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The Effect of Linear Versus Circular Vector on Enhanced Green Fluorescent Protein (EGFP) Expression in Transgenic Zebrafish (*Danio rerio*)

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Abstract: Green Fluorescent Protein (GFP) has been used as an indicator of transgene expression in living cells and organisms. In this study, a transgene construct containing the Cyto-Megalo-Virus (CMV) promoter sequences, SV40 polyA signal and the Enhanced Green Fluorescent Protein reporter gene (EGFP) was microinjected into the cytoplasm of one-cell zebrafish embryos. About 65 ng μL^{-1} circular and linearized pEGFP-N1 DNA was used in microinjection. Transgenic founders were detected by Polymerase Chain Reaction (PCR), Slot and Southern blots and Reverse-Transcriptase PCR (RT-PCR). EGFP gene expression was detected by inverted fluorescence microscope in F_0 transgenic zebrafish larvae. About 54 and 25 F_0 transgenic zebrafish were obtained after microinjection of linearized and circular gene constructs, respectively. This is the first study for generation of transgenic zebrafish via cytoplasmic DNA microinjection in Turkey. In conclusion, these results indicate that the gene expression efficiency of circular form was higher than the linearized form in F_0 transgenic zebrafish larvae.

Key words: Transgenic fish, transgenic technology, fertilized fish egg, microinjection, *Aequorea victoria*, *Danio rerio*

INTRODUCTION

The transgenic technology was first applied to fish in mid 1980's. Several transgenic fish species including rainbow trout, medaka, zebrafish etc. were used in transgenic studies (Chourrout *et al*., 1986; Brem *et al*., 1988). Especially, zebrafish has proved to be a useful model for transgenic studies. This is due to the facts that zebrafish embryos are optically transparent, develop rapidly and hatching eggs at 2-3 days postfertilization (Westerfield, 1995).

For about the 20 years, transgenic fish have been actively used in both basic and applied research. Several methods are available to monitor gene activity and protein distribution within the cells. These include the formation of fusion proteins with coding sequences for β-galactosidase and luciferase (Chalfie *et al*., 1994). Alternatively, flourescent protein GFP is a simple and sensitive detection method. GFP is a photoprotein of a bioluminescent jellyfish, *Aequorea victoria* (Bagis and Keskintepe, 2001; Chalfie *et al*., 1994). GFP used as a reporter gene in various organisms and did not appear to have a toxic effect on the cells (Bagis and Keskintepe, 2001; Takeuchi *et al*., 1999). It does not require an enzyme substrate reaction (Amsterdam *et al*., 1995; Hamada *et al*., 1998; Peters *et al*., 1995; Takeuchi *et al*., 1999) and fixation procedure, only exposure ultraviolet with suitable excitation wavelength (Chalfie *et al*., 1994). Thus, it should provide an excellent means for monitoring gene expression and protein localization in living cells (Chalfie *et al*., 1994). The objective of this study was to investigate comparison of linear versus circular vector on Enhanced Green Fluorescent Protein (EGFP) expression efficeincy in transgenic zebrafish (*Danio rerio*).

MATERIALS AND METHODS

Zebrafish maintenance and diets: Fish were spawned and cultured on a light/dark cycle essentially as described by Westerfield (1995). Non-transgenic and transgenic fish were fed on a commercial diet 3 times a day and *Artemia nauplii* once a day.

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Preparation of vector DNA: The vector construct, the 4.7 kb pEGFP-N1 plasmid containing the EGFP gene driven by the CMV promoter was purchased from Clontech Laboratories (Clontech Laboratories, Palo Alto, CA). Plasmid DNA was purified by using a commercially midiprep kit (Qiagen), resuspended in water and used in the circular and linearized forms. For linearization, the pEGFP-N1 vector DNA was digested by VspI and AflII, MBI Fermentas. A linear plasmid fragment containing CMV promoter, the EGFP reporter and the SV40 polyA signal was isolated using the EZ-10 Spin Column DNA Gel Extraction Kit (BioBasic, BS 354). The purified DNAs were quantified and diluted to 65 ng μL^{-1} in Tris-EDTA buffer (1 mM EDTA, 10 mM Tris-HCl, pH 7.4).

