*Transgenic Research* **12:** 413–424, 2003.



# High efficiency production of germ-line transgenic Japanese medaka (*Oryzias latipes*) by electroporation with direct current-shifted radio frequency pulses

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Received 15 April 2002; revised 7 November 2003; accepted 5 February 2003

*Key words:* electroporation, β-galactosidase, green fluorescent protein, transgenic fish

**Abstract**

Although there have been several studies showing the production of transgenic fish through electroporation techniques, success rates have been low and few studies show germ-line integration and expression. When electroporation has been successful, the device used is no longer commercially available. The goal of this experiment was to find an alternative efficient method of generating transgenic Japanese medaka (*Oryzias latipes*) using a commercially available electroporation device. The Gene Pulser II and RF module (Bio-Rad Laboratories, USA), along with two reporter gene constructs, were used. In contrast to other electroporation devices, which are based on a single pulse with exponential decay or square wave technology, the Gene Pulser II incorporates a direct current (DC)-shifted radio frequency (RF) signal. With this technique, over 1000 embryos can be electroporated in less than 30 min. The plasmid pCMV-SPORT-β-gal (Invitrogen, USA) was used in the supercoiled form to optimize parameters for gene transfer into single-celled embryos, and resulted in up to 100% somatic gene transfer. Similar conditions were used to generate fish transgenic for both the pCMV-EGFP plasmid (Clontech, USA) and a cytomegalovirus (CMV) driven phytase-EGFP construct. The conditions used were a voltage of 25 V, a percent modulation of 100%, a radio frequency of 35 kHz, a burst duration of 10 ms, 3 bursts, and a burst interval of 1.0 s. Seventy percent of the embryos electroporated with the pCMV-EGFP construct survived to sexual maturity, and of those, 85% were capable of passing the transgene on to their offspring. Transgenic second generation back-crossed ($BC_2$) fry were subjected to Southern blot analysis, which confirmed germ-line integration, and observation for green fluorescence protein, which confirmed protein expression. DC-shifted RF pulses are effective and efficient in the production of transgenic medaka, and germ-line integration and expression can be achieved without linearization of the transgene vector.

## Introduction

In the last decade, significant advances have been made in the application of molecular biology to aquaculture. Transgenic fish have been used to study developmental regulation of genes, improve cold tolerance, increase growth rate, and improve feed utilization (Ozato et al., 1989; for review Sin, 1997; Muir & Hostetler, 2001). In the production of transgenic fish, there have been several methods employed in the transfer of foreign DNA to the target organism. These methods include microinjection (Ozato et al., 1986; Inoue et al., 1990; Fletcher & Davies, 1991), electroporation of fertilized embryos (Inoue et al., 1990; Buono & Linser, 1991; Murakami et al., 1994; Sheela et al., 1999), electroporation of sperm (Sin et al., 1993; Symonds et al., 1994; Tsai, 2000; Venugopal et al., 1998), pseudotyped retroviral vectors (Lin et al., 1994; Linney et al., 1999), and embryonic stem cell transfer (Ma et al., 2001).

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Although microinjection has been used successfully in the production of transgenic fish and is a commonly used technique, it is not adequate for the production of the large numbers of fish needed in screening for correct transgene expression. This method is not only time consuming and labor intensive but is also limited by the physiology of fish eggs. The nuclei of fish eggs are small and difficult to visualize, the outer membrane, or chorion, hardens soon after fertilization (Ginsburg, 1963), and in many species the eggs are opaque.

Electroporation is an alternative that alleviates many of these problems and has the potential to make gene transfer more efficient. The general procedure was derived from cell culture studies in which an electrical permeabilization of the cell membrane was used to stimulate uptake of DNA (Neumann et al., 1982; Shigekawa & Dower, 1988). Initial studies involving electroporation for the production of transgenic fish used a direct current (DC) with either a pulse of exponential decay or multiple rectangular pulses (Inoue et al., 1990; Müller et al., 1993; for review Sin, 1997). Several studies have shown production of transgenic fish through electroporation. However, with traditional electroporation methods, either levels of transfer have been low with few studies showing germ-line transmission and expression (Khoo et al., 1992; Patil et al., 1994), or the device used is no longer commercially available, for example the Baekon 2000 (Zhao et al., 1993; Tseng et al., 2000; Sarmasik et al., 2002).

In an effort to improve cell poration, or electrotransfection techniques, an oscillating electric field was suggested, because the field would be less traumatic to the membrane than an ordinary square wave (Chang, 1989). Reduced trauma would increase the probability that the membrane would reseal, rather than being destroyed. Chang (1989) found that by combining the properties of a direct current field and an oscillating field to produce a new type of waveform, called DC-shifted radio frequency (RF) pulses, cell transfections were more efficient, with a higher percentage of surviving cells than with other types of pulses. This new waveform looks like a RF field riding on top of a square wave. Although this method has been tested and proven more efficient in cell culture than previously tested electroporation pulses, the proficiency of this method has not been tested with embryos.

The goal of this research was to test the efficacy of DC-shifted RF pulses to improve gene transfer into Japanese medaka (*Oryzias latipes*) embryos and to determine if germ-line integration of the transgene could be achieved. In an electric field, embryos have a threshold before permanent damage is incurred, preventing resealing of the membrane. Therefore, the ability to adjust electroporation parameters to fall below that threshold is critical. We examined the use of the commercially available Gene Pulser II and RF module (Bio-Rad Laboratories, USA) to address this issue. This apparatus provides the user with the ability to vary multiple parameters including: voltage, radio frequency (RF, the frequency of oscillations within a pulse), percent modulation (the percentage of the wave which will equal the peak–peak amplitude of the sine wave), burst duration (length of the burst), number of bursts, and burst interval (time between bursts) to optimize electroporation conditions.

