CHAPTER II

MATERIALS AND METHOD

Brood stock and manipulation

1. **Parental catfish** : Mature parental catfish *C. macrocephalus* used in this experiment were collected from the private farms in the suburb of Bangkok and neighbouring areas.

2. **Plasmid vector** : The plasmid pXGH 5 was used as an introduced gene into catfish. pXGH 5 containing a 1.9 kb fragment of mouse metallothinein-I promotor (mMT-I) fused to a 2.2 kb fragment of human growth hormone gene (hGH) and a 2.6 kb fragment of pUC 12 were obtained from Dr. Sakol Panyim 's laboratory. The physical map is shown in Figure 4.

3. **Primers** : Oligonucleotide primers specific to 186 bp of human Growth hormone gene (hGH) DNA sequence is shown Figure 5. Which specific to 150 bp of *Pangasius sutchi* growth hormone gene (cDNA) DNA sequence is shown in Figure 6. They were obtained from Biosynthesis, Inc., USA.

3.1 hGH primers

Sense strand primer

5'-CTG ACC CAA GAG AAC TCA C-3'

Antisense strand primer

5'-CCC AGT CCG GGG GCT G-3'

3.2 *P. sutchi* primers

Sense strand primer

5'-CCC TTC GAG GAT TTC TAC-3'

Antisense strand primer

5'-CTA CAG GGT GCA GTT GGA-3'

4. Artemia : Artemia, which hatched from Artemia cysts, Ocean Star International Inc., were used for fry feeding in this experiment , .

5. Fish feeds : Feed for the broodstock containing 30 % protein was obtained from UNICORD FEED Co. Ltd.

6. Chemicals

Bacterial culture medias were obtained from Difco. Ampicillin antibiotic drug, deoxynucleotide triphosphate, mineral oil were purchased from Sigma. Agarose and Protinase K were obtained from BRL. Developer and fixer were from Eastman Kodak company. Fish drug, Tetracyclin HCL were obtained from the government pharmaceutical organization. Restriction endonuclease and modified enzymes were from BRL, New England Biolabs. Nonradioactive DNA detection kit was from Boehringer Mannheim. Taq DNA polymerase produced by recombinant DNA techniques was kindly provided by Luxananil P. All other chemicals used in this study were analytical grade.

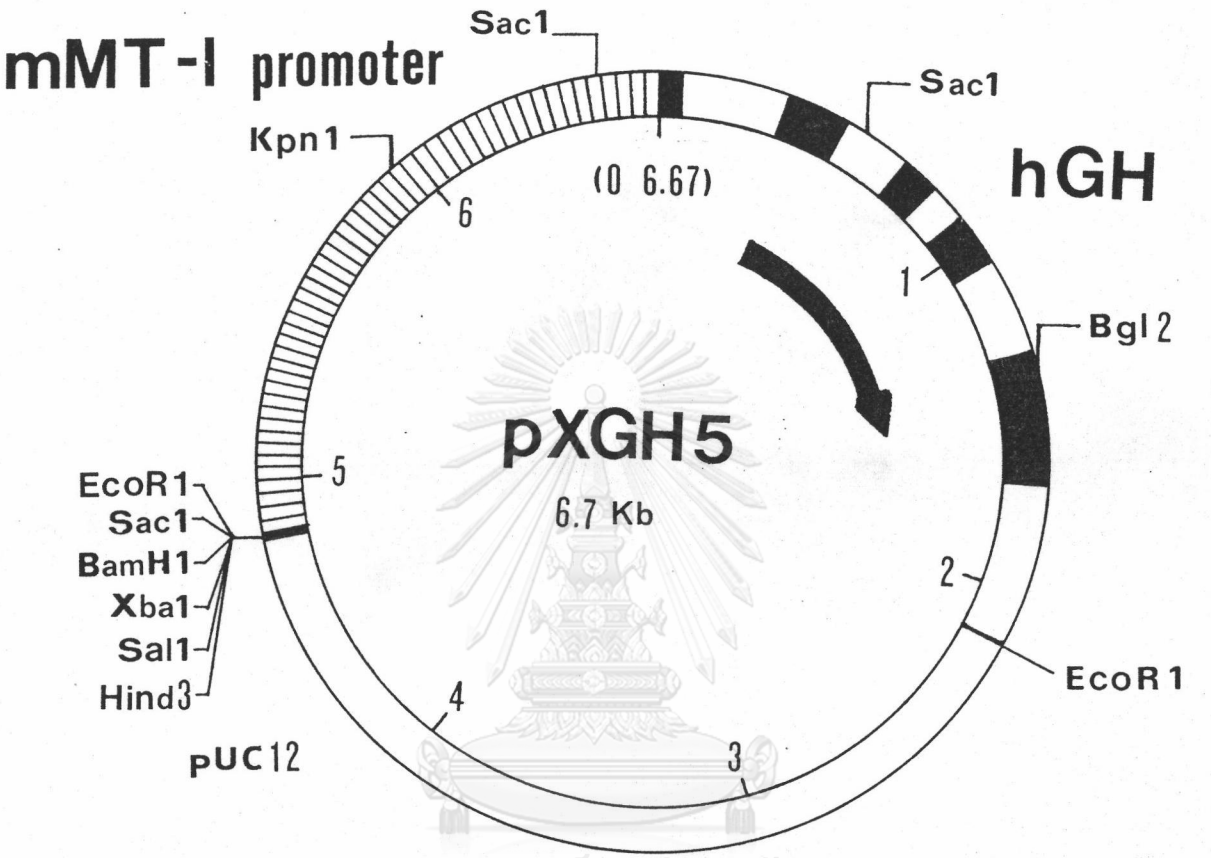

Figure 4 The physical map of plasmid pXGH 5

(Nichols Institute Diagnostics, 1988)

