ONTOGENY AND HORMONAL REGULATION OF a-AMYLASE GENE EXPRESSION IN SEABASS LARVAE, *LATES CALCARIFER*

BY

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ABBREVIATIONS

aa	amino acid
APS	ammonium persulphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bHLH	basic helix-loop-helix
bp	base-pairs (= pairs of nucleotides)
BSA	bovine serum albumin
DEPC	diethyl pyrocarbonate
DIG	digoxygenin
dph	days post hatching
DNase	deoxyribonuclease
EDTA	ethylene diaminetetraacetic acid
EGFP	enhanced green fluorescent protein
EMSA	Electrophoretic mobility shift assay
FBS	fetal bovine serum
GRE	glucocorticoid response element
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HNF	hepatocyte nuclear factor
IPTG	isopropyl-β-D-galactopyranoside
ISH	in situ hybridization
LB	medium Luria-Bertani medium
MMLV	moloney murine leukemia virus
mRNA	messenger RNA
PAGE	Poly Acrylamide Gel Electrophoresis
PBS	phosphate-buttered saline
PCR	polymerase chain reaction
PEPCK	phosphenolpyruvate carboxykinase
PTF-1	pancreatic transcription factor 1
RACE	rapid amplification of cDNA ends
RNase	riboxynuclease
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
SSC	sodium chloride-trisodium citrate solution
TAE	Tris acetate-EDTA
TBE	Tris borate-EDTA
TE	Tris-EDTA

ABSTRACT

To understand the development of digestive functions in marine fish larvae, the ontogeny and regulation of gene expression of α-amylase were studied in seabass (*Lates calcarifer*) larvae. The enzymatic activities of α-amylase and their corresponding mRNA levels were studied from hatching until 27 days post-hatching (dph). An increasing activity of amylase enzyme was recorded until 5 dph, and thereafter the activity gradually decreased and reached a steady but low level by 12 dph. To confirm this, we also studied the ontogeny of amylase gene expression. For this purpose, we cloned and sequenced a 318-bp fragment of α-amylase cDNA. Based on this sequence, a real-time reverse transcription polymerase chain reaction (RT-PCR) technique was developed to monitor the changes in the mRNA levels in the larvae. A correlation between enzymatic activity and mRNA level of α-amylase was demonstrated during the early development of seabass larvae. This suggests that the changes in α-amylase are controlled at the transcriptional level at least during the early larval development of seabass. *In vivo* thyroid hormone and cortisol treatment of seabass larvae upregulated the gene expression, suggesting the possibility of endocrine control of transcription. This led us to focus our subsequent studies on the molecular mechanisms of amylase gene expression and transcriptional regulation.

The full length cDNA was cloned and characterized. Sequence analysis showed that the coding region and the exon/intron boundaries are highly homologous to those of mammalian amylases. However, the promoter regions are distinctly divergent. To investigate the seabass amylase promoter, a series of deletion mutants were generated and fused to the luciferase reporter gene, followed by studies of their functional activity in the rat AR42J cell line. Besides identifying several potential regulatory elements based on those that had previously been identified in the human and mouse

pancreatic amylase promoter, we have identified a glucocorticoid response element (GRE). While the human and mouse pancreatic amylase promoters are highly homologous between nucleotide -160 and transcription start site, which include GRE, the 5' promoter deletion analysis revealed that the GRE of the seabass amylase promoter was located far upstream, -947 to -776 bp, of the promoter. Site-directed mutagenesis of the putative GRE, and electrophoretic mobility shift assays (EMSA) confirmed that this region was responsible for induction by dexamethasone. However, no functional pancreas transcription factor-1 (PTF-1) binding site, which is responsible for pancreas-specific transcription in higher vertebrates, was identified in the seabass amylase promoter. Instead a Hepatocyte Nuclear Factor 3 (HNF-3) binding site was found to modulate the amylase promoter expression.

A functional GRE on the amylase promoter indicates that the *in vivo* cortisol (glucocorticoid) stimulation of amylase gene expression was direct via the GRE. However no TRE (thyroid response element) was found on the amylase gene or its promoter. This suggests that the *in vivo* T_3 of amylase gene expression was indirect.

We also looked at the effect of food restriction and deprivation on amylase gene expression. Food deprivation increased amylase gene expression 5 fold. Concomitantly, body glycogen level also decreased. The findings are interpreted as a stress response whereby cortisol secretion was elevated which activated the GR-transcription system and upregulated amylase gene expression.

CHAPTER 1

GENERAL INTRODUCTION

Successful mass rearing of larvae is a basic prerequisite in commercial fish hatcheries. However, high mortality often occurs in the early larval development, particularly around the time of first feeding. Marine fish larvae have poorly developed digestive tracts at hatching (Walford and Lam, 1993). The switch from endogenous (yolk-based) to exogenous feeding is concomitant with morphological and functional transformations of the digestive tract. Among tropical marine fish species, this shift occurs very quickly compared to temperate or cold-water fish species, and develops within a few days after hatching (Sivaloganathan *et al.*, 1998). The stage during which larvae transit to exogenous energy sources is a critical period during larval development (Fig. 1.1), because it affects their survival, growth and development (Gawlicka *et al.*, 2000). Rearing of marine fish larvae in aquaculture is still dependent on the supply of live food organisms, such as rotifers and *Artemia*. However, live feed is costly and so far does not allow a standardized production protocol and cost effective production output. Therefore, compound diet substitution for live prey is desirable but so far has met with little success (Cahu and Zambonino Infante, 2001). More detailed information on the developmental changes of the gastrointestinal tract and onset of digestive enzymes secretion during the different larval stages is required in order to fully understand the nutritional physiology of larval fish for progress in the improvement of marine fish larval survival and in the development of replacement diet.

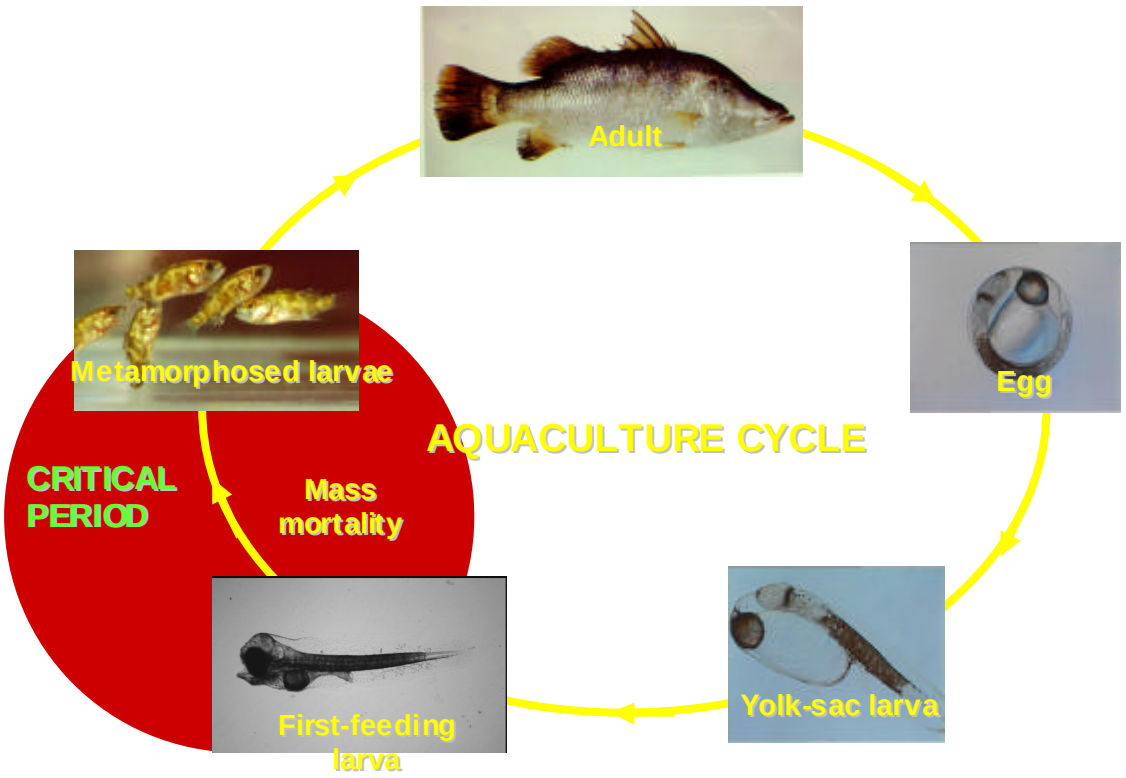

Fig. 1.1 The aquaculture life cycle for marine fish

1.1 Ontogeny of the gastrointestinal tract and digestive enzymes of marine fish larvae

1.1.1 Development of gastrointestinal tract

The transformation of the digestive system marks the transition from larva to juvenile. The juvenile has a digestive system similar to that of adults and they can be fed similar diets, consisting of either trash fish or formulated feed. However, the stomach, intestine and pancreas are under-developed at hatching and these organs undergo morphological and functional changes during the development of the larvae.

From an anatomical point of view, the stomach is somewhat developed in adult fish depending on the species. In the seabass of this study, the stomach has assumed its definite

shape on day 15; the cardiac portion of the stomach joins the pyloric portion at a sharp angle and this gives the characteristic pointed shape to the stomach. As the individuals grow, the stomach becomes larger and the ceca continue to develop. However, the form of the stomach and ceca remain unchanged in the juvenile from day 80 (Walford and Lam, 1993).

On the third day post hatch, the intestinal epithelium of seabass larvae has a regular surface. Thickening and undulations in intestinal epithelium has been observed on day 7. At around day 14, the intestinal epithelium has numerous microvilli at the luminal surface forming the brush border (Walford and Lam, 1993; Zambonino Infante and Cahu, 1994 b).

Pancreas tissue are present only in vertebrates including fish. Thus, fish can be used to investigate the phylogenic development of the pancreas. In most teleost species, the pancreas develops prior to the differentiation of the stomach and gastric gland (Govoni, 1980; O'Connell, 1981). With the exception of a small number of species, teleosts have a diffused pancreas (Harder, 1975). The morphology diverges greatly among bony, cartilaginous and agnathan fishes, and the standard pancreatic morphology present in higher vertebrates cannot be observed in some fishes. The relationship of endocrine and exocrine pancreatic cells, distribution of islets, presence or absence of principal islet and distribution of cell types within the islet can be used to compare the divergent morphology of piscine pancreas (Youson and Al-Mahrouki, 1999). In European seabass (*Dicentrarchus labrax*) larvae, the differentiation of exocrine cells and the appearance of the excretory duct occur at day 3 post hatching (dph), before the mouth opening. The presence of zymogen granules and of the pancreatic duct (called the duct of Wirsung) characterizes these events (Beccaria *et al.*, 1991). In the Japanese flounder *Paralichthys olivaceus*, gastric glands do not develop until metamorphosis, so the pancreas is the sole

exocrine organ responsible for secreting digestive enzymes during the larval stage (Miwa *et al.*, 1992). The pancreas development of the flounder is reproduced in Figure 1.2. The pancreas, which is located at the boundary between the œsophagus and intestine, is a compact organ at 3 dph. It starts to elongate posteriorly along veins on the intestine at 20 dph. After metamorphosis (45 dph), the pancreas is localized along the veins running towards the porta hepatis from the stomach, pyloric appendages and intestine. Pancreatic tissue has also begun to invade the liver along the hepatic portal vein, thereby forming a diffuse pancreas. Since the gastric glands of the stomach wall are also differentiated at metamorphosis, it has been suggested that the digestive system of the flounder assumes the adult form in the early juvenile stage following metamorphosis (Kurokawa and Suzuki, 1996). Because the digestive system of the flounder becomes equivalent to that of the adult during the early juvenile stage, this may be one of the reasons why artificial diets can be utilized by juveniles but not by larvae (Kurokawa and Suzuki, 1996). The increase in volume of the pancreas and the presence of gastric glands are obvious differences between larval and adult digestive systems. It was reported that there was no secretion of pancreatic enzymes by European sea bass larvae (*D. labrax*) fed an artificial diet even though the digestive tract was full of food (Beccaria *et al.*, 1991). These results suggest that the larvae are not dependent on exogenous enzymes for digestion, and not only the exocrine capacity but also the mechanism of regulation of the pancreas differ between larvae and adults. If artificial diets are to be developed for larvae, it will be important to understand the mechanisms by which digestive enzyme secretion is regulated in larval fish.

