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D. P. Chandler
M. Welt
F. C. Leung

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Pacific Northwest Laboratory
Richland, Washington 99352

DEVELOPMENT OF A RAPID AND EFFICIENT MICROINJECTION TECHNIQUE FOR GENE INSERTION INTO FERTILIZED SALMONID EGGS

Darrell P. Chandler, Marc Welt, and Frederick C. Leung

Battelle, Pacific Northwest Laboratories
Biology and Chemistry Department
P.O. Box 999
Richland, WA 99352

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Address all correspondence and reprint requests to:

Dr. Fred C. Leung
Battelle, Pacific Northwest Laboratories
Biology and Chemistry Department
P.O. Box 999
M/S K4-13
Richland, WA 99352

Phone: (509) 375-2169
Fax: (509) 375-6891

ABSTRACT

An efficient one-step injection technique for gene insertion into fertilized rainbow trout (*Oncorhynchus mykiss*) eggs is described, and basic parameters affecting egg survival are reported. Freshly fertilized rainbow trout eggs were injected in the perivitelline space with a recombinant mouse metallothionein-genomic bovine growth hormone (bGH) DNA construct using a 30-gauge hypodermic needle and a standard microinjection system. Relative to control, site of injection and DNA concentration did not affect egg survival, but injections later than 3-4 hours post fertilization were detrimental. The injection technique permitted treatment of 100 eggs/hr with survivals up to 100%, resulting in a 4% DNA uptake rate as indicated by DNA dot blot analysis. Positive dot blot results also indicated that the injected DNA is able to cross the vitelline membrane and persist for 50-60 days post hatching, obviating the need for direct injection into the germinal disk. Results are consistent with previous transgenic fish work, underscoring the usefulness of the technique for generating transgenic trout and salmonids.

INTRODUCTION

Recent advances in biology, biochemistry, molecular biology, and recombinant DNA technology have culminated in the genetic engineering of plants and animals, which can be defined as the permanent introduction of novel DNA constructs produced by recombinant DNA methods into the genome of a recipient organism. In vertebrates, the majority of transgenic work has been conducted with the mouse, and methods for generating transgenic mice have been particularly well established to study questions of developmental biology, immunology, neurobiology and oncogenesis (Palmiter and Brinster, '86; DePamphilis et al., '88; Hanahan, '89). The basic technique includes removing fertilized eggs from a female mouse, injecting foreign DNA into the male pronucleus of the fertilized egg, returning the injected eggs into pseudopregnant foster mothers, and testing the progeny for incorporation of injected sequences. In addition, many factors have been shown to influence the efficiency of DNA integration, including DNA form (circular versus linear), DNA concentration, buffer composition, site of injection (nuclear versus cytoplasmic), and egg source (Brinster et al., '85). Gene transfer in fish has begun largely in response to the "supermouse" of Palmiter et al. ('82), who showed that an injected mouse metallothionein-rat growth hormone (mMT-rGH) gene construct integrated into the mouse genome could produce functional protein with a pronounced physiological effect. Clearly, the ability to introduce foreign DNA into piscine species is obvious (see Ozato et al., '89 and Hew, '89 for reviews), but evidence for protein expression and transmission of foreign DNA to progeny is not as well established.

The application of mouse injection techniques to various fish species depends largely on the egg structure. Many warmwater fish eggs lack a hard and impermeable chorion which allows direct injection into the cytoplasm or germinal disk using fine glass needles (e.g., Ozato et al., '86; Brem et al., '88; Inoue et al., '89; etc.). The chorion of other species is easily removed with mechanical or chemical treatments before microinjecteion (Zhu et al., '85a,b; Hallerman et al., '88), but no successful dechorionation protocol exists for trout and other salmonids. The objective of this study, then, was to develop a simple, rapid, and efficient microinjection technique for use with fertilized rainbow trout (*Oncorhynchus mykiss*) eggs, optimize the technique, and examine some of the variables affecting egg survival.

MATERIALS AND METHODS

Isolation and purification of injected DNA

Construction of the novel mouse metallothionein-bovine growth hormone vector (pUC-mMT-bGH; Fig. 1) is described elsewhere (Leung et al., '90). Single colonies of *E. coli* strain HB101, containing the plasmid pUC-mMT-bGH, were isolated

according to routine procedures (Maniatis et al., '82) and used to inoculate 1.0-liter overnight broth cultures. Cells were collected by centrifugation and pellets were resuspended in a total of 10 ml GET [50 mM glucose, 10 mM ethylenediaminetetraacetic acid (EDTA), 2 mM Tris-HCl, pH 8.0] containing 4 mg/ml lysozyme. After a 5 min incubation at room temperature, 20 ml of 0.2 N NaOH/1% sodium dodecyl sulfate (SDS) was added and the mixture incubated on ice for 10 min. Then, 15 ml of 3 M potassium acetate was added and the mixture incubated another 10 min on ice.

The bacterial chromosomal DNA was removed by centrifugation and plasmid DNA precipitated in an equal volume of 100% isopropanol at room temperature for 20 min. Plasmid DNA was pelleted, dissolved in 10 ml TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and extracted with an equal volume of 50:50 phenol:chloroform. The two phases were separated by centrifugation, and the aqueous upper phase removed. Three molar sodium acetate (0.3 M final concentration) and 2 volumes ice-cold 100% ethanol (EtOH) were added to the aqueous phase and the plasmid DNA precipitated on ice at least 30 min. The DNA was collected by centrifugation as before, and the pellet dissolved in 1.7 M cesium chloride (CsCl) in TE. After loading the CsCl/nucleic acid solution into ultracentrifuge tubes containing 200 µl ethidium bromide (5 mg/ml), the mixture was centrifuged for 2 days at 44,000 rpm, room temperature in a Ti 35 rotor and Beckman L8-70M ultracentrifuge.

