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Strong expression of foreign genes following direct injection into fish muscle

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We report here for the first time direct injection of genes into fish muscle in vivo. Plasmids used contain either SV40 early promoter, rabbit β-cardiac myosin heavy chain promoter, human MxA promoter or an artificial promoter, fused to a chloramphenicol acetyltransferase (CAT) or β-galactosidase reporter gene. CAT assays revealed that most gene constructs were highly expressed. Histochemical analysis showed that β-galactosidase was strongly expressed at the site of injection within muscle fibres. This method provides an excellent system for testing expression of gene constructs, including those of mammalian origin, in fish muscle in vivo and has the potential for fish vaccination.

Gene injection; Fish muscle; Gene expression; MxA; *Cyprinus carpio*

1. INTRODUCTION

Transgenic organisms are of great value in providing new insights into mechanisms of gene regulation and development. However, the transgenic approach is a rather time consuming process where it may take months before results are obtained. Wolff et al. [1] reported a new method of introducing genes into mouse skeletal muscle in vivo via simple injection of plasmid DNA, and since then there has been another report of direct DNA injection into rat cardiac myocardium [2]. This approach may have implications for somatic gene therapeutics in man and animal.

We report here for the first time direct gene injection into the skeletal muscle of fish to study transgene expression. It provides a quick and simple way of testing promoter activity and fusion-gene expression in fish in vivo and can be used to test constructs in the production of transgenic fish. Furthermore, this method has the potential for vaccination in fish with gene constructs whose products can elicit a direct immune response.

2. MATERIALS AND METHODS

2.1. *Plasmids*

Chloramphenicol acetyltransferase (CAT) gene and β-galactosidase (*lacZ*) gene were used as reporter genes. The promoterless pCAT-Basic vector and the SV40 early promoter/CAT gene construct, pCAT-Control were purchased from Promega. pMHC781CAT contains the rabbit β-cardiac myosin heavy chain promoter of 781 bp length [3], inserted into the *Hin*dIII site of pCAT-Basic in the correct orientation. pMHC393CAT contains a 393 bp 5′-truncated version of pMHC781CAT. pAP42CAT contains a 120 bp artificial promoter based on a 40 bp motif derived from the interferon-inducible human MxA promoter (Chang, manuscript in preparation). p42MxACAT is a construct where this artificial promoter is linked to the human MxA promoter [4]. p0.6MxACAT contains a 600 bp version of the human MxA promoter. pCH110 is an SV40 early promoter/*lacZ* gene construct, purchased from Pharmacia. Plasmid DNA was prepared using Qiagen columns (Diagen) following the supplier's protocol. Plasmid DNA was dissolved in Dulbecco's phosphate buffered saline (PBS, Sigma).

2.2. *Injection of recombinant DNA in vivo*

The mirror variety of the common carp (*Cyprinus carpio*) were maintained in circulating aerated water at 18-20°C. Fast growing fish of around 10 cm and older fish of around 20 cm long were used for the injection experiments. Various amounts of circular plasmid DNA ranging from 12.5 μg to 100 μg dissolved in 100 μl of PBS were injected into the trunk muscle to a depth of 0.8-1.0 cm. The site of injection was immediately rostro-ventral to the dorsal fin. The fish were sacrificed 4 days after injection and muscle tissue around the area of injection was excised.

2.3. *Chloramphenicol acetyltransferase assays*

Muscle tissues were frozen in liquid nitrogen and minced using a pre-cooled mortar and pestle. The powdered muscle was transferred into a 1.5 ml tube and suspended in 200 μl of 0.25 M Tris-HCl, pH 7.8. Three cycles of freezing in liquid nitrogen and thawing in a 37°C waterbath were performed, and the supernatant was incubated for 10 min at 65°C to inactivate endogenous acetyltransferases. CAT assays were performed according to [5] with slight modifications. After centrifugation, the 200 μl of clear supernatant were mixed with 100 μl of 1 M Tris-HCl, pH 7.8, 20 μl of acetyl coenzyme A (4 mg/ml) and 2 μl [^{14}C]-labeled chloramphenicol (57.9 mCi/mmol, 0.05 mCi/ml). The CAT reaction was incubated at 37°C for 4 h. The thin layer chromatography plates (Merck) were exposed overnight. CAT activity was

