한국어병학회지 제 16권 제 3호 (2003)
J. Fish Pathol., 16(3) : 147 ~ 152 (2003)

Survey of the heterogeneous gene expression in olive flounder muscle using the luciferase reporter gene system

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The CMV promoter driven luciferase reporter gene coding plasmid (pcDNA-luc) was constructed and used as a model for DNA immunization study. Expression of the recombinant luciferase protein was confirmed *in vitro* in RTG-2 cell line before using *in vivo* study in olive flounder. In dose response study, the maximum expression of the luciferase gene was found in the group injected with 10-15μg of plasmid DNA. The kinetic study showed that the luciferase gene expression was reached at the maximum level at one day after injection and slightly decreased after then but significantly high level of expression was sustained until the conducted experiment of 7 days. In the study of tissue distribution of gene expression, it was found that luciferase gene was expressed at the significant level in immune organs such as gill and spleen, located far from the injected site, suggesting the systemic distribution of the intramuscularly injected DNA in olive flounder.

Key words : Transfection, Gene expression, Luciferase, RTG-2, Olive flounder

Introduction

The ability to introduce exogenous genes into cells provides a powerful tool to investigate gene regulation and protein function as well as to develop DNA vaccine. The use of reporter genes is an effective methodology to test for techniques of DNA introduction. Reporter genes, such as luciferase, β-galactosidase, and chloramphenicol acetyltransferase (CAT) genes, have been widely used to test the introduction of exogenous genes and to assess the expression of the transfected genes in many species. Studies have shown strong expression of these reporter genes in the muscle cells of fish injected with plasmid DNA (Hansen *et al.*, 1991;Rahman & Maclean, 1992;Anderson *et al.*, 1996;Gomez-Chiarri *et al.*, 1996). Tucker and his colleagues (2001) also have demonstrated the expression of green fluorescent protein (GFP) and CAT gene in olive flounder following DNA bombardment. However, they have only looked at the duration of expression and the pathological effect in the injected site. Thus there is insufficient information on the exogenous gene expression in olive flounder yet.

Among the reporter gene systems, CMV promoter driven luciferase gene system has been widely used to survey exogenous gene expression since CMV promoter is more efficient than other prokaryotic promoters (Lee *et al.*, 1997) and luciferase expression is easily analysed using a commercial assay kit. In some freshwater fish including *Xiphophorus* sp. (Schulte *et al.*, 1998), rainbow trout (Anderson *et al.*, 1996) and zebra fish (Heppell *et al.*, 1998), the luciferase reporter gene expression has been examined and shown that this

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reporter system is an effective method to detect exogenous gene expression in fish as well as mammals.

In this study, we used a CMV promoter driven luciferase reporter gene to characterize the expression of the intramuscularly injected plasmid-encoded gene in olive flounder such as the optimal dose of DNA, kinetics and tissue localisation of the introduced foreign gene in olive flounder.

Materials and Methods

DNA construct

Reporter gene construct used in this study expressed the firefly luciferase (luc) gene under the control of cytomegalovirus (CMV) promoter. The luc gene was obtained from the PGL3 control vector (Promega) with the restriction endonucleases of KpnI and EcoRI, and inserted into the KpnI/EcoRI site of a eukaryotic expression vector (pcDNA3, Invitrogen) at the downstream of the CMV promoter to create a new recombinant plasmid, pcDNA-luc. The constructed plasmid was multiplied in *Escherichia coli* strain DH5α cells grown in Luria-Bertani (LB) broth media, and the plasmid DNA was purified with anion-exchange chromatography columns (Plasmid Midi-Kit, Qiagen).

In vitro expression of gene

pcDNA-luc plasmid was transfected into RTG-2 cells in a 24 well cell culture plate using the Effectene transfection reagent (Gibco) by the manufacture's instruction. After incubation with the DNA-Effectene complexes, the complexes were removed and a 350 ℓ of fresh growth medium was added. Cells were cultured for 72 h and examined for luciferase expression using a commercial assay kit (Promega).

Direct gene transfer

Olive flounder (30-50g) were anaesthetized with MS-222 (3-aminobenzoic acid ethyl ester, Sigma) and injected intramuscularly to a depth of 0.5cm on the back below the dorsal fin using a 0.5 cc insulin syringe. Plasmid DNA was diluted in sterile distilled water and the amount of DNA per fish varied according to the experiment. For dose response study, 0, 0.1, 1, 5, 15 or 30μg of pcDNA-luc was injected in a volume of 20$\mu\ell$ and fish were maintained for a week before being sacrificed. For kinetic study, 10μg of pcDNA-luc DNA was injected in a 20$\mu\ell$ volume and fish were sacrificed at 0, 1, 3, 7 days after injection.

