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Efficient transient expression system based on square pulse electroporation and in vivo luciferase assay of fertilized fish eggs

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Electroporation mediated DNA transfer into fish eggs has been improved by using a train of square pulses. Fertilized eggs of African catfish (*Clarias gariepinus*), zebrafish (*Brachydanio rerio*) and rosy barb (*Barbus conchonius*) were dechorionated enzymatically followed by application of pulses. Efficiency of plasmid DNA delivery was significantly increased by applying multiple pulses on dechorionated eggs. Optimization of physical parameters such as field strength, pulse width and pulse numbers resulted in reproducible transient expression in 25–50% of embryos and larvae by using the firefly luciferase and the *E. coli* β-galactosidase (lacZ) genes both driven by CMV IE1 promoter. Temporal luceferase expression was assayed using both qualitative (sheet film) and quantitative (scintillation counting) methods in developing embryos and fry in vivo. Spatial expression of lacZ was assayed by histochemical staining. A number of embryos revealed foreign gene product also localised in the vegetal pole of the embryo.

Electroporation; Gene transfer, Fish embryo; Firefly luciferase; β-Galactosidase; Transient expression

1. INTRODUCTION

Most of the gene transfer experiments on fish have been utilizing the microinjection procedure developed for mammals [1]. This technique requires time-consuming, laborious, and in some cases technically demanding work, and results in a low rate of transgenic individuals. Development of a mass gene transfer method would be essential, provided that the advantages offered by the fish system such as high number of eggs, external fertilization, rapid and extrauterine development are to be fully exploited.

DNA technology in fish develops fast and a number of genes and regulatory sequences have been cloned and potentially useful constructs have been made, including 'all fish' constructs [2]. A trivial goal of the transient expression system is testing of such constructs and this has been mostly achieved in vitro using tissue and cell culture [3,4]. However, these experiments give little information on the temporal and spatial pattern of gene expression as dictated by the promoter sequence. Testing cloned regulatory sequences for their functionality in vivo can be performed more easily by applying an efficient mass gene transfer method on fish eggs. The simultaneous introduction of the construct into many eggs coupled with an easily assayable reporter gene would accelerate and improve the testing process.

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Another important aim for both developmental biologists and those aiming for production of genetically engineered farm species is the production of stable transgenics that contain the foreign gene integrated in the genome. Since a mass gene transfer method allows the treatment of several hundred specimens at the same time, thus the number of stable transformants will most likely be increased. It would be an evident advantage in case of species where microinjection is the limiting step of the process of transgenesis, e.g. species with tough eggs or fast development and species with short reproductive season [5].

Different gene transfer methods have already been tried on eggs, e.g. gun particle bombardment [6], sperm binding [7] and sperm electroporation [8], but the efficiency of these methods did not reach that of microinjection.

Electrical permeabilization of cell membranes is a widespread gene transfer method in prokaryotes and eukaryotic cells [9]. The first report on the application of electroporation to fish eggs was by Inoue and coworkers [10]. His results revealed low efficiency transfer as compared to the traditional microinjection protocol possibly due to the use of eggs with tough chorion. Buono and Linser [11] also achieved gene transfer via electroporation into zebrafish eggs but gene expression was not analyzed extensively.

Here we report on the application of multiple square wave pulses applied to enzymatically dechorionated eggs aiming for high efficiency transfer and expression of the gene introduced. We have used the firefly lucif-

erase gene to insure the easy detection of in vivo transient expression.

