TECHNIQUES

Targeted Gene Delivery to Differentiated Skeletal Muscle: A Tool to Study Dedifferentiation

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Cellular dedifferentiation is required for functional regeneration in salamanders. Dedifferentiating multinucleate skeletal muscle gives rise to mononucleate cells during limb regeneration. Efficient methods and tools must be developed in order to understand the molecular cues underlying dedifferentiation. Here we describe a non-viral method to express extra-chromosomal DNA exclusively in terminally differentiated muscle without the need for cell purification steps. After cytoplasmic injection of various expression vectors into myotubes or myofibres, we detect long-lasting mRNA and protein expression in up to 70% of the injected cells. The combination of the transfection protocol with live imaging allows a time- and cost-effective screen of candidate genes in terminally differentiated muscle cells of both amphibian and mammalian origin. *Developmental Dynamics 236:481–488, 2007.* © 2006 Wiley-Liss, Inc.

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INTRODUCTION

Dedifferentiation of terminally differentiated tissue is a key aspect of regeneration in salamanders such as newts (Iten and Bryant, 1973). Dedifferentiation precedes the formation of the regeneration limb blastema, a mesenchymal growth zone from which cells of the regenerate originate. Skeletal muscle is one such tissue that contributes to the formation of the blastema during limb and tail replacement following amputation (Brockes, 1997). Histological and cell tracing data suggest that during the dedifferentiation phase, multinucleated myofibres give rise to mononucleate progeny (Thornton, 1938; Hay, 1959; Hay, 1962; Lentz, 1969; Echeverri et al., 2001). This "cellularization" process involves the breaking up of multinucleate myofibres into single cells, which subsequently incorporate into the blastema. Cellularization remains unique to salamander regeneration and has been demonstrated both by implantation of labeled myotubes into the limb blastema and by direct observation of microinjected myofibres of the regenerating tail (Lo et al., 1993; Kumar et al., 2000; Echeverri et al., 2001). However, treatment of myotubes derived from mouse cell lines with small substituted purines that destabilize the microtubule network also causes cellularization (Rosania et al., 2000; Perez et al., 2002; Duckmanton et al., 2005). Furthermore, mammalian myotubes can also undergo cellularization following ectopic expression of Msx1 or treatment with extract derived from regenerating

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newt limbs (Odelberg et al., 2000; McGann et al., 2001).

The recent publication of two salamander EST databases (Habermann et al., 2004; Putta et al., 2004) extended the genomic resources that can be used in regeneration research. However, a major hurdle to understanding the mechanisms of cellularization is the lack of efficient in vitro models, which can utilize these resources in functional experiments by the over- or under-expression of various genes.

Liposome- and retrovirus-based gene delivery methods can efficiently target salamander cells (Kumar et al., 2000; our unpublished observation) but they cannot transfect/transduce differentiated muscle cells (Tsonis et al., 1996). Lentivirus can in principle transduce differentiated muscle cells but production of viruses is costly and time consuming. In addition, either promoter specificity is necessary to target differentiated muscle or the culture system needs to be purified to eliminate mononucleate contamination. Myotube purification steps often result, however, in more fragile cells leading to an increase in cell death. Microinjection directly into the nucleus of differentiated cells is one method for expressing extra-chromosomal DNA selectively in myotubes (Capecchi, 1980; Tanaka et al., 1997), but nuclear injection often results in high mortality rates.

In an elegant in vivo study, it has been shown that rat myofibres can be transfected by cytoplasmic injection of DNA constructs (Sander et al., 2000). Other experiments have shown that it is possible to attach DNA to Nuclear Localizing Signals (NLS) to increase the expression rate after cytoplasmic injection (Collas and Alestrom, 1998; Arenal et al., 2004). Therefore, we decided to test the effect of non-covalently attaching a NLS peptide to expression vectors via ionic interactions before cytoplasmic injection into myotubes or myofibres. We found that it was possible to express different NLS-associated vectors in newt myotubes and myofibres, as well as in mouse-derived myotubes, with high expression levels lasting for at least 10 days post-injection. Surprisingly, we discovered that it was not necessary to attach NLS peptides to the vector, as comparable levels of expression, with similar transfection efficiency, were also seen after cytoplasmic injection of naked DNA. Expression was exclusive to myotubes and myofibres with no expression in the surrounding mononucleate cells. Expression could be followed using RT-PCR and tracked live using fluorescent proteins. This method is, therefore, a valuable tool in describing the mechanisms of cellular dedifferentiation.

