GENE TRANSFER IN NILE TILAPIA *(OREOCHROMIS NILOTICUS)* USING HIGH – VELOCITY MICROPROJECTILES

BY

Omyma E. Khafagy* and Adel M . Tawfik**

**Genetic Engineering and Biotechnology Research Institute (GEBRI) Minufiya Univ. Sadat City,Egypt.*

*** National Research Centre ,Egypt.*

ABSTRACT

Eggs were obtained from females and fertilized artificially for experiments in the period of blastodisc formation and up to the fourth division of cleavage . Tilapia zygotes and early embryos were collected in the course of natural spawning. Tilapia fish were bombarded with high- velocity microprjectiles coverd with plasmid DNA containing sequences of B- galactosidase and neomycin phosphotransferase genes. About 65% of the eggs survived the bombardment. The activity of both transferred genes was revealed in the fish developed from the bombarded eggs . The expression and inheritance of the transgenes and phenotype differences between normal and transgenic fish are considered . The efficiency of improving the growth rates of fish is discussed . The production of transgenic fish using gene transfer technology is also discussed.Neomycin phosphotransferase gene was detected by polymerase chain reaction PCR amplification and Southern hybridization in the total DNA of tilapia.

INTRODUCTION

Several genes were introduced into fish such as the anti – freeze protein AFP (Hew and Yang .,1992), disease resistance,(Shears et al ., 1991) , somatotrophic hormone from fish , mouse , and human. Methods for gene transfer in fish are Electroporation(Inoue.,1992), Microinjection),Sperm binding(Chourrout and Perrot .,1992).Direct injection into fish muscle (Rahman and Maclean 1992), Electroported Sperm (Chen et al., 1998) and Lipofection .

The tilapia growth hormone (tiGH) cDNA was used to construct chimaeric genes expressing different levels of tiGH in vitro and in vivo (Hernandez et al ,. 1997). The use method of microprojectiles for genration of transgeneic fish has many advantages over microinjection . Microprojectile method is much faster and less demanding technically than microinjection .Foreign genetic information can be introduced into fertilized fish eggs of different species (Yoon et al .,1990). The introduced genes can be genetically integrated (Zhang et al ., 1990) and , in some cases, phenotypically expressed (Martinez et al ., 1997) . In all these works , the genetic material was introduced by the microinjection of foreign DNA into fertilized fish eggs . In this study have applied the method of (Zelenin et al .,1991) high velocity mechanical DNA injection for this purpose. The method involves the bombardment of cells with small tungsten particles (microprojectiles) covered with DNA containing the genes to be introduced (Shalnik and Orkin .,1990). The method allows one to deliver foreign DNA directly and promptly into cell nuclei without its exposure to the action of cytoplasmic nucleases (Zelenin et al ., 1991). In this work the method

was modified to transfect animal cells and demonstrated its applicability to the stable genetic transformation of NIH 3T3 mouse cells in culture (Shalnik and Orkin ., 1990). Moreover , the method makes it possible to introduce foreign genes into organ explants of rodents in vitro and also can be applied to rat liver cell transfection in vivo (Pursel et al.,1989) .

MATERIALS AND METHODS

Eggs and Embryos :

Tilapia (*Oreochromis niloticus*) were originally collected from the Ismailia Canal and bred in special aquarium , between 5 and 8 cm nose to tail length. Control fish were treated identically except that they were bombarded with the buffered salt solution alone. Eggs were collected from a gravid female by gently preeing on the ventral side of the fish and sperms were collected from 2 or 3 males in a capillary tube and stored on ice (Chrisman et al ., 1995). After 2 minutes into a dry glass petri dish water and milt were added to the eggs (18 – 20 C) with gentle stirring to enhance fertilization.

Blastula–stage embryos were harvested approximately 2 hours after fertilization. The eggs were taken in the period of blastodisc fromation and up to the fourth division of cleavage. Individual embryos were confirmed microscopically to be at the correct developmental stage . Immediately before the bombardment , the embryos were placed into a plastic dish and turned manually blastodisc up.

For one experiment approximately 200 – 500 fish embryos were taken. Survival rate was approximately 80%. Embryos were incubated in water at 18 – 25 C , additional water was added to the fertilized tilapia zygotes and embryos.

Early embryos were collected in the course of natural spawning . After spawning (after yolk sac resorption) the fry were kept in plastic aquaria for first feeding with temperature controlled at 27C.Dead embryos were counted and removed . This method was done(1997–1998) at Genetic Engineering and Biotechnology Research Institut (GEBRI) Menoufiya University,National Research Centre,Egypt NRC and Genetic Engineering Centre Faculty of Agriculture,Ain Shams University , Egypt .