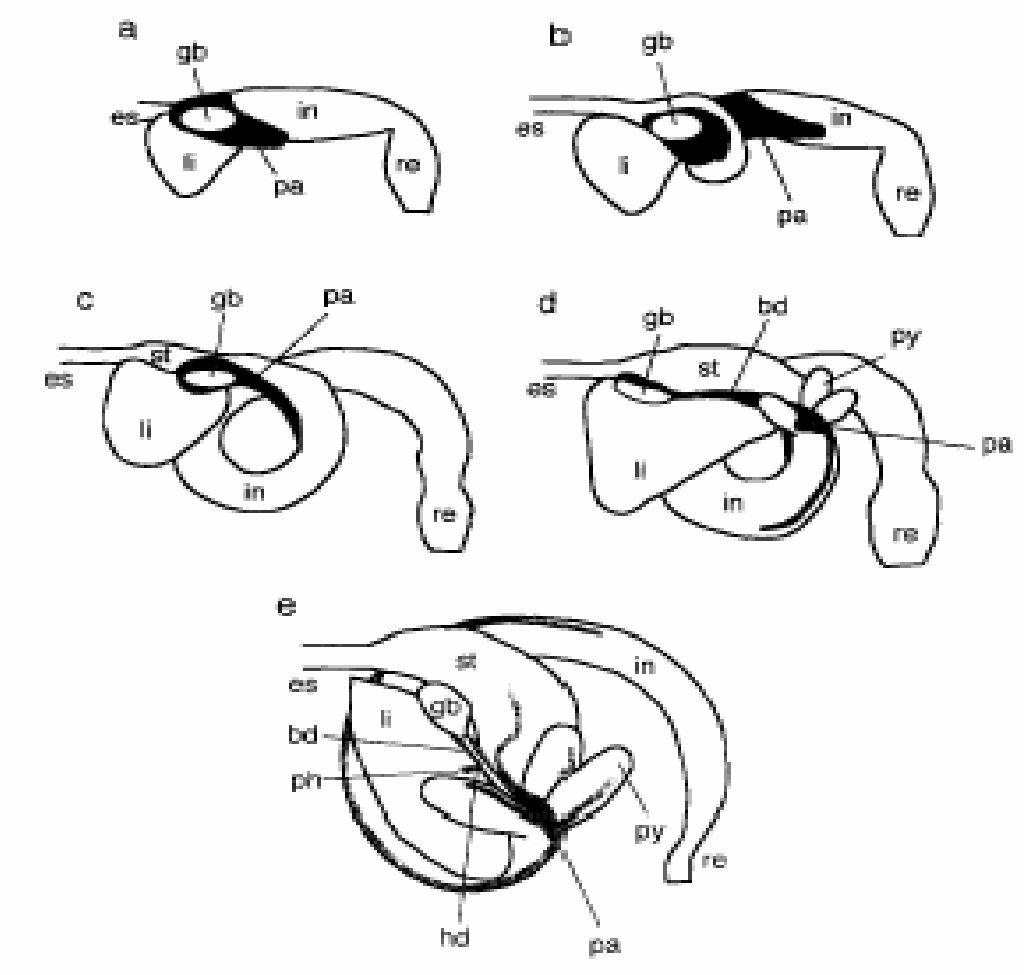

Fig. 1.2. Development of the diffuse pancreas in Japanese flounder (*Paralichthys olivaceus*). The morphology of the pancreas was reconstructed from serial histological sections and is represented schematically. Shaded areas indicate pancreatic tissue. The developmental stages PL to I follow the terminology developed by Minami (1982) for flounder larvae. (a) 3 dph, stage PL. (b) 10dph, stage A. (c) 20 dph, stage D. (d) 30 dph, stage F. (e) 45 dph (completion of metamorphosis), stage I . bd:bible duct; es:esophagus; hd: hepatic duct; in: intestine; li: liver; pa: pancreas; ph: porta hepatis; py: pyloric appendages; re: rectum; st: stomach (Reproduced from Kurokawa, 1996).

1.1.2 The onset of digestive enzymes

In recent years, there has been an increasing interest to study the development of digestive enzymes in marine fish larvae in an attempt to facilitate the choice of the optimum feeding strategy. The onset of digestive functions, associated with morphological transformations, follows a sequential chronology in developing fish like that in developing mammals. Ontogenetic development of digestive enzymes has been studied in various fish systems using enzymatic assays and immunohistochemical methodologies (Walford and Lam 1993; Oozeki and Bailey 1995; Moyano *et al.*, 1996; Baglole *et al.*, 1998; Ribeiro *et al.*, 1999). In European seabass, trypsin activity can be detected on day 3 post hatching and a sharp increase in trypsin and amylase activities coincides with the mouth opening (5 dph), and corresponds to the first secreted zymogen granules (Zambonino Infante and Cahu, 1994 b). Studies on *Clupea harengus, D.labrax* and *Solea senegalensis* suggest that the synthesis process of pancreatic enzymes is not induced by food ingestion. The specific activities of the main pancreatic enzymes follow a similar pattern during development. This pattern shows that the pancreatic digestive capacity of young larvae is very high, related to their weight, and the enzyme synthesis process is linked to age (Cahu and Zambonino Infante, 2001). Amylolytic and amylase activities, in general, decrease with fish age. This observation agrees with previous findings on carbohydrase activities in rainbow trout (Kuz'mina, 1996). In European seabass (*D. labrax*), higher amylase mRNA levels are found in young larvae than in older larvae. The coordinated decrease between amylase enzyme activity and mRNA levels of amylase suggests a transcriptional regulation of amylase expression during larval development. Furthermore, the decrease in amylase activity is observed irrespective of the

dietary glucide concentration (Peres *et al.*, 1998). This indicates that the decrease in amylase activity during larval development may be genetically programmed.

Although lipids and proteins have generally been considered to be the major substrates for energy metabolism in larval early development (Ostrowski and Divakaran 1991; Koven *et al.*, 1992), several studies show that marine fish larvae produce a large amount of α-amylase enzyme around the time of first feeding (Cahu and Zambonine Inante, 1994; Oozeki and Bailer, 1995; Martinez *et al.*, 1999). This raises the possibility that marine fish larvae may utilize carbohydrates during early development to help meet their energy requirement. Kim and Brown (2000) demonstrated that the ontogeny of digestive enzymes in the Pacific threadfin (*Polydaxtylus sexfilis*) follows a pattern in which amylase is the first to become activated, followed by lipase and protease later in development. These results indicate that carbohydrate utilization play a significant role in the earlier phases of development among some marine fish larvae, followed by a shift to protein and lipid utilization.

Although the data obtained in fish so far show that the digestive enzymes studied are qualitatively similar to those observed in other vertebrates, some assay factors, as well as the broad variety of techniques used to determine the different enzymatic activities, may cause variability in the final data (Hidalgo *et al.*, 1999). These factors are: 1) non-uniformity in the tissue used for enzymatic activity determinations results in the procedure sometimes involving the homogenization of attached glands and/or the whole digestive tract; 2) the nutritional status of the animals used in the experiments is not consistent, as the animals are killed either after starvation or at different post-feeding times; 3) the digestive tract is washed before homogenization in some cases, whereas others have used the tract including its contents for extraction; and 4) enzymes from prey also contribute

importantly to digestive capacity in larvae. It has been reported that the trypsin activity contribution of *Artemia* could amount to a maximum 5 % of the total assayed activity in 20-day-old seabass larvae (Cahu and Zambonino Infante, 1995), and the calculated contribution of *Artemia* amylase activity was more than 50 % of the total amylase activity measured in the metamorphic Atlantic halibut (*Hippoglossus hippoglossus*) larvae (Gawlicka *et al.*, 2000).

Only limited diet-related manipulation of digestive abilities may be possible (Collie and Ferraris, 1995; Peres *et al.*, 1998), the ontogenetic sequence of digestive system development appears to be genetically programmed in fishes (Buddington and Diamond, 1989; Gawlicka *et al.*, 2000; Cahu and Zambonino Infante, 2001). Therefore, finer-resolution and more definitive examination such as that afforded by enzyme gene expression and regulation using molecular approaches is desirable to clarify some aspects of larval nutritive physiology and help solve some ontogenetic questions in early fish larval development.

1.1.3 Amylase

The function of α-amylase is the hydrolysis of α-1,4 glycoside bonds in carbohydrate, such as starch and glycogen, and amylases occurs widely in nature, being found in bacteria, plants and animals. In humans, α-amylase is composed of 496 amino acids in a single polypeptide chain, which is encoded on chromosome I as part of multigene family (Gumucio *et al.*, 1988). These genes are regulated so that different isozymes are synthesized in either salivary glands or the pancreas.

The digestion of starch in humans occurs in several stages: initially the starch is partially digested by salivary α-amylase, which breaks down polymeric starch into shorter oligomers; then the partially digested starch upon reaching the gut is extensively hydrolyzed into smaller oligo-saccharides by the α-amylase synthesized in the pancreas. Finally the resultant mixture of oligosaccharides, including maltose, maltotriose, and a number of α-(1-6) and α-(1-4) oligoglucans, was degraded into glucose by α-glucosidases and this glucose is then absorbed and enters the blood-stream by means of a specific transport system.

Fish do not have a salivary gland (Yardley, 1988), and amylase is produced by pancreatic cells located in a diffuse mesentery surrounding the digestive tract (Kurokawa and Suzuki, 1996). As stated earlier, young larvae exhibit higher amylase activity than older larvae (Peres *et al.*, 1996; Ribeiro *et al.*, 1999). The decline in amylase-specific activity in dissected pancreatic segment in European seabass has been shown to be due to a decrease in mRNA coding for amylase (Peres *et al.*, 1998). These results indicate that the decline in amylase expression is transcriptionally regulated during larval development. Therefore, amylase represents a good example for illustrating an ontogenetic change in enzyme expression during larval development and offers a good marker to study pancreas development in fish.

1.2 Hormones in fish

Teleost endocrinology is a large segment of comparative vertebrate endocrinology. In recent years, there has been an upsurge of interest in this field. Domesticated fish is commonly used as experimental animals for both physiological and cell and molecular

research. Studies so far have revealed that fish larvae are in general physiologically immature with little or no capacity to produce certain hormones and enzymes, and that they are dependent to a certain extent on exogenous sources (Lam, 1994).

1.2.1 Functions for Cortisol and T_3 in fish

1.2.1.1 Cortisol

Fish do not have a discrete adrenal gland as in mammals, and the steroidogenic cells are distributed in the head-kidney region, mostly along the posterior cardinal veins and their branches. The biosynthesis of cortisol in fish is similar to that in mammals. Briefly, the synthesis involves the microsomal enzymatic pathways (Fig. 1.3), including 21-hydroxylation (P450c21), 17 α-hydroxylation (P450c17), and 3 β-hydroxy steroid dehydrogenation (3 β-HSD).

Cortisol is the major corticosteroid in teleosts. Cortisol has been shown to be involved in hatching, growth and metamorphosis during early development of teleosts (Lam 1994; Sampath-Kumar *et al.*, 1995). Gills, intestine and liver are important targets for cortisol in fish. These organs reflect the two major actions of cortisol in fish: regulation of the hydromineral balance and energy metabolism. Its effects include stimulation of protein catabolism, gluconeogenesis and hyperglycemia in response to various stress factors (such as starvation and migration). Thus, cortisol is involved in the glucose metabolism and plays an important role in the regulation of carbohydrate utilization. Because amylase activity is increased during first feeding, cortisol may be a particularly important regulatory compound in marine fish larval development. In mammalian models, the regulation of energy metabolism and hydromineral balance is

carried out by two classes of steroids, glucocorticoids and mineralocorticoids, each with its own receptor. The mineralocorticoid effects are mainly sub-served by another corticosteroid, aldosterone. The absence of the mineralocorticoid in fish suggests that cortisol serves both the functions of glucocorticoids and mineralocorticoids (Mommsen *et al.*, 1999).

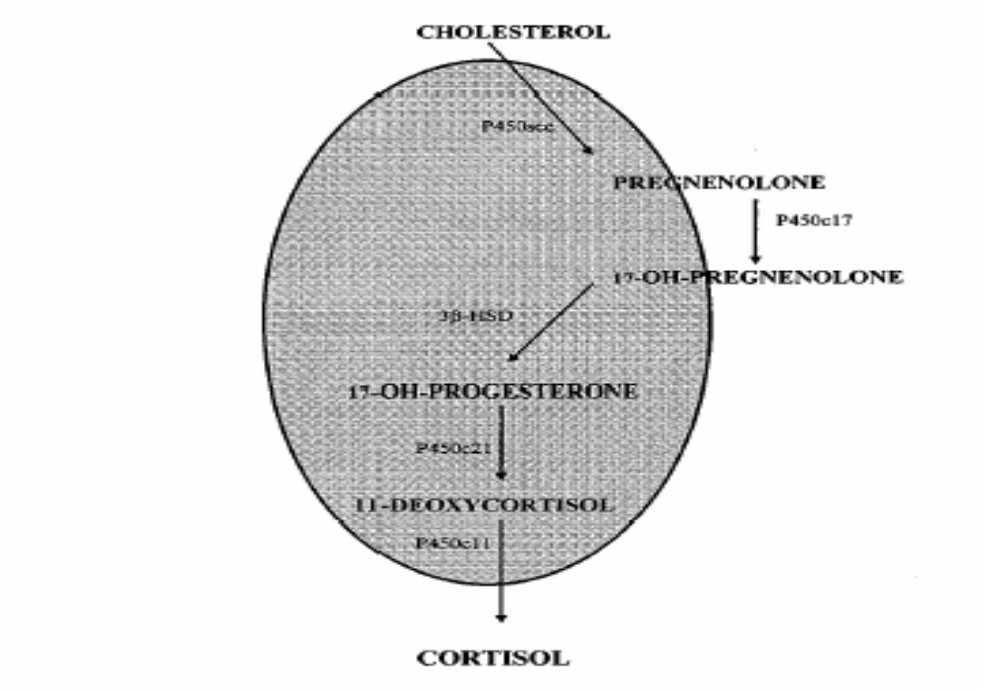

Fig. 1.3. Biosynthesis of cortisol in teleost fishes. The shaded area represents the mitochondrial compartment, whereas those reactions occurring in the nonshaded area occur within the cytosolic compartment. Abbreviations: 3β-HSD, 3β-hydroxysteroid dehydrogenase; P450s, various forms of cytochrome P450 (Reproduced from Mommsen *et al.*, 1999).

1.2.1.2 Thyroid hormones

The essential components making up the thyroid axis and their functions have largely been conserved across vertebrates. The biosynthesis of thyroid hormones (THs) occurs in the throid follicle, a single layer of epithelial cells enclosing a colloid-filled space, and thyroxine (L-T_4) is the predominant hormone secreted. T_4 has few direct actions and is considered to act principally as a precursor for triiodothyronine (T_3), which is the biologically active form of the hormone (Hadely, 1992).