RESULTS

Expression of Vectors After Cytoplasmic Injection Into Newt Myotubes

We injected two different constructs into myotubes. One construct encoded the histone2B-yellow fluorescent fusion protein (pSUPER H2BYFP), whose expression resulted in nuclear green fluorescence. The other construct encoded the red fluorescent protein (pCMV mRFP), whose expression resulted in cytoplasmic red fluorescence throughout the myotube. Vectors were injected into the cytoplasm either associated with NLS peptides (+NLS) or non-associated (−NLS). As early as 8 hr post-cytoplasmic injection of the vectors, expression of green nuclear fluorescence (Fig. 1A) or red cytoplasmic fluorescence (Fig. 1B) could be clearly detected in the myotubes. The intensity of the fluorescence increased the longer the myotubes were left in culture. We could detect fluorescence 3 days (Fig. 1C and D) and 9 days (Fig. 1E–H) post-injection. Mononucleate cells always lacked expression of the transgenes at early time points after transfection. However, at later time points a very small fraction of mononucleate cells appeared positive, but only if they were in close vicinity of an injected myotube (Fig. 1E, F; see *). Such cells may be derived by a low degree spontaneous cellularization of the myotubes. Perhaps a more likely explanation is that these cells are in the process of fusing with a transfected pre-existing myotube. Fusion is an ongoing process in differentiation medium as illustrated in Supplementary movie 1 (which can be viewed at www.interscience.wiley.com/jpages/1058-8388/suppmat). These data show that it is possible to express extra-chromosomal DNA constructs in post-mitotic myotubes after cytoplasmic injection.

Efficiency of Cytoplasm Injection

In order to determine the efficiency of the transfection protocol, we injected newt myotubes and determined the number of cells that expressed the constructs. Expression could be seen as early as 4 hr post-injection in myotubes injected with vectors + NLS, with a peak of expression at around three days (Fig. 2A). Surprisingly, fluorescent expression could be seen with comparable intensity in myotubes injected with vector − NLS (Fig. 2A). However, expression of vectors injected − NLS started 8 hr post-injection but then followed a similar temporal pattern as in the case of myotubes injected with vectors + NLS. At the peak of expression (3 days post-injection), the proportion of myotubes expressing H2BYFP or mRFP was 74 and 56%, respectively, after vector + NLS injection, compared to 71 and 63%, respectively, after vector − NLS injection. The number of myotubes expressing H2BYFP or mRFP started to decline slightly after 7 days but approximately 50% of the injected myotubes were still positive at 10 days.

We also decided to inject a mixture of both constructs, with and without associated NLS, to see if we could drive the expression of two vectors simultaneously (Fig. 2B). When both constructs were injected at the same time, fluorescent expression was observed, regardless of the presence or absence of the NLS peptide. At the peak of expression (24 hr post-injection), the proportion of myotubes expressing H2BYFP or mRFP was 38 and 8%, respectively, after vector + NLS injection, compared to 42 and 22%, respectively, after vector − NLS injection. More myotubes expressed H2BYFP than mRFP but all $mRFP^{+}$ myotubes were also $H2BYFP^{+}$. These data demonstrate that expression levels remained high for several days post-injection and peaked between one and three days. It was also possible to express two

