CHAPTER IV

DISCUSSION

The present study, plasmid pXGH 5 was used for gene transfering into early fertilized yellow walking catfish eggs. This plasmid contains mouse-Metallothionein-I promoter gene and the human growth hormone gene, (structural gene). Several successful production of transgenic fish had reported the use of this similar gene construct such as in goldfish (Zhu et al., 1985), loach (Zhu et al., 1986), channel catfish (Dunham et al., 1987) and tilapia (Brem et al., 1988). This plasmid was linearized without disruption of the human growth hormone gene function by using BamH I digestion. Whole linearized plasmid was introduced without elimination the part of pUC 12, that was also used in some transgenic fish research (Yoon et al., 1990; Zhang et al., 1990; Inoue et al., 1992; Volckaert et al., 1994). Palmiter and Brinster (1986) noted that inclusion of prokaryotic vector sequences in the plasmid DNA molecules injected into mouse eggs resulted either in lack of expression or poor expression of the foreign gene. Nevertheless Penman et al. (1990) found the egg injected with novel gene gave slightly, but not significant different than those injected with whole plasmid.

Hatching rate of microinjected embryos

From the result of Table 6, the hatching rate of microinjected egg at one-cell, two-cell, four-cell and control (uninjected egg) were 25.91 %, 29.71 %, 30.94 % and 40.67 %, respectively. Significant differences ($P<0.05$) on hatching rate of eggs was found between microinjected eggs and the control. The lower hatching rate of microinjected embryos might be due to the injury during microinjection process.

In this study, plasmid solution approximate 10^6 copies was injected into the germinal disc of eggs. Chourrout et al. (1986) suggested that higher amounts of DNA led to much lower survival of embryos. Penman et al. (1990) explained that lower amounts of DNA do not enhance the number of surviving animals but tended to give less production of transgenic fish. Coincidentally, 10^6 copies was the most common document on transgenic fish researches, especially in tilapia (Brem et al., 1988), common carp (Hayat et al., 1991 ; Zhang et al., 1990), channel catfish (Hayat et al., 1991), rainbow trout (Yoshizaki et al., 1991) and African catfish (Volckaert et al., 1994). Additionally, MacLean and Penman (1987) and Phillips et al., (1992) had studied the effect of plasmid concentration, and conclude that plasmid DNA concentration as approximate 10^6 with a range from 10^5 to 10^9 was not toxic for microinjection into fish eggs.

Volume of plasmid DNA solution injection in this study was about 240 pl at a concentration of 30 ng/μl. Some researches had injected as similar amount of plasmid DNA solution,), 250 pl in tilapia (Rahman and Maclean, 1992), 300 pl in the gilthead seabream (Cavari et al., 1993) No toxicity of

plasmid DNA was reported at these volumes. In addition, some reports had injected that higher volume but did not found the different survival of injected eggs and uninjected eggs (Yoshizaki et al., 1991; Phillips et al., 1992; Inoue et al., 1993)

Plasmid DNA was suspended in TE buffer pH 7.4 (10 mM Tris-HCl pH 7.4, 1 mM EDTA). Excess of EDTA has been reported to be toxic in fish eggs (Stuart, 1988). Many researches used similar ingredient of buffer for plasmid DNA suspension, such as in medaka (Ozato et al., 1986; Inoue et al., 1989), in tilapia (Brem et al., 1988), in goldfish (Yoon et al., 1989) and in rainbow trout (Yoshizaki et al., 1990). Penman et al. (1990) suggested that, it might be possible to increase the survival rate of injected embryos by using NT buffer (88 mM NaCl, 10 mM Tris-HCl pH 7.5) instead of TE buffer, but their results did not found significant differences between using TE buffer and NT buffer.

This plasmid solution was not mainly affected the microinjected eggs. Phillips et al.(1992) reported that there was no significant difference between sham microinjected embryo survival and plasmid DNA-microinjected embryo survival. Major factor affecting on hatching might be a physical injury of microinjection technique and the quality of eggs. Our result showed that the average hatching rate of microinjected egg at all developmental stages was 28.63 %, and uninjected egg was 41.14 %. Several researches reported that, microinjection trauma affected survival and hatching rate of eggs. Microinjection of plasmid into zebra fish egg, gave the hatching rate 50 % comparing with uninjected egg 65 % (Culp et al., 1991). The survival rate of microinjected eggs of goldfish was 50 %,comparing with 90 % of uninjected egg

(Yoon et al., 1989). About 67 % of microinjected rainbow trout eggs hatched out comparing with 89.5 % of uninjected eggs (Yoshizaki et al., 1991). In catfish, the hatching rate of microinjected egg in African catfish (*Clarias gariepinus*) as 53 %, comparing with 69 % of uninjected egg (Volckaert et al., 1994). However, in some study, the disastrous effect from the microinjection might not be found for instance, in transgenic rainbow trout (Zhang et al., 1990 ; Inoue et al., 1992), salmon (Hew et al., 1992), and northern pike (Gross et al., 1992).