Thyroid hormones (THs) are essential for metamorphosis in amphibians, and larval tissue degeneration and adult organogenesis are almost exclusively controlled by TH. Both thyroxine (T_4) and triiodothyronine (T_3) are present in eggs and in most cases, the levels decrease as development proceeds until the onset of endogenous thyroid hormone production, which usually occurs before or around yolk-sac resorption. Enhancement of T_4/T_3 levels in newly-hatched larvae through immersion or maternal injection has been shown to promote larval growth, development and/or survival in several fish species (Lam 1994). THs are also important regulatory hormones that increase epidermal mitotic rate by controlling the synthesis of specialized proteins during cell differentiation within the digestive system (Hourdry 1993).

1.2.1.3 Interaction of Cortisol and Thyroid hormone in larval development

Cortisol and thyroid hormone levels have been found to follow similar patterns throughout early development in Japanese flounder; both were present in eggs and they peaked simultaneously during metamorphic climax (DeJesus *et al.*, 1991). Cortisol and thyroid hormones are known to interact in the regulation of a range of processes within the

target tissues. Cortisol increased hepatic conversion of thyroxine (T_4) to triiodothyronine (T_3) in brook char *Salvelinus fontinalis* (Vijayan *et al.*, 1988). Cortisol has also been shown to increase conversion of thyroxine (T_4) to the active triiodothyronine (T_3) in the toad larvae *Bufo boreas* (Hayes and Wu 1995). Kim and Brown (2000) treated fish larvae with T_3 and/or cortisol, and observed increases in specific activities of amylase and serine protease throughout the experimental period. Therefore, hormonal interactions and integration need to be studied to achieve a better understanding of the endocrine regulation of digestive system development in fish.

1.2.2 Molecular mechanisms of cortisol and thyroid hormones

The treatment of fish with hormone is even more varied than the number of the fish species examined (Mommsen *et al.*, 1999). Hormone treatments have been done with different preparations (cortisol, T_3, T_4, dexamethasone) and different modes of application (single or repeated injection, at different sites, oral treatment and immersion). These varied approaches are exacerbated by a bewildering array of treatment times, ranging from days to weeks (Mommsen *et al.*, 1999). The enzyme activities were assayed using the whole larvae and then pooled for the extraction of the enzymes, therefore their specific tissue source can only be considered speculatively. It is possible, and in fact likely, that the early expression of digestive enzymes and their up-regulation by cortisol and thyroid hormones is not limited to the gastrointestinal system (Kim and Brown, 2000). The underlying diurnal and seasonal cycles associated with feeding, sexual maturation add further levels of complexity. The slight changes in experimental conditions will change the set-point of the assay. However a combination of molecular techniques, *in vitro* cell systems and whole fish physiology will allow finer resolution and more accurate,

definitive examination of ontogenetic questions, and will provide a better understanding of the multiple faces of hormone.

1.2.2.1 Mechanism of action of cortisol

Small lipophilic molecules such as steroid and thyroid hormones or the active forms of vitamin A (retinoids) and vitamin D play an important role in the growth, differentiation, metabolism, reproduction, and morphogenesis of animals. Most cellular actions of these molecules are mediated through binding to nuclear receptors that act as ligand-inducible transcription factors.

Corticosteroid hormones are hydrophobic molecules that travel bound to corticosteroid receptors (CRs) and act as transcription factors by binding to hormone response element (HRE). The mechanism of action is shown in Fig. 1.4.

The majority of DNA-binding proteins have been shown to occur as oligomers, most commonly dimers**.** It was shown that two molecules of the GR DNA-binding domain (DBD) bind to a GRE in a cooperative manner (Fig.1.5), in which binding of the first molecule enhances binding of the second (Dahlman-Wright, *et al.*, 1991). Studies of the binding of GR DBD to several variants of GRE from the TAT gene have led to a conclusion that protein-protein interactions and not changes in the structure of DNA are the major determinants for this facilitated binding.

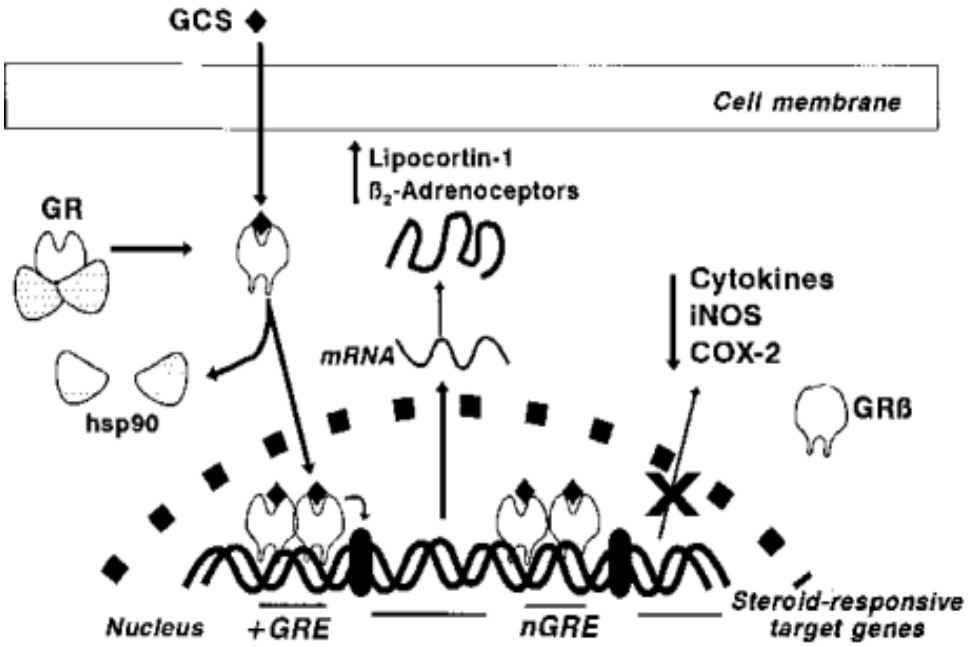

Fig. 1.4. Classical model of glucocorticoid action.

The glucocorticoid enters the cell and binds to a cytoplasmic glucocorticoid receptor (GR) that is complexed with two molecules of a 90 kDa heat shock protein (hsp90). GR translocates to the nucleus where, as a dimer, it binds to a glucocorticoid recognition sequence (GRE) on the 5´-upstream promoter sequence of glucocorticoid-responsive genes. GREs may increase transcription and negative (n) GREs may decrease transcription, resulting in increased or decreased mRNA and protein synthesis. GCS, glucocorticoid sensitive. (Reproduced from Barnes 1998).

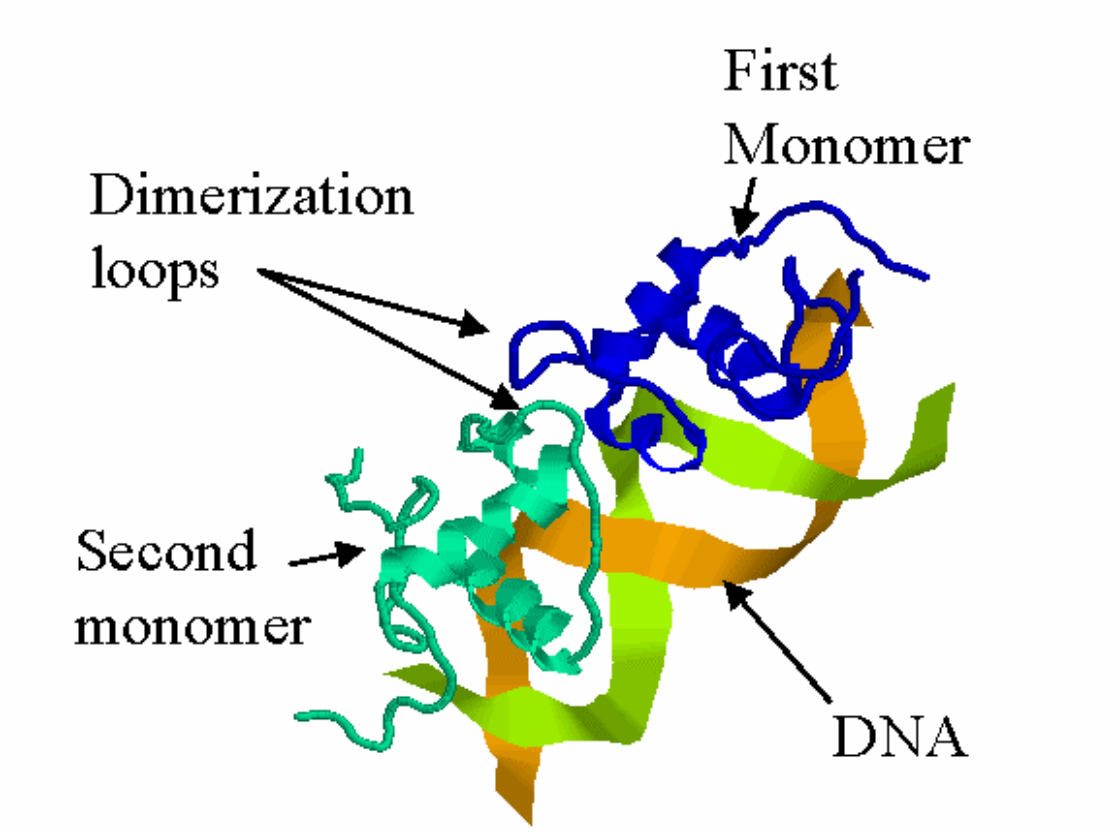

Fig. 1.5. Dimerisation of the glucocorticoid receptor occurs on binding to DNA. Interactions between the two monomers are through the dimerization loop.

Bamberger *et al.* (1996) described two types of mechanisms of GR action. The type 1 mechanism is characterized by the GR interaction with specific DNA sequences, whereas the type 2 mechanisms involves interaction of the GR with other transcription factors in the absence of specific DNA binding. The type I mechanisms represents the classic model of GR action, in which a receptor homodimer binds to short, palindrome GREs in the promoter region of glucocorticoid-responsive genes. The GRE lacks specificity as it can be bound by a variety of steroid receptors including those for progesterone, androgen and mineralocorticoids (Beato *et al.*, 1996). In addition, a series of

interactions with other transcription factors called co-activators are thought to increase the efficiency of transcription by RNA polymerase II. The list of co-activators is increasing rapidly, but already includes TFII β and steroid receptor activator 1 (SRC-1) (Bamberger *et al.,* 1996) and possibly the cAMP response-element-binding protein (CREB) (Smith *et al.,* 1996). The type 2 mechanisms of GR action inhibit rather than activate transcription. This is especially true for the anti-inflammatory/immunosuppressive effects of glucocorticoids that involve negative transcriptional regulation of immune genes, such as the collagenase and the interleukin-2 genes. The promoter lacks a GR-binding site, yet they are repressed by glucocorticoids through interaction with other transcription factors such as Jun and Fos family proteins and block their stimulatory actions on genes including those of the immune system (Bamberger *et al.,* 1996).

Glucocorticoid has been shown to regulate metabolism and induce digestive enzymes in fish. Fish are thought to have only one CR (corticosteroid receptors) type, unlike mammals which contain distinct mineralocorticoid and glucocorticoid (MR and GR) receptors. This one receptor is called a GR, consistent with the lack of a significant amount of a unique mineralocorticoid hormone in fish (Ducouret *et al.,* 1995). GR mRNAs has been detected in a large variety of rainbow trout tissues, including liver, kidney, gill, intestine, skeletal muscle and brain. Ducouret *et al* (1995) cloned a teleost fish glucocorticoid receptor which has 9 more amino acids between the zinc fingers than those seen in mammalian GRs. This unusual structure of the fish glucocorticoid receptor may be a direct consequence of the dual functions of cortisol in fish (Ducouret, *et al.,* 1995, Tujague *et al.,* 1998).

As mentioned above, studies in fish GR-transcription area will be important to help us understand the evolution of the steroid receptor family and also be important to assist us to clarify the stress responses initiated by glucocorticoids.

1.2.2.2 Mechanism of action of Thyroid hormones

Thyroid hormones (THs) exert their major effect by binding to nuclear TH receptors (TR) that act as DNA-binding transcription factors, collectively known as the nuclear receptor (NR) superfamily. TRs are involved in the regulation of a very wide range of biological processes. TR binds to DNA sequences known as thyroid hormone response element (TREs) found in the regulatory regions of a target gene (Fig. 1.6), and according to the nature of the TREs, gene expression may be enhanced or inhibited (Wu and Koenig, 2000).

The first TRs in fish were cloned from the Japanese flounder (Yamano *et al.*, 1994a; Yamano and Inui, 1995). There are four receptor transcripts; two of which corresponded to TRα and two to TRβ. This work suggests that the two flounder TRβ transcripts arise from a single gene and are generated by differential splicing, while two genes exist for the two flounder TRα transcripts. In contrast, mammals and chicken TRα arise from one gene and TRβ from the other gene, and additional receptor transcripts arise from differential splicing.