## Materials and methods

### *Japanese medaka*

Japanese medaka, of the orange-red strain, were used to produce the transgenic founder lines. Forty-two groups of mature fish were maintained at 3 males to 5 females per halved 38 l aquarium, with a 14 h light to 10 h dark artificially controlled photoperiod, and were fed three times a day with tropical flake food (Aquatic Eco-systems, USA) and live artemia. Embryos were collected every 3 days and within 15 min post-fertilization, rinsed with de-ionized water, and electroporated (see section Electroporation of medaka embryos). A different batch of mature fish was used for embryo collection for each replication of a given set of electroporation conditions. After electroporation, embryos were again rinsed with de-ionized water and hatched in an incubator adapted from that described by Blacklidge and Bidwell (1993). Fry were fed a mixture of live infusoria and larval AP100 diet (Zeigler Brothers, USA).

### *Preparation of plasmid DNA*

The reporter gene construct pCMV-SPORT-β-gal (Invitrogen, USA), for the expression of β-galactosidase, was chosen for the initial testing of somatic transgene expression due to its simplicity; one needs only to stain either the entire organism or tissues of interest in order to determine where the transgene is being expressed. The pEGFP-1 (BD Biosciences Clontech, USA) promoterless plasmid, for the expression of enhanced green fluorescence protein, was

chosen to be the backbone for a construct involving the *Aspergillus niger* phytase gene, a gene of economic and agronomic importance, due to the ability to observe protein expression in live embryos. The CMV-IE promoter was directionally cloned into pEGFP-1 at the *Kpn*I and *Bam*HI restriction enzyme sites, resulting in pCMV-EGFP.

The phytase gene from *A. niger* was amplified in a 3-step polymerase chain reaction (PCR), with the third step adding a restriction enzyme site for *Hin*dIII and the *Oncorhynchus nerka* growth hormone type I (GH1) secretion signal sequence to the 5′ end of the fragment. The reverse primer was designed to add an *Eco*RI restriction enzyme site to the 3′ end. The PCR mixture initially contained 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM of dNTP, 1 U of Elongase DNA polymerase mix (Invitrogen, USA), 20 ng of genomic DNA from *A. niger*, and 20 pmol of each of the following primers: (reverse) 5′ CCGAATTCT CAGCTAACGAAAACACTCCG 3′ and (forward 1) 5′ TTGTTTCCTGAGTCAAGGGGCAGCGCTG GCAGTAGTCCCCGCCTCGAGAAA 3′ in a total volume of 50 µl. After 20 cycles, 20 pmol of a third primer was added to the mixture (forward 2) 5′ TGCTGATGCCAGTCTTACTGGCCAGT TGTTTCCTGAGTCAAGGGG 3′ and another 10 cycles were conducted. The amplification was concluded with 10 additional cycles and the addition of 20 pmol of a fourth primer (forward 3) 5′ GCTAAGCTTCAAAGCCATGGGACAAGT GTTTCTGCTGATGCCAGTCTTACTG 3′. The resulting product was digested with *Hin*dIII and *Eco*RI, the *Hin*dIII site was end-filled, and the product was cloned into the *Bam*HI (end-filled) and *Eco*RI sites of the pOVCMV-IE construct provided to us by Dr. Robert Devlin (Canada Wildlife and Fisheries, Vancouver, BC). The pOVCMV-IE construct consists of the CMV-IE promoter, a multiple cloning site, and the *O. nerka* GH1 terminator in pBluescript II KS (Figure 1(A)). The promoter, gene, and terminator (CMV-PHY-GH1t) were digested out of pBluescript with the restriction enzyme *Not*I. The *Not*I overhanging bases were end-filled, and CMV-PHY-GH1t was blunt-end cloned into CMV-EGFP at the *Hin*dIII site (end-filled) to produce CMV-PHY-GH1t-CMV-EGFP (Figure 1(B)).

Plasmid DNA was purified using a commercially available maxiprep kit (Bio-Rad Laboratories, USA), resuspended in water, and used in the supercoiled form. Prior to use, plasmid DNA was diluted to 1 µg/100 µl in HEPES buffered saline (HBS; 10 mM HEPES, 125 mM sodium chloride, 5 mM calcium chloride, pH 8.0) and was used for five consecutive electroporations.

*Electroporation of medaka embryos*

The Gene Pulser II apparatus and RF module (Bio-Rad Laboratories, USA), along with two reporter gene constructs, were used for the optimization of electroporation in medaka embryos. For each electroporation, 400 µl of DNA-HBS solution in a 4-mm gap-width cuvette and 50 embryos were used. An initial experiment was conducted to determine the effect of each parameter on both survival and gene transfer. For this experiment, 200 different combinations of parameters and 10,000 embryos were tested with the pCMV-SPORT-β-gal construct (data not shown).

After analysis of the data resulting from these combinations, a more focused set of parameters was tested which again used the pCMV-SPORT-β-gal construct. These parameters focused on optimizing voltage, radio frequency, burst duration, and burst interval, with three replicates for each. In order to study the effect of voltage, the voltage was varied from 5 to 45 V in increments of 5 V, while holding all other parameters constant at 100% modulation, a RF of 50 kHz, a burst duration of 40 ms, 3 bursts, and a burst interval of 1.0 s. For the parameters used to determine the interaction between the RF and burst duration, the voltage, percent modulation, number of bursts, and burst interval were held constant at 25 V, 100%, 3, and 1.0 s respectively, while the values of 10, 30, 50, 70, and 90 ms for burst duration were tested for RF values of 30, 35, 40, 45, and 50 kHz. The effect of burst interval was studied by varying the values of burst interval (0.3, 0.6, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, and 6.0 s) while all other parameters were held at 25 V, 100% modulation, 25 kHz, a burst duration of 40 ms, and 3 bursts.

