CHAPTER I

INTRODUCTION

Catfish, a commercially important freshwater fish, is staple food product in Thailand for several decades. Fisheries statistics of Thailand (1991) reported that production of catfish was mainly obtained from aquaculture, with the highest aquaculture production (29.1 tons) among freshwater animals. Yellow walking catfish, *Clarias macrocephalus* Gunther., is the most commercially important freshwater catfish in Thailand. Normally, the fish take 5-6 months to reach marketable size in culture ponds. This is considered comparatively slow. Owing to this slow growth most farmers divert their interest to culture other species or the hybrid such as *C. macrocephalus* x *C. gariepinus*, which could grow very fast in pond culture. This hybrid species also exhibit higher growth and greater disease resistance than the native catfish. Nevertheless, culturing the hybrids for mass production may eventually affect the abundance of native species, especially the shortage of yellow walking catfish females. Improved performance of native species, especially growth rate should be studied to encourage the culture of native catfish for satisfying the local demand and to maintain the indigenous stocks.

Growth and development of vertebrate is normally controlled by multi-hormones, especially growth hormone. Growth hormone is produced and secreted from the anterior pituitary gland of the vertebrate. Many aquaculturists have emphasized the use of growth hormone to improve growth rate of fish.

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Interestingly the increased body weight by growth hormone treatment is due to increased tissue protein, not increased fat deposit. Direct injection of cultured fish is a procedure to increase the growth hormone and this could result in improved growth performance. However the direct injection procedure is laborious and also increase risk for bacteria infection because of the need for repeated injection (Guise et al., 1991 ; Powers et al., 1992). Transferring the growth hormone gene into the newly fertilized zygotes have been tried and could result in genome. The genome can produce growth hormone continuously at increasing frequency in other tissue cells than at low frequency by the only pituitary gland cells. With this regard, it may be a good opportunity to use this transgenic method to enhance growth rate of the yellow walking catfish, so that we can improve the growth performance of the native species for aquaculture and to maintain the native stock.

In the present study, microinjection of plasmid pXGH 5, which containing the Human Growth hormone gene fused to mouse metallothionein-1 promoter, was microinjected into the newly fertilized eggs, followed by detection of the introduced gene, and the evaluation on improvement of growth and survival performances.

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Objectives

The objectives of the present study are:

1. To study method of gene transfer in yellow walking catfish, *C. macrocephalus* by microinjection,
2. To compare survival rate of transgenesis,
3. To study an integration rate of transgenesis,
4. To compare growth performance of transgenic fish and the control.

Expected Results

1. Obtaining an information of appropriate stage for gene transferring by microinjection.
2. Better growth performance of transgenic fish.
3. Feasibility of genetic improvement of growth by fish transgenesis.

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Yellow walking catfish

Yellow walking catfish, *Clarias macrocephalus* Gunther (Figure 1), is economically important fresh-water fish in Thailand. It is known as "pla duk uey". It widely distributes in the tropical zone especially in South East Asia.

Taxonomy of yellow walking catfish is;

Phylum Chordata
Subphylum Vertebrata
Class Pisces
Subclass Teleostomi
Order Nematognathi
Family Claridae
Genus *Clarias macrocephalus*

Normally, this species mature at age of eight months to one year, however for hatchery breeding, the parent fish over one year old or over 200 grams are preferred. The female is larger than the male. They spawn during rainy season (February-October) (Ueysoonnoan, 1980 ; Na-nakorn, 1992). Mature female has special characteristics, as its abdomen is horizontal expansion and soft-touch, and genital organ is delicate shade of pink. Mature male is slightly elongate body, genital organ is elongate wart and pale pink (Na-nakorn, 1992). The picture of genitals organ of male and female catfish are shown in Figure 2. This fish can easily be artificial induced spawning by injection of hormones. Hormone which are widely used and its dosage, latency period is shown in Table 1. Induced spawning at designation time is a