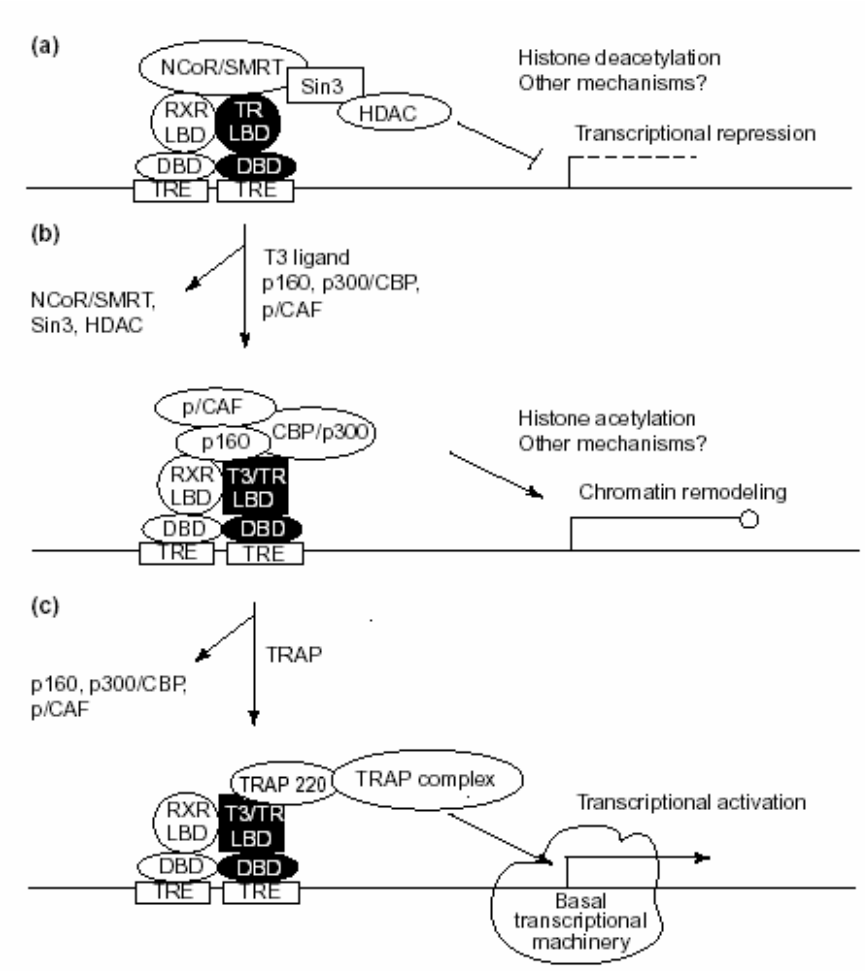

Fig. 1.6 Model of gene repression by unliganded TR and activation by liganded TR. (a) In the absence of ligand, the DNA-bound RXR–TR heterodimer interacts with a corepressor complex composed of NCoR/SMRT, Sin3 and HDAC, and actively represses gene transcription. (b) In the presence of ligand, the TR undergoes a conformational change, which results in the replacement of the corepressor complex by a coactivator complex composed of p160 proteins, p300/CBP, p/CAF and perhaps other proteins. The histone acetyltransferase activity derived from coactivators results in an 'open' transcriptionally active chromatin configuration. (c) Ligand-occupied TR then associates with the multiprotein TRAP complex, which activates transcription, perhaps by interaction with general transcription factors. Abbreviations: CBP, cAMP-response element-binding protein (CREB)-binding protein; DBD, DNA-binding domain; HDAC, histone deacetylase; LBD, ligandbinding domain; NCoR, nuclear corepressor; pCAF, p300/CBP-associated factor; RXR, retinoid X receptor; SMRT, silencing mediator for RXR and TR; TRAP, thyroid hormone-associated protein; T3, thyroid hormone; TR, thyroid hormone receptor; TRE, thyroid hormone-response element (Reproduced from Wu and Koenig, 2000).

1.3 Seabass (*Lates calcarifer*) as a model for endocrinology research in tropic marine fish

In recent years, the zebrafish has gained increasing importance as a model organism in developmental biology and genetics, as it can be used to bridge the gap between *Drosphila/C.elegance* and mouse/human genetics. Unfortunately, for some specific tasks such as the study of cortisol dynamics and metabolic regulation, zebrafish are too small to conveniently obtain data. Until appropriate molecular probes become available, large fish species must be used as models (Mommsen *et al.*, 1999). Further, marine fish have begun to receive attention due to their increasing importance in aquaculture. The present studies were carried out in a tropical marine fish, the Asiatic seabass (*Lates calcarifer*). The seabass is an euryhaline fish in the Indo-Pacific region and is intensively farmed in Asia. The eggs are pelagic and rapidly hatch into relatively poorly developed larvae around 16 hours after fertilization. Over the next two days, the larvae utilize the yolk as energy resources and organs start to differentiate and develop. On the third day post hatching (dph), the larvae start to feed on rotifers, and the larvae are called first-feeding larvae. The oil globule is still visible on 4dph, but is nearly gone by 5 dph. In Singapore, seabass are cultured in floating net cages and the hatchery-produced larvae and juveniles are exported to many countries including Japan, Malaysia, and Thailand. The broodstock spawns twice each month, predictably two days after the new moon and full moon. The physiology/endocrinology of seabass larval growth, development and health has been well studied in our laboratory for more than two decades. Similar to zebrafish, seabass embryos are transparent during early development; in 3 dph hatched larvae, rudimentary organs are easily observable. Changes in the concentrations of cortisol and triiodothyronine (T_3) have already been analyzed in eggs and developing larvae of seabass (Nugegoda *et al.*, 1994; Sampath-Kumar *et al.*, 1997).

1.4 Stress response in fish

Stress is defined as a condition in which the dynamic equilibrium of animal organisms called homeostasis is threatened or disturbed as a result of the actions of intrinsic or extrinsic stimuli, commonly defined as stressors (Chrousos and Gold, 1992). Through the stress response, an animal tries to cope with a stressor by readjusting its biological activities. This suggests the reallocation of energy, and this is reflected by the phenomenon that both neuroendocrine routes including adrenergic secretion and promotion of corticoids are also important neuroendorine pathways for the control of mobilization and allocation of energy under normal as well as stress conditions (Galbo, 1983). For the integrated stress response in fishes, the distinction between primary, secondary and tertiary responses has been introduced (Wedemeyer *et al.*, 1990; Wendelaar Bonga E.S., 1997). Primary responses are activation of brain centers, resulting in the massive release of catecholamines (CAs) and corticosteroids, whereas secondary responses are referred to as the manifold immediate actions and effects of hormones on tissue and blood level, including the mobilization of energy resources and oxygen uptake. Tertiary responses are defined as effects on the level of the organism and population, including inhibition of growth, reproduction and immune response.

Glucocorticoids are crucial for maintaining basal and stress-related homeostasis in mammals (Bamberger *et al.*, 1996). Under resting conditions, cortisol sustains normoglycaemia and prevents arterial hypotension. In the stressed state, elevated cortisol is important for central nervous system activation, increased blood glucose concentration and elevated blood pressure, all of which are important for coping with stress. Cortisol is also considered to curtail the stress-induced inflammatory/immune reaction that might

otherwise lead to tissue damage (Bamberger *et al.*, 1996). To cope with the increased energy demand, fish mobilize substrates to fuel cellular processes. One of the important metabolic roles of cortisol during stress is in the glucose-regulation and glycogen-depletion processes, both of which are important pathways for the recovery from stress. Furthermore, cortisol may also play a role in the peripheral mobilization of substrates such as amino acids and fatty acids, thereby providing precursors for hepatic gluconeogenesis in fish (Mommsen *et al.*, 1999).

1.5 Objectives of the project

In the present study, the overall objective was to investigate the ontogeny of amylase gene expression in seabass larvae and mechanism of gene regulation. There were three specific objectives:

1. To clone and sequence the α-amylase gene of seabass (both cDNA and genomic DNA), quantify the changes of its gene expression during the ontogeny of seabass larval development, and correlate these changes with the ontogenetic changes of α-amylase enzyme activity. This will be introduced and reported in Chapter 3.

2. To study the molecular mechanisms of seabass α-amylase gene expression and transcriptional regulation. The amylase promoter will be cloned and characterized, and a series of deletion mutants will be generated and fused to the luciferase reporter gene, followed by studies of their functional activity in rat AR42J cell line. Furthermore, the potential regulatory elements and hormone response elements on α-amylase promoter will be identified and confirmed using site-directed mutagenesis and Electrophoretic mobility shift assay (EMSA). Characterization of promoter will be reported in Chapter 4.

3. To study the hormonal influence on α-amylase gene expression *in vivo*. The effects of cortisol and T_3 on α-amylase gene expression during early developmental stages of seabass larvae will be discussed in Chapter 5. At the same time, the effect of starvation as a nutritional status on amylase gene expression will also be studied.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1 Animals

2.1.1 Rearing of larvae

Fertilized seabass (*Lates calarifer*) eggs were collected from floating net-cages (San Lay Marine Culture Pte.Ltd., Pulau Ubin, Singapore). The eggs were washed with sand-filtered seawater (30 ppt) and transported to the laboratory in sealed plastic bags inflated with pure oxygen. Upon arrival, the eggs were separated into buoyant and non-buoyant eggs at 30 ppt. Buoyant eggs were stocked in buckets filled with 15 L of filtered and UV-irradiated seawater. Mild aeration was provided. After hatching, larvae were transferred to 300-L fiberglass tanks (black background) containing filtered and UV-irradiated seawater. From 3 days post hatching (dph), larvae were fed once a day with rotifers (10 individuals ml^{-1}) until 12 dph and thereafter were fed with 1-day-old *Artemia* nauplii (2 individuals ml^{-1}). The water temperature in the tanks varied between 26.5° and 28.5°C during the period of study.

2.1.2 Rotifer Culture

Rotifer inoculum from Primary Production Department's Fresh Water Fisheries Section (Sewbawang Research Station, Singapore) was cultured in 15 ppt seawater in 1.5 ton tank and fed with baker's yeast twice a day. The cultures were one month old when the experiments were conducted.

2.1.3 Sampling of larvae

Sampling was carried out in the morning before feeding on 1, 2, 3, 4, 5, 6, 8, 12, 14, 15, 18, 21, 24 and 27 dph. The larval samples were collected in triplicates from tanks for each the of samplings days. Larvae collected were pooled into two aliquots (approx. 60 mg for each group). Larvae for mRNA quantification were immediately homogenized and total RNA extraction was performed, while larvae collected for enzyme assays were frozen in liquid nitrogen and then immediately stored at -80°C until assayed.

2.2 RNA extraction and analysis

2.2.1 Total RNA extraction

Total RNA from seabass embryo and larval samples was extracted using TRI reagent (Life Technologies). The samples were quickly frozen in liquid nitrogen and homogenized in 1 ml TRIzol reagent. The homogenate was incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes before adding 200 µl of chloroform. The mixture was shaken vigorously for 15 seconds and incubated at room temperature for 2-3 minutes, followed by centrifugation at 12,000 g for 15 minutes at 4°C. Following centrifugation, the mixture separated into a lower red penol-chloroform phase, an interface, and a colorless upper aqueous phase. RNA remained exclusively in the aqueous phase. The aqueous phase was transferred to a fresh tube and an equal volume of isopropanol was added. RNA was centrifuged at 12,000 g for 10 minutes at 4°C and washed with 1 ml 75 % ethanol. The RNA pellet was briefly dried and dissolved in 20 µl diethyl pyrocarbonate (DEPC)-treated water. For Real-time PCR assay, the RNA was treated with DNAase (GenHunter) at 37°C for 30 min to remove any

genomic DNA contamination. RNA samples were quantified spectrophotometrically in triplicate.

2.2.2 Poly A^+ mRNA isolation

Poly A^+ mRNA was isolated using Qligotex mRNA spin-column Kit (Qiagen) following the manufacturer's instruction. Briefly, total RNA was transferred into a 1.5 ml microcentrifuge tube and added with appropriate volume of buffer OBB and Oligotex suspension, the mixture was incubated for 3 minutes at 70°C and then placed at 30°C for 10 minutes. The oligotex:mRNA complex was centrifuged for 2 minutes at maximum speed and the pellet was resuspended in 400 µl buffer OW2. After centrifugation, the spin column was transferred to a new tube, 400 µl OW2 was added, and the column was then centrifuged for 1 minute. The spin column was transferred to another new tube and poly A^+ mRNA was eluted by adding 50 µl hot buffer OED.

2.2.3 Analysis of RNA by agarose/formaldehyde gel electrophoresis

Total RNA was separated on 1.2% denaturing agarose gel (1.2% agarose, 1×MOPS and 6% formaldehyde). Each RNA sample contained 50% formamide, 1× MOPS, and 6% formaldehyde, and was heated at 65°C for 10 minutes before loading with loading buffer. The gel was run at 75 volts in running buffer containing 1× MOPS and 3% formamide until bromphenol blue dye had migrated one-half to two-thirds the length of the gel. Gel staining was done and gel picture taken.

2.3 Polymerase chain reaction (PCR)

PCR is a powerful approach for rapid amplification of specific DNA fragments *in vitro* from a trace amount of DNA. Template DNA was amplified using 25 pmol of each primers, 2 U of PLATINUM *Taq* DNA Polymerase (Life Technologies), 0.2 mM dNTP mix, 1×PCR buffer and 1.5 mM $MgCl_2$. A typical PCR condition was as follows: Thirty-five cycles of denaturation (94°C for 30 seconds), annealing (56°C for 30 seconds) and extension (72°C for 60 seconds) were conducted with a PTC-100 thermal cycler (Peltier-effect; MJ Reseach). All reactions were initiated with 2-min incubation at 94°C and terminated with 10-min incubation at 72°C.

2.4 DNA preparation

2.4.1 Plasmid DNA miniprep

A small scale of plasmid DNA was prepared using Wizard Plus SV Minipreps DNA purification system (Promega, USA). Typically, around 10-15 µg plasmid DNA can be isolated from 5 ml of overnight bacteria culture in Luria-Bertani (LB) medium.