AGGGCACCCACGTGACCCTTAAAGAGAGGACAAGTTGGGTGGTATTTTCTGGCTGACACTCTGTG
CACAACCCTCACAACACTGGTTGACGGTGGGAAGGGAAAGATGACAACGCAGGGGCATGATCCC
AGCATGTGTGGGAGGAGCTTCTAAATTATCCATTAGCACAAGCCCGTCAGTGGCCCCATGCATAA
ATGTACACAGAAACAGGTGGGGGCAACAGTGGGAGAGAAGGGGCCAGGG **TATAAA** 5'-GGGCCC
ACAAGAGACCGGCTCAAGGATCCCAAGGCCCAACTCCCCGAACCACTCAGGGTCCTGTGGACGC
T CACCTAGC TGCAATGGCTACAG gtaagcgcccctaaaatccctttgggcacaatgtgtcctgaggggagaggcagcga cctgtagat
gggacgggggcattaaccctcaggtttggggcttctgaatgagtatcgccatgtaagcccagtatggccaatctcagaaagctcctggtccctgg agggatggag
agagaaaaacaaacagctgctggagcagggagagtgctggcctcttctgctccggctccctctgttgccctctggtttctcccca GGC TCC CGG
ACG CTG CTC CTG GTC TTT GGC CTG CTC TGC CTG CCC TGG CTT CAA GAG GGC AGT GCC
TTC CCA ACC ATT CCC TTA TCC TCC AGG CTT TTT GAC AAC GCT AGT CTC CGC GCC CAT
CGT CTG CAC CAG CTG GCC TTT GAC ACC TAC CAG GAG TTT
gtaagctcttggggaatgggtgcgcatcaggggtggcaggaaggggtgactttcccccgctgggaaataagaggaggagactaaggagctcagggtttt
tcgcgaagcgaaaatgcaggcagatgagcacacgctgagtgaggttcccagaaaagtaacaatgggagctggtctccagcgtagaccttggtgggcggtc
cttctcctag GAA GAA GCC TAT ATC CCA AAG GAA CAG AAG TAT TCA TTC CTG CAG AAC
CCC CAG ACC TCC CTC TGT TTC TCA GCG TCT ATT CCG ACA CCC TCC AAC AGG GAG GAA
ACA CAA CAG AAA TCC gtgagtggatgccttgaccccaggcggggatgggggagacctgtagtcagagcccccgggcagcacagg
ccaatgc ccgtccttcccctgcagAAC CTA GAG CTG CTCCGCATC TCC CTG CTG CTC ATC CAG TCG TGG
CTG GAG CCC GTG CAG TTCCTCAGG AGT GTC TTC GCC AAC AGC CTG GTG TAC GGC CCC
TCT GAC AGCAACGTC TAT GAC CTC CTA AAG GAC CTA GAG GAA GGC ATC CAA ACG CTG
ATG GGG gtgggggtggcgctaggggtccccaatcttggagccccactgactttgagagctgtgttagagaaacactgctgccctcttttagcagt ccag
gcc ctgacccaagagaactcaccttattcttcatttcccctcgtgaatcctctagcctttctctacaccctgaaggggagggaggaa aatgaatgaatgagaaagg
gagggagcagtacccaagcgcttggcctctccttctcttccttcactttgcagAGG CTG GAA GAT GGC AGC CCC CGG ACT
GGG CAG ATC TTC AAG CAG ACC TAC AGC AAG TTC GAC ACA AAC TCA CAC AAC GAT
GAC GCA CTA CTC AAG AAC TAC GGG CTG CTC TAC TGC TTC AGG AAG GAC ATG GAC
AAG GTC GAG ACA TTC CTG CGC ATC GTG CAG TGC CGC TCT GTG GCG
GGCAGCTGTGGCTTCTAGCTGCCCGGGTGGCATCCCTGTGACCCCTCCCCAGTGCCTCTCCTGGCC
TTGGAAGTTGCCACT CCAGTGCCCACCAGCCTTGTCCTAATAAAATTAAGTTG CATCA-3'
TTTTGTCTGACTAGGTGTCC TCTATAATATTATGGGGTGGAGGGGGGTGGTTTGGAGCA

Figure 5 DNA sequence of human growth hormone gene (hGH); underlines indicate sequences of PCR primers and their annealing position (Denoto et al., 1981)

ATGGCTAGAGTGTTGGTGGTGCTCTCTGTGGTGGTGGCGAGTTTGTT CTTTAGTCAAGGC 60

GCGACATTCGAGAACCAGCGGCTCTTCAACAACGCAGTCATCCGTGTGCAACACCTTCAT 120

CAGCTGGCTGCCAAGATGATGGATGACTTTGAGGAAGCTCTGTTACCTGAAGAACGCAAA 180

CAGCTGAGCAAGATTTTCCCCCTGTCTTTCTGCAACTCGGACTCCATCGAAGCTCCTGCA 240

GGCAAGGACGAGACCCAGAAAAGCTCTGTGCTGAAATTGCTGCACACCTCCTACCGTCTG 300

ATCGAGTCATGGGAGTTCCCCAGCAAGAACCTCGGCAACCCCAACCACATCTCAGAGAAG 360

CTGGCTGACCTGAAAATGGGCATCGGCGTGCTTATCGAGGGATGTTTGGATGGACAAACC 420

AGCCTGGATGAGAACGACTCTCTGGCTCCGCCCTTCGAGGATTTCTACCAGACCTTGAGC 480

GAGGGAAACCTGAGGAAGAGCTTCCGTCTGCTGTCCTGCTTCAAGAAGGACATGCACAAA 540

GTGGAGACCTATCTCAGCGTGGCCAAGTGCAGGAGATCCCTGGATTCCAACTGCACCCTG 600

TAG 603

Figure 6 DNA sequence of *P. sutchi* growth hormone gene (cDNA); underlines indicate sequences of PCR primers of 150 bp fragment and their annealing position

Small scale plasmid extraction

Plasmids pXGH 5 were prepared as small scale for restriction endonuclease digestion by using rapid alkaline method (Birnboim and Doly, 1979). Single colony of *Escherichia coli* JM 107, containing plasmid pXGH 5 was grown overnight with shaking at 37 °C in 3-5 ml of 100 µg/ml ampicillin-LB broth (1 % bacto-tryptone, 0.5 % yeast extract and 0.5 % NaCl). The bacterial cells were collected by centrifugating of the 1.5 ml culture in microcentrifuge tube at 5,000 g for 5 min. The cell pellet was resuspended in 100 µl of buffer I (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0) and left on ice for 30 min. Then 200 µl of freshly prepared lysis solution (0.2 N NaOH, 1 % SDS) was added, gently mixed, and left on ice for 5 min to lyse the cells. The lysate was neutralized by gently mixing with 150 µl of 3 M sodium acetate, pH 4.8 and left on ice for 1 hr. Cell debris and chromosamal DNA were seperated by centrifugation at 10,000 g for 15 min. Plasmid DNA in the supernatant was transferred into a new tube and precipitated with two volumes of absolute ethanol, kept at -20 °C for 1 hr. The DNA pellet was collected by centrifugation at 10,000 g for 15 min, washed twice with 70 % ethanol, completely airdried, and dissolved in 20 µl of TE buffer pH 7.4 (10 mM Tris-HCl pH 7.4, 1 mM EDTA). The DNA was then digested with restriction endonuclease or kept at 4 ^{0}C for further use.

Restriction endonuclease digestion

Plasmid pXGH 5 was analysed and compared with the map shown in Figure 4 by using the restriction endonuclease digestion method described by Maniatis et al. (1989). The restriction enzymes using in the present work, including their recognition sequences and recommended buffer was shown in Table 2 and Table 3. The mixture containing 5 µl of plasmid solution, 2 µl of recommended 10 x buffer, 1-2 µl of restriction enzyme, and sterile distilled water was added to make volume as 20 µl. This mixture was incubated at 37 ^{0} C for 1-2 hr. For two restriction enzyme digestions, the low salt requirement of endonuclease reaction was prior performed. After complete first digestion, the high salt requirement of enzyme was later performed with adding other ingredient salt to make optimal buffer as recommended buffer, then incubated at 37 ^{0}C for 1-2 hr. The restriction fragments were analysed by using 0.7 % agarose gel electrophoresis.

