 1997).

In the light of these reports successfully injecting hybrid proteins, we analyzed whether one could obtain directly functional protein production from plasmid based transfer in muscle *in vivo*. This was of particular interest as production and function of TTC fusion proteins have not been tested in either germinal or somatic transgenic systems in any species. The *Xenopus* system is particularly suited to development of this methodology, as somatic gene transfer in tadpole skeletal muscle with naked DNA provides high levels of protein expression during several weeks (de Luze *et al.*, 1993; Sachs *et al.*, 1997). We used constructs with and without a signal peptide to test whether secretion and uptake of the fusion protein could be improved by this means.

The results from this study provide two new main inputs to the field. It shows first, the potential of fusion protein technology in the model of amphibian metamorphosis and second, that, even in the absence of a signal peptide, the fusion protein can be released from the muscle and efficiently taken up by motoneurons.

Experimental Protocols

Animals

Xenopus laevis tadpoles were raised in dechlorinated, deionized tap water (1:2) and fed with nettle powder (Herbaplantes, Valanjou, France). Developmental stages were determined according to Nieuwkopp and Faber (1975). When tadpoles reached stages 53-54, just before prometamorphosis, different DNA constructs were injected into dorsal or caudal tail muscles.

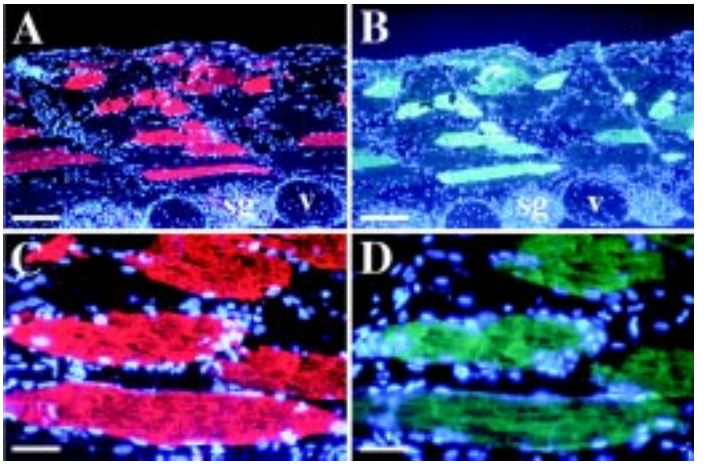

Fig. 2. Colocalization of TTC fragment and β-galactosidase in muscle fibers after injection of the different DNA constructs in dorsal tail muscle. *Three days after DNA injection, immunodetection was carried out with anti-TTC* **(A** *and* **C)** *and anti- β-gal* **(B** *and* **D)** *antibodies on adjacent slices. Anti-TTC and anti- β-gal antibodies were coupled to Cy3 and FITC-linked second antibodies respectively. Sections were counter-stained with Hoechst 33342 to localize nuclei. Colabeling is shown for muscle injected with the pCMV-IL2-LacZ-TTC (A and B) and pCMV-LacZ-TTC (D and E) expression vectors. Similar results were found with all constructs containing the TTC fragment and indicate that the fusion protein is correctly expressed in each case after transfection into tail muscle. Abbreviations: sg, spinal ganglia; v, vertebra. Bar, 200 μm (A and B); 50 μm (C and D).*

Plasmid constructions

Plasmids were propagated and purified using Endofree Plasmid Maxi kits (Quiagen, Courtaboeuf, France), and resuspended in water. Plasmids were not linearized or otherwise modified before injection.

pCMV-LacZ

This plasmid, referred to as VR-1412, was a gift from VICAL INC (San Diego; Manthorpe *et al.*, 1993). The localization of the β-galactosidase produced from the CMV promoter is cytosolic.

pCMV-LacZ-TTC

The SmaI LacZ-TTC fragment from the pGEX-LacZ-TTC plasmid (Coen *et al.*, 1997) was cloned in a pCMV vector (VR-1012, a gift from VICAL INC, San Diego) to give the pCMV-LacZ-TTC construct. The CMV promoter was the same as that in the pCMV-LacZ construct.