Cytoplasmic microinjection and analysis of transgene expression: Embryos rolled down the agarose ramp and lined up in the groove. Circular or linearized gene constructs were injected into the cytoplasm of fertilized eggs at the one-cell stage. Injections were performed under a stereo microscope (Magnification: X2) (Soif, DA 0671). EGFP expression was detected by observing 3 days old zebrafish larvae with an inverted fluorescence microscope (Carl Zeis, Axiovert 35M). Photographs were taken by camera system.

Isolation of genomic DNA and PCR analysis of integrated transgenes: The DNA was extracted from 3 and 10 days old transgenic and non-transgenic larvae. Isolation of genomic DNA was performed by using a DNA isolation kit (EZ-Spin Column DNA Isolation Kit, Biobasic) according to the manufacturer's instructions. Total genomic DNA samples were analyzed by PCR for the presence of the EGFP transgene. PCR was performed with approximately 100 ng of genomic DNA, 20 pmol of each oligonuclotide (sense and antisense primers), 2X PCR Master Mix (MBI Fermentas, Germany). The PCR cycle parameters were 30 sec at 95°C, 60 sec at 58.7°C and 90 sec at 72°C for 30 cycles. The EGFP specific primers used to amplify a 712 bp fragment of the target sequence were5'-ATGGTGAGCAAGGGCGAGGAGCTGT-3'(sense) and 5'-TACAGCTCGTCCATGCCGAGAGTGATCC-3' (antisense).

Slot and southern blot analyses: Slot and Southern blot analyses were conducted on founder F_0 transgenic and non-transgenic fish. All DNA samples were treated with RNase A (Roche) at 10 $\mu g\ mL^{-1}$ for 1 h at 37°C. Slot blot analysis was performed with 10 $\mu g\ mL^{-1}$ of genomic DNA from each fish (10 days old). Southern blot analysis was performed 20 $\mu g\ mL^{-1}$ of genomic DNA from each fish (10 days old) and genomic DNA was digested Bgl II enzymes (MBI Fermentas, Germany), electrophoresed on a 1% agarose gel and transferred to Hybond-N$^+$ membrane (Amersham) according to Sambrook *et al*. (1989). Purified circular and linearized DNA were used as a quantification and size control. Linearized EGFP gene construct was labeled with digoxigenin (DIG-DNA Labeling and Detection Kit, Roche, Germany) and used as a probe. The hybridization took place overnight at 48°C in DIG-EASY Hybridization Solution (Roche, Germany).

Total RNA isolation, first strand cDNA synthesis and RT-PCR: Total RNA was extracted from the frozen transgenic and non-transgenic larvae by using High Pure RNA Tissue Kit (Roche). First-strand cDNA synthesis was performed using 1 μg of total RNA of larvae according to the RevertAid™ First-Strand cDNA Synthesis Kit (MBI, Fermentas). RT-PCR reactions were carried out in 200 μL microfuge tubes containing 10 μL 2× PCR master mixes and 20 pmol of each EGFP gene specific primers and 1 μL of the synthesized cDNA. RT-PCR reactions were performed at the same PCR reactions conditions given before.

RESULTS AND DISCUSSION

Microinjection and production of transgenic zebrafish (*Danio rerio*): Initially, procedures for obtaining large numbers of zebrafish eggs for microinjection were established and a high frequency of germ-line transmission of injected DNA was obtained. Fluorescent cells were first observed at 3 days post fertilization. Also, flourescence and non-flourescence transgenic candidate larvae were investigated for transgene integration and expression using molecular methods. About 54 and 25 F_0