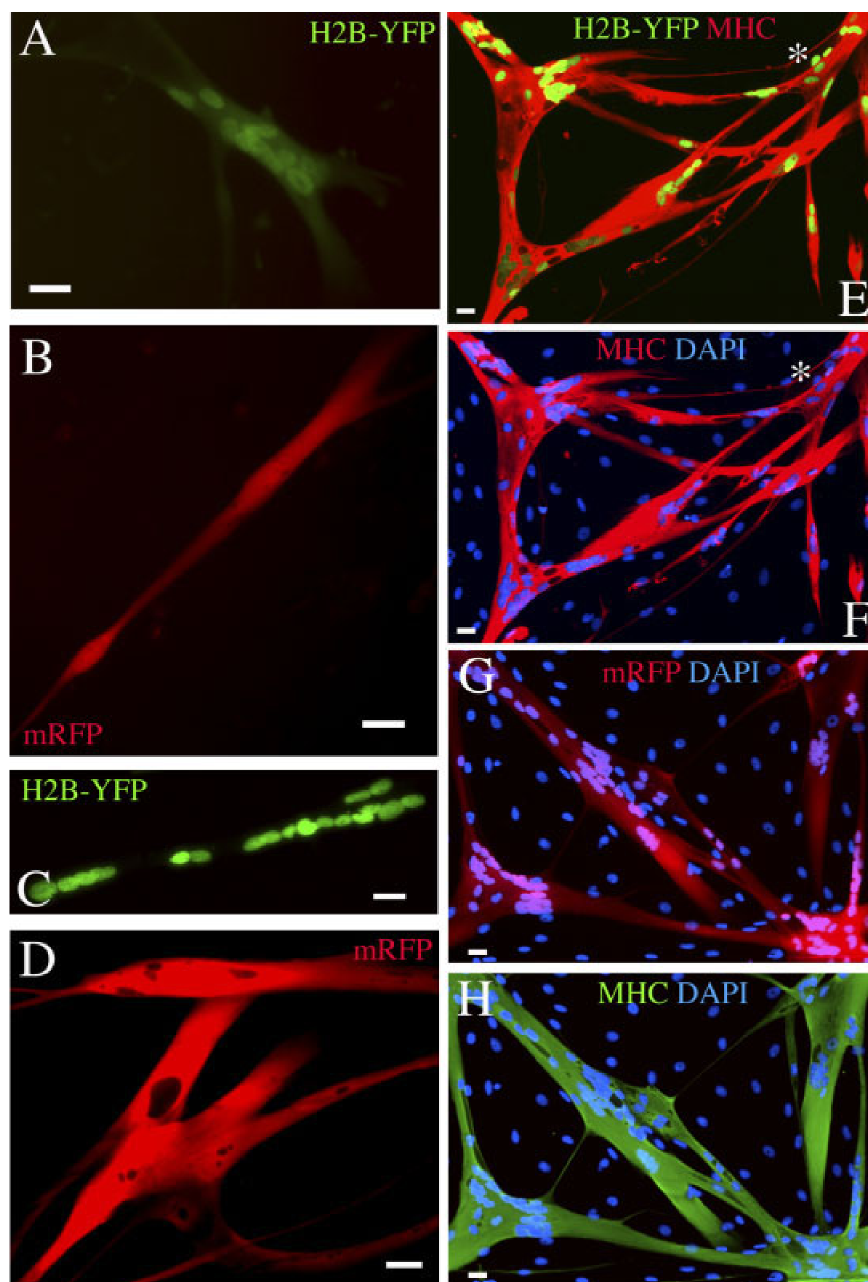


Fig. 1. Newt myotubes express fluorescent proteins after cytoplasmic injection of pSUPER H2BYFP and pCMV mRFP cDNAs. **A,B:** H2BYFP and mRFP expression at 8 hr post-injection. **C,D:** H2BYFP and mRFP expression at 3 days post-injection. **E–H:** Fixed cultures at 9 days were counterstained with MHC demonstrating expression in myotubes. Cells were counterstained with DAPI for localization of the nuclei. * marks one H2BYFP$^+$ cell in the close vicinity to a transfected myotube. This is a rare example of a mononucleate cell that is likely to be in the process of fusing to a pre-existing myotube. Scale bars = 50 μm.

vectors simultaneously, albeit with some reduction in transfection efficiency.

Cytoplasmic Injection Into Cultured Newt Myofibres

We were interested to determine whether this injection protocol could be used to express proteins in ex vivo cultured, single newt myofibres. A single myofibre culture was established by us earlier to study the regenerative plasticity of newt muscle cells (Morrison et al., 2006). Supplementary Figure 1 shows that it was possible to express the pCMV mRFP construct in cultured myofibres. Next, we determined whether we could express two different constructs simultaneously in myofibres. We used the pCMV H2BYFP vector, whose expression should result in nuclear green fluorescence, and the pCMV mRFP, whose expression should result in cytoplasmic red fluorescence. Dual expression was seen as early as 24 hr (Fig. 3A–C) and continued for 3 days (Fig. 3D–F) and 10 days (Fig. 3G–I) post-injection of vectors. We also saw an increase in fluorescent intensity with increased time in culture. We saw that, similarly to myotubes, injection of vectors − NLS into myofibres led to comparable results that we obtained with +NLS (data not shown).

The surrounding Pax7$^+$ mononucleate cells were negative (Fig. 3J–L), indicating that fluorescent expression is confined to the sarcolemma and myonuclei of the myofibres and is excluded from satellite cells (Morrison et al., 2006). This is further illustrated by the lack of H2BYFP and mRFP expression in a migrating satellite cell from a 10-day post-injected newt myofibre (arrowhead in Fig. 3G–I and magnified image in Supplementary Figure 2).

In order to test how long mRNA synthesis lasted after vector injection, we performed combined injections of both pCMV mRFP and pCMV H2BYFP, with and without associated NLS, into the cytoplasm of newt myofibres. At specific time-points, 29 myofibres were randomly picked for preparation of mRNA. The mRNA was reverse transcribed into cDNA, which in turn served as a template in PCRs using mRFP and H2B specific primers (Table 1). None of the primers recognized genomic DNA (data not shown). As GAPDH is the most appropriate normalization factor in gene expression analyses during limb regeneration (Vascotto et al., 2005), we used GAPDH to normalize the samples. Out of the 29 myofibres injected, 28 had GAPDH expression, indicating a 97% survival rate post-injection (Fig. 3M). We saw mRNA expression as early as 4 hr post-injection, which persisted for at least 7 days. Although we saw mRNA expression at 4 hr post-injection, fluorescent protein expression was not visibly detectable at this time point. In a cumulative analysis of the surviving myofibres at 5 different time points, 46% showed mRFP and