The 5 ml overnight bacterial culture containing appropriate antibiotic was harvested by centrifugation at 5,000 rpm for 10 minutes in an Eppendorf microcentrifuge. The bacterial pellet was resuspended in 250 µl Resuspension Solution. 250 µl Cell Lysis Solution was added to the bacterial suspension and mixed by gently inverting the tube 4 times. After 5 minutes incubation at room temperature, 10 µl Alkaline Protease Solution was added and incubated for 5 minutes. This mix was neutralized by adding 350 µl Neutralization Solution. The mixture was then centrifuged at 14,000 rpm for 10 minutes; the clear lysate was transferred into the prepared Spin Column by decanting. After centrifugation for 1 minute, the flowthrough was discarded and the spin column was

reinserted into the collection tube. 750 µl Column Wash Solution was then added to the spin column and centrifuged for 1 minute. After discarding the flowthrough, 250 µl Column Wash Solution was added and the wash procedure was repeated. Finally, the spin column was transferred to a 1.5 ml centrifuge tube and the plasmid DNA was eluted by adding 100 µl Nuclease-Free Water to the spin column. Purified DNA was stored at -20°C.

2.4.2 DNA Fragment recovery from agarose gel

Agarose gel used for DNA electrophoresis was prepared by adding the required amount of agarose to TBE buffer containing 0.5 µg/ml of ethidium bromide (EB). After gel electrophoresis, DNA fragments were visualized with a UV illuminator. DNA fragment was then excised using a sterile blade. The DNA was purified using QIAquick Gel Extraction Kit (Qiagen). Briefly, the gel slice containing the desired DNA bands was melted at 50°C for 10 minutes in Buffer QG and then transferred into a QIAquick spin column. The volume of the buffer QG was three times the gel slice volume. The column was centrifuged at 14,000 rpm for 1 minute and washed with 0.75 ml buffer PE; column was then stood for 2-5 minutes at room temperature and centrifuged again. QIAspin column was then transferred to a clean 1.5 ml tube and centrifuged for another 1 minute. The DNA was eluted by adding 30 µl Buffer EB or H_2O and centrifuged for 1 minute.

2.5 Ligation

DNA fragments either from PCR or from restriction enzyme digestion were ligated to appropriate linearized vectors. For pGEM-T easy vector (Promega USA), PCR products

recovered from agarose gel were cloned into the multiple cloning site (MCS). pGEM-T easy vector is cut at one site by the manufacturer with EcoR V, and a 3' terminal thymidine (T) is added to either ends. Such 3' T-overhangs at the insertion site greatly enhance the ligation of PCR fragments, which have 3' adenosine (A) overhangs in a template-independent manner by the Taq polymerase used in the PCR. Typically, the 10 µl ligation reaction mix was set up to contain 1 µl 10× ligation reaction buffer, 50 ng of pGEM-T vector, insert DNA and 1 µl T4 DNA ligase. The amount of insert DNA used made up a molar insert-to-vector ratio of 3:1. The ligation creation was incubated at 4°C overnight.

For pGL3 Luciferase (Promega) and EGFP (Clontech) reporter vectors, the ligation reaction was carried out in 20 µl of reaction volume which contained 2 µl 10× ligation buffer , insert DNA, vector DNA and T4 ligase (NEB, USA). Ligation reaction was usually performed at 16 ° C for 2-8 hours.

2.6 Restriction enzyme digestion

Restriction enzyme digestion was applied to screen recombinant clones and to harvest the insert DNA fragments, the first essential step for DNA cloning and mapping. All of the restriction enzymes in this study were purchased from New England Biolabs (NEB, USA). All restriction digestio ns were performed at 37°C for 1-24 hours with corresponding restricting buffers. The digestion result was analyzed by gel electrophoresis using appropriate concentrations of agarose gel according to the size of DNA of interest.

2.7 Transformation

2.7.1 Preparation of competent E.Coli DH5α cells

5ml of LB broth was inoculated with a single fresh colony of E.Coli DH5α cells and incubated at 37°C overnight with shaking at 250 rpm. In the following morning, 0.5 ml of overnight culture was reinoculated into a 500-ml flask containing 100 ml of LB broth and shaken at 250 rpm at 37°C until a final O.D of 0.5 was reached. The culture was chilled on ice for 15 minutes and cells were pelleted by centrifugation at 1,000g at 4°C for 15 minutes. The bacterial cells were drained thoroughly and resuspended in 30 ml of buffer RF1 (100 mM RbCl, 50 mM $MnCl_2$-4 H_2O, 30mM potassium acetate, 10 mM $CaCl_2$-$2H_2O$, 15% glycerol) with moderate vortexing. After incubation on ice for 15 minutes, the cells were pelleted and resuspended in 8 ml of buffer RF2 (10 mM MOPS, 10mM RbCl, 75 mM $CaCl_2$-2 H_2O, 15% glycerol). The competent cells were incubated for another 15 minutes on ice before an aliquot was placed into 1.5 ml microcentrifuge tubes (440 µl each, which was enough for 4 transformation reactions and a negative control) and immediately stored at -70°C. Transformation efficiency of the competent cells was usually not less than 10^6 colonies per µg of supercoiled plasmid DNA.

2.7.2 Transformation

5 µl of ligation reaction was added into 100 µl competent cells. The mixture was then placed on ice for 20 minutes. The cells were then heat-shocked at 37°C for 1 minute, after which 400 µl of LB was added. The transformed cells were incubated at 37°C for 1 hour with shaking at 225 rpm. 1/5 and 4/5 of the total reaction mixture was then plated onto a LB agar plate containing the appropriate antibiotics and incubated overnight at 37°C. Bacterial transformants that were resistant to antibiotics were randomly picked and

a small bacterial culture was prepared for the isolation of plasmid DNA. Positive recombinant DNA clones were screened by digestion with the appropriate restriction endonucleases.

2.8 DNA sequencing

2.8.1 Cycle sequencing

Cycle sequencing was carried out using ABI PRISM™ Big Dye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Perkin-Elmer). For each reaction, 100-500 ng of double-stranded DNA, 2 pmol of primer and 4 µl of BigDye reagent were mixed with ddH_2O to make up a final volume of 10 µl. The cycle sequencing reaction comprised 30 cycles of 10 seconds at 96°C, 5 seconds at 50°C and 4 minutes at 60°C. The reaction products were precipitated using 95% ethanol and sodium acetate and rinsed with 70 % ethanol twice to remove the salt.

2.8.2 Sequencing gel electrophoresis

The purified cycle sequencing products were resuspended in 3 µl of loading dye and denatured at 95°C for 2 minutes, then placed on ice. The 5% polyacrylamide gel was prepared by dissolving 18g of sequencing grade urea (Biorad) in 26 ml H_2O before the addition of 5 ml of 10× TBE buffer and 5 ml Long Ranger™ acrylamide gel solution (FMC bioproducts). The gel was polymerized with 250 µl of freshly prepared 10 % (w/v) ammonium persulfate (APS) and 35 µl of TEMED. After overnight sequencing, the sequencing data was collected and analyzed with ABI PRISM™ 377 Data Collection

program and Sequencing Analysis program (version 3.3.1). DNA chromatograms were further analyzed using Sequencher software (version 3.1.1; Gencodes Corpotation).

2.9 Real time PCR assay

2.9.1 The principle of the Real time PCR (LightCycler, Roche)

Real time PCR is a recently-developed technique and fluorescence-based (SYBR Green I) method of quantifying PCR product during amplification. SYBR Green I bind to the minor groove of the DNA double helix. In solution, the unbound dye exhibits very little fluorescence; however, fluorescence is greatly enhanced upon DNA-binding. The principle is outlined in the Figure 2.

At the beginning of amplification, the reaction mixture contains the denatured DNA, the primers, and unbounded dye; therefore minimal background fluorescence is produced and the signal is subtracted during computer analysis. After annealing of the primers, a few dye molecules can bind to the double strand DNA. DNA binding results in a dramatic increase of the SYBR Green I molecules emitting light upon excitation. During elongation period, more and more dye molecules bind to the newly synthesized DNA. If the reaction is monitored continuously, an increase in fluorescence is viewed in real-time. Upon denaturation of the DNA for the next heating cycle, the dye molecules are released and the fluorescence signal falls. Fluorescence measurement at the end of the elongation step of every PCR cycle is performed to monitor the increasing amount of amplified DNA. The unique feature of the Lightcycler Real time PCR is to perform a detailed melting curve analysis of the PCR products. The melting point of a DNA fragment depends on its individual length and its G/C content. Thus, the determination of the individual melting

temperature for a DNA fragment can be used to characterize the amplification products and discriminate between primer dimers and specific PCR products.

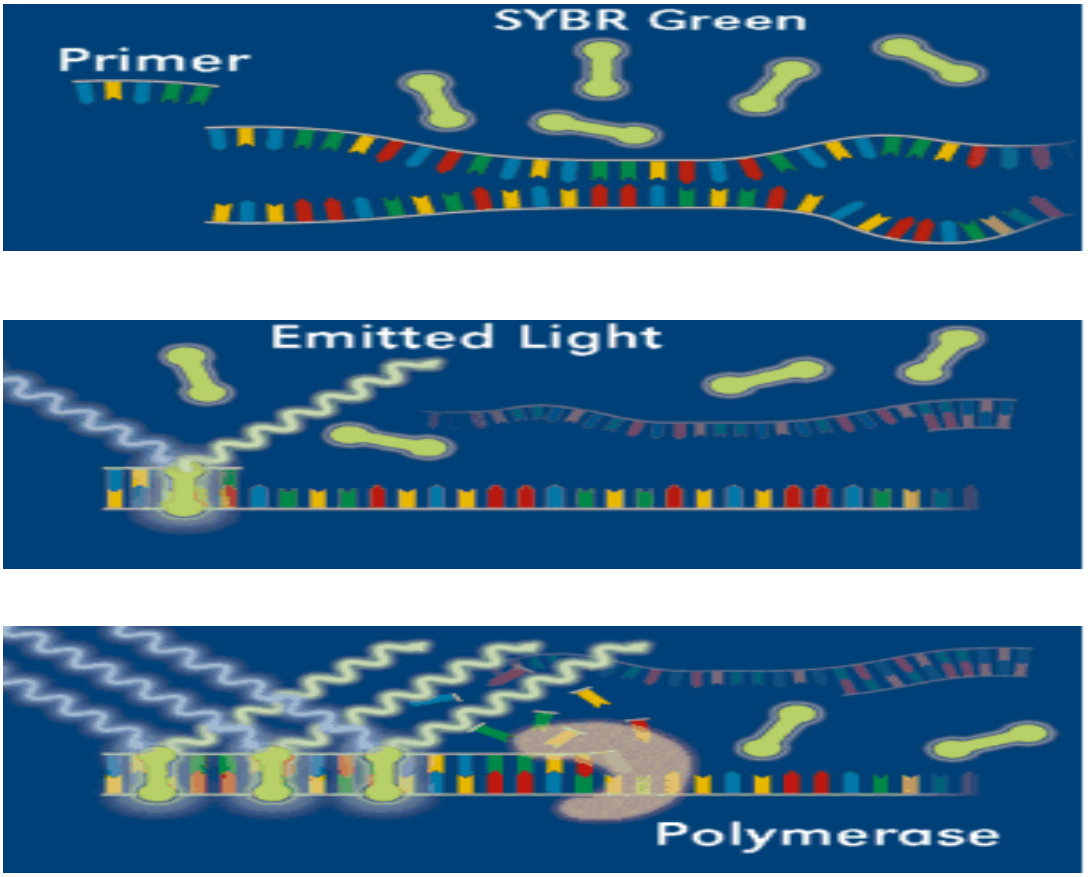

Fig. 2 The principle of the Real time PCR (from LightCycler, Roche)

2.9.2 Construction of the standard curve for seabass α-amylase

To determine the absolute copy number of the target transcript, the cloned plasmid DNA was used to generate a standard curve. The DNA concentration of the plasmid DNA was determined with a spectrophotometer at 260 nm. The copy numbers of the plasmid template were calculated according to the molecular masses of the plasmid (6.6×10^{5}/kb^{-1}

average value) and then converted into the copy number based upon the Avagadro's constant. The cloned plasmid DNA was serially diluted at a range of 2.34 fg to 36.6 pg (or 640 to 10^7 log copies). The standard curve was constructed by plotting the crossing point versus the known copy numbers of plasmids DNA. The copy numbers for the unknown samples were then calculated based on this standard curve.

2.9.3 Quantification by Real Time PCR

Optimal results of real time PCR are obtained for amplicons less than 700 bp; therefore, a 244-bp fragment of α-amylase gene was amplified in the real time PCR. One-step RT-PCR was performed using RNA Amplification Kit SYBR Green I (Molecular Probes Inc ., Eugene, OR). RT-PCR was performed in a 20 µl final volume containing 6 mM $MgCl_2$ (final concentration), 2 µl SYBR Green I, 0.5 µM of Amy 3 and Amy 4 primers (Chapter 3 Table 1), 0.4 µl RT-PCR Enzyme Mix and 1µl RNA template (1 µg/µl). Primers (Amy 3 and Amy 4) were designed to specifically amplify the seabass α-amylase gene. They were designed so as not to anneal to the DNA from rotifers and *Artemia*, which were used as seabass larval feed under stated conditions.