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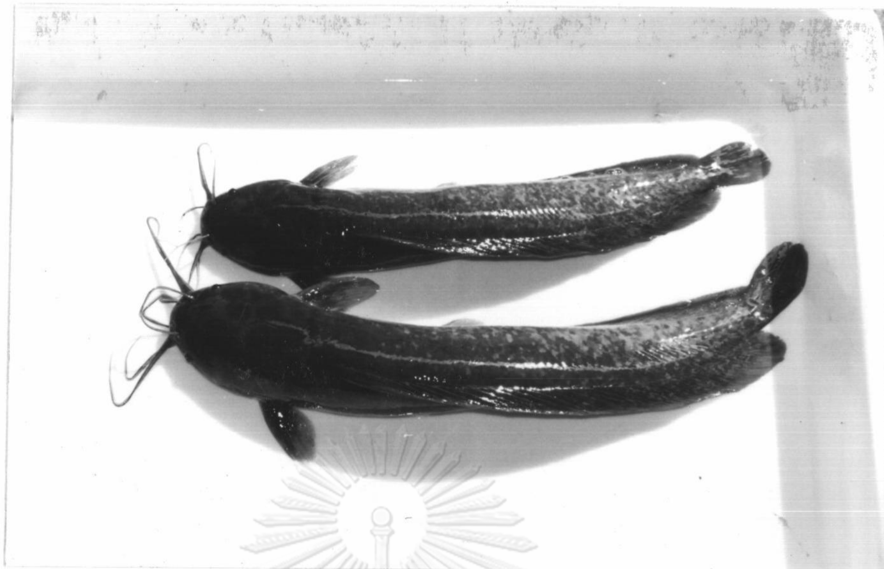

Figure 1 Matured yellow walking catfish, *Clarias macrocephalus*

A) male and B) female

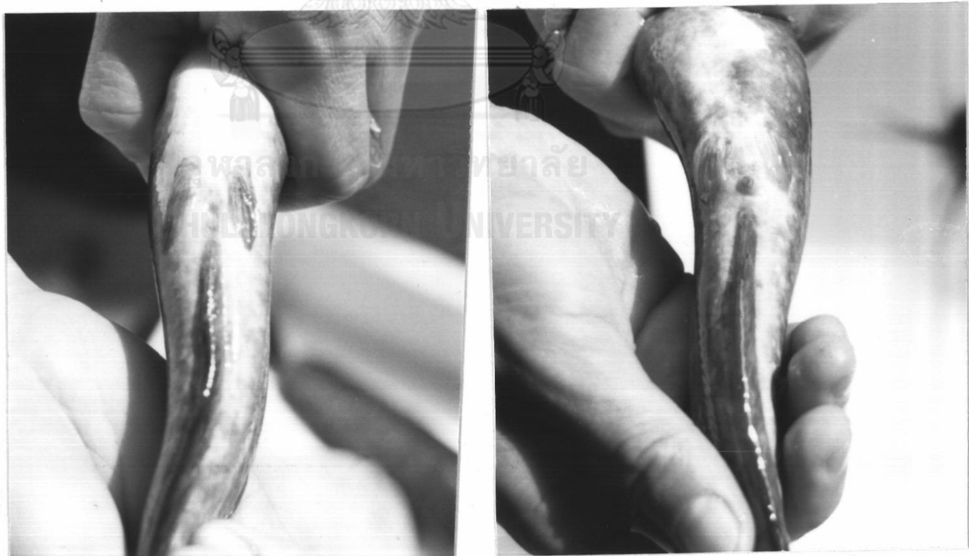

Figure 2 The genital organs of yellow walking catfish;

A) male and B) female

mechanism of eggs disjoined from the follicle and collected pooling in abdomen cavity. Method of hormone injection could devided into 2 methods, as intramuscular injection and intraperitoneal injection. However, intramuscular injection is the most effective method.

Catfish eggs are demersal and adhesive , no oil drops and high specific gravity, thick hardness of chorion and transparency (Thalchalanukij, 1978). Mature eggs look as ellipse shape. The cytoplasm movement gather with nucleus at an animal pole could be distinct as brown-orange point. Sperm of this fish could not be striped, thus fish are sacrificed and testis are removed. An appropriate method of fertilization of this fish is modified dry method (Na-nakorn, 1992). Hatching is occurred about 24-30 hours after fertilization. The newly hatched fry gathered with yolk sac do not need any feed. The fry can overturn body as the parents, sometimes tilt-wriggle, and up-down rolling. After 2-3 days of hatching, feeding of fry will begin with egg yolk or small zooplankton.

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Genetic improvement of catfish

Several methods for genetics improvement of catfish have been developed such as genetic selection, hybridization, and gene manipulation as gynogenesis, and polyploidy. Jarimopas et al. (1988) studied preliminary mass selection of yellow walking catfish for better growth, and found a slightly improvement. Jarimopas et al. (1990) had continuously studied for 4 generations and found that, selective strains got significantly higher weight gains than that of the control ($P<0.01$). They suggested that mass selection strains could give

Table 1 Dosage and latency period for each hormone uses to induce spawning of *C. macrocephalus* (Na-nakorn, 1992)

