Gene Transfer in Shrimp (Litopenaeus schmitti) *by Electroporation of Single-cell Embryos and Injection of Naked DNA into Adult Muscle*

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ABSTRACT

The genetic manipulation of shrimp is of interest for biological studies and biotechnological applications. In order to perform gene transfer experiments, chimeric genes must be designed and tested, and efficient gene transfer protocols must be established. The carp β-actin, CMV and SV40 early promoters were tested to drive the *Escherichia coli lacZ* reporter gene in shrimp. Electroporation of single-cell shrimp *(Litopenaeus schmitti)* embryos was assayed and the optimal conditions were found with a pulse amplitude of 12 kV for 6 s in 4X PBS. Transient β-galactosidase activity was detected in 19.4% of shrimp embryos after electroporation at the single-cell embryo stage and in adult muscles after naked DNA injection. The naked DNA transference to shrimp tissue was demonstrated. The functionality analysis of carp β-actin, CMV and SV40 early promoters, together with the *E. coli lacZ* gene, demonstrated that all three promoters are active in shrimp. The *E. coli lacZ* gene is considered as a useful reporter gene for transient expression studies in shrimp embryos and adult tissues.

Keywords: electroporation, *Litopenaeus*, reporter, transgenic, shrimp

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RESUMEN

Transferencia de genes en camarón *(Litopenaeus schmitti)* mediante la electroporación de embriones unicelulares y la inyección de ADN desnudo en músculo de adultos. La manipulación genética de camarones es de interés para estudios biológicos y aplicaciones biotecnológicas. Para llevar a cabo los experimentos de transferencia de genes es necesario diseñar y evaluar genes quiméricos, y se deben establecer los protocolos para transferir estos genes. Se evaluaron los promotores de β-actina de carpa, así como los promotores tempranos de CMV y SV40, en relación con la expresión del gen reportero *lacZ* de *Escherichia coli*. En el presente trabajo se ensayó la electroporación de embriones de camarón y se encontró que las condiciones óptimas fueron 12 kV durante 6 s en PBS 4X. Se detectó actividad transitoria de la b-galactosidasa en 19,4% de los embriones de camarón electroporados en estadio de una célula, y en músculo de camarones adultos inyectados con ADN desnudo. Los resultados demostraron que es posible transferir ADN desnudo a tejidos de camarón. El promotor de b-actina de carpa y los promotores tempranos de CMV y SV40, junto al gen *lacZ* de *E. coli*, demostraron ser funcionales en tejidos de camarón *(Litopenaeus schmitti)*. El gen *lacZ* de *E. coli* es considerado un reportero satisfactorio para realizar estudios de expresión transitoria en camarones, tanto en embriones como en tejidos adultos.

Palabras claves: camarón, electroporación, *Litopenaeus*, transgénico, reportero

Introduction

Aquaculture is dominated by freshwater finfish production; however, there has been an increase in the contribution of marine algae, shellfish and crustaceans to global aquaculture production over the past decade. An increased understanding of the genetics, reproduction, nutrition and physiology of target organisms is the first step to overcome constraints on the development of specific aquaculture products [1].

Gene transfer techniques offer an invaluable tool for the improvement of desirable traits, the application of DNA vaccines, and the investigation of the molecular basis of physiological processes [1, 2]. The development of expression vectors that contain genetic regulatory sequences adapted to the characteristics of each species is essential for both commercial and scientific exploitation of genetically modified aquatic organisms. As a first step, functional animal and plant reporter gene cassettes can be used to speed up the optimization of gene transfer protocols for the target organism [1]. Following gene transfer, the expression of the reporter gene is used to determine the transcriptional regulatory elements of a gene or the strength of a promoter [3, 4].

Mainly, the expression vectors employed for gene transfer in aquatic invertebrates have had heterologous regulator sequences such as β-actin promoters [5], cauliflower mosaic virus 35S promoter [6], *Drosophila* hsp70 promoter [7, 8], and Rous Sarcoma Virus long terminal repeat (RSV-LTR) [9]. Recently, homologous promoters have been employed [1]. These expression vectors were coupled to genes coding for proteins that are easy to detect using sensitive assays, such as luciferase, β-galactosidase (β-gal), β-glucuronidase and, more recently, green fluorescent protein (GFP) [1].