2. MATERIALS AND METHODS

2.1. *Collection and treatment of eggs and sperm*

Sexually mature African catfish (*Clarias gariepinus*) females were injected with dried carp pituitary (4–5 mg/kg body weight) dissolved in 0.65% salt solution approx. 10 h prior to stripping. Stripped eggs were fertilized artificially by sperm obtained from surgically removed testis. Fertilized eggs of zebrafish (*Brachydanio rerio*) were collected from natural spawning whereas eggs and sperm of rosy barb (*Barbus conchonius*) were stripped from females and males induced by light program and water change. After water hardening (approx. 5–10 min after fertilization) eggs were dechorionated by using Pronase E type XXV (No P-6911, Sigma) at a final concentration of 10 mg/ml dissolved in Holtfreter solution (3.5 g NaCl, 50 mg KCl, 100 mg $CaCl_2$, 200 mg $NaHCO_3 \cdot H_2O$ per distilled water) [12]. Eggs were incubated for 5–10 min, rinsed gently and washed in Holtfreter solution then in dechlorinated tap water two or three times.

Treated and control embryos were kept in plastic Petri dishes filled with Holtfreter's solution until hatching. Hatched larvae were reared in water until the feeding stage (5–6 days of age) and afterwards in a water recirculation system.

2.2 *Plasmids*

The 5 5 kbp construct pCMV/1 (provided by P. Gibbs, Washington State University) was produced by replacement of the β-gal gene in the pCMV/lacZ [13] with a 1823 bp long firefly luciferase gene The construct, containing the human cytomegalovirus (CMV) IE promoter and enhancer, an upstream splice site, the luciferase coding region without the upstream methionine and the SV40 polyadenylation signal, was used in circular form at a concentration of 50 μg/ml. The 6.7 kb pCMV/lacZ plasmid was also used in circular form in the same concentration as pCMV/1

2.3. *Electroporation*

Series of high voltage pulses for electroporation were generated by equipment and software constructed by L. Menczel in the Biological Research Center, Szeged, Hungary. The block diagram of the electroporator set-up is shown in Fig. 1. A computer program running on an IBM PC/AT or on an Atari Portfolio was used to generate low voltage pulses to the parallel printer port (LPT1). Pulse parameters (number of pulses, pulse length, duration between individual pulses) could be programmed in advance for different samples These pulses were used for switching on and off a high voltage transistor connected to an electrophoresis power supply (PSE-300, Biological Research Center, Szeged, Hungary). Multiple square pulses were released through an electrode chamber modified from a photometer cuvette with stainless steel electrodes (electrode distance 0.85 cm). Field strength was set by adjustment of voltage of the power supply varying between 80–120 V/cm. Pulse numbers were 1, 2, 4, 8, 16 or 24. Pulse length was 200 μs and intervals between individual pulses had 500 or 1000 μs duration.

Approximately one-hundred embryos at the one- or two-cell stage were collected in dechlorinated water into the cuvette and pulses were added. Eggs were then spread into Petri dishes for incubation in Holtfreter's solution The solution was regularly changed and gradually replaced by water.

2.4 *Analysis of luciferase expression*

Embryos of different age (few hours to one-week-old larvae) were used for the expression assays. The method of Alestrom et al. [14] with slight modifications was used for the in vivo detection of luciferase expression in the embryos. Individual embryos were placed in 750 μl microcentrifuge tubes with 100 μl luciferin sol. (500 μm luciferin, L-6882 Sigma, dissolved in Holtfreter's solution). A 15 min preincubation in this solution was followed by scintillation measurements in the same solution. Three tubes could be placed in one scintillation cuvette after the removal of the lid edge of the microfuge tubes Scintillation cuvettes were placed into a scintillation counter (Betamatic V, Contron) which was set to luminescence or 3H mode.