Agarose gel electrophoresis

Simple analysing of conformation and fragment size of DNA was carried out by agarose gel electrophoresis (Maniatis et al., 1989). 0.7 % and 3 % (w/v) agarose gel were dissolved in 1x TBE buffer (89 mM Tris- Hcl, 89 mM boric acid and 2 mM EDTA, pH 8.0), completely dissolved by heating. After a few minutes cooling, melted agarose was then poured onto a gel platform with comb position 0.5-1.0 mm and about 0.5 cm thick. When the gel was hardened, the comb was withdrawn. The DNA samples were mixed

Table 2 General formula for restriction enzyme buffer

Buffer salt	NaCl	Tris-HCl pH 7.5	$MgCl_2$	Dithiothreitol (DDT)
High	100 mM	10 mM	10 mM	1 mM
Medium	50 mM	10 mM	10 mM	1 mM
Low	0 mM	10 mM	10 mM	1 mM

Table 3 Restriction enzyme with their recognition sequences and optimal condition of the buffer salt

Enzyme	Recognition sequences	Buffer salt
Hind III	A!AGCTT	Medium
Kpn I	GGTAC!C	Low
Bgl II	A!GATCT	High
EcoRI	C!AATTC	High
BamH I	G!GATCC	High

Notes : Optimal temperature for all enzymes in the present study was 37 ^{0}C

with1/3 volume of loading dye (0.25 % bromphenol blue, 0.25 % xylene cyanol FF, 33 % glycerol in water) and loaded into the wells under 1x TBE buffer submarine. The electrophoresis was carried out from cathode to anode at constant voltage 150 volts. When the dye was migrated through 1/3 of agarose gel, the gel was then stained with 2.0 µg/ml ethidium bromide solution for 5 min and destained with a large volume of water to remove an excess ethidium bromide from the gel. DNA pattern was visualized by UV light and photographed through a red filter with Kodak-Tri X Pan 400 film .

Large scale plasmid extraction and purification by cesium chloride density gradient ultracentrifugation

A large number of plasmids was prepared by using large scale plasmid extraction (Birnboim and Doly, 1979). *E. coli* containing plasmids was activated in 5 ml of 100 µg/ml ampicillin-LB broth and incubated overnight at 37 ^{0}C. The activated cells were innoculated in 500 ml the same media and incubated at 37 ^{0}C for 6-8 hr in a shaking incubator. In order to amplify the plasmids copy, chloramphenicol (dissolved in small amount of 70 % ethanol) was added to the culture to make up final concentration of 170 µg/ml. The culture was continually incubated at 37 ^{0}C for 8-14 hr. The bacterial cells was harvested by centrifugating in a GSA rotor at 5,000 rpm for 10 min at 4 ^{0}C. The cell pellet was resuspended in 10 ml of freshly prepared lysozyme solution (50 mM glucose, 25 mMTris-HCl, 10 mM EDTA, pH 8.0 and 2 mg/ml lysozyme) and kept on ice for 30 min. Then 20 ml of freshly prepared lysis buffer (0.2 N NaOH - 1 % SDS) was added, gently mixed and kept on ice for 5 min. The cell lysate was neutralized with 15 ml of 3 M sodium acetate at pH 4.8,

gently mixed and kept on ice for 1 hr. Cell debris and chromosomal DNA was seperated by centrifugating at 5,000 rpm for 15 min at 4 ^{0}C. The plasmid supernatant was transferred into a new tube, precipitated by adding an equal volume of isopropanol and kept at room temperature for 1 hr. The plasmid was collected by centrifugating at 8,000 rpm for 10 min. The plasmid pellet was washed twice with 70 % cold ethanol, air dried, and dissolved in 1 ml TE buffer pH 7.4, kept at 4 ^{0}C for further purification process.

Purification of the large scale plasmid solution was performed by cesium chloride (CsCl) density gradient ultracentrifugation (Maniatis et al., 1979). The plasmid solution was adjusted to the final volume of 8 ml with TE buffer, then 8 g of CsCl was added to make the concentration of 1 g/ml CsCl of DNA solution, gently mixed for completely dissolved, followed by adding 0.8 ml of 10 mg/ml ethidium bromide, gently mixed. The mixture was transfered to a Beckman centrifuge tube (ultra-clear)™ and centrifuge at 45,000 rpm for 24 hr at -20 ^{0}C in a 70.1 Ti rotor of Beckman L9-70 M Ultracentrifuge. After centrifugating, two bands of DNA were visualized under UV light. The lower band consists of closed circular plasmids DNA, was collected in a syringe by puncturing the tube with a no.20 hypodermic needle. Ethidium bromide was removed from the plasmid solution by extractives with equal volume of isoamyl alcohol for 4-5 times, or until the plasmid solution was colourless. Two volumes of sterile distilled water was added to dilute CsCl. For plasmid precipitation, an equal volume of isopropanol was added and kept at room temperature for 1 hr. The plasmid pellet was collected by centrifugation at 10,000 rpm for 15 min, and washed twice with 70 % cold ethanol, air dried.

The purified closed circular plasmid DNA was dissolved in 100 µl TE buffer. The DNA concentration was determined by UV spectrophotometer (Shimadzu spectrophotometer) at wave length 260 nm. The calculation was made by using;

1 OD at 260 nm = 50 µg/ml of double-stranded DNA

Determination the flow rate for microinjection

The workplace for microinjection technique was described in Appendix 1., and Figure 7. showed the microinjecter. The filtered TE buffer was pipetted into the microcapillary (0.5 ± 0.2 µm diameter of tip) as 0.5, 1, 1.5, 2 µl by using microloader. Injection pressure nitrogen gas at 2,000 hpa was used for driving the plasmid solution, and measuring times taken.

Preparation of plasmid solution for microinjection

The plasmid was microinjected after having linearized with the restriction endonuclease BamH I. Digestion of plasmid contained of 50 µl plasmid solution, 20 µl of appropriate 10 x buffer, 4 µl of (50 x) 100 mM spermidine HCl, 10-20 µl of 10 U/µl BamH I and making volume as 200 µl with sterile distilled water, incubation at 37 ^{0}C for 2 hr or until complete digestion. After complete digestion, plasmid was precipitated by adding an equal volume of isopropanol in the presence of 0.1 volume of 3 M sodium acetate pH 5.5, kept at room temperature for 1-2 hr. The plasmid pellet was collected by centrifugation at 10,000 rpm for 15 min, and washed twice with 70 % cold ethanol, air dried, and resuspended in TE buffer. In order to remove any