Plasmid DNA :

The plasmid used in this work was kindly supplied from Dr. Inessa A. Zelenina , and Vladimir ,A. Barmintzev . Institute of Molecular Biology , USSR Academy of Sciences ,Moscow.

Plasmid containing genes of galactosidase (pRSV- *B*-gal)and aminoglycoside 3-phosphotransferase II (pSV2-neo) were taken for transfection. Plasmid DNA was prepared by standard lysozymealkaline lysis followed by CsCL gradient centrifugation. Tungsten particles 0.3-3.0 µm in diameter were coated with plasmid DNA by calcium-phosphate precipitation as in (Liu et al ., 1990) . 10µL of a DNA solution (1mg/ml) added to 10-15mg of tungsten particles was taken per shot .

Bombardment:

The shooting technology was mainly similar to that used previously for transfection of a mouse culture (Rawson et al ., 1991.) and rodent tissues in explants and in situ. The distance between the end of the barrel and the eggs to be bombarded varied frome 10 to 25 cm depending on the species whose eggs were taken for the experiments (Zelenin et al 1991.,). In our case (Tilapia) *Oreochromis niloticus* the distance between the end of the barrel and the petri dish to be shoot eggs 15 – 20 cm .

Analysis of *B*- galactosidas activity:

The activity of *B*- galactosidase was checked by a standard technique (MacGregor et al,1987) using X-gal as a substrate.The intensity of staining was detected microspectrophotometrically at 450 nm .

Selection of G418 – resistant fish :

Tilapia (*Oreochromis niloticus*) larvae and alevins were placed in 700 mg/mL solution of the antibiotic geneticin (G418 , Gibco) , kept there for 3- 6 days , and then put into fresh water . The concentration of G418 was chosen for the selection of the antibiotic resistant Individuals on the basis of preliminary experiments (Yoon et al .,1990). The embryonic and postembryonic development of the fish speciment and their death were followed up in the experiments .

Plasmid transfection and expression :

Transfection and selection for G418–resistant fish cells were performed as perviously described (Yoon et al .,1990). Expression of *B*–gal was evaluated by colorimetric enzyme assay (MacGregor et al .,1987). Lightly fixed cells (2% formaldehyde 0.2% glutaraldehyde,5 minutes, 4C) were incubated in 5 –bromo –4 – chloro – 3 – indolyl – B – D – glactoside with k ferricyanide/ferrocyanide and detergent at 37C for 24 to 48 hours . A blue color develops if the enzyme is expressed .

Polymerase chain reaction (PCR) was carried out using a commercial kit (GeneAmp , Perkin, Elmer Cetus) as recommended by the vendor in an Eric Comp twinblock thermal. Genomic DNA was separated by a standard phenol-chloroform methods and studied using amplification by the polymerase chain reaction (Maniatis et al ., 1986) with a slight modifications. The reagent mixture contained 10 mM Tris-Hcl ,

PH 9.3 at 22C,50 mM Kcl ,2.5 mM MgCl .1μ M of the direct primer , 1μM of the reverse primer ,0.1 μ g/ml of gelatin, 100 mM of each dNTP,4U of Tth polymerase for each probe , 1μ g of DNA and 100 μ L of mineral oil . Single- stranded 20 b sequences were used as direct and reverse primer .

Temperature : 1 min at 94 C , 1 min at 55 C, 2 min at 72 C . The aliquot of the mixture per probe was 50μ L without mineral oil . Amplification products were analyzed in 10% Polyacrylamide Agarose Gel (PAAG) with subsequent ethidium bromide staining . 10 μ L of the reagent mixture was applied to one electrophoretic line .

RESULTS AND DISCUSSION

The shooting technology was mainly similar to that used previously to transfect a mouse culture (Shalnik and Orkin.,1990).The destruction of fish eggs by bombardment depended to a great degree on the distance between the end of the barrel and the plate which contained the material to be bombarded as well as on the size of microprojectiles (Bio rad) and their number in a pencil .