Hormone	Sex	First injection dosage	Time (hours)	Second injection dosage	Latency period (hours)
Pituitary gland[*1]					
	Female	1	6	2	10-12
	Male	-	-	0.5	10-12
Human Chorionic Gonadotropin (HCG)[*2]					
	Female	1000	6	2000	9-11
	Male	-	-	500	9-11
Suprefact[*3] **and Moltilium M**[*4]					
	Female	[*3] 20-30		none	14-16
		[*4] 10		none	
	Male	[*3] 15		none	10
		[*4] 10		none	

[*1] dosage in body weights of the donor/body weight of the recipient

[*2] dosage in I.U./kg body weight

[*3] Trade name of Luteinizing Hormone-Releasing Hormone analogue (LHRHa), dosage in μg/kg body weight

[*4] Trade name of Domperidone, dosage in mg/kg body weight

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beneficially genetic improvement of this fish species. Progress in the manipulation of growth characteristics via selection is a slow process and will face a problem of rapid enhancement in early generations followed by slowing rate of improvement (Guise et al., 1991)

Inter-species hybridization between female *C. macrocephalus* and male *C. gariepinus*, get a hybrid that increase growth rate and disease resistance. This hybrid is widely cultured in Thailand up to now. But the progenies are sterile. Prarom (1990) studied cross-breeding of inbred lines, semi-diallel cross between Songkla province strain (Southern Thailand) and Saraburi province strain (Central Thailand). The progenies of female Songkla province strain and male Saraburi strain got a heterosis.

Polyploidy manipulations, such as triploid was induced using a cold shock technique. All of induced fry's chromosome were triploid, however, survival rate of the triploid fish was significantly lower than that of the diploid fish. Growth rate of young triploid fish at 55 days was not significantly difference from diploid fish, but at 100 days the growth rate of the triploid was significantly higher. He suggested that increase of growth regulation gene allele were effect on the better growth of triploid fish (Umnuaysith, 1990). Leka-anatakul (1992) studied triploid manipulation on this species and found that survival rate and weight gain of the triploid fish were lower than the diploid. The food conversion ratio of triploid and diploid fish were not significant differences.

Female catfish is larger than male fish. Monosex culture would take a turn for a better culture of this fish. Wattanakul (1993) had treated fish with the female hormones such as 17-β estradiol and 11-β hydroxyandrostenedione. He found no significant difference in growth rate between treated groups and untreated group. Na-nakorn et al. (1993) carried out the gynogenesis experiment to improve growth of this catfish, however the result was still questionable.

Transgenic animal

Transgenic animals are created by introducing new DNA sequences into the germ line via addition to the egg (Lewin, 1994). While the extra copies of a foreign gene generally results in increased levels of the encoded enzyme of protein, the effect on the organism into which these genes have been introduced, can be dramatic. Since the genes were incorporated into the germline, this trait was passed in true Mendelian fashion to the offspring (Guise et al., 1991). Development of transgenic animals produced by microinjection of foreign genes into fertilized eggs is now becoming a powerful tool for the study of gene expression in living animals in the field of developmental biology, oncology, animal husbandry, and others (Ozato et al., 1986)

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Gene transfer in vertebrate had been carried out first in mice (Gordon et al., 1980). Palmiter et al. (1982) had transferred a rat growth hormone gene into mouse embryos, and transgenic mice grew significantly faster and larger than control mice. From then this technology has been developed to improve economic traits in domesticated and semi-domesticated animals. Many

researches have successfully studied foreign gene transfer into unfertilized eggs, fertilized eggs and embryos of sea urchin (McMahon et al., 1985), Xenopus (Rusconi and Schaffner, 1981), Drosophila (Spradling and Rubin, 1982), mouse (Constantini and Lacy, 1981; Palmiter et al., 1982; Gordon and Ruddle, 1985; Palmiter and Brinster, 1986; Mc Grane et al., 1988), rabbits, sheep, pigs (Hammers et al., 1985), amphibians (Bendig, 1981; Etkin et al., 1984; Etkin and Pearman, 1987), chicken (Crittenden and Salter, 1991), cattle (Bendioli et al., 1991) and fish.

Production of transgenic fish

Fish is a good biological model for developmental studies, and protein producing as a part of human food (Houdebine and Chourrout, 1991). Many reports are available concerning investigations of gene transfer in either laboratory or commercially important fish. Mostly of successful gene transfers in fish are obtained by microinjection of the foreign genes into fish eggs. Zhang et al. (1990) suggested that this technology offers an effective means for rapid development of new genetic stocks of economically important livestocks or for the large scale production of novel proteins. The efficiency of microinjection procedures is a product of integration rate and survival of microinjected embryos (Hayat et al., 1991). Microinjecting manipulation has effects on survival rate and hatching rate of the embryos and eggs of fish. The cause of mortality of egg was possible that the micropipette may break or clog the zygote cytoplasm. The wound would be opened as a large hole in the chorion causing cytoplasm and yolk leakage, or the opening through which the

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micropipette penetrated may be facilitated the invasion of bacteria (Phillips et al., 1992).