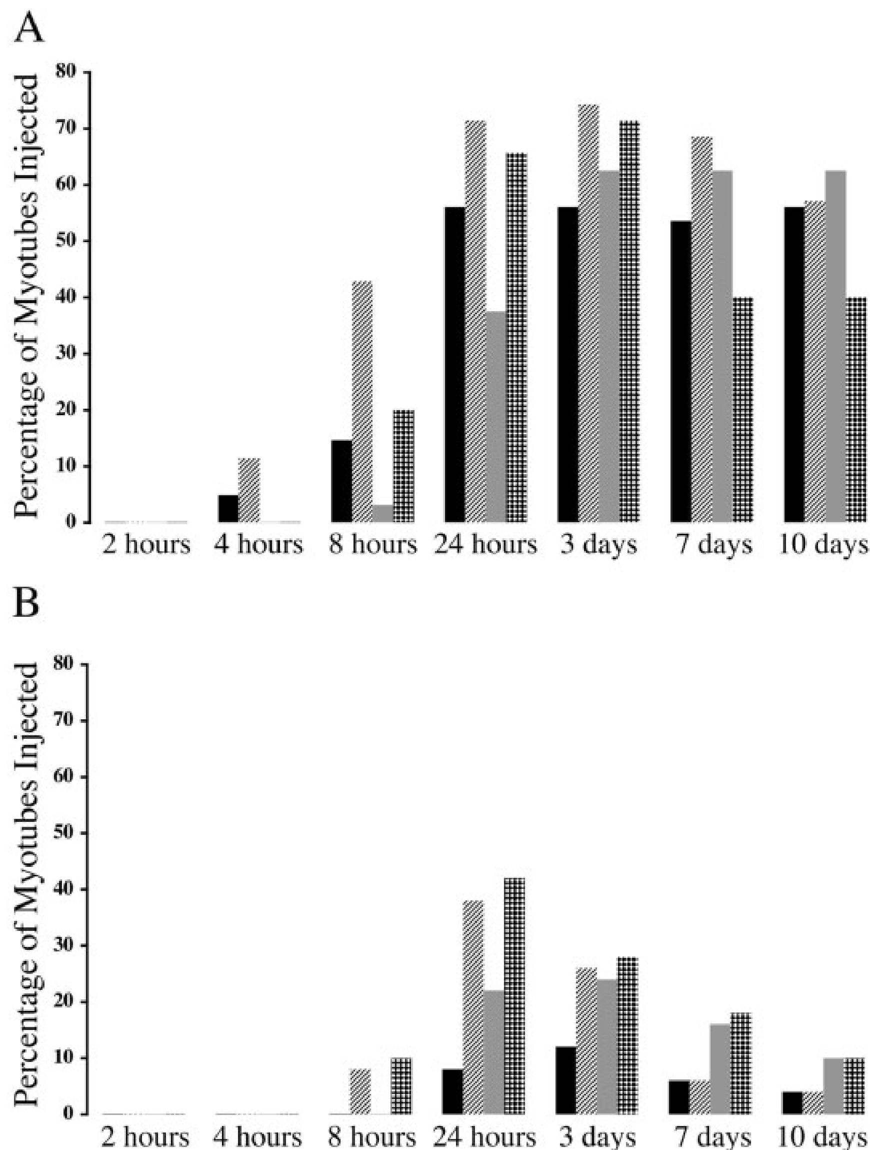


Fig. 2. Quantification of the transfection efficiency in newt myotubes. Histograms demonstrating the percentage of newt myotubes expressing H2BYFP or mRFP fluorescence after single (**A**) or combined (**B**) injection of pSUPER H2BYFP and pCMV mRFP. N = 32–41 in A and n = 50 in B for each bar. Black and striped bars, pCMV mRFP + NLS and pSUPER H2BYFP + NLS, respectively; grey and checkered bars, pCMV mRFP − NLS and pSUPER H2BYFP − NLS, respectively. All mRFP expressing cells in B also express H2BYFP.

H2B mRNA expression. These data indicate that long-lasting protein expression was not due to the stability of the expressed protein but due to ongoing mRNA transcription.