Although no significant differences in the hatching rates between various stage of egg development. Microinjection at more advanced stages seemed to favor high embryo hatching rate. High mortality of microinjection embryos at one-cell stage was affected by a reason that, newly fertilization egg formed a thin germinal disc and normally oriented up. The chorion is not very glutinous and hardeness. Direct depression of microneedle would easily penetrated through the chorion, large number of egg could be performing, but also easily injured traumatic egg cell. If the micropipette would break the egg chorion such a large hole, immediately died because of that cytoplasm or yolk leakage results. When later injection at two-cell an four-cell, the formation of germinal disc enlarges, would increase withstanding the injury of injection, hatching rate at these stages were higher than early stage. At the same time, the chorion is glutinous and hard to penetrating. The germinal disc naturally horizontal orientation and must be turned until oriented up, development interval is shorter than the early development, thus small number of egg had performed. In this study, we could determine that one-cell stage was the most appropriate developmental stage for production of transgenic yellow walking catfish by using

microinjection method. This most appropriate stage had also found in channel catfish (Hayat et al., 1991) and tilapia (Rahman and MacLean., 1992). Some contradictory results were found in the study of Rahman and MacLean (1992), when the two-cell or four-cell stage injection of tilapias increased mortality rate. Penman et al. (1990) found that the late embryonic stage injection showed reduced survival compared to the early embryonic stage injection groups. In common carp microinjection at all stages of early embryonic development did not affect on survival rate of the embryo (Hayat et al., 1991).

One of the problems in transgenic induction in fish is that the embryonic development is rapid following fertilization (Rahmman et al., 1992). The embryonic development of fertilized egg in yellow walking catfish after fertilization to one-cell, one-cell to two-cell, and two-cell to four-cell stage, at water temperature 28 ^{0}C, were about 45, 20 and 20, minutes, respectively. Manipulation at temperature 25 ^{0}C could delay embryonic development in order to allowing ample time for microinjection extending to about 60-90, 30, and 30 minutes, respectively. This delay technique had no apparent effect on egg survival. In transgenic tilapia, Phillips et al. (1992) also used low water temperature to prolong egg development during microinjection. The present study, we did not use any supported techniques during microinjection because the chorion is not too hard to penetrate by microneedle, and the germinal disc is clearly recognizable under a microscope. Microinjection carried out by injection into the germinal vesicle. The nucleus could not visualize, this seems to occur in almost transgenic fish, except the injection of medaka fish 's egg (Inoue et al., 1989).

One month survival of the microinjected fish at one-cell, two-cell, four-cell stages, and control were 52.02 %, 46.67 %, 35.51 % and 53.61 %, respectively. There were no significant differences ($P>0.05$) among the survival rates of all stages microinjected fry and control. The result indicated that microinjected trauma had no effect on the hatched fry. The similar results had been reported in common carp and rainbow trout (Zhang et al., 1990; Inoue et al., 1993). However, Inoue et al. (1989) found the 18.47 % of microinjected medaka fry was morphologically abnormal.

Detection the introduced gene

To detect the introduced gene, PCR method was used for direct detection of the gene in blood. This method is an alternative way to amplify the low copy number of gene in order to increase sensitivity of detection, simplified screening for specific DNA sequences, ability to screen large number of samples rapidly, and requiring the small amount of blood sample (Davies and Gauthier, 1992). There are quite a few defects in this method for instance. Contaminating of reaction particularly by the PCR product, which generates false positive, and a failure of the amplification reaction, which could be scored as a false negative. To eliminate PCR product contamination, all reagents used in PCR were aliquoted, the pre-PCR and post-PCR reagent, equipment and working area need to be separated. In addition, since Rajsakulchai (1992) suggested that 1 % clorox could destroy plasmid DNA within 5 minutes. 1 % clorox was then used as decontaminate for PCR technique. Due to fish stress when they are handle for blood sampling, specially, facilitated the invasion of bacteria *Aeromonas hydrophilla*. Thus after blood sampling, the fish must be immediately treated

with antibiotic drug. Houston (1990) had suggested that fish should be avoid to take blood sampling for several occasions.