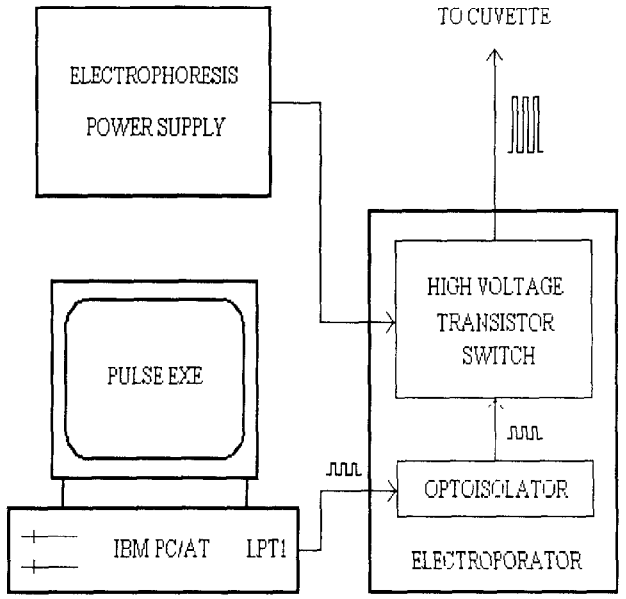

Fig. 1. The schematic diagram of the square pulse electroporator system.

Duration of measurement ranged from few hours up to 24 h. Microfuge tubes in cuvettes showing positive signal were selected and measured again for the identification of the positive individual.

Alternatively embryos were placed in the wells of 96-well tissue culture plates containing 200 μl of 500 μM luciferin and were placed on Kodak T-MAT G (5500 ASA) sheet film [15]. Exposure in dark room overnight was followed by development using the method for conventional sheet films. Neither of the two methods appeared to cause any harm to the embryos.

2.5. *Analysis of β-galactosidase expression*

Embryos at late blastula and eyed egg stage were rinsed in phosphate buffer (75 mM, pH 8.0) and fixed in the same buffer containing 1% glutaraldehyde, 2 mM $MgCl_2$ and 5 mM EGTA on 28°C for 3 h. Three times rinsing in the phosphate buffer containing 2 mM $MgCl_2$, 0 02% Nonidet P40 and 0.01% Na-deoxycholate (pH 8 0). X-gal staining was achieved at 37°C overnight in the above washing buffer containing 25 mM $K_3Fe(CN)_6$, 25 mM $K_4Fe(CN)_6$ and 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl β-galactoside).

3. RESULTS

3.1. *Experiments performed on African catfish*

Different field strength conditions were chosen in order to determine their effect on survival rates at hatching (one day at 28°C) as a means of optimizing of the electroporation parameters in experiments without plasmid DNA. Survival of dechorionated eggs is adversely affected due to the enhanced sensitivity of these embryos to the environmental circumstances. Hatching survival of normal, non-dechorionated eggs was 80 ± 10% in average of six experiments, those of dechorionated controls was approx. 60% of non-

dechorionated ones. In order to exclude the effect of dechorionation and investigate the effect of electroporation only, the survival data are expressed in percentage of dechorionated controls (Fig. 2).

More than 2500 African catfish embryos were analyzed in 3 parallel experiments. It could be clearly seen that increasing the field strengh resulted in a gradual decrease of the survival rate.

Approximately 4000 embryos of each of the three species were electroporated in the presence of plasmid DNA. The most extensive experiments were performed using eggs of African catfish where optimization of conditions revealed the most consistent results, regarding survival and percentage of luciferase-expressing individuals.

When using African catfish best results were obtained with 100 V/cm and 16 pulses with 200 μs pulse length and 500 μs pulse distances (Fig. 3). In one treatment (100 V/cm, 16 pulses) 24% of one-day-old living embryos (12/50) showed luciferase expression (100–3000 cpm) in the scintillation counter. Different group of individuals from the same treatment showed 33% expression rate at the third day of development (6/18). In a recent experiment 47.5% (19/40) of treated one-day-old embryos showed luciferase expression. A decreased rate of expression was gained with 100 V/cm and 24 pulses where approx. 22% (11/50) were found to be positive after one day of development. We observed an increased number of abnormally developing embryos among the positive ones in all experiments.