The plasmid DNA band was visualized with long-wave ultraviolet light and removed with a sterile syringe and needle. Ethidium bromide was extracted with saturated (5 M NaCl/water) isopropanol, and the cleared DNA preparation diluted with 2 volumes sterile water. Then, 2 volumes of 100% EtOH were added, the DNA precipitated as usual, and the pellet dried under vacuum. The pellet was redissolved in 600 µl TE and scanned with a Beckman DU-7 spectrophotometer.

The mMT-bGH fragment, containing the operational, metal-responsive portion of the mouse metallothionein promoter (Stuart et al., '84) and the entire translated region of the genomic bovine growth hormone gene, was isolated from pUC-mMT-bGH by digesting 10 µg plasmid with 4 µl *Bgl* I and *Hind* III (Bethesda Research Laboratories) overnight at 37°C. The reaction mixture was then electrophoresed on 0.8% low-melting agarose (SeaPlaque), and the 2.45-kb band of interest excised with a sterile razor blade. The resulting agarose band was forced through a sterile syringe and needle into 5 ml of low-salt buffer [20 mM Tris, 200 mM NaCl, 1 mM EDTA(Na_2), pH 7.4] and incubated at 65°C for 30 min. While the low-melting agarose/DNA fragment incubated, an Elutip-d (Schleicher & Schuell) was prepared by washing first in 2 ml high-salt buffer [20 mM Tris, 1.0 M NaCl, 1 mM EDTA(Na_2), pH 7.4] at room temperature, followed by 5 ml low-salt buffer at 42°C. Then, the melted agarose/DNA solution (65°C) was forced through the column such that the agarose did not solidify. The column was washed with 3 ml low-salt buffer (42°C), and the DNA eluted with 400 µl high-salt buffer into a microfuge tube. The DNA was precipitated as usual, washed with 70% EtOH, dried under vacuum and resuspended in TE pH 8.0.

Microinjection system description

The microinjection system consisted of a Wild M3C binocular dissecting microscope mounted on a magnetic stage (Model MSS 100, Micromanipulator Microscope Co., Escondido, CA) equipped with dual-color lighting. A Narishige micromanipulator allowed three dimensional movement of the injection needle, and a 250 µl Hamiliton syringe coupled with a MicroLyte SR-10 microinjector (Micromanipulator Microscope Co.) provided volume control into the nanoliter range. Fertilized rainbow trout eggs were supported in a 2% agarose petri dish containing a small depression. The injection buffer, in all cases, was 10 mM Tris, 1 mM EDTA pH 8.0 containing 0.1% phenol red to help visualize DNA injection. Injection volume was approximately 100 nl.

Egg care and handling

All eggs were obtained from a 3-year old Battelle broodstock (Battelle, Pacific Northwest Laboratories, Richland, WA; Kamloops strain). Fish were anesthetized in quinolinone and eggs fertilized by the "dry" method (Leitritz and Lewis, '80). Fertilized eggs were water-hardened at the hatchery for 30 min in 12°C Columbia River water before transport back to the lab for microinjection, where they were maintained at 10-12°C. All eggs were raised at 12°C in plastic petri dishes inside a standard refrigerator, and pre-chilled Columbia River water was changed periodically with a 25-ml pipette or gentle pouring action. Dead eggs, as indicated by a characteristic opaque white color, were counted and removed daily. All surviving fish were raised to 50-60 days post fertilization, at which time they were sacrificed and fixed in 10% neutral-buffered formalin. Some fish were used for DNA dot blot analysis, while most of the fry were paraffin-embedded and sectioned according to standard histology methods.

Development and optimization of injection technique

To simplify existing injection procedures and combine chorion puncture and DNA injection into one step, stainless steel needles of various size (27, 30, and 33 gauge) were utilized to inject fertilized, water-hardened eggs. For every injected egg, there was also a "puncture" control (punctured but no DNA injection) and an uninjected sham control.

The initial set of experiments were designed to test the effects of injection site on egg survival; the sites of injection are referred to as "top," "side," and "random" injections (Fig. 2). A "top" injection is one where the needle penetrates the chorion directly above the germinal disk. A "side" injection is one where the egg is rotated so that the vertical axis of the germinal disk

is perpendicular to the needle axis, and a "random" injection is an injection into the perivitelline space without any "aiming" of the needle. One hundred fifty eggs were injected with 10^6 copies of mMT-bGH per 100 nl in each of the orientations, 50 eggs from each of 3 different pairs of fish (trout D,E,F) per orientation.

Results from the injection site experiments were applied to the next study, which was a time-course injection series. Ninety fertilized eggs were randomly injected at various times post fertilization with 10^6 copies of mMT-bGH per 100 nl beginning at 1 hour post fertilization, 30 eggs from each of 3 different pairs of fish (trout G,H,I) per time point.

The final experiment was a DNA concentration series, where fertilized trout eggs were randomly injected with various concentrations of the mMT-bGH fragment within 3 hours post fertilization. DNA concentrations tested were no DNA, 10^4 copies/100 nl, 10^5 copies/100 nl, 10^6 copies/100 nl and 10^7 copies/100 nl. Eggs from two pairs of fish were injected, ~40 eggs per DNA concentration from trout J and ~25 eggs per DNA concentration from trout K.

Egg survival was determined as the number of eggs surviving through hatching. When all eggs in a group had hatched, the number still alive was used to calculate percent survival. Those eggs that died during hatching or small fish that died before the last egg hatched were counted as dead eggs.