Reverse Transcription was carried out at 55°C for 10 minutes, followed by inactivation of the reverse transcriptase at 95°C for 30 minutes. For PCR amplification, first template PCR was conducted for 35 cycles comprised of heating at 20°C/s to 95°C with a 1-second hold, cooling at 20°C/s to 60°C with a 10-second hold, and heating at 2°C/s to 72°C. Fluorescent product was detected at the end of each cycle. After amplification, a melting curve was attained by heating the product at 20°C/s to 95°C, cooling it at 20°C/s to 70°C, and slowly heating it at 0.1°C/s to 95°C with fluorescence

collection at 0.2°C intervals. Melting curves were used to determine the specificity of PCR and allow discrimination between primer dimers and specific product.

We used the Second Derivative Maximum method to quantify DNA concentration. This method automatically calculates the fractional cycle numbers where the fluorescence rises above background (crossing Point). The Second Derivative Maximum is the point at which the rate of change of fluorescence is fastest. It usually occurs in the cycle where the sample fluorescence can first be distinguished from background fluorescence. The most reliable point for the quantification of concentration is found by determining the cycle number at which the fluorescence rises above background (crossing point). The more starting copies in a sample, the fewer PCR cycles are required to reach this point (Morrison *et al.*, 1998).

To assess the accuracy of the quantification, negative controls (without template RNA) were included in all real time PCR amplification. Also, parallel reactions for each RNA sample were run in the absence of reverse transcriptase to confirm the elimination of genomic DNA contamination.

CHAPTER THREE

ONTOGENY OF AMYLASE GENE EXPRESSION IN SEABASS LARVAE

3.1 Introduction

Understanding of the ontogeny of digestive systems in fish is still incomplete, and attention should be focused on the onset of enzyme production. Changes in specific activity of enzymes may indicate the capacity of fish larvae to use different nutrient sources during early development, especially at the time of first feeding. Several studies have shown the possibility of marine fish larvae utilizing carbohydrate during early development to help meet their energy requirement (Cahu and Zambonine Inante 1994; Oozeki and Bailer 1995; Martinez *et al.*, 1999). It is therefore important to measure the α-amylase activity during early larval development and to understand how the amylase gene is regulated. However, most of the measurements of enzyme activity in larvae are based on homogenizing the whole larvae and measuring the enzyme in crude extracts. This enzymatic determination method can be affected by a number of factors, such as loss of enzyme activity during homogenization, interference of other carbohydrate-metabolizing enzymes (glycosidases) in the crude extract, amylase precursor activity activation during homogenization (Hidalgo *et al.,* 1999) and presence of prey/food amylase activity. Therefore it would be more reliable to correlate the enzyme activity with its gene expression.

In recent years, a number of techniques, such as Northern analysis, semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) and *in situ* hybridization have been developed to measure the levels of mRNA expression. However, certain limitations still exist, most important of which is the insensitive or inaccurate

quantitation of mRNAs when they are present at low levels (Nitsche *et al.*, 1999; Li and Wang 2000).

In this study, we have developed a robust real time RT-PCR for the direct measurement of amylase mRNA levels. This method makes use of continuous monitoring of DNA quantification to enhance accuracy and increase the sampling dynamic range (Morrison *et al.*, 1999). It employs the double–stranded DNA (dsDNA) binding dye, SYBR Green I, that allows sensitive real time quantification of the mRNA even with small quantities as low as 10 to 120 copies (Blaschke *et al.*, 2000; Simpson *et al.*, 2000).

The aim of the present study is to characterize the α-amylase gene in the seabass and to quantify the ontogenic changes in the gene expression during early development making use of real time RT-PCR method, and correlate these changes with those of α-amylase activity. It will also serve as an initial step towards understanding the overall scheme of digestion in marine fish larval development.

Amylase is a ubiquitous enzyme found in many species of bacteria, fungi, plants and animals, and has been biochemically characterized in a number of different organisms. The nucleotide sequences for amylase genes have been determined from a variety of species including bacteria, fungi, insects, mammals and plants. Thus, amylase is one of a small number of genes for which information on gene sequence and structure is available from a wide range of evolutionarily diverse species. Amylase genes are expressed in a tissue-specific manner. In humans and rodents, it occurs in two major isozymic forms: salivary (Amylase A) and pancreatic (Amylase B) amylase which are encoded by the Amylase A and Amylase B genes respectively (Nishide *et al.*, 1986; Yokouchi *et al.*, 1990). Seven α-amylase genes are clustered in the human genome: two

pancreatic amylase genes, three salivary amylase genes and two truncated pseudogenes (Gumucio *et al.*, 1988). The chicken amylase gene contains nine short to medium length introns, resulting in a locus that spans roughly 4 kb. Thus, the chicken gene is intermediate in size between its murine and *D. melanogaster* counterparts which measure about 10 kb and 2.5 kb, respectively. The positions of the introns within the coding regions of the chicken and the human pancreatic amylase genes are identical, except for intron B, which is offset by a single nucleotide in the chicken locus in comparison with the corresponding intron in the human gene (Benkel *et al.*, 1997).

However, there are few reports on the amylase genes in fish (Peres *et al.*, 1998). Until this study, the genomic sequence of a fish amylase gene had not been reported. The present study will therefore extend the molecular database on amylase gene to include a fish amylase gene sequence. We cloned and characterized a seabass α-amylase gene and a glyceraldehyde-3-phosphate dehydrogenase (GADPH) gene, and the characterization and the ontogenetic expression pattern of α-amylase gene were studied.

3.2 Materials and Methods

All general procedures, such as rearing and sampling larvae, RNA extraction and DNA preparation, ligation and restriction enzyme digestion, transformation and sequencing, real time PCR assay, are described in Chapter Two.

3.2.1 Amylase assay

Amylase enzyme activity was measured according to Metais and Bieth (1968). Briefly, 0.9% NaCl was added into the tissue or the whole larvae and then homogenised.

After centrifugation at 5,000g for 10 minutes, the aqueous layer was harvested and stored at 4°C for the next-step analysis. Starch solution was freshly prepared and mixed with buffer E2 (Na_2HPO_4/KH_2PO_4, PH 7.4), and incubated at 37°C for 5 minutes. After adding the sample, the mixture was incubated for another 30 minutes. Finally the I_2 solution was added, and the values were read in a spectrophotometer at 580 nm. Enzyme activity was expressed as specific activity, i.e., units per milligram of soluble protein. Protein concentration was determined by the method of Bradford (Bradford, 1976). All measurements were carried out in triplicates.

3.2.2 RT-PCR amplification

One μg of total RNA was reverse transcripted into cDNA using Thermoscript ™ RT-PCR kit (Life Technologies). cDNA was then amplified using 25 pmol of each primer, 2 U of PLATINUM *Taq* DNA Polymerase (Life Technologies), 0.2 mM dNTP mix, 1× PCR buffer and 1.5 mM $MgCl_2$. The degenerate primers (Amy1 and Amy2) for the amplification of the seabass α-amylase cDNA were designed from highly conserved amino acids of *Gallus gallus* (U63411), *Danio rerio* (AI 942779), *Rattus norvegicus* (J00703), *Penaeus vannamei* (X77318), *Homo sapiens* (NM_004038) and *Rana sylvatica* (U44833), amylase sequences (Table 3.1). Thirty-five cycles of denaturation (94°C for 30 seconds), annealing (56°C for 30 seconds), and extension (72°C for 60 seconds) were conducted with a thermal cycler. All reactions were initiated with a 2-minute incubation at 94°C and terminated with a 10-minute incubation at 72°C. The PCR product (318 bp) was gel purified with the Qiaquick Gel Extraction Kit (Qiagen). DNA yields after extraction were measured by agarose gel electrophoresis using Molecular Mass standard (Bio-rad).

Table3.1: Oligonucleotide primers used in RT-PCR Amplification ,Lightcycler PCR and Genomic PCR

Primer	Primers Sequence	Annealing Temp (°C)
Amy1	5'AT(CT)GT(CT)CA(CT) (CT)TGTT(CT)GA(AG)TGGC(AG)CTG 3'	58
Amy2	5'CCAGCTTCCACATG(AT)(GC)G(AT)GT(GT)TGTTC3'	61
Amy3	5'ACATTGCTGCAGAATGTGAGCGCTTC 3'	66
Amy4	5'CCACACATGTGGTTGATGACTGCGTC3'	64
Amy5	5'GGTGTGGTTTTCTCGCACATCCAG3'	66
Amy6	5'GCCCATGGATGTATTTACAGCTTGG3'	65
Amy7	5'GACTGATAACACAAAGCAGCCGTC 3'	65
Amy8	5'CATGTAGCCAACGGCCATCTTG 3'	65

3.2.3 Genomic PCR amplification

Amylase genomic DNA was amplified using two sets of primers, Amy5 and 6, and Amy7 and 8 (Table 3.1). The conditions of the amplification reactions were initial denaturation for 3 minutes followed by 28 cycles of 94°C for 1minute, 65°C for 1 minute, 72°C for 1 minute, with a final extension step of 10 minutes for the 72°C. The final reaction mixture (100 µl total volume) for each reaction was 10× Qiagen PCR buffer A, dNTPs, 1.5 U *Taq* polymerase (Qiagen, USA), 10 µm of each primer, 20 µl Q-Solution, 1.5 mM $MgCl_2$ and 2 µl of seabass genomic DNA preparation.

3.2.4 Cloning and sequence analysis

Following purification, the PCR product was ligated into pGEM-T Easy Vector (Promega), and transformed into competent *E.coli* DH5α cells. The plasmid DNA was recovered from the bacterial cells by using a Wizard Plus SV Minipreps DNA Purification Kit (Promega). The size of the cloned DNA was confirmed by restriction enzyme digestion. The insert sequence was determined using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kits and ABI PRISM 377 DNA Sequencer. Homology search was performed using BLAST (http://www.ncbi.nlm.nih.gov).

3.2.5 Cloning of full-length α-amylase gene

The 5'-RACE-Ready cDNA and 3'-RACE-Ready cDNA was generated by using the SMART^{TM} RACE cDNA Amplification Kit (Clontech). Total RNA was isolated from seabass larvae using Trizol reagent (GibcoBRL) and poly A^+ mRNA was isolated using Qligotex mRNA spin-column Kit (Qiagen). One μg of poly A^+ mRNA was converted into either 5' or 3' RACE Ready cDNA products with primers and reagents provided in the presence of Moloney Murine Leukemia Virus (MMLV) reverse transcriptase. Generation of 5'-RACE and 3'-RACE fragment was performed following the manufacture's instructions. For the amplification of the cDNA fragments containing the 5' end of mRNA, nested PCR was carried out. The cDNA of the 5' end was amplified by using the outside pair of primers: gene specific primer 1 (GSP1) (5'-CGGCAGTCACGAACCTGATTGTGCATCAC-3') and primer UPM (5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3') (Clontech). The inside pair of primers was composed of a gene specific primer 2 (GSP2)

(5’-CCACCCCCAGCTCCACACATGTGGTTGRACE-3’) and a primer NUP (5’-AAGCAGTGGTATCAACGCAGAGT-3’) (Clontech).

An initial round of PCR was carried out with GSP1 and UPM at conditions of 40 cycles of 94°C for 5 seconds, 68°C for 10 seconds and 72°C for 3 minutes. One µl of 1:100 diluted 1st round PCR product was used as the template in a nested PCR under the touchdown condition: 5 cycles of 94°C for 30 seconds and 72°C for 3 minutes; 5 cycles of 94°C for 30 seconds, 70°C for 30 seconds and 72°C for 3 minutes; 25 cycles of 94°C for 30 seconds, 68°C for 30 seconds and 72°C for 3 minutes.

The cDNA of the 3’ end was amplified by using the outside pair of primers: gene specific primer 3 (GSP3) (5’-GGGCCGACATTGCTGCAGAATGTGAGC-3’) and primer UPM (Clontech). The 3’ RACE PCR conditions were the same as 5’ RACE PCR.

RACE products were cloned into pGEM-T Easy vector (Promega) and sequenced to establish that the correct seabass α-amylase had been cloned. The full-length cDNA gene was generated by long distance (LD) PCR using the 5’-RACE-Ready cDNA as template. The specific forward primer, 5'-GGTGTGGTTTTCTCGCACATCCAG-3', was designed based on extreme 5’ end of cDNA, and the reverse primer, 5'-GCCCATGGATGTATTTACAGCTTGG-3', was based on extreme 3’ end of cDNA. The sequence of the 1.6 kb PCR product was confirmed by DNA sequencing. Sequence analysis was performed using DNAMAN software (version 4.15). The on-line servers SignalP was used to predict N-terminal signal sequences (http://www.cbs.dtu.dk/services/SignalP/).

3.2.6 Southern blot analysis

3.2.6.1 Seabass genomic DNA extraction and enzyme digestion

Total genomic DNA was extracted from seabass using the phenol:chloroform method. Restriction endonuclease digestion of genomic DNA was performed using *BamH* I, *EcoR* I and *Sac* I (New England Biolabs) correspondingly. The restriction buffer contained 10 mM magnesium chloride, 100 mM sodium chloride, 50 mM Tris-HCl (pH7.9), 1 mM dithiothreitol, and 100 mg/mL bovine serum albumin. Digestion was allowed to proceed at 37°C overnight.

3.2.6.2 DNA gel electrophoresis and blotting

Digested DNA was subjected to electrophoresis on 0.8% agarose gels in a 1X TBE buffer. After electrophoresis, the gels were treated with 250 mM HCl for 10 minutes, and were then agitated in 0.4 N NaOH/0.6M NaCl for 30 minutes. DNA was transferred to GeneScreen membrane (NEN) overnight using 20X SSC as transfer buffer. After the transfer was complete, the membrane was fixed in a UV Crosslinker for 5 minutes.