After analysis of the above parameters, the conditions which consistently produced the largest number of transgenic fish were used to electroporate embryos with the pCMV-EGFP construct. Again, 50 embryos per electroporation were added to a 4-mm gap-width cuvette containing 400 µl of DNA-HBS solution, and two replicates were conducted. The parameters used were a voltage of 25 V, 100% modulation, a RF of 35 kHz, a burst duration of 10 ms, 3 bursts, and a burst interval of 1.0 s. A burst interval of 1.0 s was chosen over a burst interval of 0.3 s, even though the average percentage of transgenic fish obtained was higher for 0.3 s. This was due to the 1.0 s value having

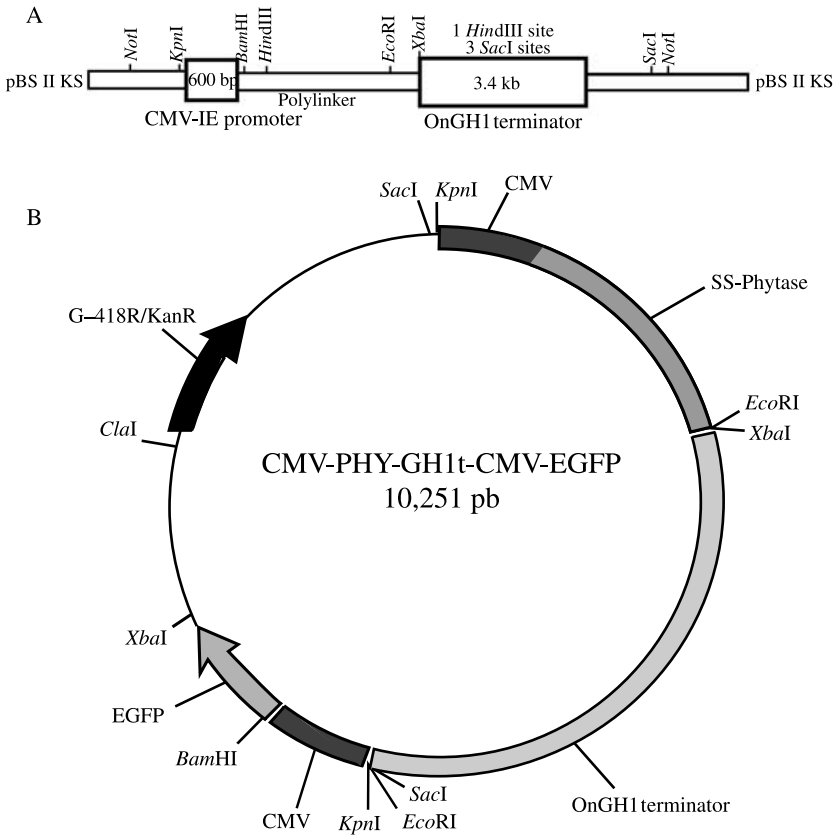


*Figure 1.* (A) pOVCMV-IE provided to us by Dr. Robert Devlin, Canada Wildlife and Fisheries, Vancouver, BC. This plasmid consists of the CMV-IE promoter, a polylinker, and the *O. nerka* type I growth hormone terminator in pBluescript KS. (B) CMV-PHY-GH1t-CMV-EGFP construct. The *A. niger* phytase gene was cloned into the pOVCMV-IE plasmid given in (A). The fragment consisting of the promoter, gene, and terminator was digested out of pBluescript KS with *Not*I, the resulting overhangs were end-filled, and this fragment was cloned into the *Hin*dIII (end-filled) site of pEGFP-1.

less variation and the results being more reproducible. These same conditions were later used to electroporate 50 embryos with the CMV-PHY-GH1t-CMV-EGFP construct.

### *Staining for β-galactosidase*

Medaka electroporated with pCMV-SPORT-β-gal were allowed to mature to 2 weeks of age post-hatching. Fry were fixed in phosphate buffered saline (PBS) with 4% glutaraldehyde for 4 h at room temperature, washed 3 times with PBS, and stained overnight at 37°C in PBS, 0.1% Triton X-100, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.05% X-gal for β-galactosidase activity. Wild type fry of the same age were used as controls. The digestive tract of wild type fish showed some staining (Figure 2), therefore, fish with blue coloration in areas other than the digestive tract were considered positive fish.

### *Generation of CMV-EGFP and CMV-PHY-GH1t-CMV-EGFP $BC_2$ fish*

Embryos electroporated with either the pCMV-EGFP or CMV-PHY-GH1t-CMV-EGFP construct were allowed to grow to sexual maturity. Each confirmed founder was back-crossed to the wild type strain to produce the $BC_1$ generation. $BC_1$ fry were tested for EGFP using the methods described below. Confirmed positive fish of the $BC_1$ were again back-crossed to the wild type strain to produce the $BC_2$ generation and confirm germline transmission.

### *Generation of CMV-PHY-GH1t-CMV-EGFP $F_3$ fish*

Two $BC_2$ fish, confirmed to be transgenic for the CMV-PHY-GH1t-CMV-EGFP construct by PCR, were selected; one male and one female. These fish