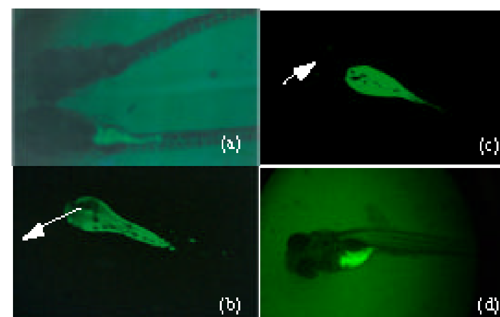

Fig. 1: Expression of linearized EGFP was observed 3 days old zebrafish larvae in different tissues. Embryo injected with pEGFP-N1 vector DNA containing EGFP gene under the control of CMV promoter was digested by VspI and AflII and viewed under a blue excitation light for EGFP observation. The expression of the linearized EGFP gene was observed at the dorsal area (A, B), yolk sack (a, b, c, d) and eyes (C)

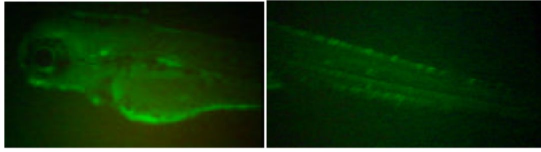

Fig. 2: Expression of circular EGFP was observed 3 days old zebrafish larvae. The expression of the circular EGFP gene construct was observed ubiquitously distributed at different parts of body (yolk sack, head, dorsal muscle of the belly and eyes) in transgenic zebrafish larvae

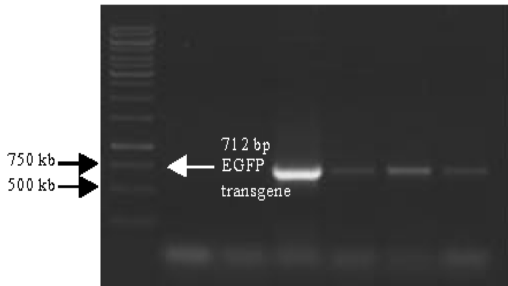

Fig. 3: Results of PCR analyses on a 1% agarose gel. Lane 1, 1 kb DNA ladder; lane 2; not including DNA; lane 3, nontransgenic zebrafish genomic DNA; lane 4, pEGFP-N1 plasmid DNA; lane 5, transgenic zebrafish DNA (line 2), lane 6, transgenic zebrafish DNA (line 3); lane 7 transgenic zebrafish DNA (line 4)

zebrafish were obtained after microinjection of linearized and circular gene, respectively. It was observed that the gene expression efficiency of circular form was higher than the linearized form in F_0 zebrafish larvae. The expression of the linearized gene was observed at the dorsal area, yolk sack and eyes (Fig. 1).

The expression of the circular gene construct was observed ubiquitously distributed at different parts of the body (yolk sack, head, dorsal muscle of the belly and eyes) in transgenic zebrafish larvae (Fig. 2).

After the microinjection of linearized form 6 F_0 transgenic fish was obtained. In this study, 520 F_1 larvae were obtained. Flourescent light emission was shown at 102 larvae (20%). Male and female F_1 transgenic fish were obtained by mating of 19 numbered F_0 female with wild-type male fish. Obtained F_1 transgenic fish were mated to each other to produce F_2 transgenic fish in which flourescent light was observed at 20%. Gene expression of EGFP was confirmed by detection of flourescent light

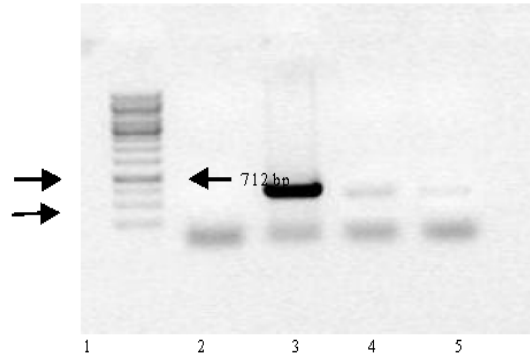

Fig. 4: Results of RT-PCR analyses. Lane 1, 1kb DNA ladder, lane 2, nontransgenic zebrafish as negative control; lane 3, pEGFP-N1 plasmid DNA; lane 4, cDNA of transgenic zebrafish (3 days old) (200 ng μL^{-1}); lane 5, cDNA of transgenic zebrafish (3 days old) (100 ng μL^{-1})