pCMV-IL2-LacZ-TTC

A pBS-LacZ-TTC construct was obtained by cloning a XmaI LacZ-TTC fragment from the pGEX-LacZ-TTC plasmid into pBluescript KS+ (Ozyme, St Quentin, France). The signal peptide sequence of mouse interleukin 2 (5'-TCTAGAAGCTTCCACC *ATG TAC AGC ATG CAG CTC GCA TCC TGT GTC ACA TTG ACA CTT GTG CTC CTT GTC AAC AGC* ATG GGG GAT CC-3'; Degrave *et al.*, 1986) was cloned as a XbaI/BamHI fragment in pBS-LacZ-TTC, in front of the fusion and in frame with the LacZ part, to give the pBS-IL2-LacZ-TTC plasmid. Then, the SmaI/HindIII IL2-LacZ-TTC fragment was cloned in the pCMV vector, giving the pCMV-IL2-LacZ-TTC plasmid.

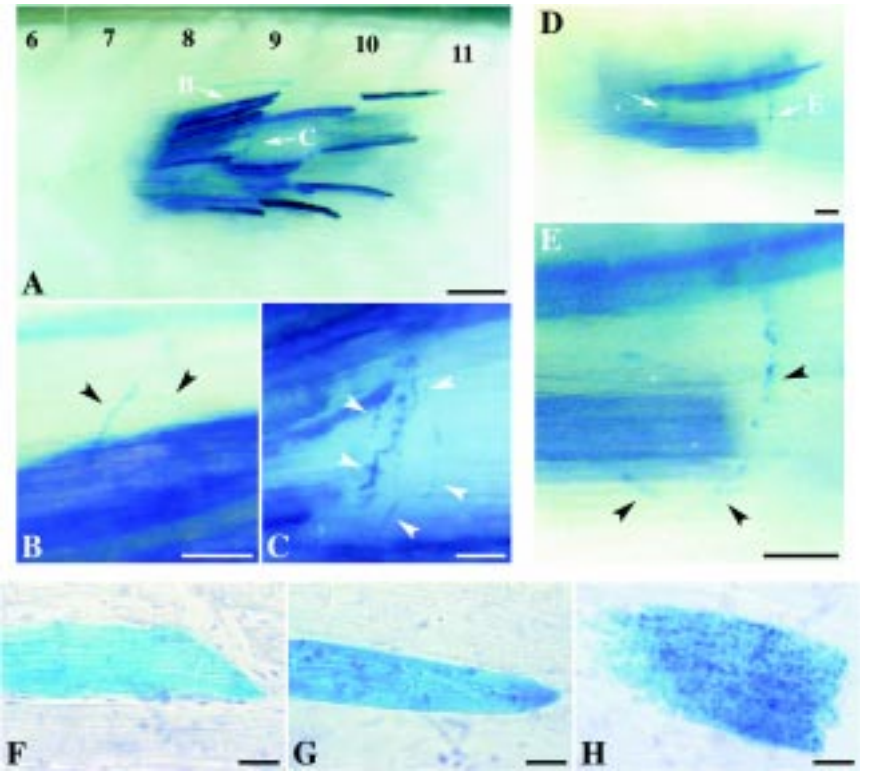

Fig. 3. Retrograde labeling of terminal motor axons one week following injection of different constructs into tadpole tail muscle (A-E) and fusion protein distribution in muscle (F-G). (A-E) *In toto X-Gal staining for muscles injected with pCMV-IL2-LacZ-TTC construct (A,B and C) and pCMV-LacZ-TTC construct (D and E) showing labeling in motoneuron axon-like structures (black and white arrows) suggesting the transfer of the fusion proteins in motoneurons from their expression site in muscle. This specific labeling in axons is absent with the control plasmid expressing the β-gal protein alone (pCMV-LacZ, data not shown).* **(F,G** *and* **H)** *X-Gal staining on muscle sections indicate that the β-gal activity shows clearly different expression patterns with (H) or without (F and G) signal peptide. The characteristic subcellular localization of the β-gal protein using pCMV-IL2-LacZ-TTC (H) suggests that the signal peptide addresses the fusion protein to the endoplasmic reticulum; a more homogeneous cytoplasmic localization is found using pCMV-LacZ (F) or pCMV-LacZ-TTC (G). Numbers indicate the myomere position from head. Bar, 0.5 mm (A); 100 μm (B-E); 25 μm (F-H).*