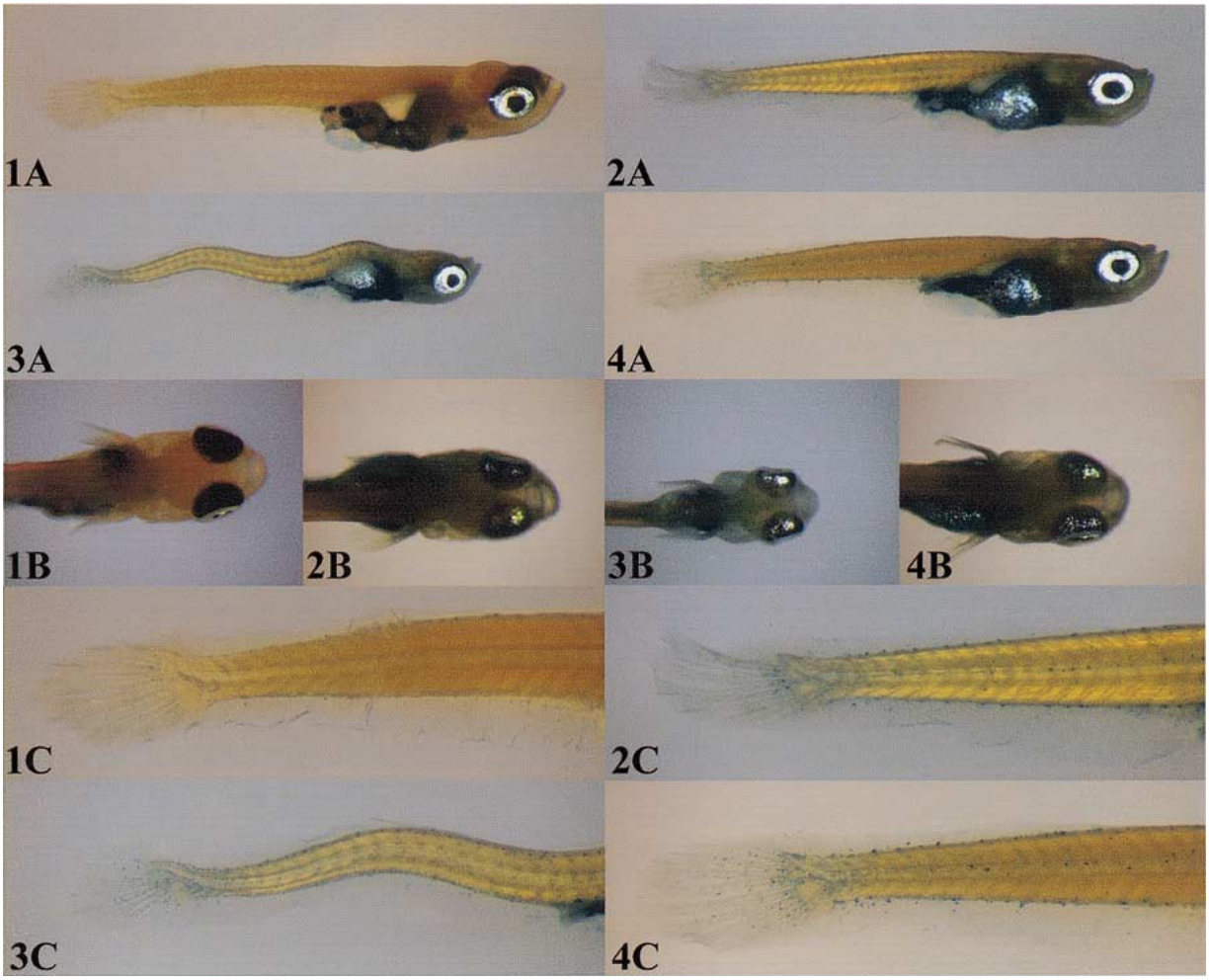


*Figure 2.* Electroporated medaka fish stained for β-galactosidase activity. Each number represents a different fish. Fish number 1 is a wild type fish (electroporated with no DNA), whereas the fish numbered 2–4 represent fish electroporated with the pCMV-SPORT plasmid. Frames 1A–4A are lateral views of each fish, frames 1B–4B are top views of each of the four fish, and frames 1C–4C are lateral views of the tail of each fish. The digestive tract of the wild type fish shows some dark staining, indicating endogenous $\beta$-galactosidase activity or cross-staining of other substrates.

were allowed to mate with each other, and the resulting clutch of eggs was collected. These eggs were hatched as previously described, and the resulting $F_3$ fry were allowed to grow to 2 months of age.

*Genomic DNA isolations*

Genomic DNA was extracted from a small clipping of caudal fin tissue for founder and $BC_1$ fish, whereas entire fry were used for testing of the $BC_2$. High molecular weight DNA was isolated from transgenic and non-transgenic medaka according to the methods of Jowett (1986). Tissue was digested in lysis buffer (100 mM Tris, pH 8.0, 1% SDS, 100 mM EDTA, 0.2 mg/ml proteinase K) at 60°C for 1 h, and DNA purified using phenol–chloroform extraction and ethanol precipitation.

*PCR for EGFP transgene*

Each PCR contained 20 pmol of each primer, 0.2 mM of dNTP, 1× PCR buffer, 2.5 mM $MgCl_2$, 1 U of *Taq* DNA polymerase, and 7 ng of genomic DNA in a total volume of 50 µl. Primer sequences were designed within the coding region of the EGFP gene to produce a 400 bp amplification product that would work well at a 69°C annealing temperature with 35 cycles. The forward primer sequence used was 5′

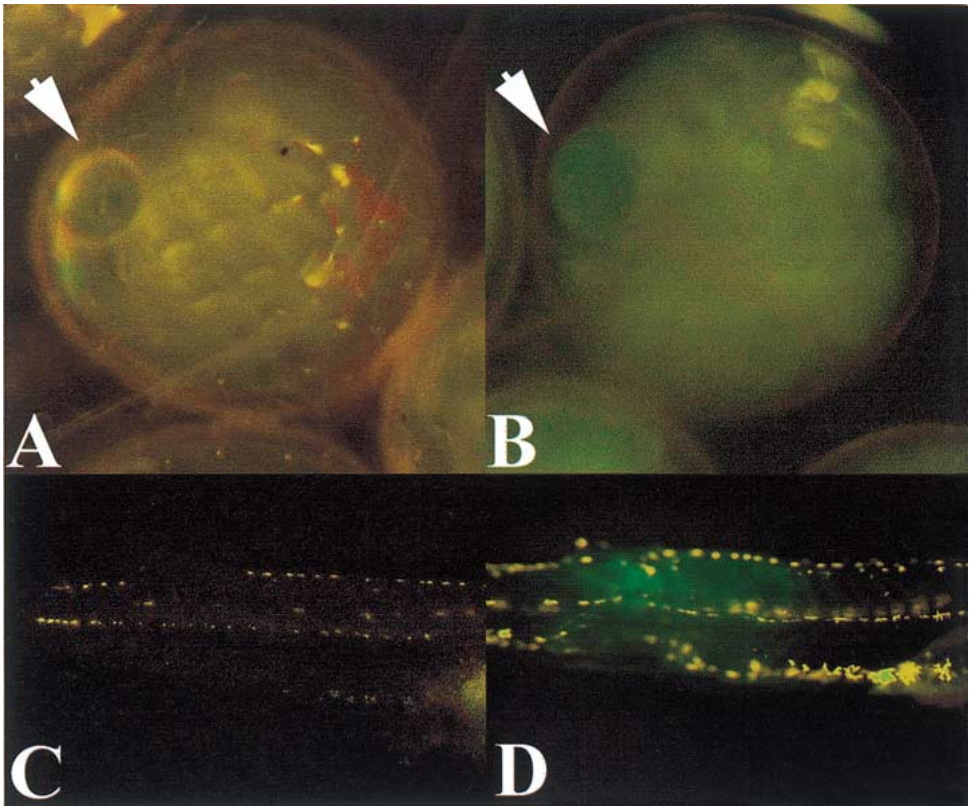


*Figure 3.* Eight-celled stage embryos (frames A and B) and lateral view of the posterior end of 2-week-old fry (frames C and D) under an ultraviolet light with a Leica GFP plus filter set. Frames A and C are of wild type medaka, whereas frames B and D are of CMV-EGFP transgenic $BC_2$ medaka. White arrows are used to indicate embryos.