Previously, it was demonstrated [10, 11] that the SV40 early promoter drove the transcription of the β-gal reporter gene, transferred by DNA microinjection and

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electroporation. The expression efficiency with these methods was 10 and 4.2%, respectively [10, 11], which suggested the use of microinjection as a more efficient method for gene transfer in shrimp.

The aim of this study was to optimize electroporation to increase its efficiency for gene transfer in single-cell shrimp embryos, as well as to demonstrate the transient expression of reporter genes after the injection of proper constructs in adult shrimp muscle.

Materials and Methods

Shrimp maintenance and embryo collection

Experiments were conducted in a hatchery facility (Santa Cruz del Sur, Camagüey, Cuba). Adult female and male shrimps were transferred to mating tanks, which were supplied with a constant filtered water flow for 2 hours. The females ready to spawn were then transferred to individual spawning tanks for continuous monitoring. Under these conditions, spawning generally occurred within 1 to 3 hours. The fertilized eggs were collected immediately after spawning.

Chimeric genes

The pCH110 plasmid contains a functional *lac*Z gene, which is expressed from the Simian Virus 40 (SV40) early promoter. This plasmid can be used as a promoter screening vector by inserting fragments into the *Hin*dIII site and monitoring β-gal expression [12]. Plasmid pBC330 is derived from the pBlueScript SK$^-$ (Stratagene, USA) and contains the carp β-actin promoter. The pβBlueCarp23 and pβBlueCarp13 plasmids were constructed by inserting the carp β-actin promoter into the *Hin*dIII site of the pCH110 plasmid in the direct and inverted orientations with respect to *lacZ*, respectively. The pCMV-*lac*Z plasmid contains a bacterial *lac*Z gene under the control of the human cytomegalovirus (CMV) early promoter (kindly provided by Dr FO Castro, Center for Genetic Engineering and Biotechnology, Havana, Cuba).

Gene transfer procedures

Buffer concentration. The embryos were incubated in different PBS buffer concentrations in order to test their influence on the survival rates. PBS buffer concentrations of 1X (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 4.3 mM KH_2PO_4, pH 7.1), 4X, 5X and 10X , were used. After 1 h of incubation, the embryos were transferred to a recipient containing filtered seawater, until they reached the nauplius stage. All embryos were treated with 0.3% urea for 5 min before incubation in their specific buffer. Embryos from the control group were transferred directly to seawater after urea treatment. One group was kept in seawater all the time as an additional control to discard the influence of the urea treatment.

Electroporation of chimeric genes into early embryos. The fertilized eggs were collected and the jelly coat was removed by incubating the eggs in 0.3% urea in seawater, at 25 ºC for 3-5 min. The circular plasmid DNA pCMV-*lac*Z was diluted in 4X PBS at 500 ng/µL. The equipment used for electroporation was constructed at the Center for Genetic Engineering and Biotechnology in Camagüey. It provides continuous and adjustable very high voltage (0-16,000 volts) and very low current (< 4 mA), and was designed to generate noncapacitor-based electric pulses, which are adjustable in their magnitude of voltage and number of repetitions. These well-defined pulses are delivered through an electrode (anode) across a distance to the DNA-egg mixture, which is contained in a 1.5 mL Eppendorf tube used as receptacle; the cathode is introduced into the DNA-egg mixture by perforating the bottom of the tube with the cathode itself. The frequency of pulse delivery is fixed to 30 s^{-1} and the pulse duration varies from 5 to 8 µs. The performance time can be regulated by a timed switch (0.3–29.7 s). The parameters used for electroporation included volume (50 µL), embryo number (150), and distance between the anode and the sample surface (d = 1 mm). The variables were amplitude (8, 12 and 16 kV) and performance time (3, 6 and 9 s), forming a matrix of 9 experimental groups. The number of pulses depended on the performance time (90, 180 and 270, respectively). Three replicates were used in each group. After electroporation the embryos were cultured in seawater for 24 h. The embryos were fixed and stained for β-gal activity detection. The samples were fixed for 1 h at 0 ºC in 0.1 M Na_2HPO_4 containing 4% paraformaldehyde and 0.2% glutaraldehyde, then washed three times at room temperature with PBS, and incubated for 1 h in PBS containing 0.2% BSA (fraction V, Sigma, St. Louis, USA) and 0.1% Triton X-100 (Merck, Germany). The samples were stained for 14-16 h at 37 ºC in a solution containing 1 mg/mL X-gal (Sigma, St. Louis, USA) in 1X PBS and 5 mM $K_3[Fe(CN)_6]$, 5 mM $K_4[Fe(CN)_6]$, and 1.3 mM $MgCl_2$.