Microinjection of DNA

Somatic gene transfer in *Xenopus* tadpoles at stage 53-54 in dorsal or caudal tail muscle was performed as previously described (de Luze *et al.*, 1993). The injection solution (1 μl per injection) contained the same amount of pure DNA for each construct (1.5 μg/μl, 3.5 μg/μl or 5 μg/μl), in 0.1 M NaCl. Two unilateral injections were performed at a given site for each animal, either in dorsal muscle (myomeres 3-7 from the head) or in caudal muscle (myomeres 8-11 from the head).

Histology, immunohistology and X-Gal staining

For histological analysis, tadpoles were sacrificed by decapitation 3 days, 1 week, 2 weeks, 3 weeks and 4 weeks after injection of the optimal concentration tested, i.e. 2x3.5 μg/μl of each plasmid in the dorsal or in the caudal tail muscle. The injected part of the tail (myomeres 1 to 8 for the dorsal injection or myomeres 1 to 15 for the caudal injection) was dissected, the skin removed, and rinsed in PBS solution.

For *in toto* X-Gal staining, the dissected tail parts were fixed with 2% paraformaldehyde (PFA) in PBS for 2 h at 4°C and then washed extensively with PBS. β-galactosidase activity was detected on fixed tails by an overnight (O/N) staining at 31.5°C in X-Gal solution as previously described (Coen *et al.*, 1997).

For visualizing fusion proteins in motoneurons, dissected tail parts were fixed with 2% PFA in PBS for 2 h at 4°C, rinsed in PBS, incubated in 15% sucrose O/N at 4°C, mounted in Tissue-tek, then sectioned transversally (15-μm) or longitudinally (10-μm) using a cryostat. For immunolabeling, sections were post-fixed with 4% PFA in PBS for 10 min at room temperature (RT) and rinsed in PBS before use. Sections were incubated with a rabbit polyclonal anti- β-gal antibody (1:1000; Cappel, Organon Tetrika, Westchester, PA) or a rabbit polyclonal anti-TTC antibody (1:500; Calbiochem) in TBST buffer (138 mM NaCl/2.7 mM KCl/0.025 mM Tris-HCl pH7.5/0.1% Tween-20) with 0.01% Triton X-100 and 1% normal goat serum (NGS) O/N at 4°C, after blocking non-specific sites 1 h in TBST buffer with 0,01% Triton X-100 and 10% NGS. Antibody detection was carried out using EnVision+kit (Dako, Glostrup, Denmark) with diaminobenzidine color development. Sections were counterstained for 1 min with hematoxilin (1:5, vol/vol) and mounted in Eukit after dehydration. For X-Gal staining, sections were incubated in X-Gal solution O/N at 31.5°C, post-fixed with 4% PFA in PBS for 10 min at RT, counterstained for 1 min with hematoxilin, and mounted in Moviol