CGTAAACGGCCACAAGTTCAGCGT 3′, and the reverse primer sequence used was 5′ GTTGTGGCT GTTGTAGTTGTACTCC 3′.

*Slot blot analysis*

Slot blot analysis was performed in the $BC_2$ generation for fish electroporated with the CMV-EGFP construct. Approximately 2 μg of DNA from each fish was blotted onto Hybond N membrane (Schleicher & Schuell, USA) with a Bio-Dot SF microfiltration device (Bio-Rad Laboratories, USA) according to Maniatis et al. (1982). Purified plasmid DNA (pCMV-EGFP) was used as a quantification control. Approximately 50 ng of $^{32}$P-labeled pCMV-EGFP DNA was used as probe. Nucleic acid hybridization protocols and washes followed the methods of Strauss (1997). Autoradiography was performed with Kodak X-OMAT film and developed using standard procedures.

*Southern blot analysis*

Southern blot analysis was conducted on a random sample of CMV-PHY-GH1t-CMV-EGFP $BC_2$ transgenic fish. A second Southern blot was performed on two parental transgenic fish and their offspring. Approximately 3 μg of genomic DNA from each fish was digested with *Kpn*I (New England BioLabs, USA), electrophoresed through a 0.8% agarose gel, and transferred to Hybond N membrane (Schleicher & Schuell, USA) according to Maniatis et al. (1982). Purified CMV-PHY-GH1t-CMV-EGFP DNA was used as a quantification and size control. Linearized CMV-PHY-GH1t-CMV-EGFP plasmid DNA was used as a hybridization probe. Hybridizations, washes, and film development were conducted as described above.

*EGFP expression*

Protein expression was detected by observing embryos and young fry with a dissecting fluorescence microscope (MZFLIII, Leica, Germany) equipped with

a camera and several GFP filter sets. Due to the high amount of auto-fluorescence of the medaka fish (Hamada et al., 1998) a Leica GFP plus filter set (excitation between 440 and 480 nm and emission at 510 nm) was used. With this filter set, the auto-fluorescence is seen as a yellow color, and actual GFP fluorescence is green (Figure 3).

*Statistical analysis*

Statistical analysis was conducted using multiple linear regression with the proc GLM option of SAS (SAS Institute, NC, USA). In each case, the hatch number, number alive at 2 weeks post-hatching, or percent transgenic served as the dependent variable and the electroporation parameters used and the resulting interactions among them served as the independent variables. Percentage data was transformed by angular transformation before analysis, as recommended by Steel and Torrie (1980).

## Results

*CMV-SPORT-β-gal*

From the initial experiment, voltage was a significant factor affecting hatch number, number surviving to 2 weeks, and the percentage of transgenic fish obtained ($P < 0.05$). The RF, burst duration, and burst interval did not affect survival, but did affect the percentage of transgenic fish obtained at the 10% level ($P < 0.1$).

The results of the more focused set of electroporation parameters are presented in Table 1. Voltage was found to significantly affect survival ($P < 0.005$). A quadratic effect of the voltage was found to affect the percentage of transgenic fish, for fish that survived to testing age, at a 16% level. In this case, the percentage of transgenic fish increased as the voltage increased from 5 to 25 V and decreased from 30 to 45 V (Figure 4). No fry hatched when the voltage used was greater than 40 V. Both RF ($P < 0.05$) and burst duration ($P < 0.001$) significantly affected the percentage of transgenic fish obtained. The interaction of the quadratic of RF with burst duration was significant ($P < 0.001$), indicating that the combination of factors needs to be examined together. In this case, the transformation frequency relative to the RF was non-linear, the shape of which depended upon the burst duration (Figure 5). The linear effect of burst interval was significant ($P < 0.05$) and did not interact with voltage, RF frequency, or burst duration. As the burst interval increased, the percentage of transgenic fish decreased (Figure 6). Only three transgenic fish were noted for burst interval values greater than 2.0 s.

*CMV-EGFP*

Only embryos electroporated with pCMV-EGFP or CMV-PHY-GH1t-CMV-EGFP were grown to sexual maturity and used to test for germ-line transmission. Electroporation conditions were 25 V, 100% modulation, 35 kHz, 10 ms burst duration, 3 bursts, and a burst interval of 1.0 s. Of the 100 embryos electroporated with pCMV-EGFP (50 from each replicate), 70 survived to sexual maturity, and all were positive for the transgene. For the first replicate, 80% of the founders were capable of passing the transgene on to their offspring, and 90% of the founders passed the transgene on to their offspring for the second replicate. Slot blot analysis (Figure 7) confirmed transmission of the transgene to the offspring, although variation in copy number was noted. Expression of the EGFP transgene was noted through observation of $BC_2$ embryos and fry for the green fluorescent protein (Figure 3), and fry were later confirmed transgenic through slot blot analysis. Early observation of embryos showed an even distribution of the green fluorescent protein throughout the entire embryo, although variation in fluorescent patterns was noted by day 5, which continued throughout the development of the fish. Expression was noted in varying tissues, although all transgenic fish showed fluorescence of the eyes and caudal fin tissues. One family of transgenic fish showed expression in the tail, just prior to the caudal fin (Figure 3(D)). All transgenic $BC_2$ medaka displayed expression of the green fluorescent protein, showing that there is not only germ-line transmission of the transgene, but also expression in subsequent generations.