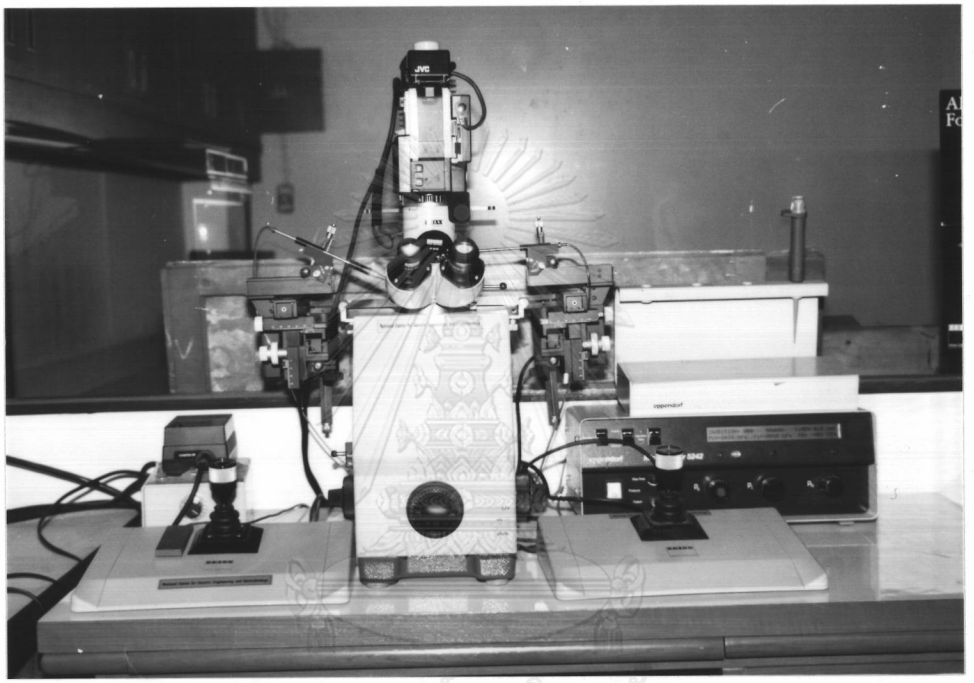

Figure 7 The workplace for microinjection

A) Microinjector Eppendorf 5242

B) Micromanipulator MR

C) Inverted Microscope

impurities, the DNA solution was filtered through a 0.2 μm sterile millipore filter and kept at 4 ^{0}C for microinjection uses.

Preparation of Fertilized eggs

Artificial breeding of yellow walking catfish was carried out using modified dry method (Na-nakorn, 1992). Sexually mature female catfish were induced to spawn by intramascular injection with 30 μg Suprefact (Luteinizing Hormone Releasing Hormone anologue, LHRHa) per Kg body weight and 10 mg moltilium M (Domperidone) per Kg body weight. Approximately 14-16 hr later, eggs were collected by mannual stripping into a cleaned dry beaker. Male fish were sacrificed, and testes were removed and ground in normal saline (0.85 % NaCl). Sperm in normal saline was mixed into eggs with gently stirring and activated by adding little well water, still stirring for dispersal of the milt. Eggs from one female were subsequently mixed and fertilized with sperm from two males. The fertilized eggs were gently rinsed several times with well water to remove excess milt and then incubated in dechlorinated tap water at 25 ^{0}C for microinjection.

Microinjection of prepared DNA into fertilized eggs

Microinjection was carried out by Eppendorf 5242 Microinjector. The injection volume was controlled by using a duration of insertion-withdrawal interval a constant plasmid flow rate. Since first claevage took place within a few minutes after fertilization, microinjection of the fertilized egg needed to begin with in 10 min after fertilization. Figures 8, 9 and 10 showed a

microinjection of plasmid DNA in one-cell, two-cell and four-cell stages, respectively. For fixing eggs were placed on petridisc by submersion in little well water. Plasmid solution was pipeted into microneedles by using microloader. During microinjection, about 240 pl containing approximately 10^6 copies of plasmids per second was delivered through the chorion and vitelline membranes into the germinal disc but did not percingly to the yolk. The microneedle with driving plasmid solution is shown in Figure 11. After injection, each stages of microinjected eggs and uninjected eggs (control egg) were removed by soft brush or dropper and separately incubated in a beaker containing 200 ml well water and incubated at 25 ^{0}C.

Incubation of embryos and frys

After microinjection for 4-8 hr, each microinjected and control egg was transferred to incubate at 27-30 ^{0}C in enameled basin containing 5 litres of well water with gently flowing dechlorinated tap water. Hatching occured approximately 24-30 hr after fertilization. The fries were then transfered to separately incubate in jars containing 5 litres of well water with gentle air supply. Hatching rate were later recorded.

After yolk sac resorption (2.5-3 days after hatching), the fries were fed with newly hatching brine shrimp or freezed brine shrimp for 3 times daily (8.00, 12.00 and 18.00 hour). Dialy maintenance included siphoning of the remaining food and feces, removal of any dead fry, and observation of the general healths was done in the morning. Survival rate was determined at one

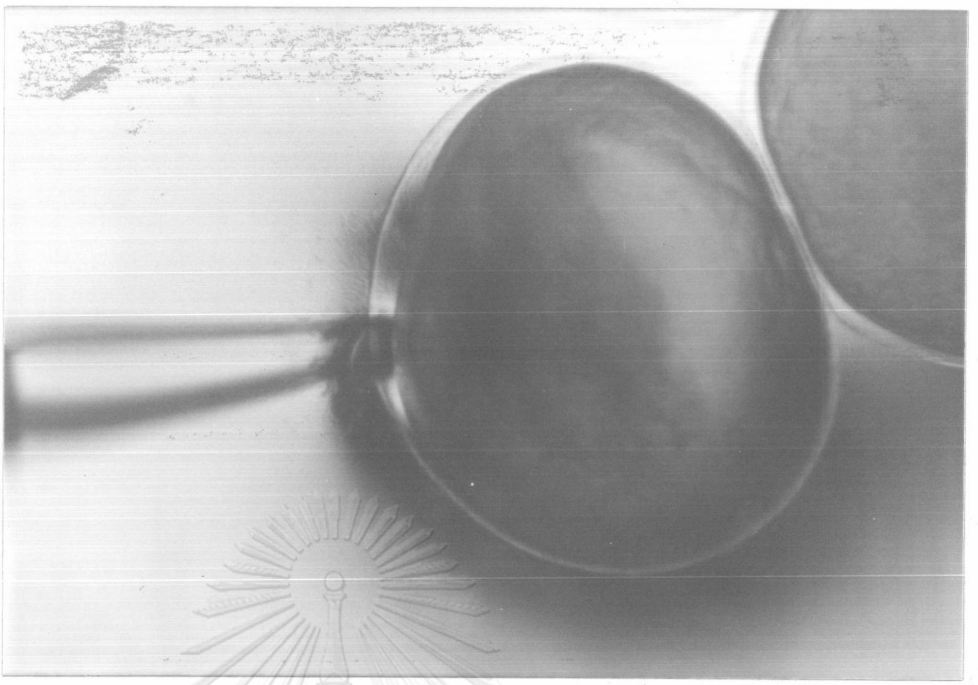

Figure 8 Microinjection of the foreign gene into one-cell stage fertilized egg of catfish