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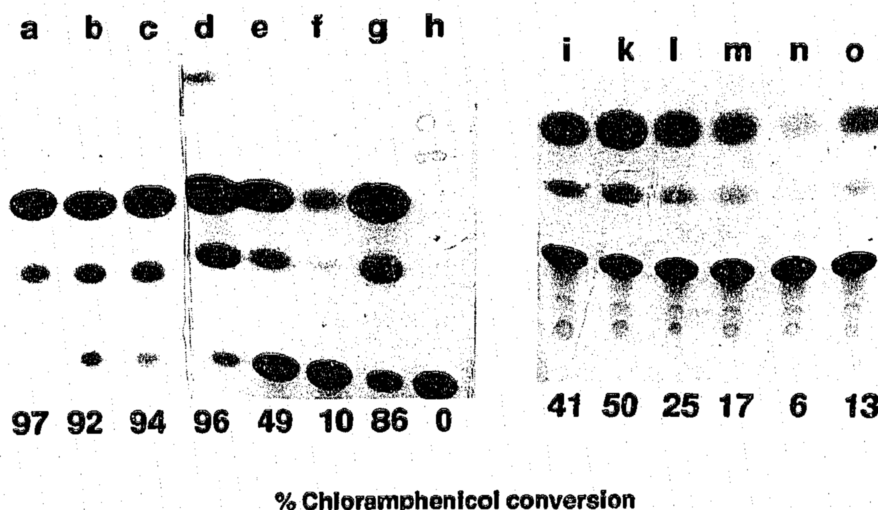

Fig. 1. Chloramphenicol acetyltransferase assays of muscle tissues injected with plasmid DNA. Details of constructs and CAT assay are as in main text. Unless otherwise indicated, 100 μg of DNA were injected. (a–h) Young carp of 10 cm in length; (i–o) older carp of 20 cm in length were injected. a, pAP42CAT; b, pMHC393CAT; c, pMHC781CAT; d, pCAT-Control; e, p42MxACAT; f, p42MxACAT with poly rI.rC; g, pMHC393CAT; h, no DNA; i, pMHC393CAT, 100 μg; k, pMHC393CAT, 50 μg; l, pMHC393CAT, 25 μg; m, pMHC393CAT, 12.5 μg; n, p06MxACAT, with poly rI.rC; o, p06MxACAT.

measured as percentage of chloramphenicol conversion to acetylated forms by densitometry (Chromoscan 3, Joyce-Loebl).

2.4. *Histochemical analysis*

Muscle previously injected with pCH110 was mounted in OCT (Bright Cryo-M-Bed, Jencons) on cork and frozen in isopentane cooled in liquid nitrogen. Serial cryostat sections (25 μm) were made and transferred onto subbed slides (2% aminopropyl triethoxysilane; [6]), fixed in 1% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.0, 1 mM $MgCl_2$, for 15 min and dried. Beta-galactosidase assays were carried out overnight as described in [7] modified for tissue sections.

3. RESULTS

3.1. *Efficiency of expression*

Following the direct injection of pCAT-Control into the muscles of young growing carp of around 10 cm in length, CAT activity obtained was more than 90% (Fig. 1, lane d), compared to 0% in non-injected muscles (Fig. 1, lane h). With the promoterless vector pCAT-Basic the background CAT activity was never above 4% (data not shown). With mammalian myosin heavy chain promoter sequences high levels of expression were obtained, yielding around 90% chloramphenicol conversion. Interestingly, we could not detect any significant difference in the level of expression of the full-length promoter construct, pMHC781CAT, and its 5′-truncated version, pMHC393CAT (Fig. 1, lanes b, c and g). The artificial promoter construct pAP42CAT which is expressed in a wide range of cell lines (Chang, manuscript in preparation), also yielded high levels of expression of more than 90% chloramphenicol conversion (Fig. 1, lane a). In the older fish we found that level of expression was correspondingly much lower than in the younger carp. With pMHC393CAT the CAT expression dropped from an average of 90% in younger carp (Fig. 1, lanes c and g) to 41% in older ones (Fig. 1, lane i). In order to test the sensitivity and optimal dosage level, we injected different amounts of pMHC393CAT in different older fish. The injection volume was maintained at 100 μl. Results shown in Fig. 1 (lanes i to m) show that CAT expression peaked at about 50 μg of plasmid DNA. However, as little as 12.5 μg of plasmid DNA were sufficient to elicit a signal (Fig. 1, lane m).

p0.6MxACAT was constitutively expressed in carp muscle (Fig. 1, lane o). With 100 μg of poly rI.rC introduced into the peritoneum 24 h after the plasmid injection, CAT expression was depressed (Fig. 1, lane n). Similar results were obtained with p42MxACAT. Co-injection of 100 μg p42MxACAT and 100 μg poly rI.rC into carp muscle resulted in a reduction of CAT expression of about 50% (Fig. 1, lanes e and f).