Injection of chimeric genes into the muscle. Four groups of three 20–30 g shrimps each, were maintained in aerated seawater at 20–22 ºC. The shrimps were injected in the ventral muscle of the third abdominal segment. Two negative control groups were used in the experiment, injected with 1X PBS and 100 µg of pβBlueCarp13, respectively. The positive control was injected with 100 µg of pCH110. The experimental group was injected with 100 µg of pβBlueCarp23. All plasmids were dissolved in 1X PBS.

Shrimps were sacrificed 72 h after injection and the abdomen and cephalothorax were separated for staining with X-gal to avoid endogenous activity [13]. The cuticles were removed before staining, which was performed as previously described for embryos, using X-gal.

Statistical analyses

The transgene expression data —represented as percentages— from different embryo groups at different amplitudes and performance times, were transformed into their square roots to carry out a Factorial Analysis of Variance (ANOVA), 3 x 3 (two factors; three levels). Thereafter, the Duncan's Multiple Range Test was applied to determine the significance level of mean differences ($p < 0.01$) [14].

Results

Transient expression of the *lacZ* transgene in shrimp embryos

The buffer concentration was critical for the embryo survival rates. The groups incubated in 4X and 5X PBS buffer had the highest survival rates: 60–70% at the nauplius stage, the same as the seawater control groups,

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with or without 0.3% urea treatment. No surviving nauplii were observed in the 1X or 10X PBS buffer.

To perform the gene transfer by embryo electroporation in the single-cell stage the 4X PBS buffer was selected, since it was the closest to seawater salinity. After shrimp embryo electroporation no differences in the survival rates were found, compared to the control groups (60–70%).

The transient expression experiment showed that the CMV early promoter was able to drive the transcription of the *lacZ* reporter gene. The best results were obtained with the following conditions: 12 kV amplitude and a performance time of 6 s (Table). In these conditions 19.4% of the embryos treated showed β-gal activity after X-gal staining. No enzyme activity was detected in the control group.

The percentages of transformed embryos always increased with the performance time for each amplitude value, except for 12 kV in the longest performance time (9 s), where no transformed embryos were obtained (Table).

Transient expression in adult shrimp muscle

Three animals were used in all the groups. At the end of the experiment one shrimp from each group died, except for the pβBlueCarp 23 group. All the shrimps injected with pCH110 and pβBlueCarp23 plasmids showed intense β-gal activity in muscles 72 h after the injection of *lacZ*-expressing constructs in the tissue. The shrimps injected with 1X PBS or with the pβBlueCarp13 plasmid showed no X-gal staining of muscles (Figure).

Discussion

The efficiency of gene transfer procedures is essential for the success of gene transfer to living organisms. Electroporation was evaluated as an effective method to introduce genes into shrimp embryos. The absence of survivingl shrimp embryos after 1 h of incubation in 1X PBS could be the cause of the high variation in the survival rates found by Pimentel *et al*. [11], which ranged from 6 to 71% compared to the control group. The stable survival rates in the present study (60–70%), even after electroporation, suggest a better adaptation of the embryos to osmolarity in 4X PBS when used as electroporation buffer. These results are in agreement with the survival rate of 75% obtained for *Penaeus japonicus* after the electroporation in seawater [15].