3.2.6.3 Probe labeling

1 µg DNA was randomly labeled with DIG-11-dUTP using DIG-High Prime, a 5× concentrated labeling mixture of random hexamers, dNTPs mix containing alkali-labile DIG-11-dUTP and Klenow enzyme (Roche Molecular Biochemicals). The yield of DIG-labeled Probe was estimated by preparing a serial dilution of the labeled probe and dilution of a DIG-control DNA as described in the protocol. Visual comparison of the spot intensities of the control and probe allowed estimation of labeled probe concentration and yield. A probe dilution with the same intensity as one of the control dilutions has the same concentration.

3.2.6.4 Hybridization

Nonspecific background was reduced through prehybridization for 30 minutes at 42°C in a hybridization buffer using DIG Easy Hyb (Roche Molecular Biochemicals). Hybridization then proceeded at 42°C overnight with rolling in a hybridization incubator (Stuart Scientific, UK) in the same buffer containing 25 ng/mL DIG-labeled probe. The probe was denatured by boiling at 100°C for 5 minutes before adding to prewarmed hybridization buffer.

3.2.6.5 Posthybridization washes and immunological detection

Membranes bearing transferred genomic DNA were washed twice for 5 minutes each in 250 mL 2X SSC and 0.1% SDS at 67°C. The membranes were then subjected to a final wash in 0.5X SSC and 0.1% SDS for 2×15 minutes at 67°C. After hybridization and stringency washes, the membrane were rinsed for 5 minutes in washing buffer, incubated at 37°C for 30 minutes in blocking buffer, then incubated in a 1:10,000 dilution of anti-DIG–Alkaline-Phosphatase, and washed twice for 15 minutes each at 37°C in washing buffer. After equilibration in detection buffer, the membranes were applied with 1 ml ready-to-use CSPD and incubated for 10 minutes at 37°C. Results were recorded by exposing the membranes to X-ray film (Kodak) for 15-25 minutes.

3.2.6.6 Stripping and reprobing

The probe hybridized on the membrane was stripped away by washing the membrane in 0.2 M NaOH containing 0.1% SDS at 37°C for 2×15 minutes. After rinsing

thoroughly for 5 minutes in 2×SSC, the membrane was air-dried and was ready for reprobing.

3.3 Results

3.3.1 Amylase enzymatic activity during larval development

Amylase enzymatic activity during larval development in seabass is shown in Figure 3.1. Amylase activity was almost undetectable on 1 dph (day post hatching), but could be observed from 2 dph onwards. After the onset of feeding, amylase activity increased and peaked at 5 dph, then decreased progressively from 6 dph to 12 dph and thereafter remained at a low level until the end of the study.

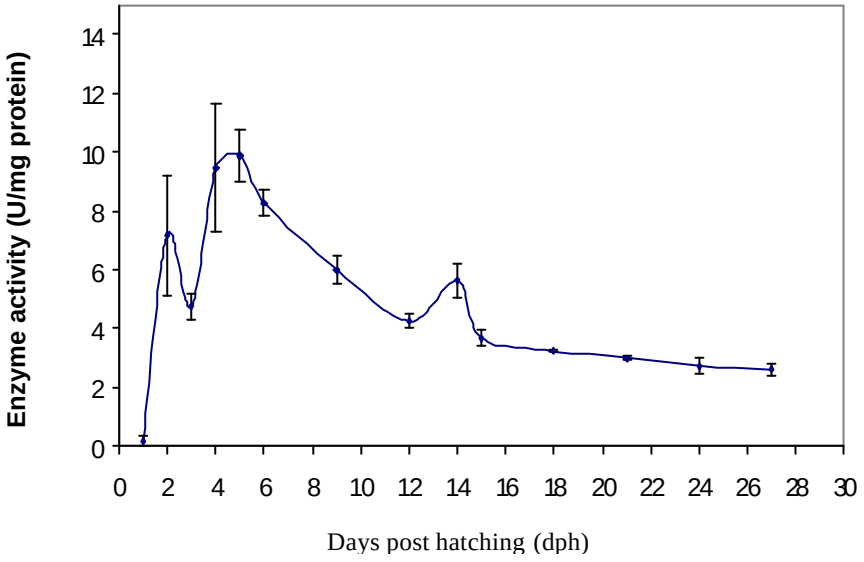

Fig 3.1. Specific activity of amylase during larval development in seabass (*Lates calcarifer*). Data presented as mean±S.D. of three samples of pooled larvae.

3.3.2. Cloning of a 295 bp fragment of seabass glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene

PCR amplification resulted in a 295 bp product whose identity as GADPH was confirmed by sequencing (Fig. 3.2A) (GeneBank accession number: AF322254). The amino acid sequence of the cloned product shows high homology to other species glyceraldehyde-3-phosphate dehydrogenase (Fig. 3.2B). Initially, the purpose of cloning this house-keeping gene was to use it for semi-quantitative RT-PCR. However, GADPH expression was not consistent in the seabass larval development (Fig. 3.2C); therefore it was not suitable for routine application as house-keeping purpose. Another house-keeping gene expression, β-actin, was also not consistent during early development stage (Thellin *et al* 1999), and was not useful for quantitative assay in this study.

A

```
1          CATGGTCTACATGTTCAAGTATGACTCCACTCACGGCGTGTGGAAGCATGGAGAGGTGAA
1            M  V  Y  M  F  K  Y  D  S  T  H  G  V  W  K  H  G  E  V  K

61         GGCCGAGGGCGGCAAGCTGGTCATCGGCAACATGCACATCACGGTCTTCCACGAGAGGGA
21           A  E  G  G  K  L  V  I  G  N  M  H  I  T  V  F  H  E  R  D

121        CCCCGCCAACATCAAATGGAGCGATGCTGGCGTCGACTACGTGGTGGAGTCCACCGGTGT
41           P  A  N  I  K  W  S  D  A  G  V  D  Y  V  V  E  S  T  G  V

181        GTTCACCACCATCGAGAAGGCCTCTGCTCACCTGAAGGGCGGAGCCAAGAGGGTGGTGAT
61           F  T  T  I  E  K  A  S  A  H  L  K  G  G  A  K  R  V  V  I

241        CTCTGCTCCCAGTGCTGATGCTCCCATGTTCGTCATGGGCGTA
81           S  A  P  S  A  D  A  P  M  F  V  M  G  V
```

B

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Seabass      MVYMFKYDSTHGVWKHGEVKAEGGKLVIGNMHITVFHERDPANIKWSDAGVDYVVESTGVFTTIEKASAH  70
Rainbowtrout MVYMFKYDSTHGRF.HGEVKAEGGKLVIDGHKITVFHERDPANIKWGDAGATYVVESTGVFTTIEKASTH  69
Mouse        MVYMFQYDSTHGKF.NGTYKAENGKLVINGKPITIFGERDPTNIKWGEAGAEYVVESTGVFTTMEKAGAH  69
Human        MVYMFQYDSTHGKF.HGTVKAENGKLVINGNPITIFQERDPSKIKWGDAGAEYVVESTGVFTTMEKAGAH  69

Seabass      LKGGAKRVVISAPSADAPMFVMGV  94
Rainbowtrout LKGGAKRVVISAPSADAPMFVMGV  93
Mouse        LKGGAKRVIISAPSADAPMIVMGY  93
Human        LQGGAKRVIISAPSADAPMFVMGV  93
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C

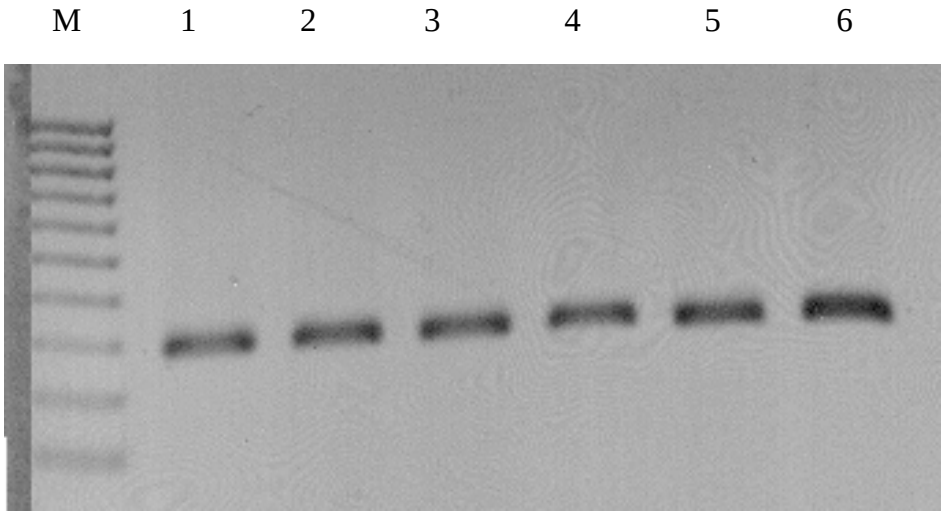


Fig. 3.2 (A) The sequence of seabass GADPH gene. (B)Aligment of amino acid sequences of GADPH from seabass (AF322254), rainbow trout (AB 066373), Mouse (XM_14423), and Human (CAA37794). Residues conserved in 50% of the sequences are shaded. (C) Agarose gel electrophoresis of RT-PCR product of GADPH in seabass larvae. Seabass GADPH Gene: Genebank accession No AF322254. Lane1-lane 6:Day 1-day 6 GADPH expression pattern. Samples were larvae at 1 dph (lane 1), 2 dph (lane 2), 3 dph (lane 3), 4 dph (lane 4), 5dph (lane 5) and 6 dph (lane 6). A 100 bp ladder (Bio-rad) was used as marker (lane M).

3.3.3 Cloning of a 318-bp fragment of seabass α-amylase cDNA

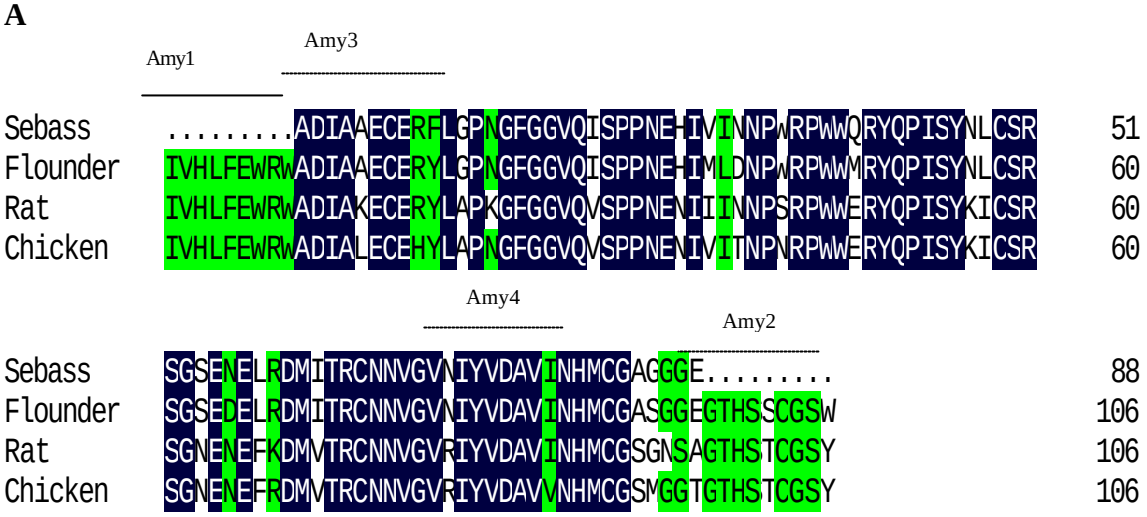
PCR amplification of cDNA prepared from 2-dph larvae using Amy1 and Amy2 primers resulted in a 318-bp product whose identity as α-amylase was confirmed by sequencing (GeneBank accession number AY007592). The amino acid sequence of the cloned product shows high sequence identity with pancreatic amylases and pancreatic amylase precursors, followed by the salivary amylases, of other species. The deduced amino acid sequence of sea bass α-amylase shows 86% identity to the α-amylase of *Pleuronectes americanus*, 76% to *Rattus norvegicus*, and 75% to *Gallus gallus* sequence (Fig. 3.3A). By agarose gel electrophoresis for the RT-PCR reaction, a single amylase-specific product is generated using primers Amy3 and Amy4 (Fig. 3.3B).

3.3.4 Quantification of mRNA using Real Time PCR

3.3.4.1 Real time RT-PCR

For specific and efficient amplification using LightCycler instrument, it is essential to optimize the $MgCl_2$ and primer concentration (Fig 3.4). Under the optimized primers condition in this study, the optimal concentrations for $MgCl_2$ were 6 mM (pink curve), or 7 mM (green curve), as they shared similar amplification pattern. From melting curve analysis, the "melting peak" of these two concentrations was also similar. The left-hand curve was the control reaction without amylase template, therefore only primer dimers formed and melted at a lower temperature. When there was amylase template in the reaction under 5, 6 and 7 mM $MgCl_2$, only specific PCR product was amplified, and no primer dimers were detected on left-hand side (Fig. 3.4B).