It appears to be a great deal of similarity, or homology, among the growth hormone proteins of fish and human from comparing the conserved amino acids in mature growth hormone (Watahiki and Yamamoto, 1989). So almost dissimilar of growth hormone between human and piscine was chosen to be amplification the target of 186 bp fragment in human growth hormone introducing gene, and a set of primer were designed, in order to avoid the amplification of non designed fragment from fish genome, or false positives result occurring. Figure 5 shown human growth hormone gene sequence consists of five exons and four intervening sequences. One primer annealed to a 19 bases sequence of the fourth intervening sequences at position 98 to 116 or then called 5' end primer, and 3' end primer, annealed to a 16 bases sequence of the fifth exon at 15 to 30 position with equaling of melting temperature (Tm) 58 ^{0}C. However this set of primer could detect only some part of the human growth hormone gene.

Preliminary study in specificity of this part of primers in pXGH 5 plasmid amplification showed the satisfying PCR result at the lowest of template of 5 pg. The expected band 186 bp could be intensively visible. A small fragment about 40 bp in size is clearly visible in the reaction containing very low or no template. This small fragment is possible the primer-dimer formation, because its length is very closed to the sum of two primers. The formation of primer-dimer was observed in low annealing temperature, high enzyme, high primer concentration and very low copies of the initial template (Innis and

Gelfand 1990; Saiki, 1989). Anyway, in this experiment, occurrence of the dimer does not interfere the interpretative result. Furthermore, the primer-dimer band can serve as an internal control indication of successful amplification in the individual reaction tube. At annealing temperature 50 ^{0}C, the non specific bigger bands was also indistinctly visible. This may be a possibility caused by too low of annealing temperature or high concentration of primers or jointly interaction.

Since the fact that blood is composed of many components may affect PCR reaction. Our observation agrees with the believe that hemoglobin, in red blood cell, can inhibit PCR reaction (Keller et al., 1990). The boiling time, 20 minutes, is excess to lyse cell, to release DNA, to denature the nuclease and to precipitate hemoglobin (Tirasophon, 1991). Blood was lysed and observed for obtaining DNA patterns. DNA patterns obtaining from the first process, which were modified from method of Davies and Gauthier (1992), were not consistent. When the lysate blood was boiled, some random DNA was trapped in the pellet of cell debris. This process was not used for blood preparation prior PCR, because some trapped DNA could not annealed with the primer, and may cause failure of the DNA amplification or false negative. In the same reason in the second process, which was modified from the method of Tirasophon (1991), only consistently DNA arrounding the pellet of cell debris was dissolved, but not some of detained DNA in the pellet. While in the third process, most DNA in the cell was dissolved in water before boiling. Thus the third process was considered to be a appropriate method for blood cell lysis prior PCR detection, because the almost of DNA in the cell was analyzed.

The PCR product from the blood lysised with NaOH seems to be more intensively visible than lysis with SDS. When blood was lysed with NaOH, pH of PCR reaction was about 9.2. This result was possible that the property of the lab produced taq polymerase which has a maximum activity between pH 8.0-9.5 (Luxananil, 1992). Blood volume used in this experiment was 2.5 µl. This was an appropriate volume for the template of PCR detection because blood template at 5 and 10 µl gave too high intensities to distinct the expected 186 bp, while blood template as 1µl is too small amount, that was easy to error pipetting.

Optimization of PCR condition

Concentration of primers used in the PCR reaction is one of the major parameters affecting the amplification product. High primer concentrations may promote mis-priming of the primers that occasionally bind to the genomic DNA, increase accumulation of non-specific product, and may increase the primer-dimer artifact (Innis and Gelfand, 1990). Under estimation of the primer concentration will result in lower yield of the PCR product. In our experiment , the suitable concentration at 0.1 µM considered to be a more deletion of the non-specific PCR product than use 1 µM primers concentration and reduced primer-dimer formation with did not significant reducing the yield of 186 bp product.