Nine larvae that were found to give measurable signals of light emission in the scintillation counter parallel with four non-positive ones were placed on sheet film at one day of age and measured again for comparison (Fig. 4). Two embryos (#5 and #6) showed sufficient luciferase activity for detection after 30 min exposure to the film whereas 6 embryos showed obvious signs of light emission after overnight exposure. In two cases (#2 and #8) the signal may be explained by reflection of the light of embryos in the neighboring well of the plate.

The overall expression rates were investigated by measurements at different stages of development. Results of these experiments are shown in Table I. Expression was first detected at 3–4 h of development and was found to reach the 3000 peak (the maximum level measurable with our machine) gradually at different times but mostly by 10 h. Levels of expression showed a wide fluctuation in individual embryos (Table I).

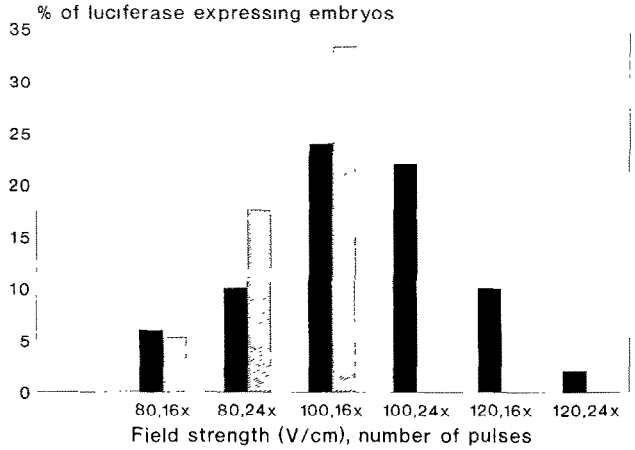

Fig. 3. Expression of the luciferase gene in African catfish embryos and larvae electroporated in the presence of pCMV/1. 50 embryos from each batch treated were incubated in 500 μM luciferin in Holtfreter's solution then measured in the scintillation counter for light emission. Black bars represent the percentage of embryos expressing luciferase after one day of development. Hatched bars represent percentage of embryos expressing luciferase after three days of development.

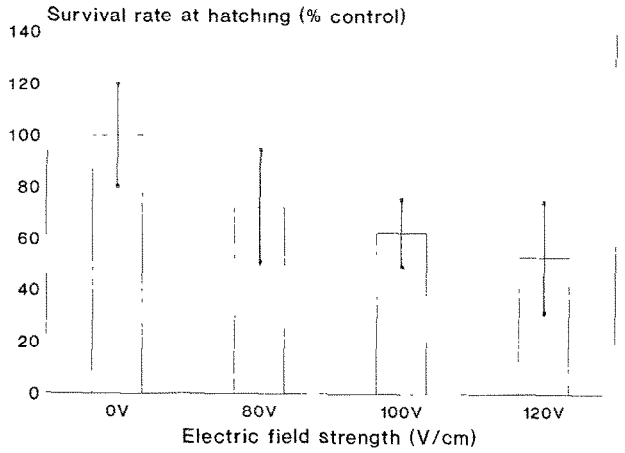

Fig. 2. Effect of square wave electric pulses on survival of African catfish embryos at hatching. Survival is expressed in the percentage of the average of dechorionated controls with standard deviation (± S.D.) measured in three series of experiments.