*CMV-PHY-GH1t-CMV-EGFP*

Southern blot analysis was used to test for chromosomal integration of the transgene in CMV-PHY-GH1t-CMV-EGFP $BC_2$ fish. A random sample of $BC_2$ fish from five different founder lines was initially tested. Southern blot analysis of putative transgenic $BC_2$ fish suggests that the CMV-PHY-GH1t-CMV-EGFP transgene was heritable and that individual $BC_2$ fish inherited from 10 to 30 copies of the transgene from their respective founders (Figure 8). An analysis of $F_3$ fish generated from a cross, involving two transgenic $BC_2$ fish, also indicated that the transgene

*Table 1.* Average effects of electroporation parameters on mortality and the percentage of transgenic fish obtained out of number electroporated and out of number alive at testing, while percent modulation and number of bursts are held constant at 100% and 3, respectively

| Voltage (V) | RF frequency (kHz) | Burst duration (ms) | Burst interval (s) | Average % mortality | Average % transgenic | Average % transgenic at testing |
|---|---|---|---|---|---|---|
| 5 | 50 | 40 | 1.0 | 52.7 | 16.7 | 35.2 |
| 10 | 50 | 40 | 1.0 | 56.0 | 6.0 | 13.6 |
| 15 | 50 | 40 | 1.0 | 41.3 | 25.3 | 43.2 |
| 20 | 50 | 40 | 1.0 | 56.0 | 32.7 | 74.2 |
| 25 | 50 | 40 | 1.0 | 62.7 | 21.3 | 57.1 |
| 30 | 50 | 40 | 1.0 | 72.7 | 7.3 | 26.8 |
| 35 | 50 | 40 | 1.0 | 94.0 | 0.7 | 11.1 |
| 40 | 50 | 40 | 1.0 | 97.3 | 1.3 | 50.0 |
| 45 | 50 | 40 | 1.0 | 100 | 0 | 0 |
| 25 | 30 | 10 | 1.0 | 32.0 | 26 | 38.2 |
| 25 | 30 | 30 | 1.0 | 62.0 | 16.7 | 43.9 |
| 25 | 30 | 50 | 1.0 | 79.3 | 13.3 | 64.5 |
| 25 | 30 | 70 | 1.0 | 92.7 | 5.3 | 72.7 |
| 25 | 30 | 90 | 1.0 | 98.7 | 0.7 | 50.0 |
| 25 | 35 | 10 | 1.0 | 44.0 | 48.7 | 86.9 |
| 25 | 35 | 30 | 1.0 | 74.0 | 14.0 | 53.8 |
| 25 | 35 | 50 | 1.0 | 89.3 | 3.3 | 31.3 |
| 25 | 35 | 70 | 1.0 | 90.7 | 1.3 | 14.3 |
| 25 | 35 | 90 | 1.0 | 100 | 0 | 0 |
| 25 | 40 | 10 | 1.0 | 59.3 | 34.7 | 85.2 |
| 25 | 40 | 30 | 1.0 | 59.3 | 17.3 | 42.6 |
| 25 | 40 | 50 | 1.0 | 82.7 | 2.7 | 15.4 |
| 25 | 40 | 70 | 1.0 | 92.7 | 2.0 | 27.3 |
| 25 | 40 | 90 | 1.0 | 92.0 | 2.0 | 25.0 |
| 25 | 45 | 10 | 1.0 | 32.0 | 30.7 | 45.1 |
| 25 | 45 | 30 | 1.0 | 64.7 | 14.7 | 41.5 |
| 25 | 45 | 50 | 1.0 | 72.7 | 8.0 | 29.3 |
| 25 | 45 | 70 | 1.0 | 88.7 | 0.7 | 5.9 |
| 25 | 45 | 90 | 1.0 | 100 | 0 | 0 |
| 25 | 50 | 10 | 1.0 | 44.7 | 16.0 | 28.9 |
| 25 | 50 | 30 | 1.0 | 66.7 | 14.0 | 42.0 |
| 25 | 50 | 50 | 1.0 | 84.0 | 13.3 | 83.3 |
| 25 | 50 | 70 | 1.0 | 91.3 | 2.7 | 30.8 |
| 25 | 50 | 90 | 1.0 | 100 | 0 | 0 |
| 25 | 25 | 40 | 0.3 | 86.7 | 10.0 | 75.0 |
| 25 | 25 | 40 | 0.6 | 94.7 | 0.7 | 12.5 |
| 25 | 25 | 40 | 1.0 | 87.0 | 4.0 | 30.8 |
| 25 | 25 | 40 | 1.5 | 91.3 | 3.3 | 38.5 |
| 25 | 25 | 40 | 2.0 | 84.0 | 1.3 | 8.3 |
| 25 | 25 | 40 | 3.0 | 77.3 | 0 | 0 |
| 25 | 25 | 40 | 4.0 | 90.0 | 0 | 0 |
| 25 | 25 | 40 | 5.0 | 86.0 | 0.7 | 4.8 |
| 25 | 25 | 40 | 6.0 | 90.0 | 0 | 0 |

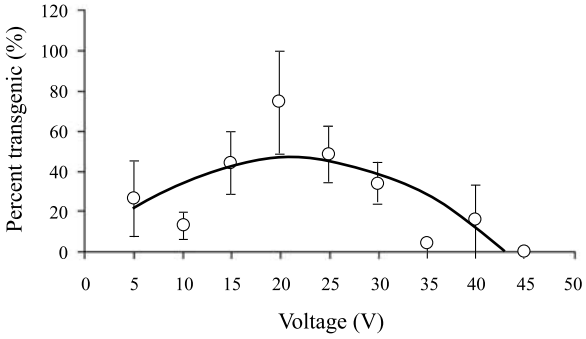


*Figure 4.* Effect of voltage on the percentage of transgenic fish. The open circles represent the percentage of transgenic fish (ρSE), as a function of number of fish at testing. The smooth line through the data points represents the non-linear trend of the data.

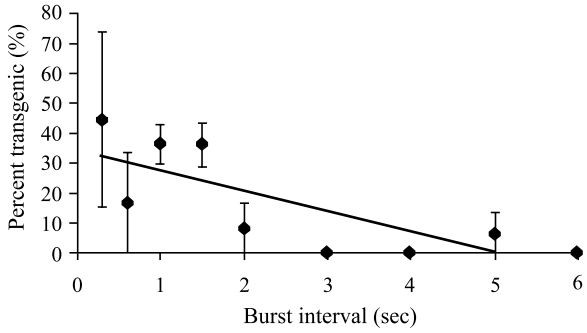


*Figure 6.* Effect of burst interval on the percentage of transgenic fish obtained. The solid circles represent the percentage of transgenic fish (ρSE) as a function of number of fish at testing. The line through the data represents the linear trend of the data for the percentage of transgenic fish to decrease as the burst interval increases.