Initial heating for 5 min was used to ensure complete denaturation of the complex genomic DNA prior starting PCR cycle as recommended by Innis and Gelfand (1990). PCR reagent mixture was added to the reaction at 75 ^{0}C in order to prevent reannealing of genomic DNA. Denaturation temperature at 95 ^{0}C in PCR cycle is used for ensuring separation of the genomic DNA. Even

though taq polymerase is a thermostable enzyme, but too high temperature has the effect on the activity of taq polymerase enzyme. The lab-produced taq-polymearase shows stability since approximately 50 %, 67 % and 75 % of the activity lost after 5 min heating at 85 ^{0}C, 90 ^{0}C and 95 ^{0}C, respectively (Luxananil, 1992). In addition using a high concentration, 2 units/50 µl reaction and only 30 number of cycles, could adequate for leaving enough activity until finish the last amplification cycle. These also suggested that for amplification reactions involving DNA samples with high sequence complexity, such as genomic DNA, usually 1 to 4 units per 100 µl (Saiki, 1989).

Generally, the annealing temperature depends on the primer length and base composition (G + C) content of the primers (Saiki, 1989). Increasing the annealing temperature will enhance discrimination against incorrectly annealed primers and reduced misincorparation at 3'end primers (Innis and Gelfand, 1990). Stringent annealing temperature will help to increase specificity. The present study, the stringent conditions for annealing 55 ^{0}C in the PCR cycle can be used to avoid non specific priming of the primers. Innis and Gelfand (1990) also suggested an applicable annealing temperature at approximately 5 ^{0}C below the true Tm of the amplification primers. Minimizing the incubation time during the annealing will limits the opportunities for mispriming, so using with the short annealing temperature, 15 seconds in the PCR cycle could be deletion the non specific band.

Appropriate extension temperature of this taq polymerase enzyme was 70-75 ^{0}C (Luxananil,1992). In this study using at 72 ^{0}C, that was in the interval appropriate temperature of extension. Since allowing 1 min for each kilobase of

sequence is almost certainly excessive (Saiki, 1989). Another optimization the PCR condition would be the extension time, because over long time for extension may produce the bigger non specific product. Since the expected product was the small fragment as 186 bp in size, the extension time as 15 seconds could be enough to completely synthesized this fragment with did not occur the nonspecific bigger band.

Heparin used as blood anticoagglulant during blood sampling of the fish. Inhibition of heparin in PCR began at a concentration of 0.1 I.U./ml reaction (Tirasophon, 1991). Thus, in the present study a final concentration of 0.05 I.U. heparin / ml blood was used to avoid inhibition of heparin in the PCR reaction.

Under the present optimization condition, the target DNA could be amplifier from as little as 5 pg or $6.8 * 10^5$ copies, lower than the introducing copies. Additionally, using this condition can amplify the target product from growth hormone gene of genome in human blood. The result showed that at very low template is small volume of human blood, the expected 186 bp could not be found from the ethidium bromide staining gel, but it could be found by using nonradioactive detection.

Amplification of 150 bp in fish blood

Due to high homology among growth hormone proteins of fish, a pair of primers were designed from *P.* sutchi growth hormone gene complementary with equal melting temperature (Tm) 54 and 56^0C. The result showed that this set of primers could amplify the deisgnation band of 150 bp from genome in fish

blood using the same condition with change to the appropriate annealing temperature at 50 ^{0}C. The growth hormone gene is naturally low as 1 copy per haploid genome. The sensitivity of this optimal condition of PCR method could detect the introduced gene, if the integration of introduced gene has accomplished as little as least at 1 copy per haploid genome.

In analysis of introducing gene, PCR methods were used with a human growth hormone gene set of primer. The 186 bp amplification products from all blood samples of microinjected fish could not be found from the ethidium bromide staining gel. But the sensitvity of the detection could exhibited the hybridization signal with the expected size 186 bp product by using nonradioactive detection. The sensitivity of nonradioactive detection (chemiluminescence) generally is 10-100 folds higher than visualization of the staining gel (Mannheim Biochemica, 1989). When comparing the amplification PCR product of fish growth hormone gene from the same volume of blood. The hybridization and chemiluminescence detection result indicated that number of copies of introduced gene may be less than 1 copy per haploid genome integration. Similar incidence integration was also reported in Ozato et al. (1986), Stuart et al. (1990), Zhang et al. (1990), Yoshizaki et al. (1991), and Gross et al. (1992). These results suggested that both integrated and non-integrated cells coexisted mosaically in the host fish.

The failure to detection by PCR indicates that, the obtaining optimal condition was not suitable to detection a very little introduecd gene. The low integration of introduced gene in genomic could be detected, using PCR methods combined with hybridization. This method would be powerful to detect low

level integration of foreign gene in fish. It may use as rapidly preliminary screening detection, but it is not designed for quantitative analysis. Improving of the optimal condition for detection by PCR should be additionally investigated of some factor such as magnesium ion, deoxynucleotide concentration (dNTP), buffer pH, primer concentration, and PCR cycling number.