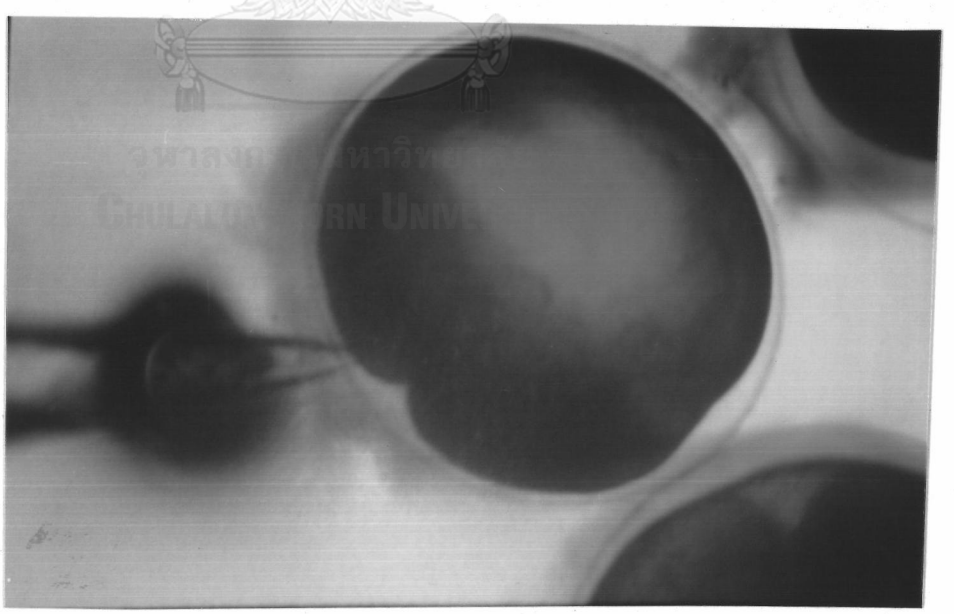

Figure 9 Microinjection of the foreign gene into two-cell stage fertilized egg of catfish

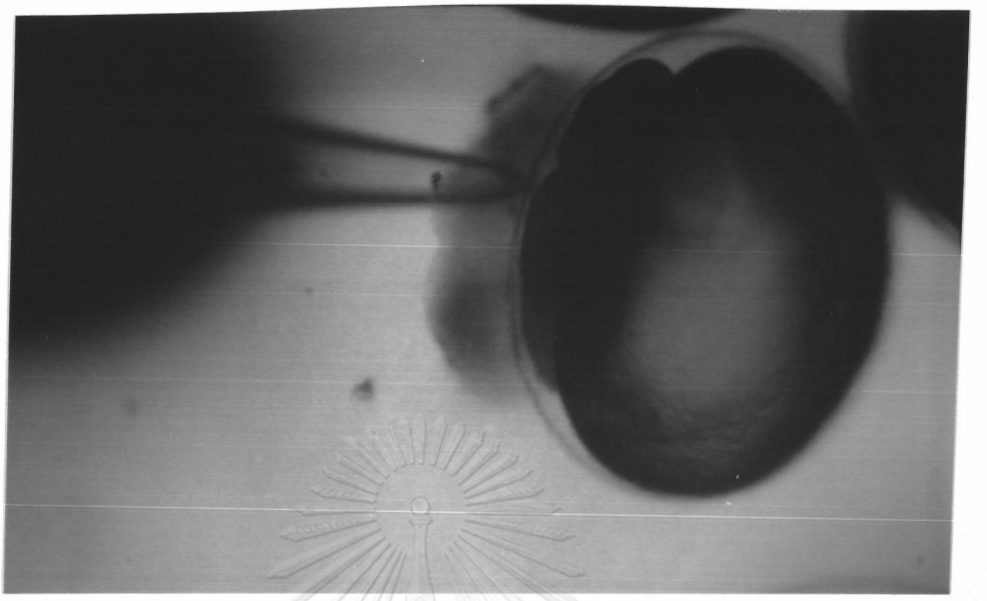

Figure 10 Microinjection of the foreign gene into four-cell stage fertilized egg of catfish

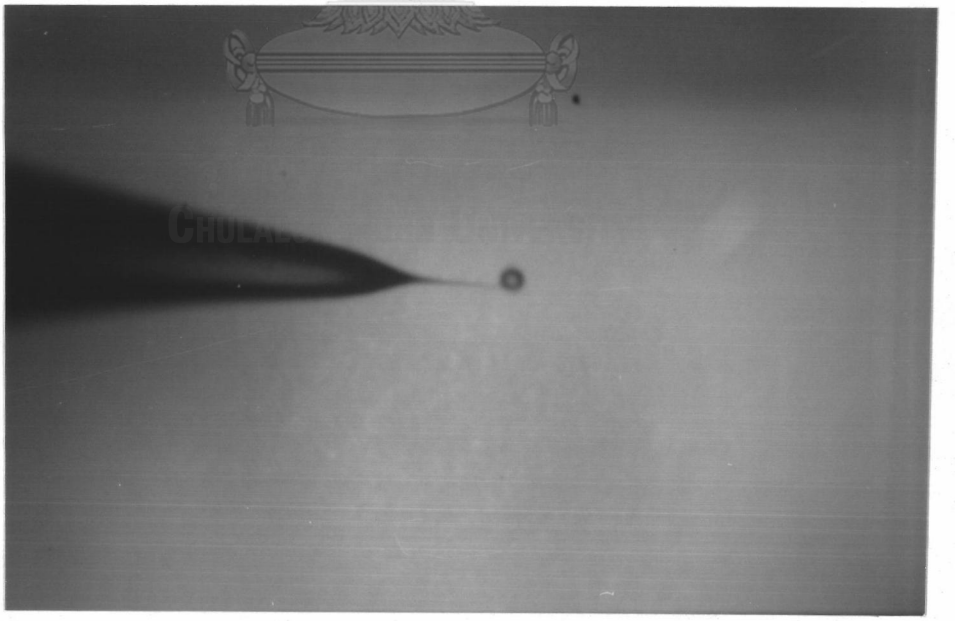

Figure 11 The microneedle (about 0.5 ± 0.2 μm of diameter) driving plasmid solution

month after hatching and then the fish were transferred to the next experiment.

Fish culture and weight measuring

For growth experiment, the injected fish and control fish of the same spawner were separately reared in cages (30 * 45 *30 cm^3) at similar densities (Figure 12). Fish were fed with commercial catfish feed pellets (Unicord Feed Co. Ltd.) twice a day. The proximate content of feed is crude protein 30 %, carbohydrate 46 %, fat 4 %, fiber 8 % and moisture 12 %. Daily maintenance included siphoning of the remaining food and feces, replacing new well water and net cleaning was done during the morning. When the fish grew bigger, the space was limited. The fish were moved into new larger cages to maintain optimum space conditions. Fish of each group were weighed and its age counted from the day hatched out.