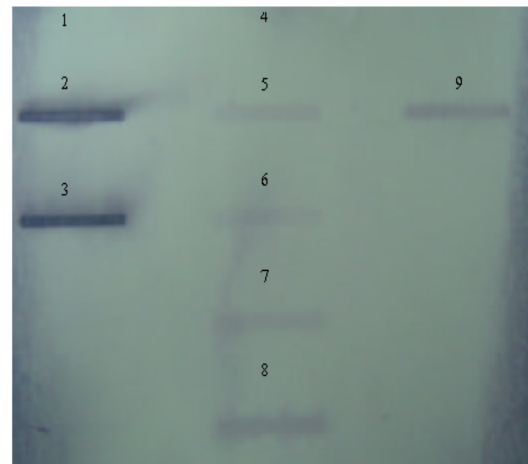

Fig. 5: Results of slot-blot analyses; 1. nontransgenic genomic DNA as negative control (10 μg); 2. pEGFP-N1 plasmid DNA; 3. Afl II digested pEGFP-N1plasmid DNA ; 4, 1X copy; 5, 5X copy; 6; 10X copy; 7, 20X copy; 8, 50X copy; 9, transgenic zebrafish DNA (10 μg) (line 2)

in zebrafish larvae. The EGFP gene integration to fish genome was detected by PCR analysis (Fig. 3).

Besides, RT-PCR results indicated that EGFP gene was stably transcribed in transgenic zebrafish (Fig. 4). Slot Blot analysis showed that one of the transgenic fish carry about 50 copies of foreign DNA (Fig. 5). Southern Blot analysis was used to test for presence of transgene in the transgenic fish (Fig. 6). Here, we report success in rapidly obtaining large numbers of eggs for injection and also report a high frequency of germ-line transmission of an

Fig. 6: Results of southern-blot analyses. Lanes 1-3 transgenic zebrafish (10 days old) DNA (20 μg); lane 4, VspI-AflII fragment of the plasmid (EGFP DNA); lane 5, pEGFP-N1 plasmid DNA. All DNA samples were RNase treated before agorose gel electrophoresis. The filters were hybridized with an EGFP-specific DNA probe. The DNAs were digested with an BglII restriction enzyme

injected linearized and circular EGFP forms. Gene expression efficiency of circular form is higher than the linearized form (Haobin *et al*., 2000). Moreover, only F_0 transgenic fish using circular gene construct was expressed examinations over the first 48 h following microinjection showed that the linear DNAs and circular forms were expressed at about equivalent rates but thereafter the circular form has given higher rates of expression (Ekici, 2007; Ekici *et al*., 2007; Haobin *et al*., 2000). After microinjection of linear form 6 F_0 transgenic fish were obtained which maintained the transgene expression after mating of F_0 transgenic fish with wild-type fish. In concordance with Stuart *et al.* (1988) and Alimuddin (2002) it was found that these F_1 transgenic fish was mating each other to produce F_2 transgenic fish which flourescent light observed at 20% (Alimuddin, 2002; Culp *et al*., 1991; Stuart *et al*., 1988).

Expression of the gene construct contaning the coding sequence for the EGFP was determined in whole body of the transgenic zebrafish. Mosaic expression was observed in zebrafish because foreign DNA construct was injected into cytoplasm. Finally, as reported in other studies, we confirm here that EGFP gene expression has no toxic effects in zebrafish embryos.

In this study a transgenic zebrafish, carrying the EGFP gene expression unit was generated. One founder identified by PCR, RT-PCR, Southern and Slot blot analyses were used to establish transgenic zebrafish lineage. Non-radioactive DNA labeling and detection system was applied for the detection of copy number of the transgene sequences. Although, radioactive labelling system is too sensitive for transgene detection it is to harmful for human health. In this study it was shown that non-radioactive DNA labeling and detection system is sensitive enough for detection of low copy number sequences. Copy number of transgene was detected in 4 and 7 μg of genomic DNA used in Slot Blot experiments.