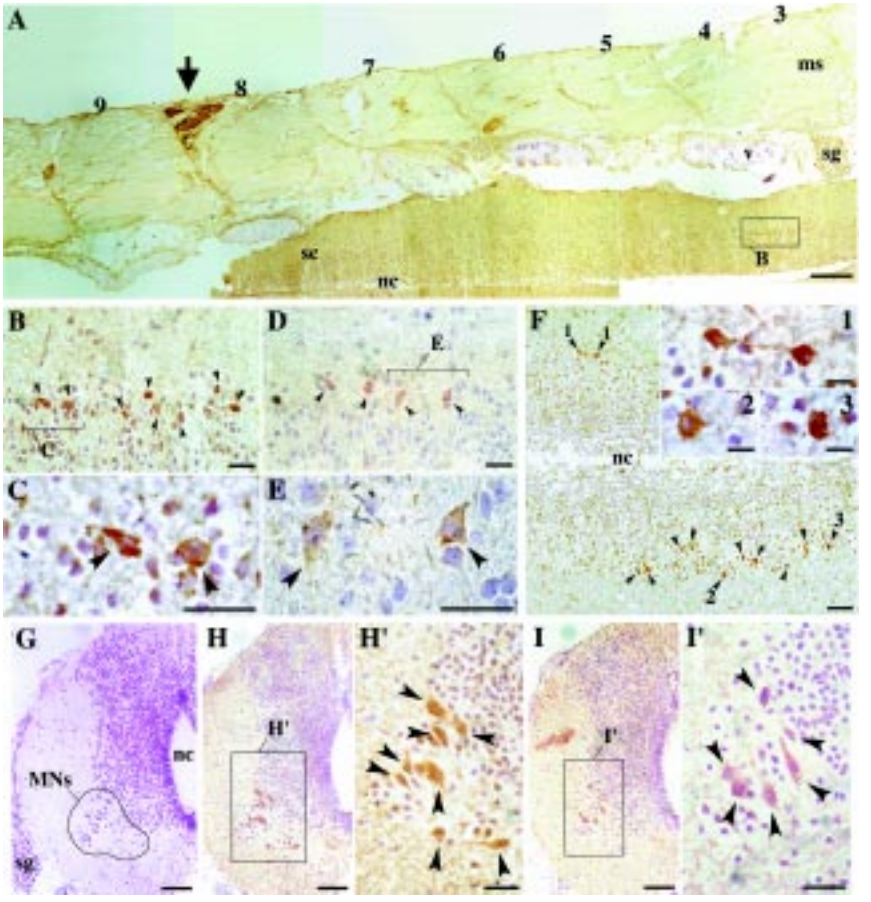

Fig. 4. Retrograde labeling of motoneuron soma after 4 weeks expression in muscle for the pCMV-LacZ-TTC fusion construct. (A) *Immunodetection with anti-β-gal antibody show a strong labeling, principally in myomere 8, and positive motoneurons at the level of the 4th myomere (more visible in B, at a higher magnification). Only a few labeled fibers are seen in this section. Most of the labeled fibers are not in the same plane as the labeling in the spinal cord because the injection site is located more ventrally. Colocalization of LacZ and TTC fragment was evident on adjacent sections immunolabeled with anti-β-gal* **(B** *and* **C)** *and anti-TTC* **(D** *and* **E)** *antibodies respectively. The labeled motoneurons (black arrows) are strongly positive for LacZ or TTC fragment.* **(F)** *The contra-lateral staining, carried out with anti- β-gal antibody, shows positive motoneurons on both sides of the spinal cord (black arrows), with strong staining of the motoneuron somata (see 1,2 and 3).* **(G,H** *and* **I)** *Transverse sections unambiguously show the motoneuron ventral localization (black arrows): compare histology (G) with LacZ labeling (H and H') and the TTC labeling (I and I') on 3 adjacent sections. Abbreviations: ms, muscle; sc, spinal cord; sg, spinal ganglia; v, vertebra; nc, neural canal, MN, motoneuron. Bar, 200 μm (A); 50 μm (F,G,H and I); 25 μm (B-E,H' and I'); 10 μm (1,2 and 3).*

before light microscopy analysis. For X-Gal staining on muscle sections, the same protocol was applied to longitudinal 25-μm sections.

For immunohistology on muscle, dissected tail parts were fixed with 4% PFA in PBS for 3 h at 4°C, rinsed in PBS, incubated in 15% sucrose O/N at 4°C, mounted in Tissue-tek, then sectioned longitudinally (10-μm). Sections were incubated with a mouse monoclonal anti-β-gal antibody (1:400; Sigma) or the anti-TTC antibody (1:1000) with 2% BSA/0.02% Triton X-100 in PBS for 2 h at RT, after blocking non-specific sites 1 h in the same buffer. Labeling was visualized using fluorescent secondary antibodies: Cy3, goat anti-rabbit IgG (1:500; Amersham, Les Ulis, France) or anti-mouse IgG with extravidin-fluorescein isothiocyanate (1:200; Sigma). Sections were counterstained with Hoechst 33342 (Sigma), mounted in Moviol and observed with epifluorescence.