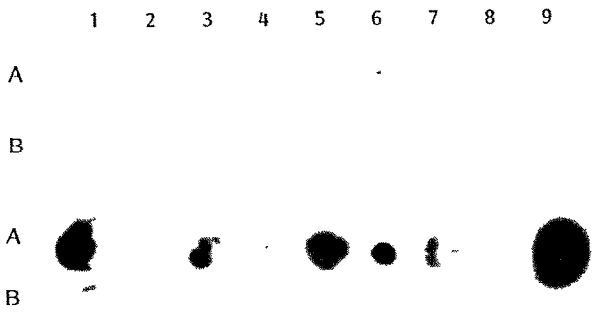

Fig. 4. Detection of light emission of developing African catfish eggs electroporated in the presence of pCMV/1 Eggs were electroporated with 100 V/cm and 16 pulses than incubated in 500 μM luciferin solution in 96 well tissue culture plate on Kodak T-MAT G sheet film for 30 min (top) and 12 h (bottom). Row A: larvae previously shown to be positive in scintillation counting. Measured scintillations 2 h prior to film exposure were in the order from left to right: 140, 300, 3000, 40, 40, 20, 190, 50, 3000 cpm, respectively. Row B: larvae found negative in scintillation measurement.

Table I

Dependence of luciferase expression in electroporated African catfish embryos on their time of development

Developmental time (h)	Expression value* (cpm)	S.D.**
1	10	0
4	159	486
7	458	544
10	956	1263
13	867	1255
16	895	1228

Embryos electroporated with pCMV/1 were incubated in 500 μM luciferin in Holtfreter's solution and their light emission was measured in the scintillation counter during their early development.

*Results are expressed as the average of 47 luciferase expressing embryos. Maximal value measurable on scintillation counter is 3000 cpm.
**Standard deviation.

From approximately 900 non-dechorionated and partially dechorionated catfish eggs with 0.1% Trypsin solution (only the external layer of catfish chorion is removed in 20 min) none showed any luciferase activity after electroporation in the presence of pCMV/1 and similarly no expression was found in approx. 300 eggs electroporated without plasmid DNA. Additionally no luciferase expression was detected in several thousand treated fish when exponential decay pulses were used on either dechorionated or non dechorionated eggs of African catfish and zebrafish applied from a capacitor discharge system (data not shown).

A hundred catfish embryos were electroporated in the presence of PCMV/LacZ plasmid (100 V/cm, 16 pulses) and stained for visualizing β-galactosidase expression. Eleven embryos out of 60 contained blue cells at late blastula stage and 10 out of 35 was found still containing the foreign enzyme at one day of age, whereas no staining was detected in the appropriate non-treated controls (Fig. 5).

3.2. *Experiments performed on zebrafish and rosy barb*

To examine the applicability of the technique to other species, fertilized and dechorionated eggs of zebrafish (*Brachydanio rerio*) and rosy barb (*Barbus conchonius*) were also electroporated at different field strengh conditions in the presence of DNA. In the case of zebrafish both 80 and 100 V/cm field strength with 16 pulses resulted in 20% positives measured at gastrula stage. A more extensive experiment was performed on rosy barb eggs with a wide range of different parameters. The effect of number of pulses was assayed when 1, 4, 8, 24, 32 pulses were applied at 100 V/cm field strength. Best expression rates were gained with 24 and 32 pulses (15/30 = 50% and 6/14 = 42%, respectively) measured on film at one day of development (Fig. 6); however, survival rates at hatching (two days at 28°C) were far less than in case of catfish (approx. 10%), which may have