3.2. *Histochemical analysis*

Cells staining blue were distributed along the path made by the injection needle (Fig. 2a). In most sections, muscle fibres are clearly seen to be expressing the *lacZ* gene (Fig. 2b). In sections from non-injected muscles no *lacZ* expression was found (data not shown).

4. DISCUSSION

Apart from producing transgenic animals via microinjection of DNA into fertilized eggs, methods of transferring genes into live animals in vivo require either retroviral vectors [16,17] and/or liposome carriers containing DNA [18], or a receptor-mediated soluble DNA carrier system [19,20]. Benvenisty and Reshef [21] reported on the injection of calcium-phosphate co-precipitated DNA into the peritoneum of newborn rats. Animals used were either fetal or newborn mice and rats,

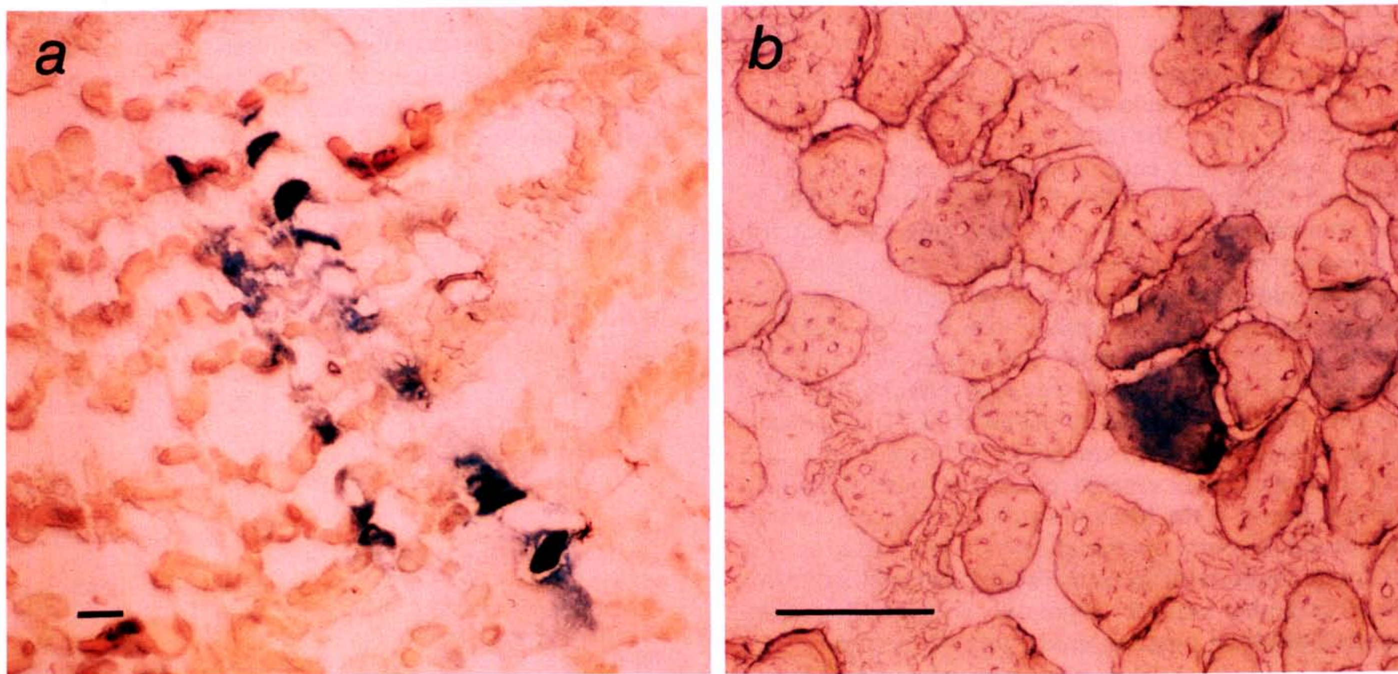

Fig. 2. β-Galactosidase expression in carp muscle sections. *LacZ* expressing muscle cells are stained blue. Bar = 100 μm.

and expression was mostly found in liver and spleen. Wolff and co-workers [1] were the first to report direct gene injection of pure DNA or RNA into skeletal muscle of mice in vivo, and later gene expression in rat myocardium following injection of reporter gene constructs was also reported [2]. Our findings of high levels of expression following direct injection of foreign genes into fish skeletal muscle demonstrate that this method is not restricted to mammals. The fact that expression obtained was as high as in mammalian cell lines using the same construct and the same CAT assay protocol (92% chloramphenicol conversion for pCAT-Control in human L132 cells [4]) may reflect on a mechanism of gene transcription and translation involving highly similar factors in mammals and fish. The histochemical analysis revealed that the constructs were expressed in distinct muscle cells in the injection area (Fig. 2b). It is not known how differentiated muscle cells take up the injected DNA. As shown in Fig. 2a, cells along the path of the introduced injection needle are very likely to express *lacZ*, suggesting that cell membranes are damaged during injection, facilitating DNA uptake. Another possibility is the presence of high amounts of calcium ions in the sarcoplasm which may facilitate DNA uptake.