Results and Discussion

The most important finding in this work is that when the TTC protein is synthesized *in situ* in *Xenopus* muscle, it can reach and pass the neuromuscular junction from whence it can undergo retrograde transport. This provides the first demonstration of the synthesis and transport of a TTC fusion protein produced directly from exogenous DNA in a vertebrate system. This is an advance over a previously published work (Coen *et al.*, 1997) where the β-gal-TTC protein was produced *in vitro* from a bacterial expression vector before the hybrid protein was intramuscularly injected into the tongue of mice to be retrogradely transported into hypoglossal motoneurons innervating the tongue. Here, it was not the hybrid protein but the plasmid DNA coding for the hybrid protein that was injected directly into the muscle. There are many advantages to injecting DNA rather than protein. First, skeletal muscle provides protein production from injected DNA over long periods with a high level of expression of the transgene. Second, injecting DNA is more straightforward and has lower experimental error than injecting protein. Third, there is no need to purify the fusion protein for each construct using the bacterial expression vector; DNA constructions are easy to prepare and to test in the model using somatic gene transfer. This facilitates testing of specific cell expression in muscles or neurons.

Optimization of fusion protein expression in muscle

We used the control construct pCMV-LacZ to test the effect of increasing DNA concentration and injection volume on muscle expression of β-galactosidase. On the one hand, we tested the effect of increasing DNA concentrations (1.5, 3.5 and 5 μg) in a constant volume (1 μl) per injection. On the other hand, we tested the effect of increasing volume injected by carrying out two injections of each DNA concentration in the same region. Results obtained with a single injection were always less efficient in terms of number of muscle fibers transfected as compared to double injections. Increasing DNA concentration also increased efficiency. As shown in Figure 1 (compare A and B), the area of plasmid expression extended over 3 or 4 myomeres in each case, but the number of fibers expressing the transgene was dependent on DNA concentration. Expression 3 days post-injection (p.i.) showed the numbers of muscle fibers expressing the β-galactosidase to be much greater with increasing concentration (2x3.5 μg/μl). An approximate count showed that raising DNA concentration from 2x1.5 to 2x3.5 μg/μl increased five fold the number of fibers expressing protein (data not shown). Using concentrations higher than 3.5 μg/μl (5 μg/μl tested) did not improve the efficiency of transfection (data not shown). Thus, in all of the following experiments we used unilateral, double injections of DNA at 3.5 μg/μl (i.e. 7 μg/site).

Expression of the fusion proteins in transfected muscle is stable over a long period

Two types of fusion construct, with or without a signal peptide were used. The IL2 signal peptide has been shown to direct secretion of fusion proteins *in vitro* (Dee *et al.*, 1990). We tested whether it could enhance release and uptake of the β-gal-TTC protein. Expression of the fusion proteins encoded by each of the constructions was analyzed in muscle 3 days p.i. using immunocytochemistry for LacZ and TTC. Expression from the pCMV-IL2-LacZ-TTC and pCMV-LacZ-TTC constructs (Fig. 2) resulted in strong immunoreactivity for both components of the fusion proteins, colocalized in the cytoplasm of numerous polynuclear muscle fibers, showing that the fusion protein is expressed efficiently in muscle fibers from the transgenes. In all cases, fusion protein localization was confined to the muscle fiber cytoplasm and no fusion protein was found in the connective tissue or in any non-muscle cell type. This observation is important because it underlines the specificity of the muscle production and labeling. It should also be noted that transfection had no deleterious effects on muscle morphology, with no degeneration or inflammation being observed at any time point. Moreover, expression was extremely stable. The high level of expression seen at 3 days p.i. for all the constructs was maintained at uniformly high levels for at least one month p.i. (the latest time point analyzed, see Figs. 3,4 and 5).

The TTC fragment is necessary and sufficient for release and retrograde transport of the fusion protein

Transfections for each construct were carried out as described above, but expression times were extended to test whether migration into axons occurred. Localization at one week p.i. was followed by *in toto* X-Gal staining for each construct. The results show that fusion proteins produced in muscle had entered motoneuron axons at this time point. As seen in Figure 3, we observed strongly labeled axons emerging from muscles injected with each construct containing the fusion protein: the pCMV-IL2-LacZ-TTC (Fig. 3A-C) and pCMV-LacZ-TTC (Fig. 3D and E) constructs. Similar results were found in axons innervating muscles injected with another LacZ-TTC construct with the NGF signal peptide (data not shown). In contrast, no labeled axons were ever observed, at any time point, in tadpoles injected with the control construct pCMV-LacZ, lacking the TTC fragment.