Extraction of genomic DNA from \ trout fry

Yolk sacs were removed from those fish used for dot blot analysis and the carcass cut into small pieces in a 1.5-ml microfuge tube. The tissues were homogenized in 600 µl sterile water, cells vortexed for 1 min and then pelleted at 2800 x g in a microcentrifuge. The tubes were blotted dry and the cells vortexed and spun, dehydrated and rehydrated, with the following solutions: 20%, 50%, 80%, 95%, 100%, 95%, 80%, 50%, and 20% EtOH, sterile water, and Tris-HCl pH 7.6. The final cellular pellet was resuspended in 1.0 ml TNES (10 mM Tris, 0.12 M NaCl, 0.1 mM EDTA, 0.5% SDS, pH 7.5) containing 500 µg/ml proteinase K (Sigma) and the suspension incubated at 50°C overnight. Then, 25 µl 5 M NaCl was added to each tube and the mixture centrifuged for 10 min at 4000 x g to remove cellular debris. The supernatant was decanted and saved, and 2 volumes of cold EtOH added to precipitate the DNA. The resulting precipitate was pelleted at 15,000 x g for 15 min, the supernatant decanted and the pellet dried under vacuum. The pellet was redissolved in 400 µl TE pH 8.0, extracted 2X with 50:50 phenol:chloroform and 2X with chloroform:isoamyl alcohol (24:1). The aqueous phase was saved, and DNase-free RNase added to a final concentration of 100 µg/ml. The solution was incubated at 37°C for 30 min, extracted 2X with chloroform, EtOH precipitated as usual (-20°C), pelleted, and dried under vacuum. The resulting pellet was resuspended in 150 µl TE pH 7.6, and DNA concentrations were determined by Hoechst H-33258 staining (Labarca and Paigen, '80).

Hybridization, washing, and autoradiography

Thirty nanograms of the small *Pvu* II-*Eco*RI fragment in Exon V of the bGH gene (550 bp, Fig. 3) was labeled with ^{32}P-dCTP (10 µCi/µl, 3000 µCi/mM, Amersham) by nick translation to a specific activity of 5×10^8 cpm/µg. Labeled DNA was precipitated in 100 mM Spermine and herring sperm DNA, and redissolved in 200 µl 0.2X SET (1X SET= 1% SDS, 10 mM Tris-HCl, 5 mM EDTA, pH 7.5).

Six µg genomic DNA from rainbow trout fry was aliquoted in separate microfuge tubes, and the total volume adjusted to 200 µl with 0.2 M NaCl. The DNA was precipitated overnight and the resulting precipitate pelleted as usual. The pellet was resuspended in 8 µl 2 M NaCl, 0.1 M NaOH and gently boiled for 5-10 min. The contents were collected at the bottom of the tube by a brief centrifugation, and gently mixed with a quick vortex at low speed. Five microliters from each sample (3.75 µg DNA) was applied to a piece of nitrocellulose paper; the membrane was air dried and rinsed briefly in 2X SSC (1X SSC = 150 mM NaCl, 15 mM Sodium citrate), and then baked between two sheets of Whatman 3-mm filter paper for 2 hours at 80°C. The membrane was prehybridized for 30 min at 45°C in 50% formamide, 4X SSC , 4X Denhardt's, 20 mM NaH_2PO_4, 1.0% glycine and 150 µg/ml herring sperm DNA, pH 7.5. After 30 min, the prehybridization solution was removed and 9.5 ml of hybridization solution [50% formamide, 3X SSC, 50 mM NaH_2PO_4 (pH 6.5), 100 µg/ml herring sperm DNA, 10% Dextran sulfate, pH 7.5] containing 100 µl of the radioactive probe was added. The filter was then hybridized at 45°C overnight.

After hybridization, the filter was washed once in 2X SSC, 0.5% SDS, 0.1% sodium pyrophosphate at room temperature, followed by three washes, 15 min each at 68°C with constant agitation, in the same solution. The filter was then washed once for 15 min in 0.5X SET, 0.1% Sodium pyrophosphate at 68°C with constant agitation, air dried and exposed to x-ray film for 7 days at -70°C with intensifying screens.

Statistical Analysis

Overall survival results were analyzed by a X^2 test for equal proportions, and multiple comparisons of survival percentages analyzed by a Tukey-type comparison according to Zar ('84). Levels of significance were set at $p<0.05$.

RESULTS

Injection technique

Of the three needle sizes tested, only the 30-gauge provided adequate volume and puncture control with satisfactory survivals. The 33 gauge, in all cases, was too small and became clogged with chorionic proteins after only a few injections, while the 27 gauge was too large and resulted in extraordinarily high mortalities after several days of egg incubation (data not shown). The basis for a successful injection with the 30-gauge needle is outlined in Figure 4. If the bevel of the needle is completely submerged beneath the chorion, then all volume control is lost and the DNA solution flows uncontrolled out of the puncture wound and over the surface of the egg. In addition, the completely submerged needle is not easily removed from the injected egg, requiring the use of forceps to remove the egg from the needle which results in high egg mortality. If, on the other hand, the bevel of the needle is only partially or half-sunk into the chorion of the egg, then there is an equalization of pressure across the chorion so that a controlled, smooth flow of DNA solution is injected into the perivitelline space. Furthermore, a partially sunk bevel allows the needle to be withdrawn without incident, minimizing extraneous damage to the egg. With the microinjection system described previously and the injection technique outlined above, it is possible to inject up to 100 eggs/hour by one person working alone.

Sites of injection

Survival of individually injected groups is outlined in Table 1. Survival of sham controls was ≥95% in all cases, while puncture controls and injected eggs showed extreme variability between egg groups. Overall, injection site did not affect egg survival (for injected groups, X^2=0.1371, 2 d.f., p>0.95), with ~30% average survival for each of the orientations. Due to the rapidity with which eggs could be randomly injected, the random injection technique was utilized for all subsequent injections, even though the effects of injection site on DNA uptake had not been established.