Statistical analysis

To test the difference on hatching rate, survival rate and growth rate between microinjected and control fish, the statistical analysis were carried out by using statistical computer software. The general linear model was computed to compare hatching rate and survival rate, and the linear regression was computed to compare the growth rate by using SAS/STAT™ (SAS/STAT™, 1989)

Figure 12 Rearing cage of microinjected fish and control of the same spawner

Amplification of 186 bp fragment from pXGH 5 by PCR

Primer designation

a) Primer designation of human growth hormone gene

A pair of primers for Polymerase Chain Reaction (PCR) were designed from human growth hormone DNA sequences. Dissimilar of growth hormone gene between human and piscine was majority consideration in order to prevent the amplification of non designed fragment.

b) Primer designation of fish growth hormone gene

A pair of primers (PCR) were designed from *Pangasius sutchi* growth hormone gene complementary DNA sequences, which have been in Dr. Sakol's laboratory.

Preliminary PCR condition

The original PCR conditions were taken from a pair of hGH designed primers. The pXGH 5 plasmid was used as a template at concentrations; 1, 5, 10, 100 pg and 1 ng. The PCR mixture of 1x PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01 % w/v gelatin), 200 μM each of (dATP, dTTP, dGTP, dCTP), 1 μM of each primers and 2 units of taq polymerase enzyme were added. Sterile distilled water was added to make a final volume as 50 μl. The reaction mixture was overaid with 2 drops of mineral oil, and performed for 30 cycles in a DNA Thermal Cycler Machine

(Perkin Elmer Cetus), which consisted of denaturation at 95 ^{0}C for 1 min, comparison the annealing temperature between 50 ^{0}C and 55 ^{0}C for 1 min, and extension at 72 ^{0}C for 1 min. One-fifth (10 µl) of the PCR product was analysed by 3 % agarose gel electrophoresis.

Blood sampling

Blood samplings were obtained when the body length of fish was more than 7 cm by using 1 ml syringe with needles no. 21g * 1 1/2 containing 20 µl of 6 I.U. heparin. To collect blood sample, the needle was penetrated from anal fin to the caudal vessels. Figure 13 showed the blood sampling from the caudal vessels. Then 100 µl of blood was taken, homogeneously mixed and aliquoted to test. After blood sampling, fish were individually maintained in well water with 30 ppm tetracyclin HCl and 0.5 % NaCl for 5-7 days. Daily replacement of new treated water was done every morning. Some dead fish were collected and freezed at -20 ^{0}C for further experiment.

Blood preparation by non-processing method

Blood cells were lysed with 0.001 % SDS (Tirasophon, 1991) or 10 mM NaOH (Davies and Gauthier, 1992). Three processes of blood cells lysis had been performed. First, all fish blood (100 µl) was mixed with 0.001 % SDS or 10 mM NaOH, and immediately heated at 100 ^{0}C under mineral oil layer for 20 min, cooling and then spun for 1 min. 10 µl of supernatant was diluted with sterile distilled water to make a final volume 50 µl. Second process, 5 µl of fish blood was mixed with 0.001 % SDS or 10 mM NaOH,

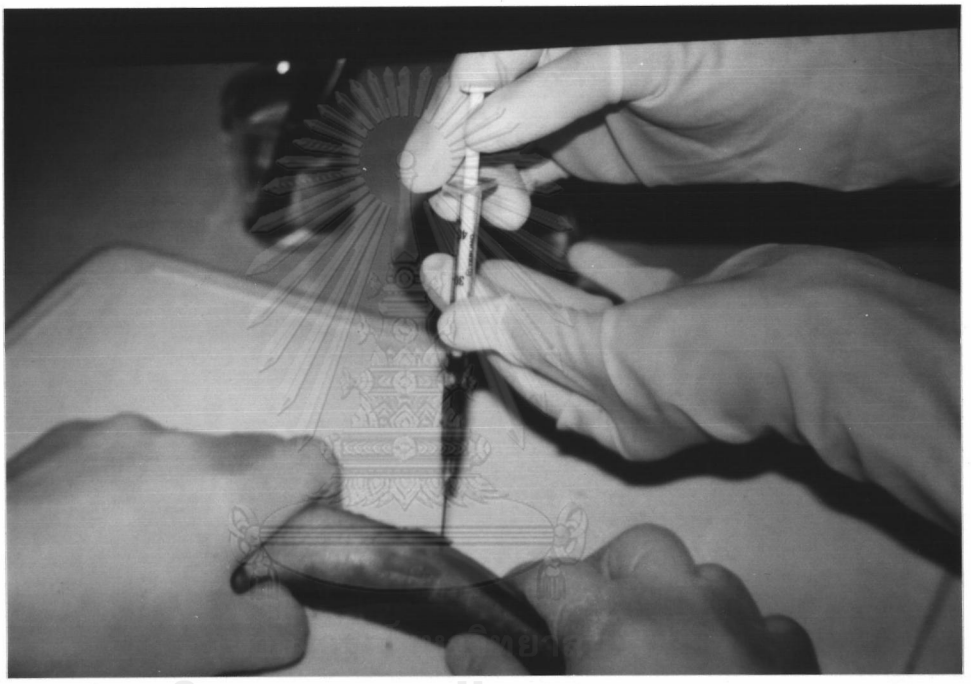

Figure 13 The blood sampling from the caudal vessels

(The anal fin is up in this illustration)

immediately heated at 100 ^{0}C under mineral oil layer for 20 min, 40 µl of sterile distilled water was added. The last procedure, 5 µl of fish blood was mixed with 0.001 % SDS or 10 mM NaOH, subsequently mixed with 40 µl of sterile distilled water, immediately heated at 100 ^{0}C for 20 min. DNA patterns of blood cells from the three processes were analysed by one-fifth of each reaction loading in 3 % agarose gel electrophoresis.

Optimization of PCR conditions for detection the introduced gene.

The optimal conditions for detection of introduced gene using a pair of hGH primers were determined. In order to improve the efficiency of the amplification and to decrease the non-specific designed DNA amplification, the following factors: lysis solution, blood amount, primers concentration, annealing temperature, annealing time, extension time and heparin effects were optimized.

Blood lysis solution for PCR and quantity of blood

Two replications of 1, 2.5, 5 and 10 µl of blood sample were lysed with and equal volume of 0.002 % SDS and 20 mM NaOH. Sterile distilled water was added to make 30 µl. The lysis reaction could be rared on ice before or immediately heated at 100 ^{0}C under 2 drops mineral oil layer for 20 min. 5 pg of linearized pXGH 5 was added into a replicate for positive control. The 20 µl PCR mixtures as in preliminary PCR condition was added at 75 ^{0}C and the PCR reaction was performed for 30 cycles, consisting of denaturation at 95 ^{0}C for 1 min, annealing at 55 ^{0}C for 1 min and extension at 72 ^{0}C for 1 min.

One-fifth (10 µl) of the PCR product was analysed by 3 % agarose gel electrophoresis.