4.1. *A range of constructs is expressed in fish muscles*

Previous findings [3] revealed that the rabbit β-cardiac myosin heavy chain (MHC) promoter functions efficiently in fused muscle cells derived from primary chicken skeletal myoblasts. The 5′-truncated version yields even higher expression than the full-length promoter. However, CAT activity was negligible in non-muscle cells. We demonstrated in our experiments that both, the full-length and a 5′-truncated version of this promoter, were highly expressed in fish muscles at a similar level (Fig. 1, lanes b, c and g). There is hardly any report on transgenic fish expressing the protein products of mammalian promoter-driven constructs unlike the use of viral or avian regulatory sequences [8–11]. High levels of CAT expression of the highly specific mammalian myosin heavy chain promoter constructs in fish muscles suggest that the mechanisms of gene regulation in differentiated piscine and mammalian muscle cells are not entirely dissimilar.

Mammalian Mx genes are expressed in a wide range of tissues following induction either with interferons (IFNs) or with double stranded poly rI.rC. Murine Mx1 expression had been shown to occur mainly in mononucleated cells [12]. Evidence for the existence of piscine Mx genes was demonstrated by [13] who isolated a genomic DNA fragment from a freshwater fish which is homologous to murine Mx1 gene and whose mRNA expression in liver is inducible by double stranded RNA. We used human MxA promoter constructs for our experiments which have been shown to be inducible in human L132 cells but constitutively expressed in murine LM cells [4]. MxA based promoter constructs (p42MxACAT and p0.6MxACTA) injected into fish muscles were constitutively expressed (Fig. 1, lane e and o) and did not show positive inducibility by poly rI.rC. The expression was, on the contrary, reduced when we

either co-injected poly rI.rC or injected it 24 h after DNA injection (Fig. 1, lane f and n). dsRNA is a potent inducer of IFN-genes. There is some evidence for the existence of a piscine β-IFN-like gene [14], and also for an IFN-gamma protein [15]. A possible explanation for our results is that the induction of IFNs occurred too early and that they enter a refractory phase before the plasmid DNA was in a suitable cellular environment to be transcribed. However, it is also possible that fish IFNs may not recognize the heterologous human MxA promoter in the same way as its own, and further studies are necessary to elucidate this phenomenon. CAT expression in fish muscle following injection of pAP42CAT (Fig. 1, lane a) was of a similarly high level as with pMHC393CAT and pMHC781CAT constructs (Fig. 1, lanes b, c and g) and pCAT-Control (Fig. 1, lane d). This artificially created promoter, derived from a 40-mer found in the MxA promoter sequence, has been expressed in every mammalian cell line tested so far (Chang, manuscript in preparation). Interestingly, this short promoter sequence is not only recognised in mammalian cell lines but also in fish muscle in vivo.

4.2. *DNA dosage and age of fish affect levels of expression*

Not surprisingly, our results demonstrated that the level of foreign gene expression is dependent on the amount of DNA used for injection. 50 μg of DNA was about the optimal level in our experiments, increasing the amount to 100 μg did not result in a a higher level of CAT activity (Fig. 1, lanes k and i). We assume that DNA is taken up only by a limited number of cells either due to cell membrane damage or other, as yet, unknown physiological conditions of the cells. Furthermore, the physiological condition of the animals seems to be an important factor. Younger and fast-growing fish showed much higher levels of CAT activity than the older ones (Fig. 1, lanes b and i).

4.3. *Vaccination potential*

We have demonstrated that direct gene injection into fish muscle is an easy and efficient method for studying gene expression. Wolff's report [1] showed that DNA was present extrachromosomally up to 6 months following injection. It would be of valuable interest to determine the time span in which injected DNA could remain active. If it is comparably long, this direct injection is a potentially powerful means for fish vaccination. If disease resistance or other genes are introduced under the regulatory control of a muscle-specific or non-specific promoter, containing the DNA sequence for a leader peptide, proteins could be produced in muscle cells, secreted, and subsequently elicit a specific immune response. This would revolutionalize vaccination and open up new approaches for studying disease resistance.

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