Time-course injections

Overall time-course results, plotted in Figure 5, show a sharp decrease in egg survival with increasing time of injection post fertilization. Extreme variability in egg quality between individual females also occurred during the time-course series (Table 2), but the same general trend was observed with all groups of injected or punctured eggs. A maximum survival of 90% was

obtained for injections within 2-3 hours post fertilization, although high survivals can be obtained to 4 hours post fertilization. Many egg groups became contaminated with naturally occurring river pathogens, which resulted in 0% survival (Table 2). In those cases where egg mortality was a result of fungal contamination, the results as shown in Figure 5 were adjusted to account for the nonprocedural mortalities. The overall test for equal proportions among injected groups in the adjusted data showed a highly significant difference (X^2=108.6, 6 d.f., p<0.001), and multiple comparisons between proportions revealed that both the 1- to 2-hour and 2- to 3-hour injected groups were significantly different from each other and all other injected groups (p<0.001).

DNA concentration

Two separate groups of eggs were injected, but trout J eggs were adversely affected by some unknown factor. Not only was egg mortality high and fertilization low (Table 3), but most of the eggs that survived to the eye-up stage or hatching (including uninjected sham controls) were grossly deformed, showing severe scoliosis, underdeveloped cardiovascular systems and missing eyes. Even though high mortality and deformity rates occurred in uninjected controls, the trout J data indicate no effect (X^2=4.0127, 4 d.f., p>0.25) of DNA concentration on egg survival. Trout K eggs, taken alone (X^2=4.8202, 4. d.f., p>0.25) or both groups taken together (overall X^2=0.2359, 4 d.f., p>0.99) also support the hypothesis that DNA concentration did not affect egg survival.

Injected 0-3 hr - introduce variability? see above

Dot blot analysis of injected fry

Results from a dot blot analysis of 77 injected fry are shown in Figure 6. Positive controls were tail blots from transgenic mice known to be carrying the bGH gene, and negative controls were 3.75 µg genomic DNA taken from uninjected control trout. Three individuals (4%) hybridized with the radioactive bGH probe (#20, 69, 80), indicating that the injected DNA fragment (mMT-bGH) was still present in those three fish and that the microinjection technique is capable of introducing foreign DNA into the developing egg. The dot blot, as shown, has been repeated with identical results.

no fig 6

DISCUSSION

One of the major limitations in transgenic fish work is the speed with which eggs can be treated and the relatively low survival of injected eggs. In those species lacking an egg chorion or where the chorion can be easily removed by mechanical or enzymatic manipulations, application of mouse injection techniques using glass capillary needles works well (e.g., Vielkind et al., '82; Zhu et al., '85a,b; Ozato et al., '86). In trout and other salmonids, however, lack of a dechorionation protocol coupled with a very hard and impermeable egg chorion prevents direct injection into the germinal disk. To overcome the chorion barrier, Rokkones et al. ('85) published a brief note on the injection of DNA into fertilized atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) eggs which was later modified by Chourrout et al. ('86) to become the principal method of trout egg injection. Generally, a small hole is bored in the chorion directly above the germinal disk. Then, a glass capillary needle is inserted into the germinal disk, where the DNA solution is deposited. Brem et al. ('88) also described a one-step injection through the micropyle of the fertilized egg, where the DNA solution is deposited into the perivitelline space or germinal disk. Both techniques have successfully introduced foreign DNA into developing trout embryos, but they are both extremely slow (30 eggs/hr) and labor intensive. Recently, Penman et al. ('90) attempted to address the relative merits of previous injection techniques, DNA concentrations, buffers and DNA structure on the survival and frequency of DNA uptake in microinjected rainbow trout eggs, but survival of injected eggs was very low (0-40%) and frequency of DNA uptake was generally 5-10%, consistent with previous transgenic fish work.

In this report, the high survival rates (up to 100%) of several injected groups indicate that the stainless steel needle is a useful alternative to the "traditional" two-step injection technique, comparing favorably with many of the published reports. The success of the injection technique depends critically on proper penetration of the needle, and the ability to inject 100 eggs/hr helps overcome some of the effects of poor egg quality. The random injection technique was used in all experiments because eggs could be quickly injected, but the effect of egg orientation on DNA uptake has not been established. Therefore, the possibility remains that top or side injections, which deposit the DNA at the highest concentration closest to the developmental focal point, result in a greater incidence of DNA uptake.

The time course series revealed a distinct window of opportunity that results in maximum survival, and the 90% average survival within 2-3 hours post fertilization is one of the highest reported to date. The results also showed that the trout eggs were able to withstand chorion puncture up to 4 hours post fertilization, but it is not known whether the sharp decrease in survival for injected groups at 3-4 hours post fertilization results from DNA toxicity during a developmentally sensitive period, an artifact of relatively small sample size (3 egg lots), or some other factor. The survival results were consistent with expectations, because the longer an egg water hardens, the harder the chorion becomes and the more static pressure exists

within the perivitelline space. Puncture of the chorion, then, becomes progressively more difficult and increasingly disruptive with time, resulting in more egg damage than occurs shortly after fertilization. The noticeable and significant drop in sham control survival percentages after 5-6 hours post fertilization [p ($q_{\alpha,\infty,7} > 6.36$) <0.001] also indicates that the egg-handling technique has a negative impact on egg survival with increasing time. As with the orientation experiment, however, maximum DNA uptake and integration might occur at a time that does not correspond with maximum egg survival.