From previous results microinjection was considered more efficient for gene transfer in shrimp, since the embryos expressed β-gal in only 4.2% of the electroporated shrimp embryos, as well as a high variation in the survival rates [11]. However, microinjection only permits manipulation of a small number of embryos: 50–75 eggs could be microinjected in 40 min [10, 16].

Table. Percents of β-galactosidase activity expression in electroporated shrimp embryos.

Performance time (s)	Pulse amplitude (kV) 8	12	16
3	$0.6^{a} \pm 0.9$	$5.6^{bc} \pm 0.9$	$0^{a} \pm 0.0$
6	$8.1^{bc} \pm 0.9$	$19.4^{d} \pm 2.7$	$3.1^{b} \pm 0.9$
9	$11.2^{cd} \pm 1.8$	$0.0^{a} \pm 0.0$	$5.6^{bc} \pm 0.9$

Percent of electroporated shrimp embryos that expressed β-galactosidase activity ± standard deviation. Each value is the mean of three determination; a, b, c, and d denote significant differences between means ($p < 0,01$).

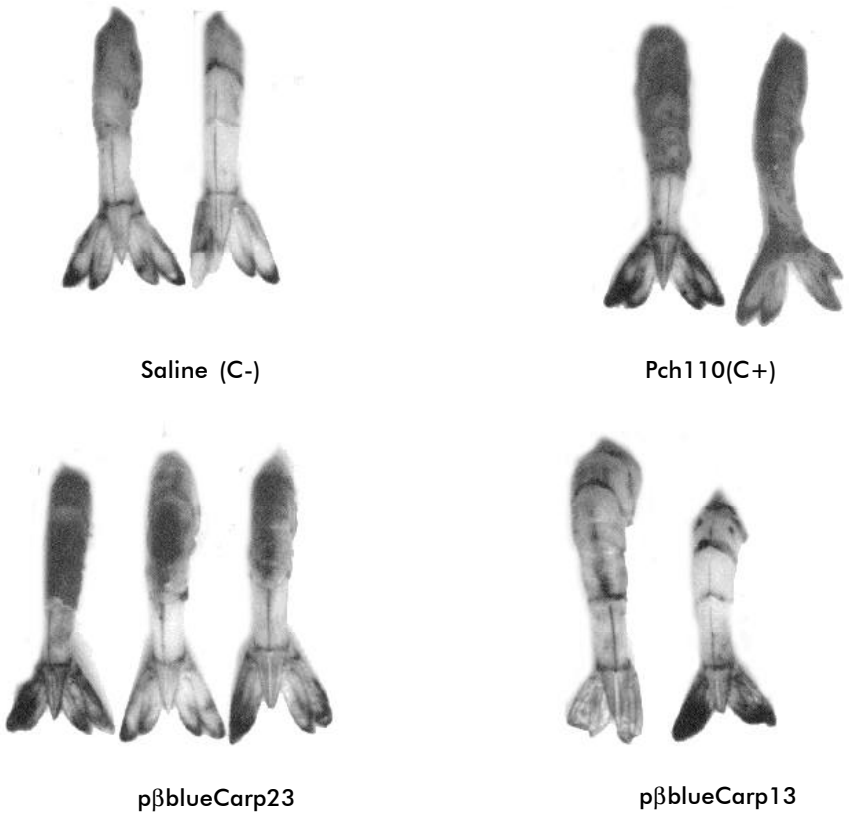

Figure. Transient expression of the *lacZ* reporter gene in adult shrimp (*Litopenaeus schmitti*) muscle. Shrimp abdomens were injected with 1X PBS buffer, pCH110 (C+), pβBlueCarp23 or pbBlueCarp13 and stained for b-galactosidase activity. (See color picture in page 272).