Culp et al. (1991) microinjected the plasmid DNA into fertilized zebrafish eggs. They found the hatching rate of 50 % of injected egg compared with 65 % of control eggs.

Yoon et al. (1989) reported a successful transfer of the neo gene into newly fertilized goldfish eggs by microinjection. Survival rate of microinjected fish was ranged from 10 to 50 %, compared to 90 % in uninjected control groups.

Yoshizaki (1991) microinjected carp α-globin gene into the cytoplasm of fertilized rainbow trout eggs, with about 67 % of microinjected eggs hatched out and grew normally, comparing to 89.5 % of non-injected eggs. Integration rate of carp α-globin gene into genome were about 40 %.

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Transgenic fish by microinjecting of chicken δ-crystalline gene into the oocyte nucleus of medaka *Oryzias latipes* had been studied by Inoue et al. (1989). About 50 % of the microinjected oocytes developed to 7-days old embryos, and the exogenous gene were detected in 10 of 30 embryos examined.

Zhang et al. (1990) microinjected the recombinant plasmid (pRSV-rtGH cDNA) containing the Rous sarcoma virus-long terminal repeat (RSV-LTR) promoter linked to rainbow trout growth hormone complementary

DNA (rGH cDNA) into fertilized carp eggs (*Cyprinus carpio* Linnaeus). They found that hatching and survival rate for microinjected embryos (37 %) were not different (P>0.05) with the control. The integration rate was 5.5 %.

Hew et al. (1992) also found that the survival rate of the injected salmon eggs were 80 %, which was similar to the non-injected control eggs.

Inoue et al. (1991) microinjected a DNA solution into cytoplasm of fertilized eggs of rainbow trout with a micropipette. The hatched-out rates of injected and uninjected eggs were 75 % and 72 %, respectively.

Not only successful gene transfer in the foregoing fish but also carried out in trout (Chourrout et al., 1986), loach (MacLean and Penman, 1990), zebrafish (Stuart et al., 1990), catfish (Powers et al., 1991), Northern pike (Gross et al ., 1991).

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Helpful technique for microinjection

Most of fish eggs, after fertilization and contact to water, egg chorion become harden an opaque. It may make difficulty and limit the ability to visualize the target for injection. Another possible strategy to avoid the though egg chorion is to complete egg microinjections immediately after fertilization and before water hardening begins (Penman et al., 1990). Tilapia eggs were cooled to 20 ^{0}C to delay time to first cleavage beyond two hours and also to slow water hardening. This procedure had no significant effect on egg survival and allowed ample time for microinjections (Phillips et al., 1992). In addition, three

difference techniques are used to overcome the problem of water-hardening of the chorion and for helpful microinjection.

Guise et al. (1991) suggested that microinjection is an efficient method for the transfer of DNA into fish, especially, if the target nucleus can be visualized by dechorination of the egg. Microinjection depends upon the ease of removal or softening of the egg chorion. To achieve, gene transfer by microinjection of fish eggs, some techniques need to be developed. The first method was to remove the chorion of eggs by manually dissection (Ozato et al., 1986; Stuart , 1988) or to use enzymatic treatment to dechorinate partially softened chorion. The enzymes used in this purpose might be 0.25 % trypsin for goldfish eggs (Yoon et al., 1989), 1 mM glutathione solution (pH 8.0) for rainbow trout eggs (Yoshizaki et al., 1991) and 0.5 mg/ml pronase for zebrafish eggs (Culp et al., 1991). However, some fish eggs such as salmonid eggs are resistant to enzyme treatment. In some species in which this technique is utilized, the chorion is thin and the perivitelline space is relatively large (Jiang, 1993).

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The second, microinjection was performed by two-step; the chorion was drilled manually using a broken pipette and then insert a microneedle for introducing of DNA solution into egg via the opening. This method have mostly performed in salmonid eggs. Disadvantage of this method may cause by harmfully drilled with a large hole in the chorion. The cytoplasm and yolk may leak and damage to the eggs (Chourrout et al., 1986 ; McEvoy et al., 1988 ; Rokkones et al., 1989).

The third method is to inject egg through the chorion at the micropyle the opening which sperm enters, such as in tilapia egg (Brem et al., 1988), salmon egg (Fletcher et al., 1988). This method requires that the micropyle should be easily visualized. Injection through the micropyle is a simple and harmless manipulation to overcome the chorion barrier. However, this method may interfere normal fertilization of eggs (Davies et al., 1990).