The problems encountered with Trout J eggs remain unexplained. The deformities and mortalities may have resulted from a water change during the time of neural tube formation and blastopore closure, but other egg lots in the same developmental stage were unaffected. An initial suspicion that 0.1% phenol red adversely affected egg survival was dispelled with the DNA concentration experiment, since in both egg lots the NO DNA group had similar survivals with other DNA dose groups. The DNA concentration range tested is consistent with other transgenic fish work, but only represents injections of 0.3 fg-0.03 pg DNA/egg. Stuart et al. ('88) showed that cytoplasmic injection of 90 pg DNA/zebrafish embryo is lethal, a figure 300 times greater than the maximum concentration injected into the rainbow trout eggs. However, Stuart et al. injected foreign DNA into the cytoplasm of zebrafish embryos, which do not contain a chorion or perivitelline space and are orders of magnitude smaller than trout and salmon eggs. Penman et al. ('90) also showed a trend toward reduced survival of \ trout eggs with increasing DNA concentration, but a separate experiment revealed no such trend; it is possible, then, that the reduced mortalities with increasing DNA concentration represented a time effect similar to that described here. Because the DNA must cross the yolk (vitelline) membrane before "uptake" or integration when injected into the perivitelline space of the fertilized trout egg, it may be possible to inject DNA concentrations in excess of 10^{10} copies/100 nl, thereby enhancing the probability of DNA uptake.

In conclusion, we have developed a fast and efficient injection procedure for gene transfer *in vivo* in rainbow trout. The stainless steel injection technique should also be applicable to other salmonids and any fish eggs that possess a hard and impermeable chorion, aiding in the development of transgenic trout and salmonids. A 4% DNA uptake rate is comparable to previous work in the field, underscoring the usefulness of the technique in developing transgenic fish. That the described injection technique generated positive individuals also indicates that the injected DNA is able to cross the vitelline membrane and persist for 50-60 days post hatching, obviating the need for direct injection into the germinal disk. To further optimize the technique, however, a number of other parameters should be investigated, including buffer composition and pH, injection volumes, DNA size, form and end structure, and higher DNA concentrations.

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Table 1. Summary of trout eggs microinjected in different sites. Values are percent survival to hatching. D,E,F = Egg lots; O = Overall.

	Site of Injection								
Egg[a]	Top			Side			Random		
Lot	Inj.[b]	Punc.[c]	Con.[d]	Inj.	Punc.	Con.	Inj.	Punc.	Con.
D	73%	80%	98%	67%	69%	94%	72%	54%	94%
E	4%	16%	88%	12%	18%	90%	6%	0%	94%
F	0%	54%	100%	4%	22%	100%	10%	64%	96%
O	28%	51%	95%	28%	36%	95%	29%	39%	95%

[a] D,E,F = Egg lots; O = Overall results
[b] Inj. = Injected groups
[c] Punc. = Puncture control, no DNA injection
[d] Con. = Uninjected sham control

Table 2. Summary of trout eggs injected at various times post-fertilization. Values are percent survival to hatching.

Egg Lot[a]	Time of injection post-fetilization (hrs.)											
	0-1			1-2			2-3			3-4		
				Inj.	Punc.	Con.	Inj.	Punc.	Con.	Inj.	Punc.	Con.
G	Water-Hardening			63%	90%	100%	88%	91%	97%	27%	77%	97%
H				83%	73%	93%	83%	77%	97%	19%	77%	97%
I				84%	87%	100%	100%	87%	100%	93%	87%	100%
O	-	-	-	77%	84%	98%	90%	85%	98%	46%	79%	98%
Egg Lot	4-5			5-6			6-7			7-8		
	Inj.	Punc.	Con.	Inj.	Punc.	Con.	Inj.	Punc.	Con.	Inj.	Punc.	Con.
G	41%	41%	100%	16%	48%	100%	-	-	-	-	-	-
H[b]	0%	0%	93%	10%	0%	70%	17%	3%	70%	13%	13%	83%
I	43%	67%	100%	43%	37%	100%	57%	43%	97%	50%	37%	97%
O	29%	36%	98%	23%	29%	90%	37%	23%	83%	32%	25%	90%

[a] G,H,I = Egg lots; O = Overall results.

[b] Low values for trout H injected and puncture controls (4-5, 5-6, 6-7 hours) resulted from fungal contamination.

Table 3. Summary of trout eggs injected with various DNA concentrations. Values are percent survival to hatching.

Egg Lot[a]	DNA Concentration (Copies/100 nl)														
	NO DNA			10^4			10^5			10^6			10^7		
	Inj.	Punc.	Con.	Inj.	Punc.	Con.	Inj.	Punc.	Con.	Inj.	Punc.	Con.	Inj.	Punc.	Con.
J	7%	10%	10%	12%	10%	35%	10%	5%	12%	5%	1%	8%	17%	0%	17%
K	58%	33%	100%	60%	45%	100%	60%	60%	-	64%	41%	-	36%	48%	-
O	26%	18%	34%	28%	22%	48%	27%	23%	12%	26%	16%	8%	25%	18%	17%

[a] J,K = Egg lots; O = Overall results.

FIGURE LEGENDS

Figure 1. Restriction map of pUC-mMT-bGH.