No difference in axonal uptake of the fusion protein was seen when using constructs with or without a signal peptide, although the subcellular distribution of the proteins was distinct. As seen in Figure 3F and G, pCMV-LacZ and pCMV-LacZ-TTC respectively showed a homogeneous cytoplasmic distribution, whereas the addition of a signal peptide, pCMV-IL2-LacZ-TTC gave a more punctate distribution of the fusion protein (Fig. 3H), characteristic of endoplasmic reticulum associated proteins. Furthermore, using constructs with the signal peptide did not affect the time at which labeling appeared in the terminal axons.

These results show that the TTC fragment is necessary and sufficient to provide for release of the β-gal protein from muscle without a signal peptide and to account for retrograde transport of the fusion protein into motoneurons. That the retrograde transport of the protein was due to the TTC fragment is shown by the failure to find any such transport with proteins produced from constructs lacking the TTC coding sequence. Moreover, our results show that the addition of a sequence coding for a second protein component along with that encoding the TTC fragment does not interfere with the function of either protein and, in particular, does not hinder the movement of the TTC fragment to and across the neuromuscular junction. This complex itinerary requires the fulfillment of a series of events including protein synthesis and processing, release from the muscle (by an as yet undefined process) and then uptake at the motoneuron/muscle synapse. What is more, the process is sufficiently efficient as not to be improved by the addition of a signal peptide.

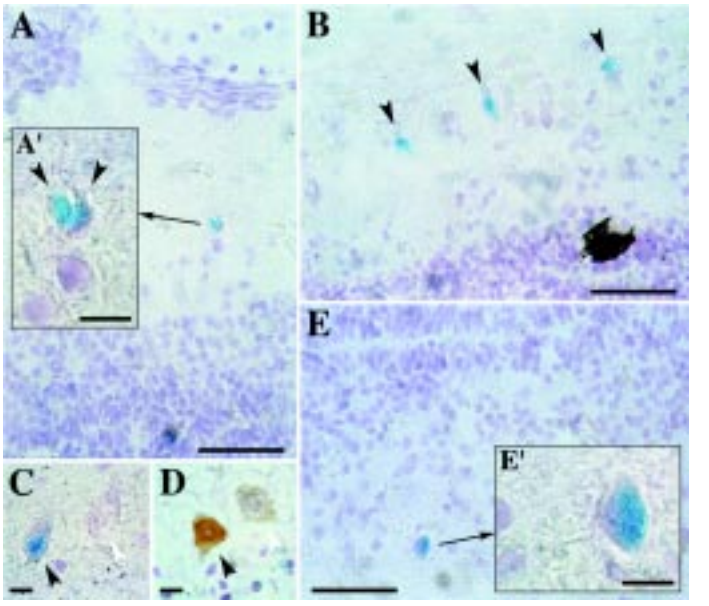

Fig. 5. Detection of β-galactosidase activity in motoneurons after 4 weeks expression in muscle for the pCMV-LacZ-TTC fusion construct. *Histochemistry with X-Gal shows β-gal activity to be present on the ipsilateral side* **(A, A'** *and* **B)** *and on the contra-lateral side* **(E** *and* **E')**. *Labeled motoneurons are indicated with black arrows.* **(C** *and* **D)** *Positive motoneurons (black arrow) can be adjacent to negative cells (white arrow). Bar, 50 μm (A,B and E); 10 μm (C,D,A' and E').*