CONCLUSION

In this study, transgenic fish production technology was established at the first time in Turkey and EGFP transgenic zebrafish was obtained by using this technology. Procedures for obtaining large numbers of zebrafish eggs for microinjection were established and a high frequency of germ-line transmission of injected DNA was obtained. In the present study, evidence was given for the first time that coding sequence for the GFP gene could be integrated, stably transcribed and expressed in whole body of the transgenic zebra fish. Further breeding programs in the laboratory are being performed to expand EGFP carrying and expressing transgenic lineage to establish a useful model to the scientists for investigating human gene functions. The result of this study will give an opportunity to produce transgenic zebrafish lineages.

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REFERENCES

Alimuddin, 2002. Introduction and expression of foreign Δ6 desaturase-like gene in a teleostean fish. M.Sc. Thesis, Graduate School of Fisheries Science, Tokyo University of Fisheries.

Amsterdam, A., S. Lin and N. Hopkins, 1995. The *Aequorea victoria* green fluorescent protein can be used as a reporter in live zebrafish embryos. Dev. Biol., 171: 123-129.

Bagis, H. and L. Keskintepe, 2001. Application of green flourescent protein as a marker for selection of transgenic mouse embryos before implantation. Turk. J. Biol., 25: 123-131.

Brem, G., B. Brening, G. Horstgen-Schwark and E.L. Winnacker, 1988. Gene transfer in tilapia (O. Niloticus). Aquaculture, 68: 209-219.

Chalfie, M., Y. Tu, G. Euskirchen, W.W. Ward and D.C. Prasher, 1994. Green fluorescent protein as a marker for gene expression. Science, 263: 802-805.

Chourrout, D., R. Guyomard and L.M. Houdebine, 1986. High efficiency gene transfer in rainbow trout (*O.mykiss*) by microinjection into egg cytoplasm. Aquaculture, 51: 143-150.

Culp, P., C. Nüsslein-Volhard and N. Hopkins, 1991. High-frequency germ-line transmission of plasmid DNA sequences injected into fertilized zebrafish eggs. Proc. Natl. Acad. Sci. USA., 88: 7953-7957.

Ekici, A., 2007. A study on the gene (GFP) transfer in fertilized zebrafish (*Danio rerio*, Hamilton-Buchanan 1822) eggs. Ph.D. Thesis, Istanbul University, Istanbul, Turkey.

Ekici, A., D. Aktoprakligil, M. Timur and H. Bagis, 2007. The effect of linear and plasmid EGFP gene construct which used in generating transgenic Zebrafish on the transgene integration. Poster Presentation. Aquaculture Europe 2007, Istanbul, Turkey.

Hamada, K., K. Tamaki, T. Sasado, Y. Watai, S. Kani, Y. Wakamatsu and K. Ozato, 1998. Usefullness of the medaka beta-actin promoter investigated using a mutant GFP reporter gene in transgenic medaka (*Oryzias latipes*). Mol. Mar. Biol. Biotechnol., 7: 173-180.

Haobin, Z., C. Shangping, S. Yonghua, W. Yaping and Z. Zuoyan, 2000. Time course of foreign gene integration and expression in transgenic fish embryos. Chinese Sci. Bull., 45: 734-739.

Peters, KG., P.S. Rao, B.S. Bell and L.A. Kindman, 1995. Green fluorescent fusionproteins: Powerful tools for monitoring protein expression in live zebrafish embryos. Dev. Biol., 171: 252-257.

Sambrook, J., E.F. Fritsch and T. Maniatis, 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbour Laboratory Press, New York, pp: 238.

Stuart, G.W., J.V. McMurray and M. Westerfield, 1988. Replication, integration and stable germ-line transmission of foreign sequences injected into early zebrafish embryos. Development, 103: 403-412.

Takeuchi, Y., G. Yoshizaki and T. Takeuchi, 1999. Green fluorescent protein as a cell-labeling tool and a reporter of gene expression in transgenic rainbow trout. Mar. Biotechnol., 1: 448-448.

Westerfield, M., 1995. The Zebrafish Book: A Guide for the Laboratory use of Zebrafish (Danio Rerio). University of Oregon Press, Eugene, OR, pp: 385.