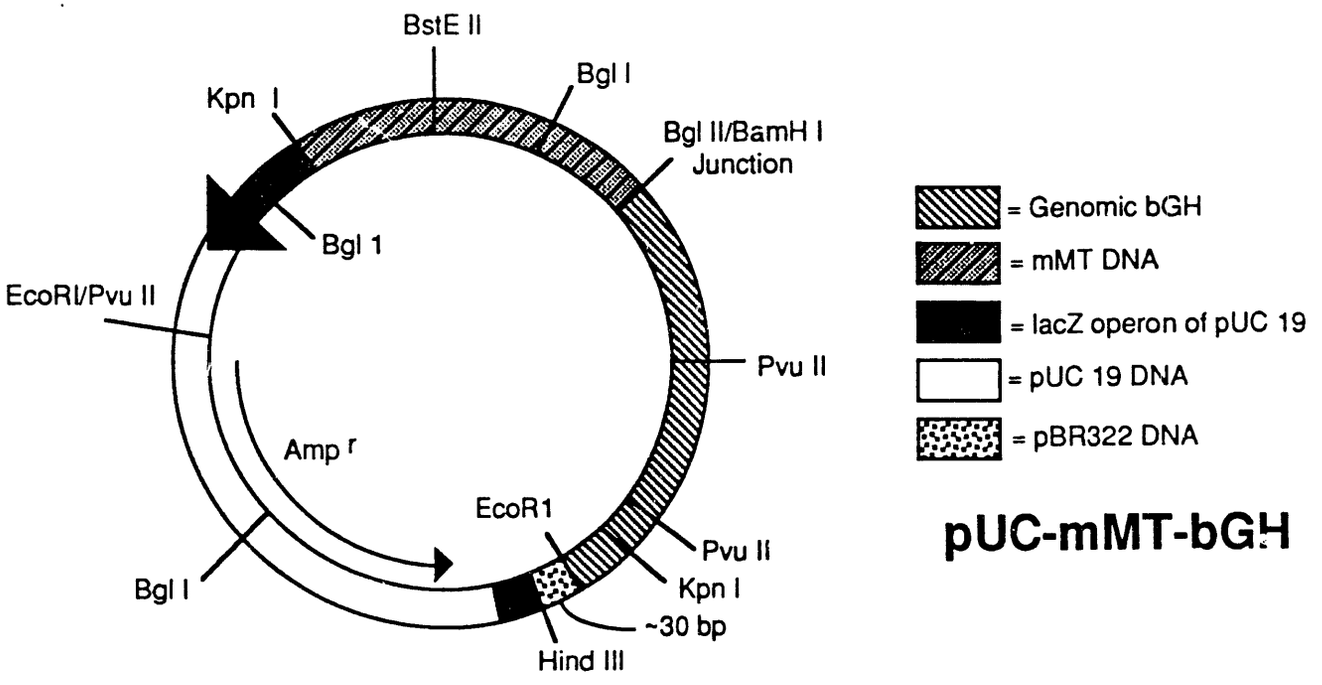
Figure 2. Description of top, side and random injection sites.

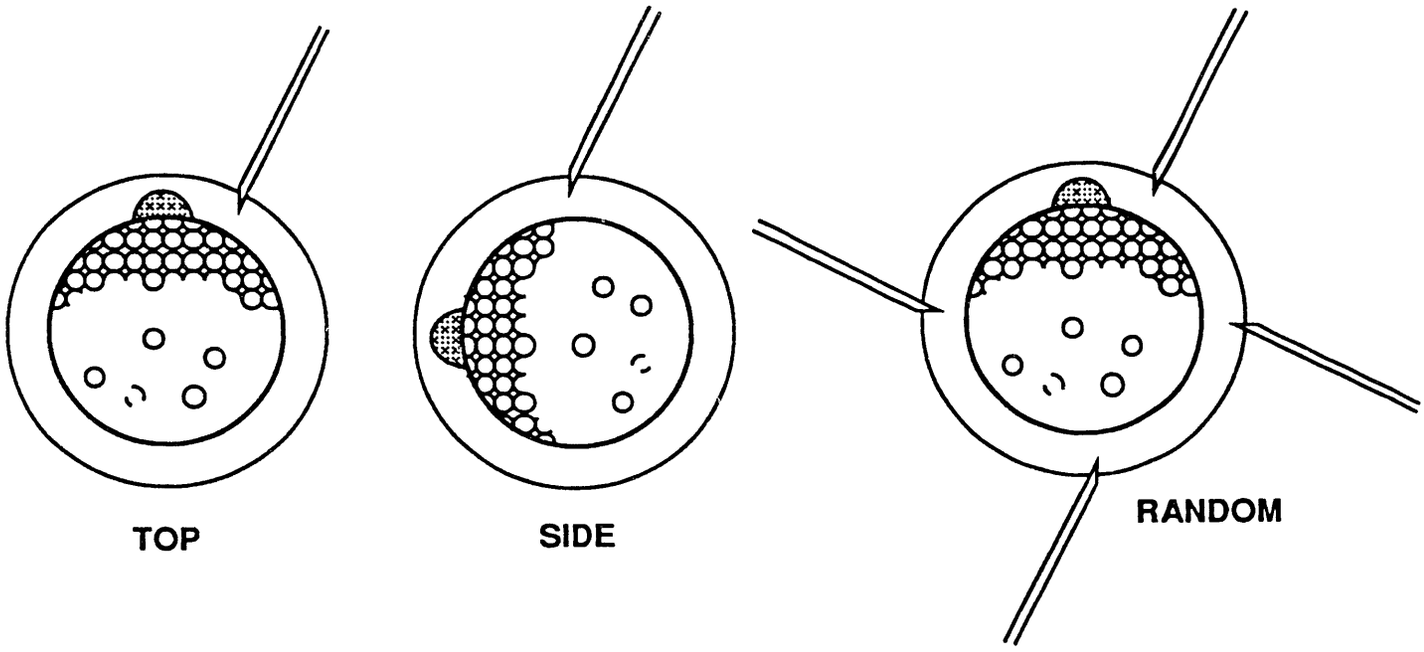
Figure 3. Linear restriction map of the bGH gene in pUC-mMT-bGH. Exons are boxed and numbered. The small *Pvu* II to *Eco*RI fragment (3' end) was nick translated for probing dot blots.