Primer concentration and annealing temperature

2.5 µl of fish blood were lysed with an equal volume of 20 mM NaOH, and subsequently adding with 25 µl sterile distilled water, heating at 100 ^{0}C under 2 drops of mineral oil layer for 20 min. 5 pg of linearized pXGH 5 was added for the positive control. The PCR mixtures with two different primer concentration as 1 and 0.1 µM was added at 75 ^{0}C. The PCR reactions were performed for 30 cycles as the same manner condition with two different annealing temperature at 50 ^{0}C and 55 ^{0}C. One-fifth of the PCR products was analysed by 3 % agarose gel electrophoresis.

Annealing and extension time with concentration of primers

Preparations of blood samples and the mixtures were performed as the previous same manner. The PCR reactions were carried out for 30 cycles with two different annealing times, 15 sec and 60 sec, and two different extension times, for 15 sec and 60 sec. One-fifth of the PCR products was analysed by 3 % agarose gel electrophoresis.

Heparin effect

2.5 µl of fish blood in the present 1 I.U. heparin/ml blood was lysed with 2.5 µl of 20 mM NaOH, adding with 25 µl sterile distilled water,

heating at 100 ^{0}C under mineral oil layer for 20 min. 5 pg of linearized pXGH 5 was added for the positive control. The 20 µl PCR mixtures containing 1x PCR buffer 200 µM of each dNTP, 0.1 µM of each primers and 2 units of taq polymearase enzyme was added at 75 ^{0}C. The PCR reaction were performed for 30 cycles, which consisted of denaturation at 95 ^{0}C for 1 min, annealing at 55 ^{0}C for 15 sec, extension at 72 ^{0}C for 15 sec. One-fifth of PCR products were analysed by 3 % agarose gel electrophoresis.

Amplification of 186 bp fragment in human blood

Human blood in the presence of heparin 1 I.U./ml blood was lysed with an equal volume of 20 mM NaOH and mixed with sterile distilled water to make volume as 30 µl, heating at 100 ^{0}C under mineral oil layer for 20 min. The PCR reaction was done by adding, PCR mixtures within the conditions as in heparins effect method. One-fifth of PCR products was analysed by 3 % agarose gel electrophoresis and 1.5 % agarose gel electrophoresis with Southern blot hybridization confirmation.

Amplification of 150 bp fragment in fish blood

Samples of *C. macrocephalus* and *P. Sutchi* blood were performed as the previous same manner. The PCR mixture containing 1x PCR buffer, 200 µM of each dNTP, 0.1 µM of each fish growth hormone gene primers, and 2 units of taq polymerase enzyme was added at 75 ^{0}C. The PCR reactions was performed for 30 cycles which consisted of denaturation at 95 ^{0}C for 1 min,

annealing at 50 ^{0}C for 15 sec, extension at 72 ^{0}C for 15 sec. One-fifth PCR products were analysed by 3 % agarose gel electrophoresis.

Detection of introduced gene by PCR method

2.5 µl of microinjected and control fish blood samples were lysed with an equal volume of 20 mM NaOH, mixed with 25 µl of sterile distilled water, heating at 100 ^{0}C under mineral oil layer for 20 min. The heated lysed blood was then tested or kept at 4 ^{0}C for further working. The PCR reactions were carried out by heating at 100 ^{0}C for 5 min, adding the PCR mixtures as described in heparin effect method into the reaction at 75 ^{0}C, and performed for 30 cycles consisting of condition as described in heparin effect method. One-fifth of PCR products were analysed by 3 % agarose gel electrophoresis.

After PCR detection, the result was confirmed by hybridization with nonradioactive probe. 10 µl of PCR products were confirmed by dot blot and hybridization. 10 µl of PCR products in 1.5 % agarose gel electrophoresis were confirmed fragment size by southern blot and hybridization.

Nonradioactive DNA labelling

a) Digoxiginin-11-dUTP labelled DNA probe by random primed labelling

pXGH 5 plasmid DNA was labelled with digoxigenin-11-dUTP by using the random primed DNA labelling (Mannheim Biochemica, 1989).

600 ng - 1 µg of linearized pXGH 5 DNA was denatured at 95 ^{0}C for 10 min, and quickly chilled on ice. 2 µl of hexanucleotide mixture, 2 µl of dNTP labelling mixtures (1 mM each of dATP, dCTP, dGTP, 0.65 mM dTTP and 0.35 mM Dig-dUTP,pH 6.5) and 1 µl of klenow enzyme (2 units) was mixed, made up volume to 20 µl with sterile distilled water. The mixtures was incubated at 37 ^{0}C for at least 1 hr but not more than 20 hr. The reaction was stopped by adding 2 µl of 0.2 M EDTA solution pH 8.0. The labelled DNA was precipitated with 2 volumes of absolute ethanol in the presence of 1/10 volume of 3 M Sodium acetate pH 5.5, kept at -20 ^{0}C overnight, and centrifuged at 10,000 rpm for 15 min. The pellet was washed with 50 µl of 70 % ethanol, air dried, and redissolved in 50-100 µl of TE buffer pH 7.4, kept at -20 ^{0}C for futher uses. For hybridization, 25 µl of DNA probe solution was added in prehybridization solution.

b) Digoxiginin-11-dUTP labelled DNA probe by specific primer labelling

100 ng of linearized pXGH 5 DNA was denatured at 95 ^{0}C for 10 min and chilled on ice. Reaction mixture containing 100 ng of, 5 µl of 2 µM specific primer, 2 µl dNTP labelling mixture (1.25 mM each dATP, dCTP, dGTP, 1 mM dTTP and 0.25 mM dig-11-dUTP), 1 µl of 10 mg BSA/ml, 7 µl of TM buffer (250 mM Tris-HCl, 25 mM $MgCl_2$, 50 mM 2-mercaptoethanol, pH 8.0), 7 µl of 1 M HEPES pH 6.6, 1 µl of 2 units/µl klenow enzyme and sterile distilled water to make volume up to 50 µl was incubated at 37 ^{0}C for 2 hr. The reaction was stopped by adding 50 µl of

250 mM EDTA pH 8.0. For hybridization, 25 µl of DNA probe solution was added in prehybridization solution.

Southern blot procedure

In order to transfer DNA fragment from gel agarose eletrophoresis to membrane (Gene Screen Plus™) was carried out by southern capillary blot procedure (Gene Screen Plus™, 1987). The DNA fragment in agarose gel was incubated in 0.4 N NaOH - 0.6 N NaCl for 30 min and neutralizing with 1.5 M NaCl - 0.5 M Tris-HCl pH 7.5 for 30 min. The membrane was cut to exact size of gel and placing on side B. The membrane was equilibrated with sterile distilled water and subsequently in 10x SSC solution (1.5 M NaCl, 0.15 tri-sodium citrate) for 15 min. The blotting papers should be larger than the gel, were placed into a container filled with 10x SSC. Two pieces of filter papers were cut to the same size of the blotting papers, soaked in 10x SSC and placed on the blotting papers. The agarose gel was placed on filter papers. The membranes was carefully placed on the gel so that side B was contacted with the gel and avoiding any air bubbles were traped between the gel and membrane. Two pieces of filter papers were cut to the same size of membrane and placed on. The blotting papers, which were same size of membrane, placed to 2-3 inches stack towels on the top of filter papers, and pressed with appropriate weights. The DNA was allowed to transfer for 16-24 hr with frequently changing of towels papers. When it is complete, the towels papers and filter papers were carefully removed and the membrane was carefully lifted away from the gel. The membrane was immersed in 0.4 NaOH for 60 sec for denaturation of immobilized DNA,

and then neutralized in an excess of 0.2 M Tris-HCl pH 7.5 - 2x SSC for 30 min. The membrane could then be used directly for hybridization or stored at 4 ^{0}C for later hybridization.