Expression of Vectors After Cytoplasmic Injection Into Mammalian Myotubes

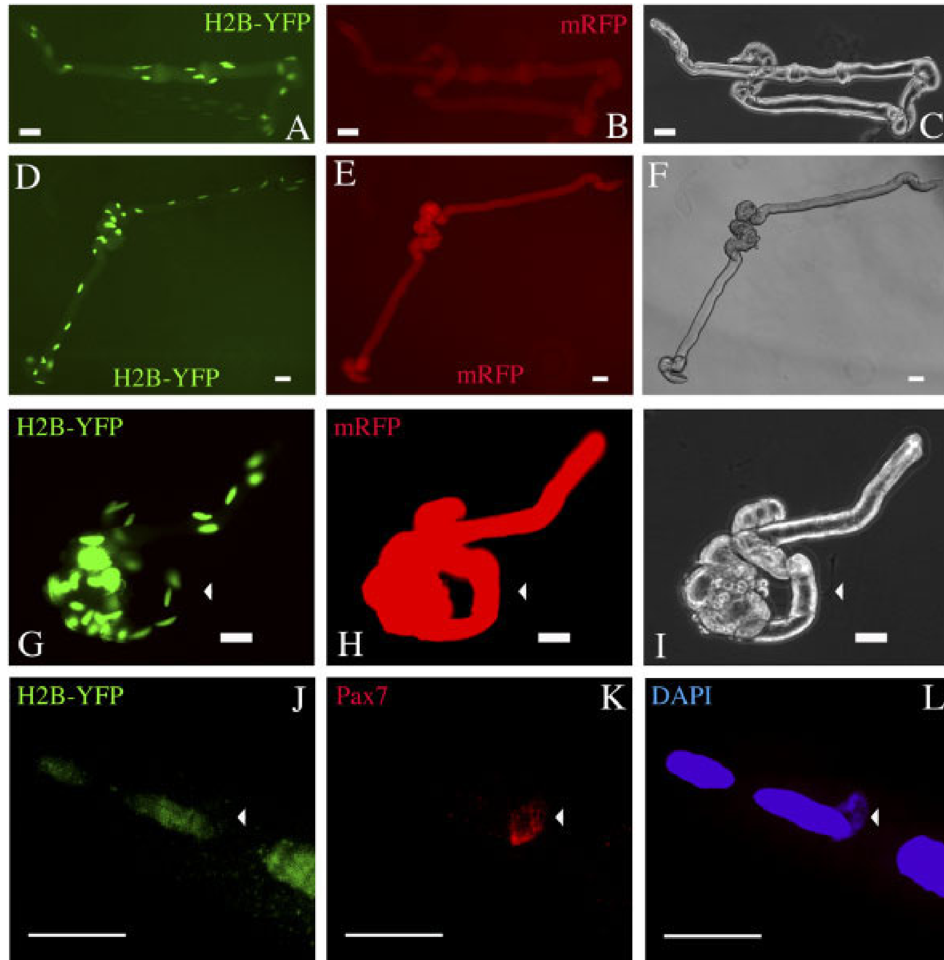
The ability of differentiated newt muscle cells to express DNA following cytoplasmic injection led us to consider whether mammalian myotubes possess the same ability. Therefore, we decided to inject cultured mouse–derived myotubes with pCMV H2BYFP, with and without associated NLS. We could see nuclear expression of the H2BYFP protein after 24 hr (Fig. 4A–C) and continuing up to 9 days in culture (Fig. 4D and E). As for the newt cells, we saw the same level of expression regardless if there was a NLS associated to the vector or not (Fig. 4F). However, there was a decreased efficiency of expressing mouse myotubes, as compared to newt cells (Fig. 2A). At the peak of expression (3 days post-injection), the proportion of myotubes expressing H2BYFP, with or without associated NLS, was 20 and 18%, respectively. We did observe that mouse myotubes were much more sensitive to the injection procedure as compared to newt myotubes, resulting in increased cell death. These results demonstrate that differentiated mammalian myotubes can express proteins of interest after cytoplasmic injection, but the transfection efficiency was lower as compared to their newt counterparts.

DISCUSSION

Here we devised a gene expression method to functionally manipulate the molecular cues driving cellularization and dedifferentiation of skeletal muscle. We used cytoplasmic injection of vectors encoding fluorescent pro-

Fig. 3. Newt myofibres express fluorescent proteins after cytoplasmic cDNA injection. Fluorescent protein expression at 24 hr (**A–C**), 3 days (**D–F**), and 10 days (**G–I**) post-injection. The arrowhead in I shows a cell migrating from the myofibre that has no corresponding fluorescence (as seen by the arrowheads in G and H), indicating it is of satellite cell origin (see also a magnified image in Supplementary Figure 2). **J–L:** Confocal photomicrograph showing expression of H2BYFP in syncytial myonuclei and the lack of expression in a $Pax7^{+}$ satellite cell. Cells were counterstained with DAPI for localization of the nuclei. Scale bars = 50 μm. **M:** Newt myofibres were randomly taken at the specified time-points for RNA preparation and subsequent RT-PCR. + and − signs indicate cDNA strand synthesis with and without the presence of the reverse transcriptase enzyme, respectively. The expression of mRNA could be seen as early as 4 hr, with expression lasting for 7 days post-injection. The arrowhead indicates a live myofibre even though the GAPDH expression is weak. The black arrow indicates a dead myofibre lacking GAPDH expression.