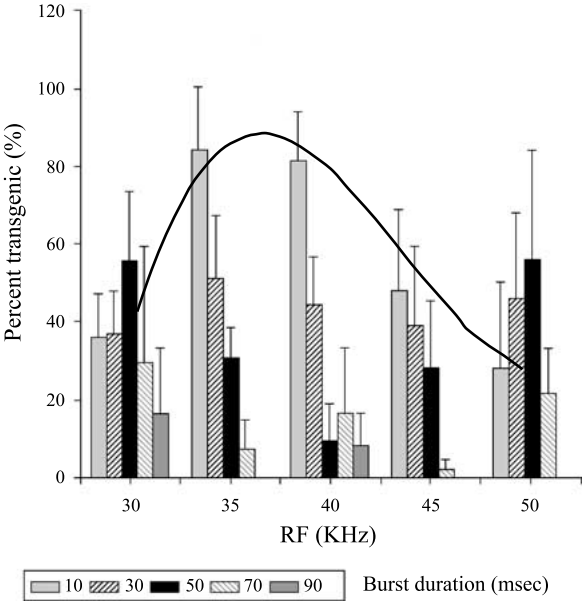


*Figure 5.* Effect of RF and burst duration on the percentage of transgenic fish obtained. Bars represent the percentage of transgenic fish (ρSE) for a given burst duration (in ms) and a given RF. The smooth line graph represents the non-linear trend in the data for a 10 ms burst duration.

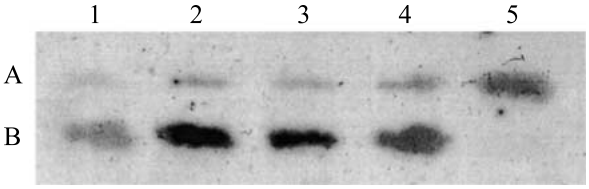


*Figure 7.* Slot blot of CMV-EGFP $BC_2$ medaka fish. Row A is of plasmid DNA controls representing 1, 2, 3, 5, and 10 genomic transgene copies. Row B contains 2 Πg of genomic DNA from CMV-EGFP $BC_2$ medaka samples. B1–B4 are of positive samples, whereas B5 is non-transgenic. Sample B1 has approximately seven transgene copies, B2 and B3 each have approximately 20 transgene copies, and B4 has approximately 10 transgene copies.

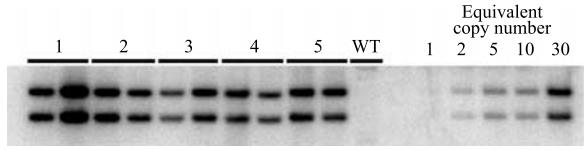


*Figure 8.* Southern blot of 10 CMV-PHY-GH1t-CMV-EGFP $BC_2$ fish from five different founder lines and a wild type control. Genomic DNA was digested with *Kpn*I and separated on a 0.8% agarose gel. Plasmid DNA was also digested with *Kpn*I and separated by gel electrophoresis to provide two bands of 4.8 and 5.5 kb which served as quantification controls, representing 1, 2, 5, 10, and 30 genomic DNA copies. The smaller fragment represents the CMV-EGFP portion of the construct, while the larger fragment contains the CMV-PHY-GH1t portion.

was heritable, with individual $F_3$ fry inheriting from 0 to more than 30 copies of the transgene (Figure 9). The transgene hybridized intensely to the probe, and with short exposure of the blot to film (Figure 9(A)), no additional bands were visible. However, upon prolonged exposure of the blot to film (Figure 9(B)), two weakly hybridizing bands appeared. These bands were inherited from the $BC_2$ father, and probably represent flanking genomic DNA at the location in which the transgenes were inserted. Similar insertion sties were not identified for the $BC_2$ mother. This does not preclude the possibility that extra chromosomal transgenes were present and transmitted to the $F_3$ offspring. However, the relatively consistent hybridization signals seen in segregating $F_3$ fish, and the heritability of the weakly hybridizing bands suggest that the $BC_2$ parents had stable sites of integration that contain multiple copies of the transgene.

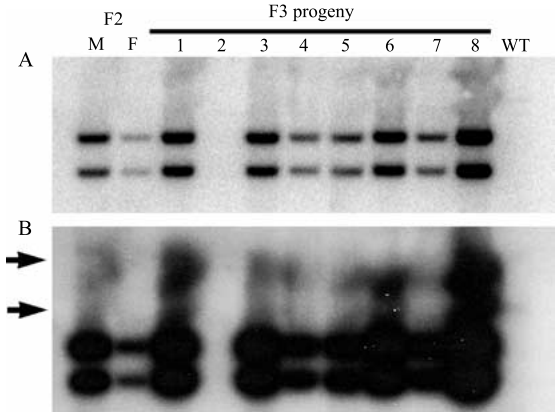


*Figure 9.* Southern blot of a male and female CMV-PHY-GH1t-CMV-EGFP $BC_2$ fish and their $F_3$ progeny. Although one progeny looks like the wild type sample, with no transgene present, other progeny have similar banding patterns and intensity levels as the father or mother, and four offspring seem to have banding patterns that are a combination of both parents. (A) Short exposure of blot to film shows two distinct bands for all but two samples (including the wild type) of 4.8 and 5.5 kb representing the CMV-EGFP fragment and the CMV-PHY-GH1t fragment, respectively. (B) A longer exposure time shows two additional bands present in the father fish and several of the progeny. These bands are approximately 6 and 9 kb in size and most likely represent insertion sites of the transgene into the chromosomal DNA.