Retrograde transport of the fusion protein is a slow, time-dependent process

To determine the kinetics of fusion protein transport to the spinal cord motoneurons, we used histochemistry and immunocytochemistry for LacZ and TTC detection applied to longitudinal and transversal section of tadpole tails. We found that it took four weeks for the motoneuron cell bodies to show detectable positive staining for the fusion protein components. Despite this relative long delay, we found the process to be highly reproducible and efficient as motoneuron staining was found in every animal successfully injected. When injections were carried out in the caudal tail muscle (myomeres 8-11) motoneuron cell bodies positive for β-gal and TTC proteins were found in the spinal cord at the level of myomeres 4-7 (Fig. 4A). This corresponds to the anatomical distance between caudal motoneuron terminal and their cell bodies (Westerfield and Eisen, 1985; Nishikawa and Wassersug, 1988). As for the *in toto* labeling (Fig. 3), no labeling, and therefore no retrograde transport, was ever observed in the spinal cords of tadpoles injected with the control construct lacking the TTC fragment. Again no advantage was seen when using construct with the IL2 signal peptide.

To unambiguously identify positive reactive neurons in the motor-column, we used immunocytochemistry for LacZ and TTC on adjacent sections. Motoneuron cell bodies positive for Lac-Z (Fig. 4B and

C) and TTC (Fig. 4D and E) were seen with similar staining patterns and intensities in such adjacent sections.

The labeling revealed an interesting feature: that of labeling of the motoneurons on the contra-lateral side, at the same level as those motoneurons on the injected side (Fig. 4F). Labeling on successive transverse sections showed unambiguously that the staining for lac-Z (Fig. 4H and H') and TTC (Fig. 4I and I') was localized in the motoneuron cell bodies of the spinal cord motor-column (in the ventral region of the spinal cord, Fig. 4G).

A final confirmation used was histochemical revelation of β-galactosidase activity. As shown in Figure 5, cell bodies showing β-gal activity were found in the same areas displaying immunoreactivity on both sides of the spinal cord. The specificity of both the immunocytochemistry and the histochemistry is illustrated in Figure 5C and D, where positive and negative cells could be found immediately next to one another with each method used.

Although the process is efficient, it is a slow, time-dependent process. Even if the protein can be detected in the motoneuron terminals at 1 week p.i. (Fig. 3), it takes the better part of a month for the sufficient amounts of the fusion protein to accumulate in the motoneuron cell body to allow detection by immunocytochemistry and/or histochemistry. One factor that might explain the long time course for fusion protein uptake is that this process could be correlated with synaptic activity, which is the case for the native tetanus toxin (Matteoli *et al.*, 1996). In *Xenopus laevis* (in contrast to *Rana* species) tail movements related to swimming are mainly the results of muscle activity in the most caudal part of the tail (Nishikawa and Wassersug, 1988), with little contribution from the more anterior part of the tail, which is only recruited for turning or accelerating. We transfected the anterior caudal and the dorsal tail muscles of the tadpole. As these muscles will have little motor activity and thus low synaptic activity, this in turn will limit uptake of the fusion protein.

Whatever the cause of this extended time frame for detection in motoneuron cell bodies, it is amply compensated for by the fact that the episomal production of protein can be maintained in skeletal muscle of both mice and amphibians for several months (Wolff *et al.*, 1992; de Luze *et al.*, 1993). This engenders stable muscle protein levels and allows one to observe the process, and its consequences, over long time periods.

Taken together, these results make a clear contribution to the study of TTC fusion protein properties in that, to date, only the uptake at the neuromuscular junction of TTC fusion proteins synthesized *ex vivo* and injected into muscle has been clearly demonstrated (Fishman and Savitt, 1989; Beaude *et al.*, 1990; Coen *et al.*, 1997; Figueiredo *et al.*, 1997). The data show that the TTC fragment contains all the structural information needed for correct cellular processing, packaging and release from the muscle. This latter finding in itself is intriguing and clearly calls for further investigation to determine the process of cellular release.

A number of interesting applications are raised by these observations. A first possibility is the use of this fusion protein as a genetic marker. The advantage of the fusion gene is that it derives from a single genetic entity that is amenable to genetic manipulation and engineering. The feasibility and usefulness of this approach is reinforced by the elegant recent demonstrations of neuronal mapping with use of plant lectins (wheat germ agglutin and barley lectin) expressed in transgenic mice and *Drosophila* (Horowitz *et al.*, 1999; Yoshihara *et al.*, 1999). In contrast to the TTC fusion proteins, these lectin fusion proteins provide principally anterograde transport and thus reveal sensory and mainly centrifugal projections. To our knowledge, the TTC fragment is the only non-viral marker that can be used for a genetic approach providing retrograde mapping motor of centripetal projections. Certain viruses have been tested in this context, e.g. Herpes simplex viruses or rhabdoviruses, but their toxic effects in neurons and complex genetics are serious limitations (Kuypers and Ugolini, 1990). For the moment, TTC is the only protein that fulfils all the properties required for use as a retrograde tracer for genetic applications: it enters neurons efficiently, is specific to this cell type and transport is unambiguously unidirectional (in contrast to lectins that show mainly anterograde but also some retrograde movement).