Fig. 4. Mouse myotubes express fluorescent proteins after cytoplasmic cDNA injection. Fluorescent protein expression at 24 hr (**A–C**) and 9 days (**D,E**) after injection with pCMV H2BYFP. C is an overlay image of A and B showing localization of the fluorescent protein to the nuclei of a myotube. Cells in D and E were fixed and counterstained with MHC and DAPI, to visualize the myotubes and nuclei, respectively. Scale bars = 50 μm. **F:** Histogram demonstrating the percentage of C57BL6 myotubes expressing H2BYFP after injection of pCMV H2BYFP. Black and grey bars, vectors associated with or without NLS, respectively. n = 40 for each bar.



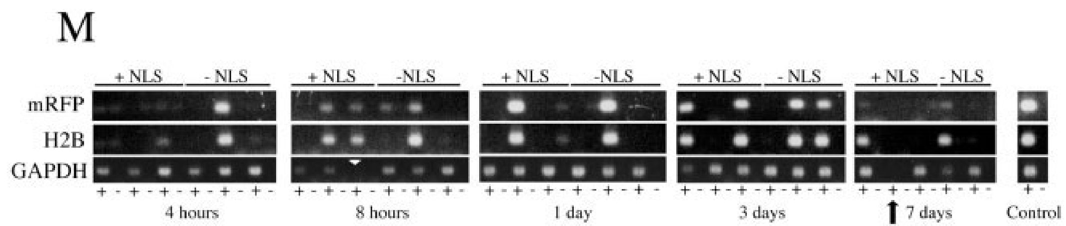


Fig. 3.

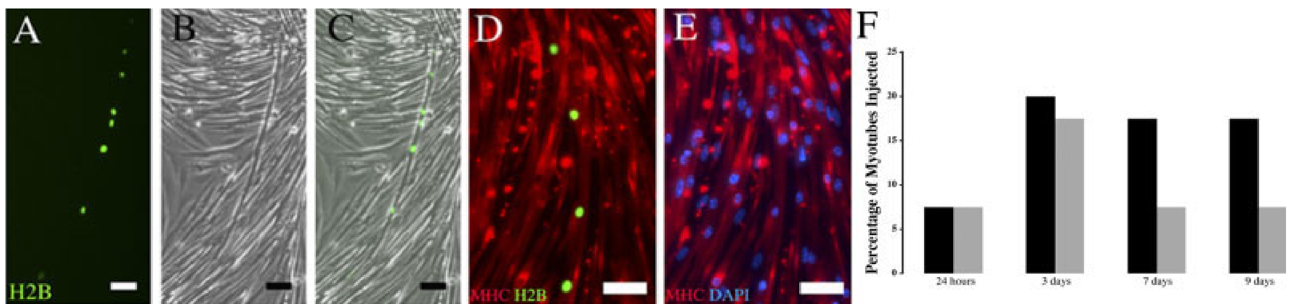


Fig. 4.

TABLE 1. Forward and Reverse Primers Used in the PCR for GAPDH, mRFP, and Histone H2B, Following cDNA Synthesis From Newt Myofibre mRNA

Gene	Primers
GAPDH fwd	5′-TGTGGCGTGACGGCAGAGGTG-3′
GAPDH rev	5′-TCCAAGCGGCAGGTCAGGTCAAC-3′
mRFP fwd	5′-CCTCCTCCGAGGACGTCATCAA-3′
mRFP rev	5′-CCTTGGTCACCTTCAGCTTGGC-3′
H2B fwd	5′-CCCGAAAAAGGGCTCCAAGAAG-3′
H2B rev	5′-AGGGTGGACCTGCTTCAGAACCTT-3′

teins to demonstrate the validity of our protocol. We show that cytoplasmic injection combined with live imaging is a simple and versatile way for rapid screening of candidate genes during cellular dedifferentiation.

Due to the direct application into the myotubes or myofibres, the requirement of specific promoters is made redundant and several hundreds of cells can be injected in a few hours. Compared to bulk transfection techniques, there is no need for cellular purification steps to remove mononucleate contamination. Compared to viral vectors, which can be time-consuming and costly to produce, this method only requires the sub-cloning of the gene of interest, and subsequent purification, before it can be used.