The extracted genomic DNA gave hybridization signal as same result as the PCR detection. The integration of gene transfer obtained in this study by using micronjection method at various stages, at one-cell stage, two-cell stage, and four-cell stage were 5 of 99 (5.05 %), 2 of 31 (6.45 %), and 1 of 12 (8.5 %), respectively. It is a similar incidence comparing to the previous reports; 5 % in fin tissue of zebrafish (Stuart, 1988), 6 % in whole fish samples of tilapia (Brem et al., 1988), 5.5 % by analysing in pectoral fin of common carp (Zhang et al., 1990), and 7% in whole fish of goldfish (Yoon et al., 1990). However, if foreign DNA was inject into the nucleus of oocytes, a good yield of transgenic animal could be produced such as in medaka fish, with 16% integration rate (Ozato et al., 1986)

Several reports explained that the factor affecting on integration was amount of DNA, and form of DNA. The high success rate of integration in transgenic rainbow trout might be a microinjection of large number (10^7) of copies of linear DNA (Yoshizaki et al., 1991). Chourrout et al., 1986 obtained high transformation 75 % after DNA injection of $2*10^9$ copies in rainbow trout. Mostly linearized form of plasmid was used for microinjection. This form could highly incorporate within chromosome and had short persistence in cell, (Chourrout et al., 1986; Penman et al., 1990). However Winkler et al. (1991)

found contradictory result that higher expression of the introduced DNA was obtained in transferred circular plasmid rather than in linearized plasmid.

Positive result on dot blots and PCR analysis was not neccessary to indicate genomic integration, but the assay gave only the persistence of the injected sequence. It is therefore essential to follow dot blotting by proper southern blotting of positives samples. The persistence of the injected DNA may be cause of an over estimation of integration rate. In this study blood of juvinile fish was detected. The extrachromosomal DNA might probably be integrated into the genome. Non-integrated form would be degraded and the transgenic DNA appeared to be only an integrated form. Therefore, this detection result would be the true integration rate.

Mosaic

In the present study, the DNA detected result indicated that the introduced gene did not occur in all tissues. This indicated obtaining a transgene mosaicism. Founder fish which was microinjected at one-cell stage were mosaic indicating that injected DNA did not usually integrate immediately. The integration step occured later in more cell stage. It was high posible that the embryonic development of this yellow walking catfish was faster than the integration rate of foreign DNA. Another possibility was high frequencies of mosaic transgenic fish generated when linearized DNA are injected into the egg cytoplasm (Tewari et al., 1992). However, due to a small number of tested fish, we could not make a conclusion of mosaicism rate in this study.

Growth improving of the injected fish

Human growth hormone gene was introduced to yellow walking catfish embryos by microinjection for the purpose of improving genetics on growth characteristics. The growth rate of induced transgenic fish derived from at one-cell and two-cell microinjected eggs had higher than the control fish. While the growth rate of induced transgenic fish derived at four-cell stage microinjected egg was similar to the control fish. It might be the effect from the transferring of foreign growth hormone gene. This suggestion could explain by hypophysectomized human growth hormone transgenic common carp. Cui and Zhu (1993) suggested that the hGH transgene was expressed in tissues other than in the pituitary. Comparing form mosaic founding, that the introduced gene was detected in more tissues of one-cell and two-cell microinjected fish. It can be explained that increasing weight gain might be a function of how many tissues and which particular tissues were expressed by the introduced gene (Gross et al., 1992). From both results of survival rate and integration rate, we can conclude the appropriate developmental stage for production of transgenic yellow walking catfish by microinjection the foreign gene into eggs were at one-cell stage and two-cell stage.

The growth acceleration in this study was not high as in previous reports in common carp (Zhang et al., 1990) and rainbow trout (Inoue et al., 1993). This transgenic yellow walking fish were not extreme growth rate comparing to the control. It may due to low copy of integration rate of introduced gene and high mosaic integration. Because mouse-Metallothionein-I could be function well in fish cell, it was possible to obtain higher expression of the foreign

growth hormone gene by inducing expression with heavy metal (Inoue et al., 1992; Kinoshita et al., 1994). Our experiment did not treat the microinjected fish with heavy metal.