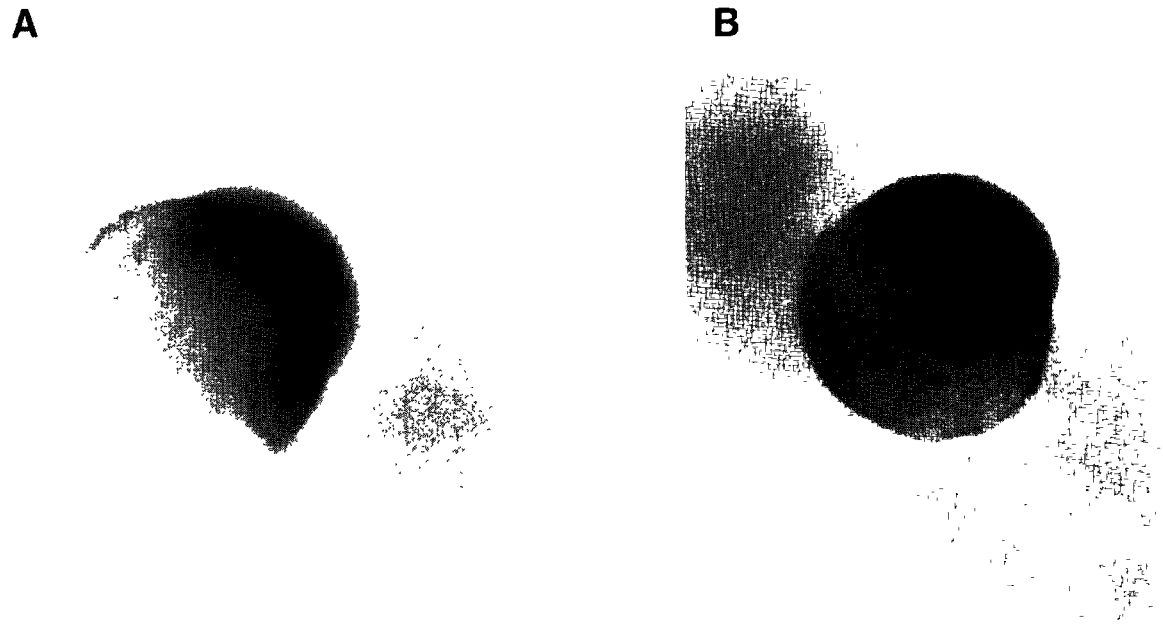

Fig. 5. African catfish embryos electroporated in the presence of pCMV/LacZ at 100 V/cm with 16 pulses. Blastula embryos fixed at 5 h of development showing expression of β-galactosidase stained with X-gal. Dark areas show the presence of β-galactosidase activity in the animal pole cells (A) and in the yolk (B).

been due to the high number of pulses causing irreversible membrane deterioration. Since a serious loss of non-electroporated controls was observed as well (20% survival), poor incubation conditions may also be suspected.

4. DISCUSSION

The use of electroporation for the production of transgenic fish has been reported earlier but the efficiency did not reach that of microinjection [10]. Buono and Linser [11] have reported efficient electroporation of non-dechorionated eggs of zebrafish as revealed by dot blots. We were unable to gain any luciferase expressing catfish and zebrafish using non-dechorionated eggs either when using exponential or square wave pulses.

The fact that non-dechorionated eggs did not show luciferase activity might be explained by the mycopolysaccharide based multilayer chorion which may be a barrier to these pulses, or the number of copies of the plasmid taken up were insufficient for the detection of their activity.

We have demonstrated the success of a square wave electroporation system that utilizes enzymatically dechorionated eggs of several fish species and allows the parallel delivery of foreign genes into a high proportion of fertilized eggs. The system requires a simple and commercially available machine (M-Elektro, Szeged, Hungary) which is connected to a computer and a power supply both of which can be found in most labs.

Our results compare favourably with those obtained by microinjection as far as early transient expression levels are concerned. On average they show similar levels of expression in three species to those produced by microinjection in our laboratory (T. Papp, unpublished results). What makes this system more attractive than microinjection is its capacity. Several hundred fertilized and dechorionated eggs of catfish and few thousand of those of zebrafish or rosy barb can be treated at once and can easily be selected for the luciferase activity afterwards. We have incubated individual eggs from morula to hatching in 100 μl of luciferin diluted in Holtfreter's solution without any visible harm to the developing embryos.