In most cases, pronuclei are not visible. Microinjection of exogenous materials into fertilized fish eggs has been performed in cytoplasm. Exceptionally, microinjection into oocytes, this is the only method that currently allows injection of foreign DNA solution into egg nuclei in fish. Microinjection into oocyte nuclei is rather easy because the chorion is soft and the oocyte nucleus is clearly seen near the animal pole of an immature oocyte. This method has been used only in medaka but is presumably applicable to other species in which oviposition cycles have been studied (Inoue et al., 1992).

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Suitable egg developmental stages for microinjection

After fertilization, the eggs or embryos usually develop into one cell, two cell, four cell and continuously develop into a fry. Time of egg development of fish usually depends on species and temperature. In microinjection a key factor for survival rate, integration rate and transgenic mosaicism is stage of egg development.

Hayat et al. (1991) determined the most appropriate developmental stage for maximizing production of transgenic channel catfish (*Ictalurus*

punctatus) and common carp (*Cyprinus carpio*) embryos. They found that hatching rate significantly decreased when channel catfish embryos were microinjected at later stages compared to earlier stages. But in common carp, developmental stage did not affect survival of the microinjected embryos. They concluded that late one-cell and early two-cell stage were preferably appropriate stages for maximizing production of transgenic channel catfish and in common carp early one-cell stage and two cell-stage proved to be best period for integration of foreign DNA.

Rahman et al. (1992) reported production of transgenic Tilapias, *Oreochromis niloticus*. The hatching success varied due to the developmental stage injected. Microinjection at one cell stage had higher hatching rate than at two-cell and four-cell stages.

Brem et al. (1988) reported that number of Tilapia (*O. niloticus*) containing foreign DNA had the highest in a group injected at 21-24 hours after spawning, when hatching occurred approximately 4 days.

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Zhang et al. (1990) reported that survival rate of the microinjected carp (*Cyprinus carpio*) at one cell, two cell, four cell and non-microinjected control were not significant difference. They also noted that the percentage of foreign gene integration varies considerably depending upon early embryo development at injection. The two-cell stage had highest integration. Transgenic fish derived from one-cell injected embryos showed weight gain above the control.

Gene constructs used for transgenic fish production

Gene constructs used for transgenesis mainly contain promoter gene and structural gene, which are obtained from eukaryotic genes or prokaryotic genes. Most researchers working on transfer of fish gene usually use mammalian growth hormone gene (GH) connected with promoter/enhancer regions of mammalian origin (Guise et al., 1991).

Having a regulatable promoter may be particularly advantageous for timely expression (Palmiter et al., 1982). Promoters gene have been defined in terms of their abilities to initiate transcription in suitable test systems (Lewin, 1994). Identification of active promoters in fish is an important practical aspect of developing gene transfer. Inoue et al. (1992) suggested that variety of promoters and enhancer derived from mammalian genes and viruses are available. Most promoter genes used for introducing are mouse metallothinein-I promoter (mMT-I) (Zhu et al., 1985; Chourrout et al., 1986; Dunham et al., 1987), Simian virus 40 (SV 40) (Stuart, 1988), and Rous sarcoma virus (RSV) (Yoon et al., 1990; Zhang et al., 1990).

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Metallothioneins (Mts) are a family of low molecular weight proteins containing heavy metals. It is known that the Metallothionein gene expression induced by heavy metals such as zinc, cadmium and copper or corticosteroids (reviews by Inoue et al., 1992). In producing transgenic fish, the regulatory elements of metallothionein promoter have been received much interest because of the responsiveness of Metallothionein promoters to heavy metals (Kinoshita et al., 1994). Using the mMT-1 promoters Inoue et al. (1992) tried to achieve the

expression of foreign genes, including growth hormone gene. Recently, Hong et al. (1993) isolated and functionally tested the promoter of the rainbow Metallothionien-B gene to obtain information on its specificity. They found that this promoter was highly suitable for the production of transgenic fish.