High variation of size in same sibling of both microinjected fish and control fish, were found in this experiment. It was normally found in catfish culture. In this study, possible genotypes of this transgenic fish might be:

1) The nontransgenic fish
2) Mosaic transgenic fish as less than one copy per haploid genome integration.
3) Mosaic integration in various tissues which were detected, even in blood obtained the incorporation of foreign gene but not all tissue incorporation (fish no. 217), and even non-incorporation of foreign gene in blood but in other tissue obtained the integration, and
4) Completely transgenic individuals possibly obtain but was not detectable.

Fortunately this study found the introduced gene in gonad tissue, that can possible transmission through mendelian fashion. Gene transfer by using microinjection can become the valuable tool for genetic improvement of commercially interested characteristics on fish. This method may improve better growth than that of polyploidy manipulation (Leka-anatakul, 1992), mono-sex culture (Wattanakul, 1993), and especially could transmission to descendent generation. The transgenic techniques is considered to be useful in the future research of improvement of growth in cultured fish. However, our transgenic

fish did not gave dramatic growth improving as well as hybridization with *C gariepinus* but can be used for good stock of hybridization breeding.

Abnormal fish in microinjection

Some of fish derived from microinjection were found morphology abnormality, showing in Figure 39. Abnormalities may be caused by a incorparation of recombination exchange with some existing piece in a chromosome. Presumably, the genomes of eukaryotes were so large that much of the DNA was essentially redundant and therefore random pieces up to 10 kilobases might loose without any genetic implication. Some deletions by transgenic incorporation may be fetal and contribute to the reduced viability of injected eggs and embryos (MacLean and Penman, 1990).

Biosafety

The present study, fish was strictly detained in the cage, maintained with vigilance from natural ecosystem. The fish would not allow to leave to natural water. The transgenic fish would be consumable and would not harm to consumer after cooking, because the foreign DNA was denatured digested by heating and enzyme. Transgenic fish raising was greater ethical, environmental and safety concerns. There is less confidence about the effectiveness of proposed guidelines to control transgenic organisms outside of the laboratory. On the available widence it is suggested that transgenic fish will not have unprecedented impacts on natural ecosystem; their impacts will affect on natural fish species (Hallerman and Kapuscinski, 1992).

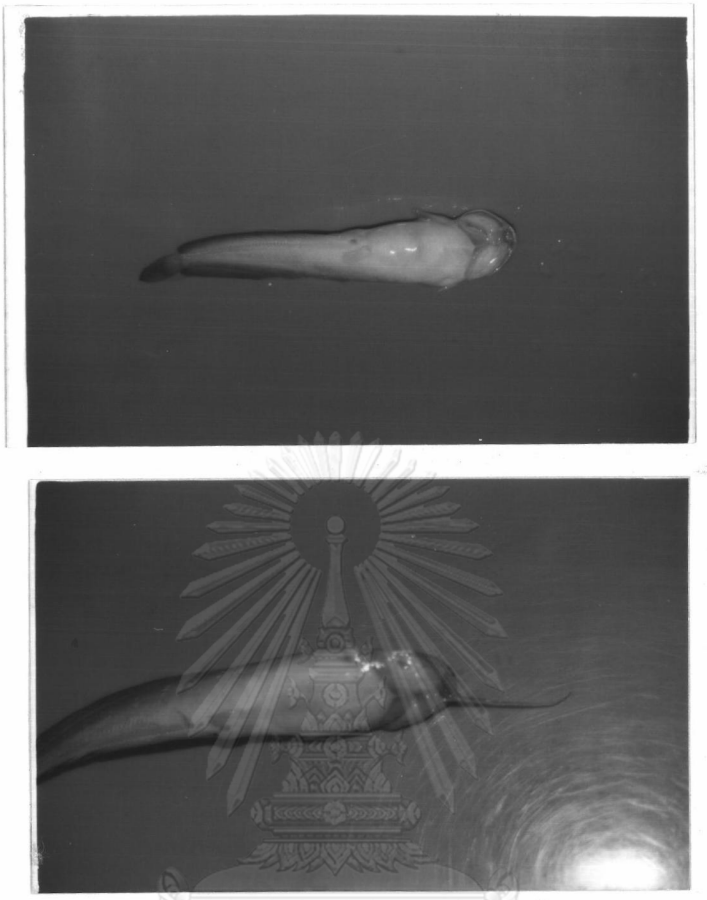

Figure 39 The abnormal fish derived from microinjected egg

A) abnormal gill B) abnormal mouth