Detection of lucuferase activity

Luciferase expression was measured through its light emitting activity in the presence of the luciferin substrate. Anaesthetized olive flounder was killed and dissected into the injected muscle (i.e. a piece of muscle tissue approximately 0.4 cm^3 including the injected site) and other tissues (e.g. gill and spleen). Samples were put into 1.5 ml microtubes with 500$\mu\ell$ of chilled cell lysis reagent (Promega) and then homogenized with micropestles. Homogenized tissues were centrifuged at 15,000Xg for 15 min at 4°C and 20$\mu\ell$ supernatant was mixed with 100$\mu\ell$ of luciferase assay reagent (Promega). The emission of light was measured in a 96 well plate with a luminometer (Victor 2, Wallac). The integrated sum of light units for 10s was measured and represented as relative light units (RLU).

Statistical analysis

Kruskal-Wallis test was performed to know whether there are differences between samples and followed by Mann-Whitney comparison test to find where the difference exists ($P<0.05$).

Results and Discussion

CMV promoter driven luciferase gene coding plasmid (pcDNA-luc) was constructed to study exogenous gene expression in olive flounder and the expression was confirmed *in vitro* before using for *in vivo* study. RTG-2 cells were used for the transfection since flounder cell line is not available yet. The pcDNA-luc plasmid was transfected into RTG-2 cells and the successful expression was observed (Fig. 1), expecting that the plasmid would be fully operated in olive flounder *in vivo*.

It is important to decide an optimal injection dose of DNA for the highest expression of the injected gene. Heppell and Davis (2000) have demonstrated in their review paper that the typical doses for fish fall in the range of 1-50μg of DNA in a volume of 10-50$\mu\ell$, but the optimal dose of DNA probably varies according to the species. Unfortunately, there is no report about the optimal injection dose of DNA in olive flounder yet. Therefore, in this study, we tried to find the optimal dose of DNA to induce the highest expression of the injected gene in olive flounder using the pcDNA-luc plasmid. Olive flounder muscle injected with pcDNA-luc showed a dose dependent increase of the luciferase gene expression for doses of 0.1-10μg. Injection of the greater amount of DNA (30μg) did not significantly increase the activity further (Fig. 2). Thus the optimal dose of DNA for the injected gene expression was 10-15μg. This is a higher amount than 0.1-1μg of plasmid encoding a luciferase gene reported by Heppell et al. (1998). They have demonstrated that the dose dependent increase of plasmid encoding a luciferase gene at the doses between 0.1 and 1μg in trout and zebra fish and no significantly increased activity observed further at a greater amount of DNA than 10μg. This difference could be due to the species difference as mentioned above by Heppell and Davis (2000).

The kinetic study showed that the maximum expression of the luciferase in the injected muscle was found after one day, and then slightly decreased thereafter but a noticeably high level of expression

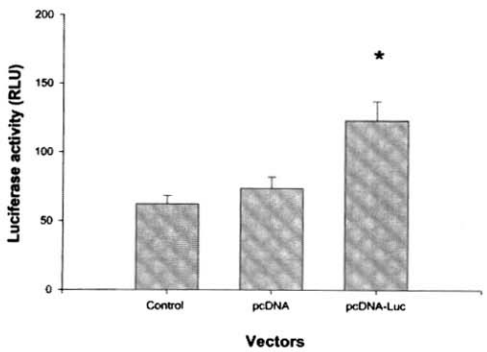

Fig. 1. Luciferase expression in RTG-2 cells transfected with pcDNA-luc vector or pcDNA vector as a negative control. pcDNA-luc vector was conjugated with the Effectene reagent and transfected into RTG-2 cells. Transfected cells were lysed after 72 h and assayed for luc expression. * Significantly higher than control analysed by Mann-Whitney test ($P<0.05$).

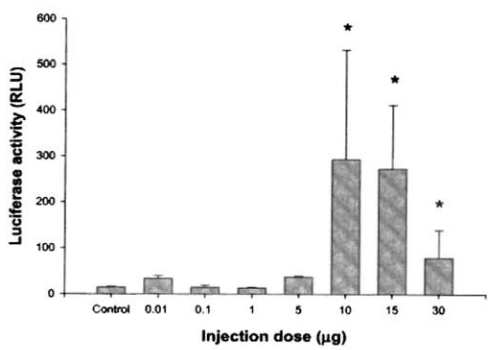

Fig. 2. Dose response of luciferase expression in olive flounder injected with the various doses of pcDNA-Luc vector (0.1-30μg) intramuscularly. Enzymatic activity was measured 7 days after injection. Bars represent the mean (n=4) and T bars represent S.E. of the mean. * Significantly higher than control analysed by Mann-Whitney test ($P<0.05$).