The classical view of a structural gene is a unique component of the genome, the only sequence coding for its protein product (Lewin,1994). Structural gene is commercially interesting characteristics as mostly on growth hormone gene and a few on disease resistance gene. In all vertebrates, normal growth is controlled by intricate interactions among several hormone factors including anabolic steroids, thyroid hormones, insulin, insulin-like growth factor (IGF) and growth hormone (GH). Of all these hormones, growth hormones plays the most important role. Growth hormone is a single chain polypeptide of about 22 kD, produced by the somatotrophs of the anterior portion of the pituitary gland. It is released into the circulation and exerts stimulating influences over growth and development (Jiang, 1993). Growth hormone is required for normal growth and development of pre-adult vertebrates (Cavari et al., 1993). Growth hormone gene for fish transgenesis may obtain from homiotherms such as human growth hormone gene (Brem et al., 1988; Chen et al., 1990; Hayat et al.,1991), bovine growth hormone gene (Phillips et al., 1992; Gross et al., 1992), and rat growth hormone gene (Penman et al., 1990; Rahman et al., 1992), or from fish gene as well as complementary DNA (cDNA) such as rainbow trout growth hormone gene, cDNA (Zhang et al., 1990; Inoue et al., 1992; 1993), Chinook salmon growth hormone gene, cDNA (Gross et al., 1992; Zhang et al., 1993), and *Lates calcarifer* growth hormone gene, cDNA (Cavari et al., 1993). These constructs of the growth hormone

genes have been fused to a promoter before insertion into fish eggs. The gene will be expressed at a high frequency in other tissues, where the introduced gene was integrated. Such as mammalian metallothionien promoter would be expressed the product by the liver cells (Jiang, 1993). While disease resistance gene was still be sequencing and cloning, and have been introducing into fish. In 1985, first fish gene sequence, the antifreeze protein gene of the winter flounder, became available and was used for the production of freeze-resistant salmon (Fletcher et al., 1986).

Some transgenic fish researchers concerned about marker gene in order to convenience assay cost, sensitivity and accuracy. Marker gene whose products are easily detectable and useful to analyze activities of promoters in transgenic animals (Inoue et al., 1991). A gene conferring a selectable phenotype would ultimately be utilized as one component of a construct which also bears a gene economic importance. Individuals exhibiting the levels of marker-gene expression would be likely to exhibit the expression of the economically important gene (Yoon et al.,1990). The gene lac Z and chloramphenicol acetyltransferase (CAT) are commonly used (MacGregor et al., 1990).

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The plasmid vector pXGH 5, which is transient expression, contains mouse Metallothionein-I promoter (mMT-I) fused to human Growth hormone gene structural sequences, and is useful for high-level regulated expression of hGH in a variety of cell types. The mMT-I promoter fragment also contains its CAP site as well as a 64 bp of 5'-unstranslated region. The fusion mMT-I/hGH mRNA contains 64 mMT-I nucleotides and 817 hGH nucleosides

(Nichols Institute Diagostics, 1988). Plasmid pXGH 5 can be used to optimize transfectability of most cell types, and is also valuable as a normalization plasmid (Nichols Institute Diagostics, 1988).

Integration

Transgenic animals usually integrate foreign DNA into one of the host chromosome at early stage of embryonic development (Gordon and Ruddle, 1985). If foreign DNA was injected into the nucleus of oocyte, a good yield of transgenic animals would be produced (Houdebine and Chourrout, 1991). Since fish eggs were microinjected into cytoplasm, thereby integration rate of introduced gene in fish chromosome is low. Introduced gene might be integrated into fish chromosome or still persistence in fish cell. It is also postulated that the fusion of the nucleus-like structure with the cellular nucleus during embryo development allows the foreign DNA to be integrated into the host genome, which may be protected against degradation. Ozato et al. (1986) reported that the amount of foreign DNA recovered from the transformed embryos was 10^3 to 10^5 times greater than the estimated amount of injected DNA. This indicates extensive replication of exogenous DNA sequences in medaka embryos. Typically, the microinjected DNA is rapidly amplified after fertilization, regardless of the origin and the sequence of the foreign DNA, but only a small proportion of the replicated DNA is maintained after gratula stage. Winkler et al. (1991) also observed a large part of injected DNA replicated transiently in the early stages, mostly in an extrachromosomal form. A limited number of copies persist in later stages,

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possibly in an integrated form in the African catfish. The presence of the injected transgenes in supercoiled, open circular, closed circular and multimeric forms has been detected almost throughout the embryogenesis in loach, goldfish, medaka and tilapia. However the sequences are rapidly converted into a high molecular weight form at hatching indicating that other forms had been degraded (Pandian and Marian, 1994). Form of plasmid may affect integration in transgenic fish. Palmiter et al. (1982) chosen the linearized fragment for injecting into mice embryos because fragments integrated into host DNA are more efficient than supercoiled plasmids. MacLean and Penman (1990) suggested that circularized DNA is almost certainly more resistant to enzymatic degradation in the cell, but is less available for recombination insertion into the genome of the egg.