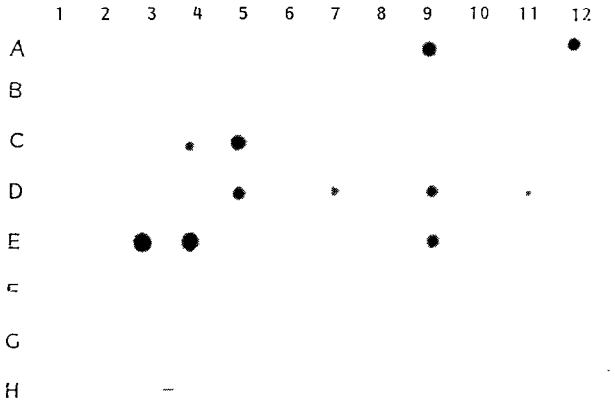

Fig. 6. Frequency of luciferase expression in one-day-old electroporated rosy barb eggs detected on sheet film after 12 h exposure (from blastula to 20 somites stage). Conditions of incubation were as in Fig. 4 Electroporation conditions were as follows:
A1-C12 = 100 V/cm, 8 pulses;
D1-F6 = 100 V/cm, 24 pulses;
F7-G8 = 100 V/cm, 32 pulses;
H1-12 = non-electroporated controls.

As described in section 3, temporal expression patterns were very variable. From the results gained by scintillation measurements we can conclude that individual embryos show a wide range of variation in the onset and duration of expression (see S.D. data in Table I). Expression was found to start only after 3–5 h of development which is around the blastula stage of these species. This is mainly in agreement with findings in medaka (*Oryzias latipes*) by Chong and Vielkind [16] and with the midblastula transition theory described in *Xenopus* [17] according to which nuclear genes are only expressed from the midblastula stage of the embryo.

We have not found the a great reduction of transient expression by 3 days of development but later events were assayed only occasionally. In one case seven-day-old larvae were measured and one individual was found to be expressing the transgene. This embryo was a non pigmented mutant (data not shown). Other individuals may not have been found positive due to the pigmentation which may be an obstacle to light measurement.

The two methods for in vivo luciferase assay were compared in our experiments. Both have advantages that make them a useful tool for expression analysis. The film exposure method takes a longer time since a sufficient duration of exposure to the film is required for the visualization of the signals of individual embryos (from several hours to a day). On the other hand, the scintillation measurement protocol provides almost instant results and allows fast detection and early selection based on expression which is easily quantified. However, it gives information about the momentary dose of activity. A further limitation of the latter is that some scintillation counters (e.g. our Betamatic V, Contron) are unable to count single photons above a certain limit and thus quantification of activity in the most strongly expressing individuals becomes impossible.

Interestingly, we found that in some cases both luciferase and β-galactosidase expression were also localised in the vegetal pole of the egg, i.e. in the yolk sac. This was confirmed by experiments in which we have dissociated the embryonic cells away from the yolk sphere of 5 catfish embryos at early stage where embryonic cells have a distinct brown colour while the yolk is yellow. The yolk sacs remained intact (although syncytial cells may not have been separated by this method) and could be incubated in luciferin and measured in the scintilla-

tion counter. Most surprisingly, very high expression levels were measured (2000–3000 cpm). Similarly, X-gal staining also revealed sign of β-galactosidase activity in the yolk of some of the embryos (Fig. 5B). This is difficult to explain since there is no evidence for the presence of transcriptional and translational machinery in the yolk sac of the fish embryo. Nevertheless, we cannot exclude the possible transport of transgene product into the vegetal pole of egg. Experiments have been in progress in our lab to describe the movements of exogenous DNA and its protein product during the early development of fish eggs (Papp et al., manuscript in preparation).

The method described in this paper may not be restricted to transient expression studies. Integration of foreign DNA in the genome of the embryo is more dependent upon other factors than the way in which the genes are introduced into the cells. These factors include the form, size, sequence and structure of the foreign DNA and the affinity of the recipient cell (cell cycle phase, activity of recombinases, repair systems, etc.). Such integration events could occur in our experiments as well, and Southern blot DNA hybridizations should answer these questions. If this proves true, the method may be used as a mass gene transfer method for the production of transgenic lines which can be useful both in developmental genetics and in the genetic improvement of farm fish species by the introduction of desirable traits.

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