## Discussion

### *CMV-SPORT-β-gal*

Although a strong, ubiquitous promoter was used to drive the expression of β-galactosidase, the fish presented in Figure 2 displayed a high degree of mosaicism within the founder lines. Since the required amount of time for DNA to become stably integrated into the host genome is not always equivalent to the time required for cleavage of the embryo, it is possible for the cells to have undergone several cleavages before the DNA was integrated. In this instance, not all cells would contain a complete copy of the transgene; this in turn could lead to localized expression within tissues that contain the transgene (Stuart et al., 1988). Since other researchers have noted variable expression patterns in the founder lines, or expression in the founder line with no expression in the subsequent offspring, due to failure of the transgene to become properly integrated into the genome (Patil et al., 1994), it was important to look for expression patterns in non-mosaic fish by observing expression in the subsequent transgenic offspring. Since the protocol for staining fish for β-galactosidase required euthanization of the fish, the pCMV-EGFP reporter gene construct was utilized to test for germ-line transgene expression.

### *CMV-EGFP*

Although early observation of embryos showed an even distribution of the green fluorescent protein throughout the entire embryo for both $BC_1$ and $BC_2$ fish, variation in fluorescent patterns was noted by day 5 and continued throughout the development of the fish. In all fish found to contain the transgene, expression of the EGFP gene was observed in the eyes and caudal fin tissues. Although expression patterns were similar for fish from the same line, some variation existed between founder lines. In all cases, expression was not ubiquitous.

Early studies with constitutive regulatory sequences, which would be expected to cause expression throughout the entire organism, have shown that mosaic-type expression can occur, even in $F_1$ fish (Tsai et al., 1995). Chou et al. (2001) reported similar results with the CMV promoter and suggested that epigenetic modification might have been the cause of the reduced expression. It has been suggested that this variable expression might also be due to the presence of unintegrated DNA (for review Iyengar et al., 1996). In order to determine which suggestion was correct, integration of the transgene needed to be tested.

### *CMV-PHY-GH1t-CMV-EGFP*

Many researchers agree that proof of transgene integration is difficult to obtain, especially in fish. This is because many studies involving transgenics in fish have shown that the transferred DNA is rapidly replicated extra chromosomally during embryogenesis, leading to the formation of tandem arrays or concatamers (for review Fletcher & Davies, 1991; Iyengar et al., 1996). This rapid replication corresponds to the rapid DNA synthesis occurring within the embryo at this time (for review Fletcher and Davies, 1991; Iyengar et al., 1996). However, after gastrulation, the amount of transferred DNA has been shown to decrease rapidly, and is thought to be due to degradation of unintegrated DNA sequences (Stuart et al., 1988).

In an attempt to show chromosomal integration of the transgene, Southern blots were utilized. Figure 8 shows that although there was slight variation in copy number between fish of the same founder line, the variation between founder lines was greater. Since integration in each founder was an independent event, it would be expected for copy number to vary between

founder lines. The variation noted for samples from the same founder line (specifically 1, 3, and 4) could be due to two factors. First, the transgene could be integrated into two different chromosomes; this would result in some offspring obtaining only one of either transgene and some offspring obtaining both. The other alternative is that there are some integrants plus several more persisting plasmids or concatamers, which are being passed on to the offspring. In all samples, concatamers are being formed, a relatively common occurrence in transgenics which explains stability and the large number of repeating units of the plasmid. Unfortunately, the weak presence of only one genome equivalent copy of the transgene is almost undetectable, making the observation of junction fragments impossible.

In an attempt to overcome this difficulty, two transgenic fish were crossed and the parental and offspring DNA tested for heritability of copy number and junction fragments (Figure 9). In this example, junction fragments are pointed out by the presence of arrows in the figure. This figure shows that there are junction fragments for the male $BC_2$ fish and that the copy number is heritable. $F_3$ fish number 2 has received no transgene copies and looks like the wild type fish. The high copy number for $F_3$ fish number 1, 6, and 8 could only be explained by the transmission of the transgene from both the male and female $BC_2$ fish, whereas $F_3$ fish number 3 and 5 have copy numbers and junction fragments similar to the male and $F_3$ fish number 4 and 7 have copy numbers similar to the female. No junction fragments were noted for the female $BC_2$ fish, this could be due to two reasons. Either the junction fragments were too weak for detection or the female contains several copies of plasmids or concatamers. The detection method used here was not sufficient for differentiation between these possibilities. Regardless, heritability of the junction fragments shown in Figure 9 for the male $BC_2$ fish in a Mendelian fashion clearly demonstrates genomic integration of the transgene.

*Summary*

The use of radio frequency pulses is an efficient method for the production of transgenic Japanese medaka fish. It is an extremely simple and time efficient technique that results in a high percentage of transgenic fish. We were able to electroporate over 1000 embryos in less than 30 min and were also able to optimize conditions such that repeated use of a given set of electroporation parameters gave consistent results with 100% of the fry that hatched being somatically transgenic. These experiments resulted in 80–90% of these fish being germ-line transgenic with offspring that exhibited transgene expression, an important concern in transgenic research.

We were able to create transgenic medaka fish using three different constructs. Since each of the constructs used was of a different size (7.8 kb for CMV-SPORT-β-gal, 4.8 kb for CMV-EGFP, and 10.3 kb for CMV-PHY-GH1t-CMV-EGFP), and since similar electroporation parameters resulted in successful gene transfer, electroporation using RF pulses should be efficient in the creation of transgenic fish with any transgene. Since supercoiled DNA was used in the production of the transgenic fish, the time required to purify DNA for electroporation was greatly reduced. This combination of time reduction in both purifying the DNA for gene transfer and in the gene transfer itself greatly enhanced the rate of production of transgenic fish for this experiment.

With the increasing role biotechnology plays in aquaculture and agriculture, gene transfer techniques must be capable of being applied to economically important genes and species. Our lab is currently using similar methods to optimize gene transfer through electroporation in tilapia (*Oreochromis niloticus*) embryos. Although somatic presence of the transgene has been confirmed through dot blot and PCR in the tilapia fry, experiments involving transgene integration and expression still need to be completed. We anticipate that these methods can be employed in other fish species as well to facilitate the production of transgenic fish.

## Acknowledgements

The authors would like to thank Dr. S. Dean Rider, Jr. for his helpful conversations, his assistance with preparing the photographs, and his initial review of this manuscript. This work was supported by the U.S. Department of Agriculture (USDA grant 98-35205-6762).

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