Expression

Basic expression systems contain a promoter element to drive transcription of the foreign DNA, the coding sequence, and the signals require for efficient processing of the transcript. In general, the level of expression of the foreign gene does not correlate well with copy number, probably due to influences from flanking DNA (position effect) (Houdebine and Chourrout, 1991). Transient expression of foreign genes takes place between 12 and 72 hours after introduction of the DNA into the cells, when the plasmid vector is in nuclear, but not integrated into the host cell genome (Harvey et al., 1992). Although it is generally known that a number of factors control gene expression in eukaryotic cells, only a few details have been undertaken to understand the various aspect of expression in transgenic

animals (Pandian and Marian, 1994). Zou et al. (1993) explained the expression of foreign genes during embryogenesis as follows:

1. Before gastrula stage, the replication rate of injected genes was higher than their degradation, and the amount of foreign DNA increased.

2. Foreign genes that were either integrated into host genome or unintegrated were successfully expressed during embryonic development.

3. Excessive foreign gene copied and the protein product coded by protein gene made the embryos to be abnormal or lethal.

Most the transgenes were generally poorly expressed, nevertheless, several successful fish transgenesis had also detected expression at level of mRNA (Yoon et al., 1989), enzyme (Stuart et al., 1990; Yoon et al., 1990), protein (Ozato et al., 1986; Inoue et al., 1991) and visible characters (Chen et al., 1990).

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Transmission

The transgene appears to be stable incorporated and transmitted to the F1 generation in standard Mendelian fashion. There is few information on transmission of transgenic fish, because some economically fish have long lifespan such as salmonids and cyprinids. Some experimental fish as zebrafish and medaka, their generation time is short, and could be easily followed the transmission. Obtaining a high frequency of germ-line transmission of injected DNA is possible. It seems likely that the inherited plasmid DNA sequences are integrated, since they could be passed to the F1 generation with

Mendelian ratios (Culp et al. 1991). The introduced DNA was found in spermatozoa of transgenic fish such as in zebrafish and trout. However in the F1 offspring, only 20-50 % and 7-30 % in zebrafish and trout, respectively, harbored the foreign genes (Guyomard et al., 1989; Stuart, 1988). The low proportion of transgenic animals among the F1 fish confirmed that the progenitors were mosaic in their germ cells. Zhang et al. (1990) reported that transmission of rtGH gene into the F1 carp offspring also confirmed the mosaic nature of the founder transformants. Yanzhang et al., (1993) found that the progeny of a cross between a P1 transgenic female and a control male showing only 22.7 % of F1 fish expressed the gene from the female and got a heavier body weight was than control.

Transgenic mosaics

Mosaicism means the condition existing when tissue of different genetic make-up occur in the same organism. The transgenic individuals either carry the foreign DNA in only a proportion of their cells or in different patterns of integration are observed in different cells (Guyomard et al., 1989). Since transgenics mosaicisms occur when the incorporation of the microinjected gene into the fish genome takes place at the two cell stage or later (Ozato et al., 1986), the microinjected embryos at two-cell, four-cell or later would be mosiacs. A method with regard of mosaicism have been produced by microinjection of the novel gene either before or at one-cell stage. However, cause from the persistence of extrachromosomal foreign DNA, delay of gene integration may produce this mosaicism (Lovel-Bodge, 1985). Mosaic parents with some transgenic germ cells would pass the novel gene to a

portion of their progenies (Yoon et al.,1990). Cavari et al. (1993) observed the mosaic expression pattern in transgenic gilthead seabream fish, and found that tested fish were mosaic. Founder fish were mosaic in all cases studied indicating that injected DNA does not usually integrated immediately (Culp et al., 1991). High frequencies of mosaic transgenic trout are generated and can transmit the foreign DNA to a minority of their offspring, suggesting that their germline is also mosaic (Tewari, 1992).

Other methods for gene transfer

Several methods for gene transfer have currently been investigated. These methods are sperm binding, electroporation and lipofection.

Sperm Binding

Sperm binding or adsorption of plasmid DNA to fish sperm, with the utilizing of DNA/sperm complex to fertilize the egg, is a possible mode of production of transgenic fish. Infectious hematopoietic necrosis virus can be transmitted vertically via this technique of sperm binding

Electroporation

The technique of electroporation for gene transfer has been done successfully in bacteria, mammalian tissue culture, and plant protoplasts. These conditions still generate sufficient changes in membranes to allow uptake of DNA. Dechorination appears to be necessary if electroporation is to have a significant chance of success.

Lipofection

Lipofection for gene transfer depends on the encapsulation of DNA within a phospholipid bilayer. The encapsulated DNA is delivered by membrane fusion of the liposome with the cell (fish eggs) membrane (Guise et al., 1991).