As shown in Table 1, the amplitude of 12 kV was better than 8 kV, whereas the lowest results were obtained for 16 kV. It was also seen that better results were achieved with the increase of the performance time. The groups treated with 12 kV for 9 s were the only exception, which were expected to be the best conditions according to Table. On the contrary, no transformed embryos were observed for this treatment. We suggest that the decrease in expression was not due to the toxic levels of the plasmid in the cell, or the performance time and pulse amplitude combination, because the embryos viability was not affected by this condition, neither by 16 kV and 9 s. Maybe a high DNA uptake into the cell nucleus occurs under these conditions, which may inhibit the transcriptional machinery [17]. Contrary to Preston *et al.* [15], these findings had less variation. It is thought to be related with the electric features of the equipment used for gene transfer: very high voltage and very low current are produced, which could be enough to induce membrane pores that keep the integrity of the egg. Furthermore, the anode tip is not in contact with the DNA-egg mixture; the electric arc goes through the air phase and the buffer to the cathode tip in a process that generates an induced current which produces the membrane pores. The small amount of eggs that are directly exposed to the main electric pulses does not survive. On the contrary, Preston *et al.* used an electroporation system that generates a low voltage

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(40–100 V/cm) [15] and a higher current in a single application pulse. The embryos were placed between two aluminum plates, so that all the embryos were exposed to the electric discharge generated by a capacitor and applied to the slot cuvette aluminum sides [15].

In this study all the established conditions were fulfilled, and transformation was achieved in 19.4% of the embryos (around 750 eggs in 40 min). Thus, this procedure proved to be useful for mass embryo treatment.

In spite of Preston *et al.* [15], who found that microinjection was the most efficient method to transfer DNA into shrimp embryos, these findings showed that this type of electroporation is more efficient. It is also discussed that electroporation and biolistics seem to be better adapted to obtain transgenic invertebrates because of the low integration frequency of the DNA introduced in these species [16].

Since Pimentel *et al.* [11] used 50 ng/μL of plasmid in shrimp electroporation experiments, we consider that the new DNA and salt concentrations influenced the higher percent of transformed embryos obtained in this assay with the same method. It can be assumed that proper integration levels could be achieved varying the DNA concentrations, thus making this method very efficient for both transient and stable gene transfer studies *in vivo*.

Significant advances have recently been made in the generation of vectors that conduct an efficient long-term expression of transgenes *in vivo* [18]. Wolf *et al.* [19] were the first to report direct injection of pure DNA or RNA into skeletal muscle of mice *in vivo*. Later on, the expression of reporter gene constructs in carp skeletal muscle after injection was reported as well [20]. The *lacZ* gene, which was expressed from carp β-actin and SV40 early promoters following direct injection into shrimp muscle, has proven that this method is not only restricted to mammals and fish. The muscle is the preferred target for gene delivery because it is accessible, well vascularized, and able to express and process secreted proteins [21].

An intense blue color was detected in all shrimp muscles injected with pCH110 and pβBlueCarp23. These results corroborate the findings of histological studies after intramuscular injection of tilapia with pCH110 and pβBlueCarp23 plasmids [22].

Nevertheless, no differences in intensity were observed. So, when the goal of a study is the comparison of a promoter regulatory activity, quantitation of the gene products is strongly recommended. Furthermore, no endogenous β-gal activity was observed in the muscles of the control groups. The backgrounds observed in the shrimp telson may denote a possible bacterial infection (Figure).

In the past two decades DNA has proven to be an effective means of generating protective cellular responses [23]. The intramuscular injection of naked DNA could be used to protect shrimps against different diseases at reproduction stock scale. It could be a valuable tool to improve the reproducer health and reduce egg and larval infections that cause loses from mortality. This method can also be employed to test promoter functionality and gene product expression *in vivo*. Thus, genetic constructions can be evaluated in shrimp muscles before developing stable transformation experiments in this species.

These results have also shown that CMV and SV40 promoters early, as well as the carp β-actin promoter, can be used in the construction of expression vectors for gene transfer studies in shrimp. In addition, the *lacZ* gene is functional in shrimp tissues and can be used as a reporter gene in transfer experiments. These results strongly suggest that electroporation of single-cell embryos and DNA injections into muscle are effective means for gene transfer in shrimp.

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