Another important characteristic of the β-gal-TTC fusion protein is its apparent stability. It could therefore be applied to the analysis of muscle/motoneurons interactions in physiological or pathological situations. Although it is generally accepted that an overriding component involved in motoneuron survival is the production of growth factors from the muscle innervated, it is also becoming more and more established that their interdependence is complex, with other contributing elements entering into play. For example, in the absence of skeletal muscle, trophic signals produced by other tissues are sufficient to support survival of about 10% of embryonic spinal motoneurons (Grieshammer *et al.*, 1998). The models most often used currently involve the creation of transgenic mice in which muscle development is blocked by elimination of key myogenic genes (Grieshammer *et al.*, 1998; Kablar and Rudnicki, 1999). Such studies show that the great majority of motoneurons deprived of their target undergo apoptosis (Grieshammer *et al.*, 1998; Kablar and Rudnicki, 1999). In each of these powerful approaches the target muscle is removed early in embryogenesis or never develops. One advantage of the amphibian metamorphosis model is that a pre-existing differentiated motoneuron/muscle system undergoes massive remodeling, recalling certain events in some neuromuscular pathologies.

However, despite its experimental potential, there is limited data on the amphibian model. Indeed, even in the dramatic case of tail regression during amphibian metamorphosis, the determinants and the kinetics of muscle and motoneuron death have yet to be established. Recent work (Berry *et al.*, 1998a,b) suggests that muscle apoptosis and degradation of the extracellular matrix results in neuron "murder". However, alternative scenarios can be proposed in which the demise of at least certain neurons might precede apoptosis of the muscles innervated, or the events could be simultaneously provoked by common factors. These equally plausible hypotheses can be verified by injecting given muscles with the fusion protein DNA and then following the persistence of the fusion protein in each cellular compartment over metamorphosis. Moreover, if the β-gal marker protein were to be replaced by the fluorescent marker GFP, the events could be followed 'on line' (at least in the more transparent part of the tail) in the freeliving tadpole. Finally, when the construct is injected relatively early in development (stages 53-54) into dorsal muscle myomeres, the protein reaches motoneurons innervating primary fibers that will be replaced by adult fibers after metamorphosis. This allows one to follow synaptic plasticity and target reorganization by these motoneurons, a feature that appears to be much used during the extensive remodeling that occurs during metamorphosis.

A further extension of the approach could be in the context of gene therapy in the nervous system. TTC could act as a carrier to target specifically macromolecules to neurons. In such a case, the lacZ

reporter would be replaced by a gene encoding a therapeutic protein. Superoxide dismutase (SOD) has already been used *in vivo* fused to the TTC fragment (Figueiredo *et al.*, 1997) and shown to maintain most of its enzymatic function. The advantage of the TTC system is that superficially placed muscles could be injected so as to deliver proteins to less accessible neuronal targets. This could be useful for treating neurodegenerative and motoneuron diseases such as amyotrophy lateral sclerosis (Rosen *et al.*, 1993), spinal muscular atrophies (Lefebvre *et al.*, 1995; Roy *et al.*, 1995), or deficiencies such as neurodegenerative lysosomal storage diseases (Wolfe *et al.*, 1992; Sango *et al.*, 1995). The overriding advantage here being that expression of the transgene is stable over the long term.

In conclusion, we show that fusion proteins can be produced in defined muscles by a somatic plasmid DNA transfer approach. The proteins so produced are functional, being released from the muscle and undergoing retrograde transport to the innervating motoneurons. This demonstration consolidates the established usefulness of this somatic gene delivery method.

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