Detection of gene transfer

Detection of transgenic offspring from such treated fish would depend on selection of the growth enhancement phenotype or on the use of a selectable marker. Since some transgenic fish might not be expression, phenotype detection and selectable marker hardly determined incorporation rate. Almost detection of introduced gene was previously done by hybridization with specific probes. This detection method has high confidence, but much time consuming. Alternatively, the Polymerase Chain Reaction (PCR) was designed to amplify a specific DNA sequences between two oligonucleotide primers (Saiki, 1989). Figure 3 shows schematics diagram of the PCR. By this method it is possible to detect as little as one copy of the target DNA out of a complex DNA background. Additionally, ability to screen large numbers of samples rapidly and also at low cost is particularly important. The use of PCR for detecting the introduced gene was studied in many transgenic fish researches (Inoue et al., 1993 ; Du et al., 1992).

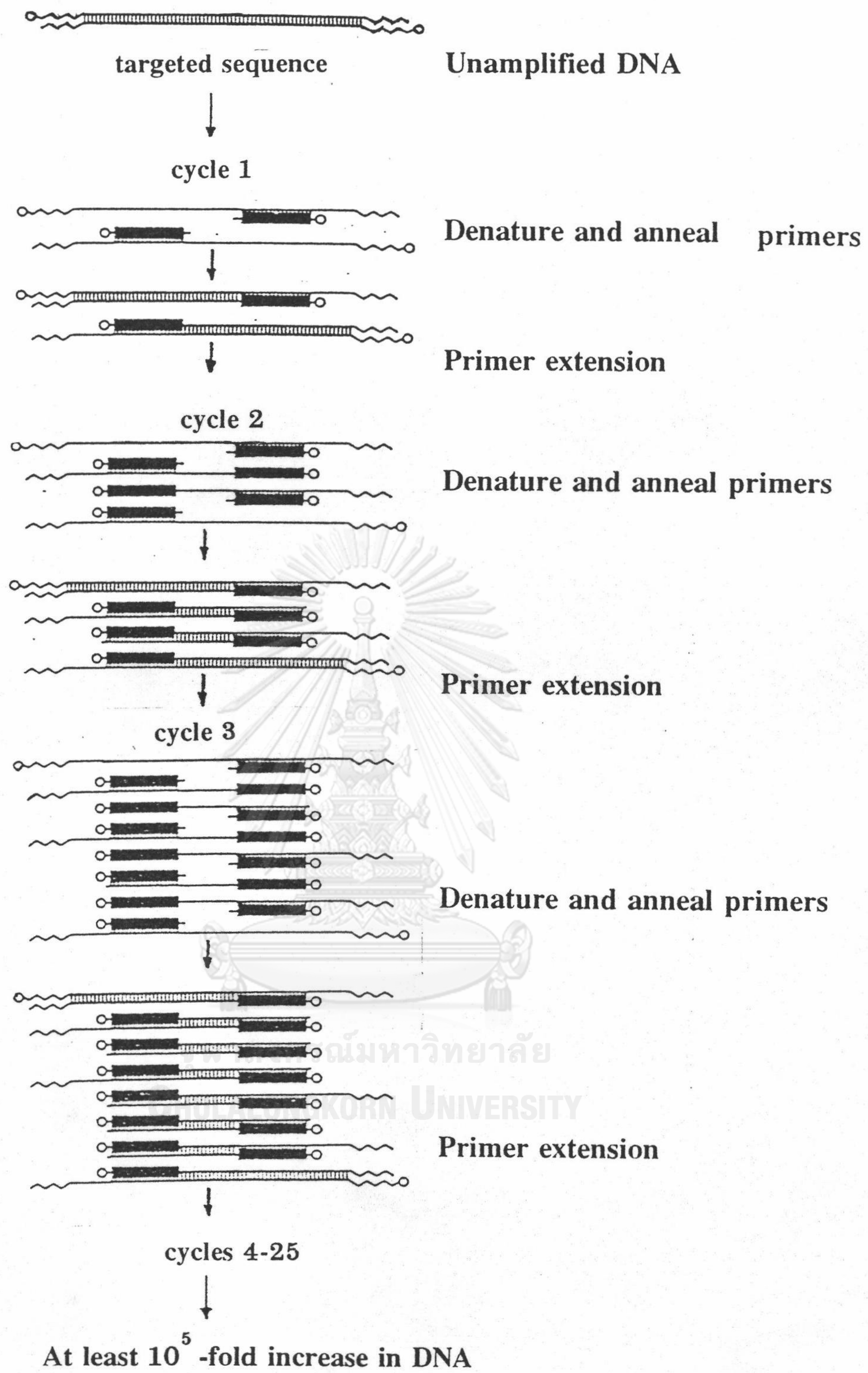

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Figure 3 The schematic diagram of the polymerase chain reaction