The following conditions for egg bombardment and efficient cell transfection were chosen by varying these parameters : the distance between the end of the barrel and the Petri dish 15 – 25 cm for eggs . A mixture of tungsten particles 0.1 - 1.5 μ m in diameter was taken to shoot small eggs and of 1.0 – 3.5μ m for bigger eggs . The total weight of particles per shot was the same in both cases . When the cells were bombarded under these conditions , about 65% of fertilized eggs of all fish taken for experiments survived bombardment . In the second series of experiments ,

Developing indivduals were analyzed for the activity of *B*- glactosidase 3 day after the bombardment with microprojectiles which had carried the corresponding gene. In about 5% of the larvae studied , a positive reaction for the enzyme activity was revealed (50 times exceeding the control) . Thus the gene for *B*- glactosidase was shown to be transferred by high velocity microprojectiles into fish fertilized eggs and expressed in their larvae . In the third series of experiments , we studied how a Neomycin phosphotransferase gene introduced into tilapia influenced its resistance to antibiotic geneticin (G418) as (Yoon et al 1990). During the first two days of the incubation with (G418) ,we found no differences (non–bombarded) and experimental (bombarded) fish . Differences appeared only by the 3 rd – 5th day. At the end of 3 – 6 days destruction commenced in both the control and experimental (bombarded individuals . At that period a G418 solution was replaced by running or repeatedly changed water, the destruction of fish embryos went on but its dynamics differed in the control and in the experiment . As one found the survival of specimens with introduced neo gene was higher than in control ones .

These experiments have thus demonstrated that the introduction of the neomycin phosphotransferase gene made some of the developing embryos resistant to the action of G418. It means that the introduced gene was expressed in their cells . Our results are consistent with the data of experiments in which the neo gene was introduced into goldfish (*Carassius auratus*) eggs zebrafish (*Brachydanio rerio)* eggs, and medaka *(Oryzias Latipes*) eggs by a microinjection technique (Chrisman et al., 1995).The fourth series of experiments was concerned with detecting the neumycin phosphotransferase gene in the DNA of tilapia larvae resistant to G418. Hundred larvae were analyzed by PCR technique in this series . In two cases fish were shown to contain sequences similar to those of the neo gene (Fig. 1). The results of amplification experiments were confirmed by electroblotting and a standard hybridization technique.(Fig.1). In the last series of experiments , transfected alevins which had survived after the G418 treatment were analyzed for a neo DNA sequence . In all our cases the presence of a neo DNA sequence was revealed by PCR. Our experiments have thus shown the applicability of high – velocity microprojectiles to the introduction of foreign DNA into developing fish embryos . The reasonable survival , the expresstion and the presence of the introduced gene during a rather long time of development indicate that the method can be used for creation of transgenic fish .

REFERENCES

Chen,J.,Tsay,T.,Shie,J.,Chern,R.,Chen,JD.,Tsay,TT.,Shie,JM.,Chern,RH. (1998): Generation of transgenic tilapia by using cryopreserved electroporated sperm to mediate gene transfer.J.Fisheries, Taiwan.25:4,265-272.

Chrisman,C.L.,Thomas,T.CH.,and Orania , M. A.(1995): Integration expression and germ–line transmission of foreign growth hormones genes in medaka (*Oryzias Latipes*). Mol Marine Biol Biotechnology 1(4/5),366-375.

Chourrout Daniel and Perrot Elizabeth (1992): No Transgenic rainbow Trout produced with sperm incubated with linear DNA Mol Marine Biol Biotechnology 1(4/5),282 – 285 .

Hernandez O., Guillen I ., Estrada MB., Cabrera E., Pimental R., Pina JC., Abad Z., Sanchez V ., Hidalgo Y . and Fuente J. (1997): Characterization of transgenic tilapia lines with different ectopic expresstion of tilapia growth hormone . Mol Marine , Bio and Biotech .6:4,364 – 375 .

Hew C.L. and Yang D.S.C.(1992): Protein interaction with ice Eur J Biochem 203:33-42.

Inouei Koj (1992): Expression of reporter genes introduced by microinjection and electroporation in fish embryos and fry Mol Marine Biol Biotechnology 1 (4/5) , 266 – 270

Liu.Z., Moav,B., Faras,A.J., Guise,K.S., Kapuscinsk,A,R. and Hackett,P.B. (1990): Functional analysis of elements affecting expression of the B-actin gene of carp.Mol Cell Biol 10:3432-3440 .

MacGregor,G.R., Mogg, A.E., Bruke, J.F., and Caskey ,C.T.,(1987): Histochemical staining of clonal mammalian cell lines experssing E.coli B- glactosidase indicates heterogeneous expression of the bacterial gene .Somatic Cell Mol Genet 13 : 253 – 265 .

Maniatis , T ., Fritsch, E. F., and Sambrook, J. (1986): Molecular Cloning : A Laboratory Manual .(Cold Spring Harbor Laboraory ,Cold -Spring Harbor ,New York).

Martinez , R .,Lieonart , R ., Fuente ,J,de,la ., and De,La, Fuente,J.(1997): Characterization of transgenic tilapia lines with defferent ectopic expresstion of tilapia growth hormone. Molecular Marine ,Biology and Biotechnolgy .6:4 , 364 – 375 .