The size of all constructs used here was approximately 5,000 bp (equivalent to 3.3 MDa), which is considerably higher than the cut-off point for passive diffusion, suggesting that DNA injected into the cytoplasm should not have been able to cross the nuclear membrane. Based on previous results (Collas and Alestrom, 1998; Arenal et al., 2004) we tested transfection efficiency employing NLS-associated vectors. Surprisingly, we discovered that DNA association via non-covalent ionic binding of a NLS peptide was unnecessary, as naked DNA was also expressed at high levels after cytoplasmic injection. This was unexpected considering that except during mitosis, when the nuclear envelope disassembles, the only mode of nuclear entry of macromolecules is through the nuclear pore complex (NPC) (Ryan and Wente, 2000). The aqueous channel of the nuclear pore complex allows free diffusion of molecules less than 50–70 kDa, but the translocation of larger karyophilic proteins and DNA requires an active process (Featherstone et al., 1988). It remains to be clarified whether our observation reflects a specific nuclear architecture in salamander cells or that the size limit of the nuclear pore complex is higher than previously determined. The fact that post-mitotic mammalian cells can also be effectively transfected by cytoplasmic injection argues for the latter possibility. It would also be interesting to determine the generality of this phenomenon by testing other post-mitotic cell types such as neurons. The forces generated from injecting a solution into the cytoplasm is an unlikely explanation for nuclear delivery, as electron microscopy studies using cytoplasm-injected gold-particles into rat myotubes resulted in no nuclear-located expression (Dowty et al., 1995). There is evidence to suggest that nuclear import of plasmid DNA is sequence specific, with plasmids containing the regions of the SV40 enhancer/early promoter being targeted to the nucleus in the absence of cell division (Dean, 1997). As only two of the vectors used here contain such a sequence, it seems that this cannot be the sole reason for nuclear uptake of vector DNA in our model system.

With increasing amounts of molecular information, such as cDNA and EST databases, suitable methods and tools need to be developed that can help to utilize these resources. We have devised such a tool that can be used with both salamander and mammalian muscle cells and can help delineate the molecular cues of dedifferentiation during regeneration. Combined with live imaging after expression of fluorescent fusion proteins, transfected cells could be tracked without fixation. Based on our results, expression of interfering RNA may also become testable by injecting RNAi vectors such as the pSUPER RNAi vector system™ (Brummelkamp et al., 2002) used in this study. Taken together, we show a versatile method for a rapid screen of gene function during cellular dedifferentiation.

EXPERIMENTAL PROCEDURES

Animals

All experiments were performed according to European Community and local ethics committee guidelines. Adult red-spotted newts, *Notophthalmus viridescens*, were supplied by Charles D. Sullivan Co., Inc., and maintained in a humidified room at 15–20°C. Animals were anesthetized by placing them in an aqueous solution of 0.1% ethyl 3-aminobenzoate methanesulfonate salt (Sigma, St. Louis, MO) for 15 min, before myofibre isolation (see below).

Cell Culture

Newt A1 myogenic cells and myofibres were cultured as described previously (Ferretti and Brockes, 1988; Morrison et al., 2006). Prior to injection, the newt A1 cells were left in differentiation medium for 5 days, whereas myofibres were cultured for 4–5 days, to allow for proper attachment to the culture plate. Mammalian myoblasts from C57BL6 mice were isolated from single myofibre derived satellite cell cultures as described previously (Rosenblatt et al., 1995). C57BL6 myoblasts were cultured in proliferation medium consisting of DMEM supplemented with 20% Fetal Calf Serum, 10% Horse Serum, 1% Chicken Embryo Extract (AB Göteborgs Termometer fabrik), 2% Glutamax (Invitrogen) and 1% Penicillin/Streptomycin (Invitrogen). When the cells reached 90–100% confluency, the proliferation medium was replaced with differentiation medium consisting of 1% Horse Serum, 2% Glutamax, and 1% Penicillin/Streptomycin. The cells were left in differentiation medium for 4 days, prior to injection. Cells were cultured at 37°C with 5% CO_2.

DNA and NLS Attachment

Three vectors were used in this study: (1) pSUPER H2BYFP (a kind gift from Christina Karlsson, Karolinska Institutet, CMB and Claire Acquaviva, Wellcome Trust/CRC UK Gurdon Institute, UK) was modified from the pSUPER RNAi system™ (Brummelkamp et al., 2002), with a Histone2B protein fused to Yellow Fluorescent Protein. This is a control vector and has no knockdown capabilities. (2) pCMV H2BYFP (a kind gift from Claire Acquaviva, Wellcome Trust/CR UK Gurdon Institute, UK) was modified from pEYFP vector (Clonetech, Palo Alto, CA), with Histone2B fused to the YFP cassette. (3) pCMV mRFP (a kind gift from Jacques Neefjes, Netherlands Cancer Institute, Amsterdam, The Netherlands) was modified from pEGFP-N1 vector (Clonetech) with a monomeric red fluorescent protein replacing the EGFP. All vectors were purified using a Qiagen Maxi Prep Kit (Qiagen, Chatsworth, CA). Thermo Electron Corporation synthesized a synthetic polypeptide containing the NLS of the SV40 antigen T, PKKKRKVED-PYGGC. The NLS peptide was reconstituted in distilled water at a concentration of 1μg/μl. The synthetic NLS peptide was bound, via ionic interactions, to the vectors at a molar ratio of 100:1, in the presence of 0.25M KCl, for 30 min at room temperature. Negative controls replaced the NLS with distilled water.