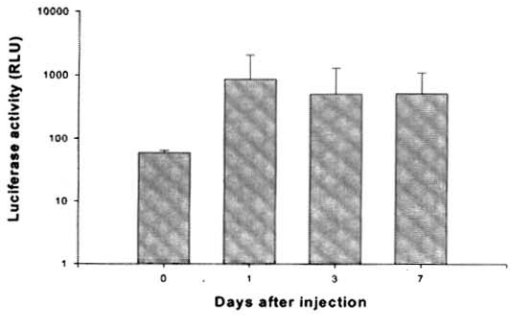

Fig. 3. Kinetics of luciferase expression in muscles of olive flounder injected intra-muscularly with 10μg pcDNA-luc plasmid. Enzymatic activity was measured at 0, 1, 3, and 7 days after injection. Bars represent the mean (n=4) and T bars represent S.E. of the mean. * Significantly higher than control analysed by Mann-Whitney test ($P<0.05$).

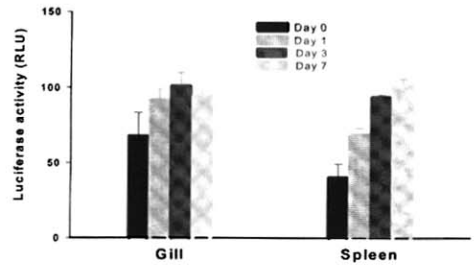

Fig. 4. Tissue distribution of the luciferase gene expression in olive flounder injected with 10μg of pcDNA-Luc vector intramuscularly. Enzymatic activity was measured in gill and spleen at 0, 1, 3, and 7 days after injection. Bars represent the mean (n=4) and T bars represent S.E. of the mean. * Significantly higher than control analysed by Mann-Whitney test ($P<0.05$).

was observed until the end of experiment of 7 days (Fig. 3). Peak activity of the luciferase gene injected into the fish muscle was varied upon fish species or researchers. For example, the maximum expression of the luciferase gene was found at 7 days in rainbow trout (Anderson et al., 1996) or 2.5 days in *Xiphophorus* sp. and zebrafish (Heppel et al., 1998). In a study counting β-gal positive muscle fibres at different times after intramuscular administration of pCMV-lacZ, the number of β-gal positive muscle fibres was started to increase from 7 days and stable until 21 days in gold fish (Kanellos et al., 1999).

The site of protein synthesis is an important issue in DNA vaccination methodology. Thus we tested the luciferase expression in two immune organs (gill and spleen) as well as in the injected site of muscle in olive flounder (30-50g). Even though luciferase gene was much more highly expressed in the injected site of muscle than gill and spleen, it seemed that protein production could take place in different tissues over the time since the luciferase activity was gradually increased in other organs over the experimental period of 7 days (Fig. 4), allowing for different compartments of the immune response to be involved in these immune organs. This result is in agreement with Anderson et al. (1996) and Heppell et al. (1998) who reported that, in small fish between 0.2 and 2g, expression of the luciferase reporter gene injected intramuscularly was detected systemically in different organs, including gills, spleen and kidney, but was the highest in muscles. However, Boudinot et al. (1998) have reported that expression of G protein gene from the viral hemorrhagic septicemia virus was mostly restricted to the site of injection, that is in myocytes as well as in cells infiltrating the muscle tissue or epithelial cells lining small capillaries in trout between 150 and 200g. The tissue location of the injected gene expression seems to be different in the different sizes of fish. Differences between large and small fish are most likely due to more rapid spread of the injected DNA from the injected muscles in small fish, but there may also have been variations in the protocols, the sensitivity of assays and the animal models used.

In conclusion, this study was conducted to char-

acterise the expression of the intramuscularly injected heterologous gene in olive flounder using a reporter system, luciferase. The results showed a strong and continuous expression of CMV promoter driven luciferase reporter gene in muscle cells of olive flounder injected with plasmid DNA. Even though the expression of the injected DNA was the highest in muscle, the gene expression doesn't seem to be restricted to the inoculated site (muscle) since the gene expression was appeared to be gradually spreaded into the immune organs (e.g. gill and spleen) over the experimental time of 1 week, suggesting that the distribution of the intramuscularly injected DNA could conduct a systemic immune response in various organs as a successful vaccine in olive flounder.

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Manuscript Received : August 14, 2003
Revision Accepted : October 04, 2003
Responsible Editorial Member : Myung-Joo Oh
(Yosu Univ.)