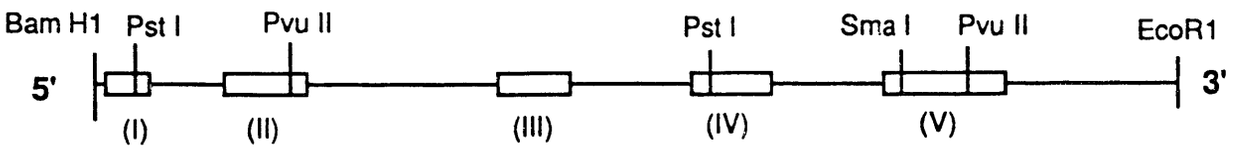
Figure 4. Description of correct injection technique with the 30-gauge needle. If bevel is completely submerged beneath the chorion, volume control is lost and DNA solution flows freely out of the egg and over the chorion. If bevel is partially submerged, DNA solution flows smoothly into the perivitelline space.

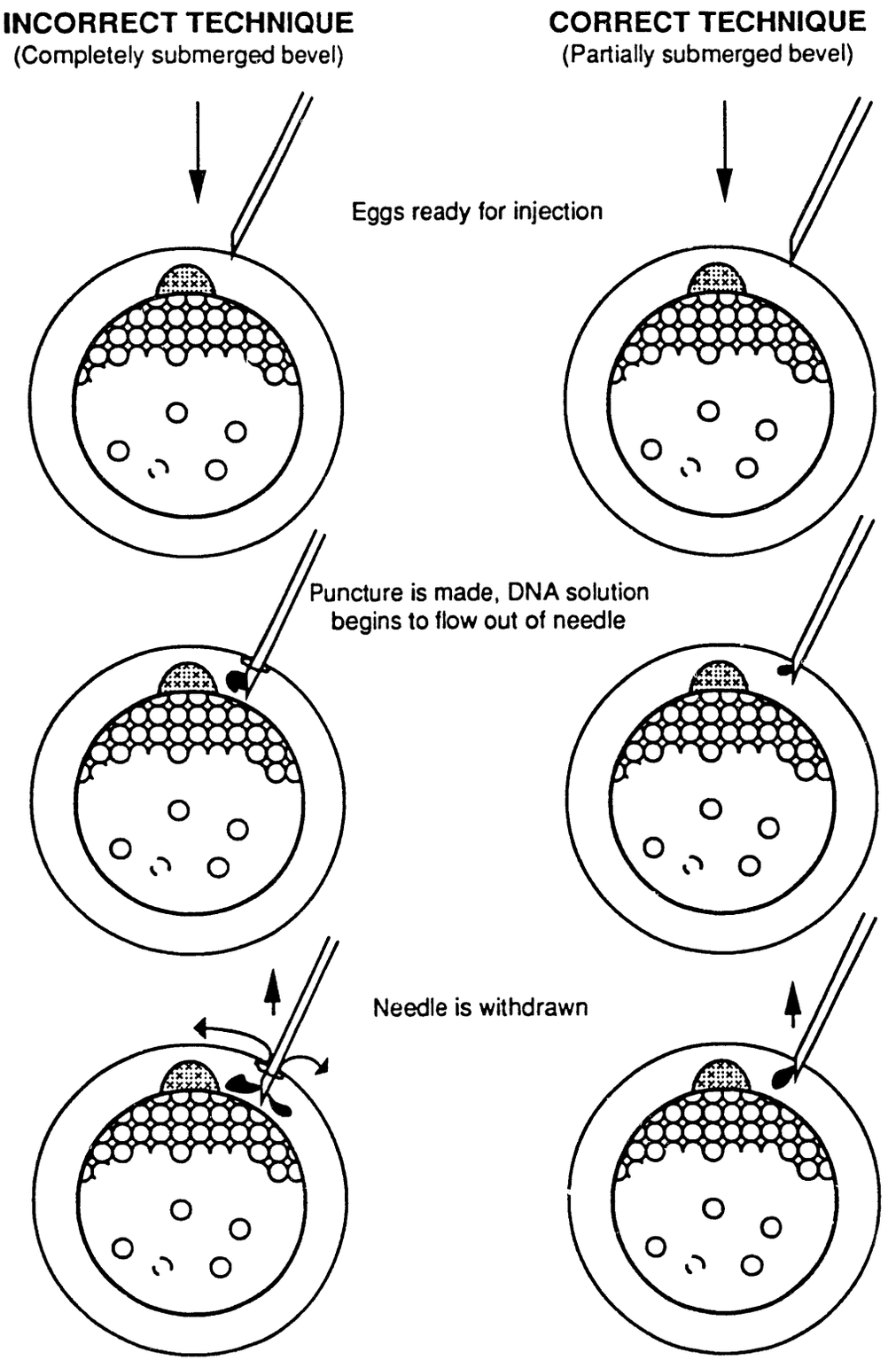
Figure 5. Overall survival of randomly injected eggs at various times post fertilization.