Dot blot procedure

DNA dot-blot analysis (Gene Screen Plus™, 1987) was used to detect the presence of the introduced gene within the genome of fish. The membrane (Gene Screen Plus membrane) and 2 filter pads were cut for appropriate sizes, soaked in 0.4 M Tris-HCl pH 7.4 for 30 min. DNA was denatured in 0.25 N NaOH for 10 min and chilled on ice. Denatured DNA was diluted in 0.125 N NaOH, 0.125 x SSC. The membrane and filter pads were placed in manifold and clamping tightly. The diluted DNA was added to the wells of manifold. The DNA was remained on the membrane by applying a slight suction. The membrane was removed and transferred to 0.5 N NaOH two times, gently agitated for 5 min. The membrane was incubated in 1 M tris -HCl pH 7.4 two times for 5 min and subsequently in 0.5 M tris-HCl, pH 7.4 - 1.5 M NaCl two times for 5 min. The membrane was blotted dry at room temperature and used directly for hybridization or stored at 4 ^{0}C for later hybridization.

Hybridization

The DNA-bound membrane from dot blot or southern blot was hybridized as following protocols (Gene Screen Plus™, 1987). The DNA-bound membrane was prehybridized with 10-15 ml of prehybridization (PHB) solution (50 % v/v formamide, 5x SSC, 5x Denhardt's solution (0.1

% filcoll 400, 0.1 % polyvinylpyrolidine, 0.1 % BSA fraction V, 5 mM EDTA), 20 mM tris-HCl pH 7.5) and also 2 % SDS in sealed plastic bag. Heated-denatured sonicated salmon sperm DNA was added to make a final concentration of 100 µg/ml of PHB solution. All air bubbles in plastic bag containing the membrane were removed. After sealing, the bag was incubated at 42 ^{0}C at least 1 hr with gentle agitation. Hybridization solution was prepared by adding the heat-denatured DNA probe to the PHB solution. The membrane was incubated in hybridization solution for overnight at 42 ^{0}C with gently agitation. After hybridization the membrane was washed twice in an excess volume of 3 x SSC for 15 min at room temperature, and twice in 0.1 x SSC-0.1 % SDS at 55 ^{0}C for 30 min. the membrane could then be used directly for detection or stored at 4 ^{0}C for later detection.

Rehybridization

Membrane could be rehybridized with new DNA probed as following protocols (Gene Screen Plus™,1987). The washed membrane was not be completely dry to prevent inreversible binding of probe. The membrane was incubated in 100 ml of 0.4 NaOH at 42 ^{0}C for 30 min with agitation. Then the membrane was incubated in 100 ml of 0.1 x SSC, 0.1 % SDS, 0.2 M tris-HCl pH 7.5 at 42 ^{0}C for 30 min with agitation. The membrane was blotted with filter papers, and used directly for hybridization or stored at 4 ^{0}C for later hybridization.

Detection of hybridization

After hybridization with nonradioactive DNA probe, the membrane was detected by chromogenic detection or chemiluminescent detection (Mannheim Biochemica, 1989).

a) Chromogenic detection ;

The hybridized membrane was washed briefly in buffer 1 (100 mM tris-HCl and 150 mM NaCl, pH 7.5), incubated for 1 hr with buffer 2 (0.5 % w/v blocking reagent in buffer 1) and washed again briefly in buffer 1. The antibody alkaline phosphatase conjugate was diluted to 150 mU/ml in buffer 1. Then, the membrane was incubated in the diluted antibody-conjugate solution for 30 min, subsequently washed to remove unbound antibody twice in buffer 1 for 15 min. The membrane was equilibrated for 2 min in buffer 3 (100 mM tris-HCl, 100 mM NaCl and 50 mM $MgCl_2$, pH 9.5) and incubated in the dark with freshly prepared of colour solution (45 µl NBT solution and 35 µl x-phosphate solution in 10 ml buffer 3). NBT solution is composed of 75 mg/ml nitroblue-tetrazolium-chloride in 70 % v/v dimethylformamide and x-phosphate solution in 50 mg/ml 5-bromo-4-chloro-3-indodyl phosphate,toluidinium salt in dimethyl formamide. When the blue precipitate of the desired bands were detected, the reaction was stoped by washing the membrane in TE buffer pH 8.0 for 5 min and the result was recorded by photographing the membrane.

b) Chemiluminescent detection

The membrane was blocked and conjugated with antibody as described in chromogenic detection, but the step of coloration was replaced by luminescing. The membrane was incubated in 10 ml diluted AMPPD solution (100 µl AMPPD in 10 ml buffer 3) for 5 min in sealed plastic bag. After the membrane was blotted, it was sealed in transparent plastic bag, and then expossed to X-rays film for 1-2 hr.

Genomic DNA extraction from blood and tissues

High molecular weight DNA was extracted from tissues samples and clotted fish blood by using genomic DNA extraction (Brem et al., 1988). Samples were homogenized in 9 volumes of 50 mM Tris-HCl, 100 mM EDTA, 100 mM NaCl, pH 8.0 using mannual homogenizer, and transferred into incubation tube. The mixture was lysed with 0.5 % SDS and 100 µg protinase K/ml , gentle flip-flop, and incubated in 55 ^{0}C for 8-18 hr. After complete digestion, samples were extracted twice for 15-20 min with an equal volume of phenol/chloroform/isoamyl-alcohol (25:24:1) and extracted again for 15 min with an equal valume of chloroform/isoamyl-alcohol (24:1). If aqueous phase was not clear, phenol/chloroform extraction could be performed again. The aqueous phase was precipitated with an equal volume isopropanol in the presence of 1/10 volume 3 M sodium acetated (pH 6.0). Genomic DNA was hooked by sterile pasteured pipette or collected by centrifugation at 5,000 rpm for 20 min and washed twice with 70 % ethanol, resuspended in TE buffer and left overnight at 4 ^{0}C. High molecular weight DNA was analysed by 0.7 %

agarose gel electrophoresis. DNA concentration was determined by UV spectrophotometer at 260 nm using the formula :

1 OD 260 nm = 50 μg/ml of DNA concentration.

10-15 μg of DNA prepared in this manner was then directly used for dot blot analysis.