Pursel V.G., Pinkert C.A., Miller K.F., Bolt D.J.,Campbell R,G., Palmiter R.D. and Brinster R.L. (1989): Genetic engineering of livestock .Science 244,1281 –1288.

Rawson , C., Loo , D . , Hedstrom , O ., Schmidt , E ., and Barnes, D.(1991): Death of serum – free mouse embryo (SFME) cells caused by EGF deprivation.J Cell Biol 113:671-680 .

Rahman Azizur and Maclean Norman (1992): Fish transgene expression by Direct injection into fish muscle.Mol Marine Biol and Biotech1(4/5),286–289.

Shalnik,D.,and Orkin,S.(1990): Simple screening method for transgenic mice using polymerase chain reaction . Biotechnology 8:1-32 .

Shears,M.A ., Fletcher, G. L ., Hew, C .L., Gauthier,S., and Davies,P.L.,(1991): Transfer , expression ,and stable inheritance of antifreeze protien genes in Atlantic salmon (*Salmo salar)* . Mol Marine Biol Biotechnology 1:58 – 63 .

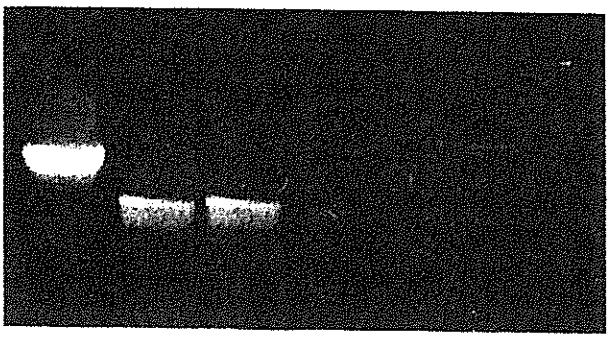
Yoon,S.J.,Hallerman,E.M.,Gross,M.,Liu,Z.,Schneider,J.F.,Aras, A.J., Kapuscinski, A.R. and Guise,K.S.(1990): Transfer of the gene for neomycin resistanse into goldfish (*Carassius auratus)* Aquaculture 85:21-33.

Zelenin V. Alexander, Alimov A Andrey, ,Barmintzev A Vladimir, Beniumov O, Alexei , Zelenina A Inessa, Krassnov M, Alexei and Kolesnikov A. Viktor (1991): The delivery of forein gene into fertilized fish eggs using high velocity microprojecties . Volume 287 , number 1,2,118 – 120 Federation of European Biochemical Societies00145793 91/53 – 50 .

Zhang,P.,Hayat,M.,Joyce,C.,Gonzalez.,Yoon,S.J.,Hallerman,E.M.,Gross,M.L..,Schneider,J.F.,faras,A.J.,Kapuscinski,A.R.,and Guise,K.S.(1990): Transfer of the gene for neomycin resistanse into goldfish (*Carassius auratus*). Aquaculture 85:21-33.

ACKNOWLEDGMENTS

The authors would like to express their thanks to Dr.Vladimir. A. Barmintzev and Dr. Inessa. A. Zelenina Institute of Molecular Biology. USSR Academy of Sciences. Moscow for their kind support providing the material and advice during the course of this study



Fig,1: Transfected neomycin phosphotransferase gene in the DNA of Tilapia larvae (100) analyzed by PCR fractionated on 10 % agarose gels , results by blot hybridization . Lane 1, positive control (plasmid DNA pSV2-neo) , Lanes 2,3 and 6 fish DNA (positive results) , lanes 4 and 5 DNA from untransfected fish .

الملخص العربى

استخدام الطلقات المدفعية فائقة السرعة فى النقل الجينى لأسماك البلطى النيلى.

أميمة خفاجى

*معهد بحوث الهندسة الوراثية والتكنولوجيا الحيوية - جامعة المنوفية - مدينة السادات - المنوفية - جمهورية مصر العربية.

بعد الحصول على البيض المخصب صناعيا لأسماك البلطى النيلى تم حقنه فى مرحلة مبكـــرة جدا من النمو الجنينى بجين النيومسين حيث حقن ما يقرب من ٦٥ % من البيض المخصب وتـــم متابعـــة نشاط وفعل الجين الذى تم فحصه بعد عدة ايام من النمو الجنينى وباستخدام تقنية اكثار المادة الوراثيـــة عن طريق جهاز ال PCR وتهجين المادة الوراثية امكن اكتشاف وجود التتابعات المحددة للجين الذى تم حقنه مما يمكن من الحصول على اسماك محورة أو معدلة وراثيا باستخدام هذه الطريقة.