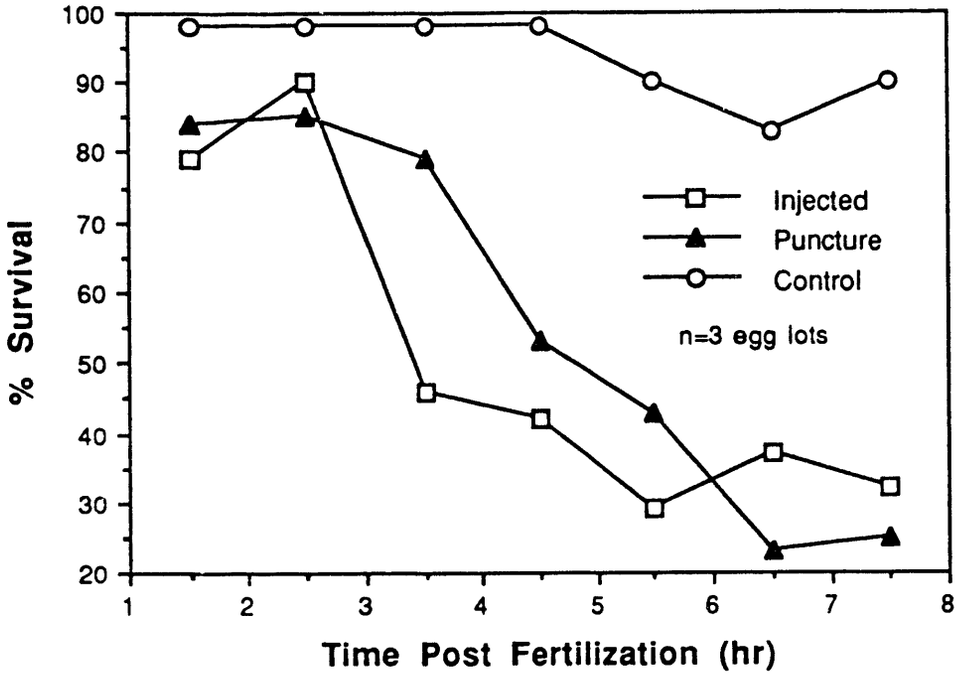
Figure 6. Dot blot results from microinjected rainbow trout fry. Each dot represents 3.75 μg genomic DNA. Positive controls (+) were tail blots from transgenic mice known to be carrying the bovine growth hormone gene.

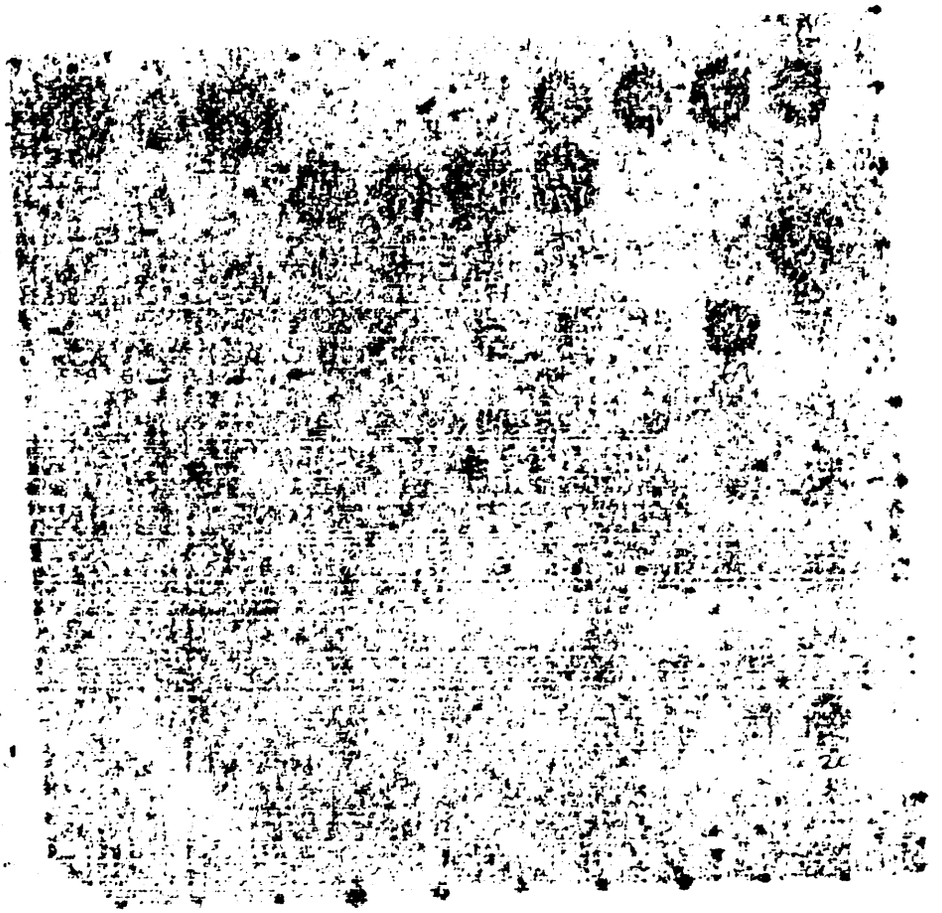

BstE II
Bgl I
Kpn I
Bgl II/BamH I
Junction
Bgl 1
EcoRI/Pvu II
Pvu II
Amp r
EcoR1
Pvu II
Bgl I
Kpn I
~30 bp
Hind III
= Genomic bGH
= mMT DNA
= lacZ operon of pUC 19
= pUC 19 DNA
= pBR322 DNA
pUC-mMT-bGH


TOP
SIDE
RANDOM


Bam H1
Pst I
Pvu II
Pst I
Sma I
Pvu II
EcoR1
5'
3'
(I)
(II)
(III)
(IV)
(V)


INCORRECT TECHNIQUE
(Completely submerged bevel)
CORRECT TECHNIQUE
(Partially submerged bevel)
Eggs ready for injection
Puncture is made, DNA solution
begins to flow out of needle
Needle is withdrawn


100
90
80
70
60
50
40
30
20
% Survival
1
2
3
4
5
6
7
8
Time Post Fertilization (hr)
Injected
Puncture
Control
n=3 egg lots



b6H

END

DATE FILMED

01/03/92