Microinjections

Microinjection was performed using a micromanipulator (InjectMan; Eppendorf) working with a pump (FemtoJet; Eppendorf), with which the microscope was equipped. The injection sample was loaded into a "FemtoTip" needle (Eppendorf) by using a microloader (Eppendorf). For the injection of newt myotubes, an injection pressure of 30 hPa, a maintenance pressure of 30 hPa, and an injection time of 0.1 sec was used. For the injection of mouse myotubes, an injection pressure of 50 hPa, a maintenance pressure of 30 hPa, and an injection time of 0.1 sec was used. For the injection of myofibres, two injections at a pressure of 950 hPa, a maintenance pressure of 50 hPa, and an injection time of 1.2 sec were used. Under the phase contrast view using the ×20 objective, the faint movement of fluid in the cytoplasm indicated an injected cell. No fluid movement was ever localized exclusively in the nuclei. DNA concentrations of 4 μg/μl and 1μg/μl were used for injection into newt and mouse myogenic cells, respectively. When simultaneous injection of cDNAs was performed, cDNAs at a concentration of 4 μg/μl were mixed at a ratio of 1:1 prior to injection.

Immunohistochemistry

Newt and mouse myotubes were fixed with 2% paraformaldehyde, blocked with 20% Goat Serum, 3% Bovine Serum Albumin, 0.1% Triton X-100 in PBS, followed by incubation with mouse monoclonal anti-myosin heavy chain IgG (MF20; Developmental Studies Hybridoma Bank). The primary antibody was detected with appropriate species-specific Alexa Fluor-conjugated secondary antibodies (Invitrogen). Nuclear expression was detected using 100 ng/ml DAPI (Sigma-Aldrich). The protocol for immunofluorescent staining of newt myofibres with mouse monoclonal anti-Pax7 IgG (Developmental Studies Hybridoma Bank) were followed as previously described (Morrison et al., 2006). Controls omitting the primary antibody were performed in parallel resulting in the absence of staining.

RNA Extraction and cDNA Synthesis

RNA was extracted using the RNeasy microkit (Qiagen) according to the instructions of the manufacturer, using 75 μl RLT buffer and the carrier RNA and DNase I supplied in the kit. For every RNA sample, one myofibre was picked, with the aid of an inverted microscope, using a horse serum–coated glass capillary attached to a syringe through plastic tubing. The myofibre was then expelled directly into the RLT buffer. The cDNA synthesis was performed in a 20-μl reaction, using 5 μl of RNA, 0.5 μM dNTPs, 750 ng anchored oligo$(dT)_{20}$, 10 μM random nonamers, 1× first strand buffer, 40 U Rnase out, 1 mM DTT, and 200 U Superscript III (Invitrogen). RNA, primers, and dNTPs were first incubated at 65°C for 5 min and then chilled on ice. After addition of the other reagents, samples were incubated at 25°C for 5 min, followed by 60 min at 50°C. The reaction was stopped by heat inactivating at 70°C for 15 min. For every RNA sample, a negative control, lacking reverse transcriptase, was also performed.

PCR

For the PCR, the cDNA was mixed with 0.2 μM of each primer (see Table 1), 0.2 mM of each dNTP, 2 mM $MgCl_2$, 1× PCR buffer with $(NH_4)_2SO_4$, and 0.75 U Taq polymerase (MBI Fermentas) in a 25-μl reaction. After an initial denaturation step at 95°C for 2 min, the samples were put through 35 cycles of 94°C for 15 sec, 55°C for 30 sec, and 72°C for 1 min, followed by a final elongation step at 72°C for 10 min. The products were run on a 2% agarose gel containing ethidium bromide and visualized on a UV table.

Microscopy and Image Processing

A microscope (Axioplan 2; Carl Zeiss MicroImaging, Inc.) with Openlab 3.1.7 software (Improvision Ltd.) was used for brightfield and fluorescence microscopy analyses. A LSM 510 Meta laser microscope with LSM 5 Image Browser software (both Carl Zeiss Microimaging, Inc.) was used for confocal analyses. Images were taken at room temperature and were further processed using Photoshop (Adobe) according to *Developmental Dynamics* guidelines.

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