A



B

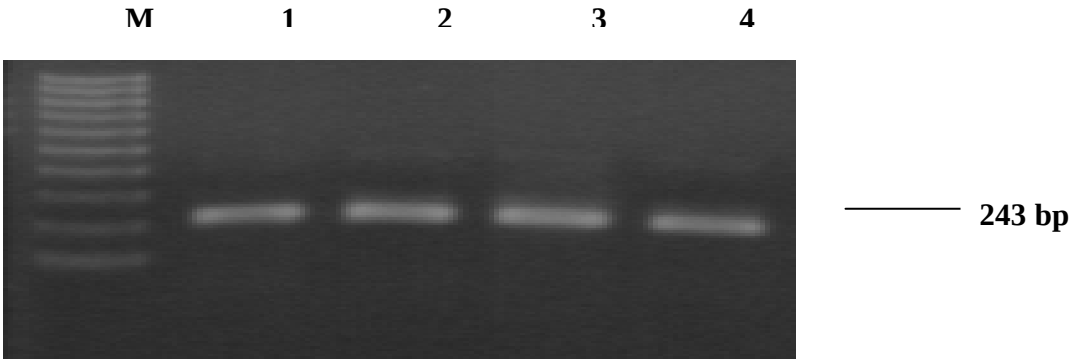
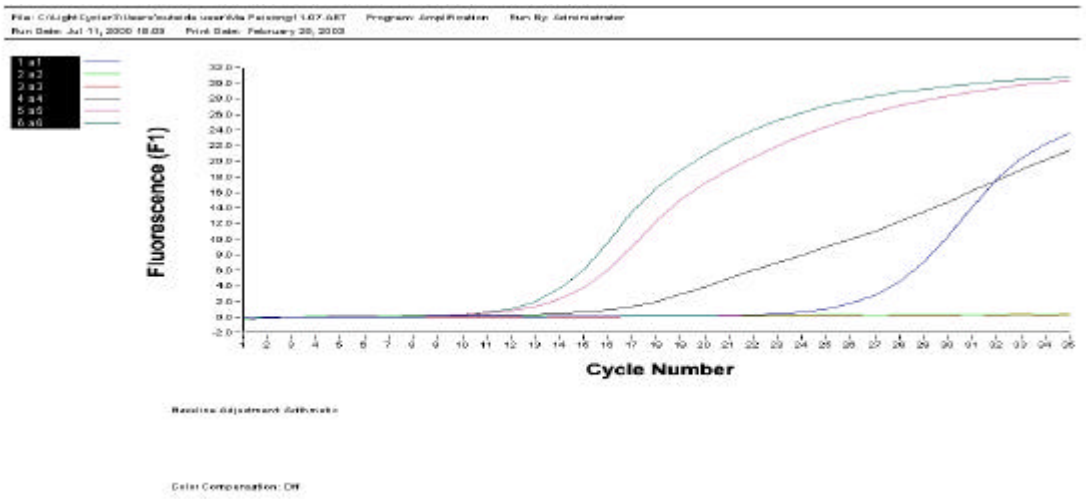


Fig. 3. 3 (A) Alignment of amino acid sequences of amylase from seabass, winter flounder, rat and chicken. The positions of primers Amy1, Amy2, Amy3 and Amy4 used for PCR are indicated by arrows. Residues conserved in 75% of the sequences are shaded. (B). Agarose gel electrophoresis of RT-PCR product of amylase in seabass larvae. Samples were larvae at 3 dph (lane 1), 4 dph (lane 2), 5 dph (lane 3) and 6 dph (lane 4). Amplification products (243 bp) from reactions using primers Amy 3 and Amy 4 were resolved on 1% agarose gel using a 100 bp ladder (Bio-rad) as marker (lane M).

A



B

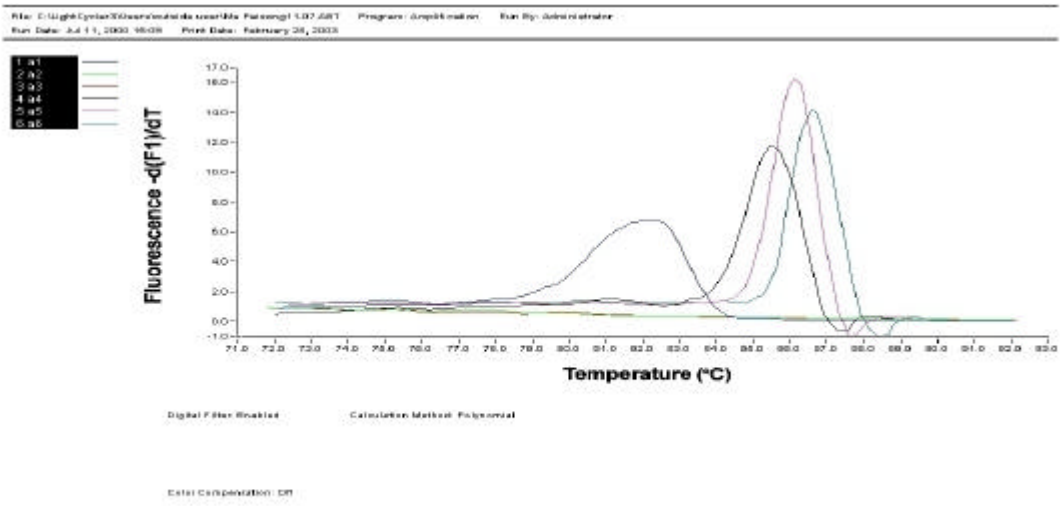


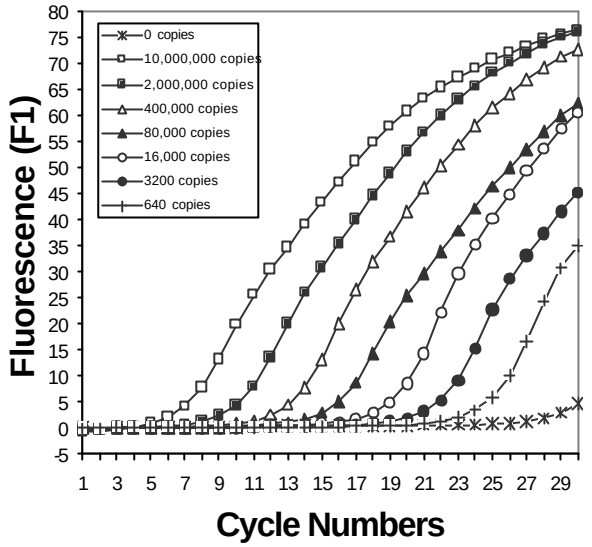
Fig. 3.4 Titration of $MgCl_2$ concentrations using LightCycler instrument. The concentrations of $MgCl_2$ vary from 3-7 mM. A): Amplification curves. B) The melting curve analysis. Left-hand side curve amplified without amylase template and the right-hand side group of three curves were amplified with 5, 6 and 7 mM $MgCl_2$.

3.3.4.2 Quantification

Using a series of diluted plasmid DNA as templates, a standard curve for α-amylase was obtained using Lightcycler real-time PCR (Fig 3.5). Since the crossing point decreased linearly with increasing amount of plasmid DNA copy numbers (Fig. 3.5 A) and since all our tested samples were located within this linear amplification range, the copy numbers of α-amylase transcripts in our test samples could be determined by using the standard curve (Fig. 3.5 B).

The quantitative changes in mRNA level in developing sea bass larvae are shown in Figure 3.6. The mRNA could be detected as early as 1 dph. There was a significant decrease at 2 dph; this was followed by a marked increase at 4 dph and remained high until 6 dph. These levels decreased until 14 dph and remained at low levels thereafter. There was a correlation between amylase mRNA level and enzyme activity. However, there appeared to be a phase shift in the enzyme activity. This was particularly clear in the first 4 days after hatching. Thus, the mRNA level was high at 1 dph, low at 2 dph, and high at 3 dph; while the enzyme activity was undetectable at 1 dph, high at 2 dph, low at 3 dph, and high at 4 dph. This suggests a delay in the translation of amylase mRNA. There was sharp decrease in mRNA level after 12 dph; however the protein level decreased more gradually, further indicating a delay in the translation of the amylase mRNAs. Since we have not studied the actual time course, we cannot be certain that the delay is 1 day as indicated here.

A



B

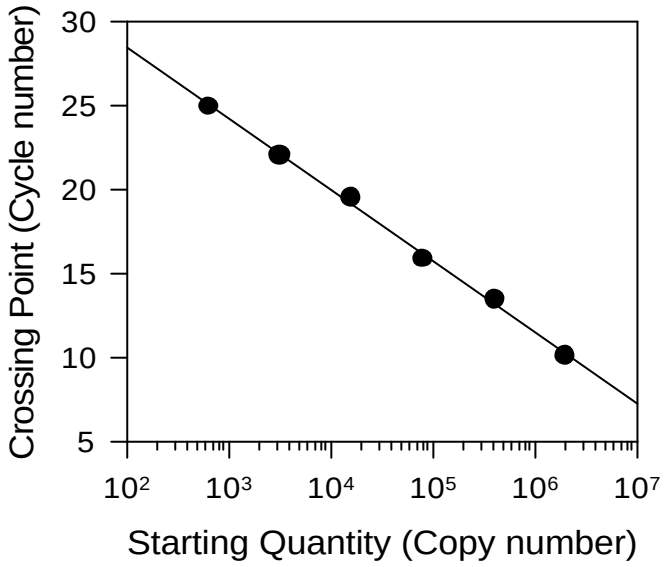


Fig. 3.5. Real time PCR of standard curves using a cloned plasmid DNA as template. (A), Amplification from zero to 10 million copies of plasmid DNA. (B) Calibration curves obtained by correlating crossing point and plasmid copy number from amplification A.

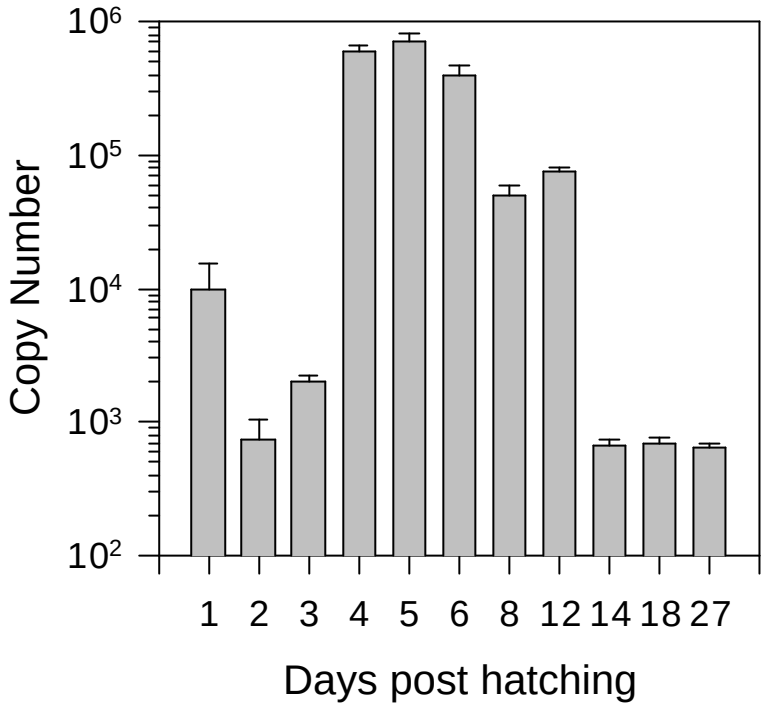


Fig 3.6. Real time PCR analysis of α-amylase mRNA expression during seabass larval development. Data were analyzed based on the crossing point of each sample and converted into the copy numbers according to the standard curve obtained in Fig. 3.5B.

3.3.5 5'- and 3'- rapid amplification of cDNA ends of seabass α-amylase gene

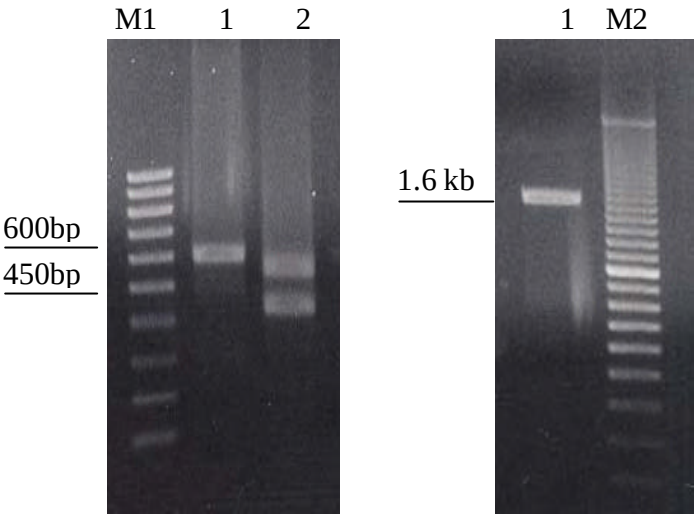


Fig. 3.7 5'- and 3'- rapid amplification of cDNA ends of seabass α-amylase gene. (A) 5' RACE. (B) 3' RACE. Lane 1, DNA 100 bp ladder; Lane 2, 5' RACE with primers GSP1 and UPM; Lane 3, nested PCR with primers GSP2 and NUP. (B) Lane 1, 3' RACE with primers GSP3 and UPM; Lane 2, DNA 1 kp ladder.

Based on a partial seabass cDNA sequence of α-amylase isolated by us (Genebank accession No: AY007592), 5' and 3' rapid amplification of cDNA ends (RACE) was performed in order to isolate seabass full length cDNA. A 450 bp 5'-RACE fragment was obtained, and 3'-RACE yielded a PCR fragment of 1,600 bp. After 5' RACE and 3' RACE products were characterized by sequencing, the full length of amylase cDNA sequence was generated using the Long Distance PCR (LD PCR). The LD PCR yielded a 1,620 bp cDNA sequence consisting of an open reading frame (ORF) of 511 amino acids including a putative 15 amino acids signal peptide (Fig. 3.9). The Genebank accession No of seabass amylase gene is AF416651. The 5' UTR region contains a length of 28 bp, while there is poly A tail downstream of the stop codon.

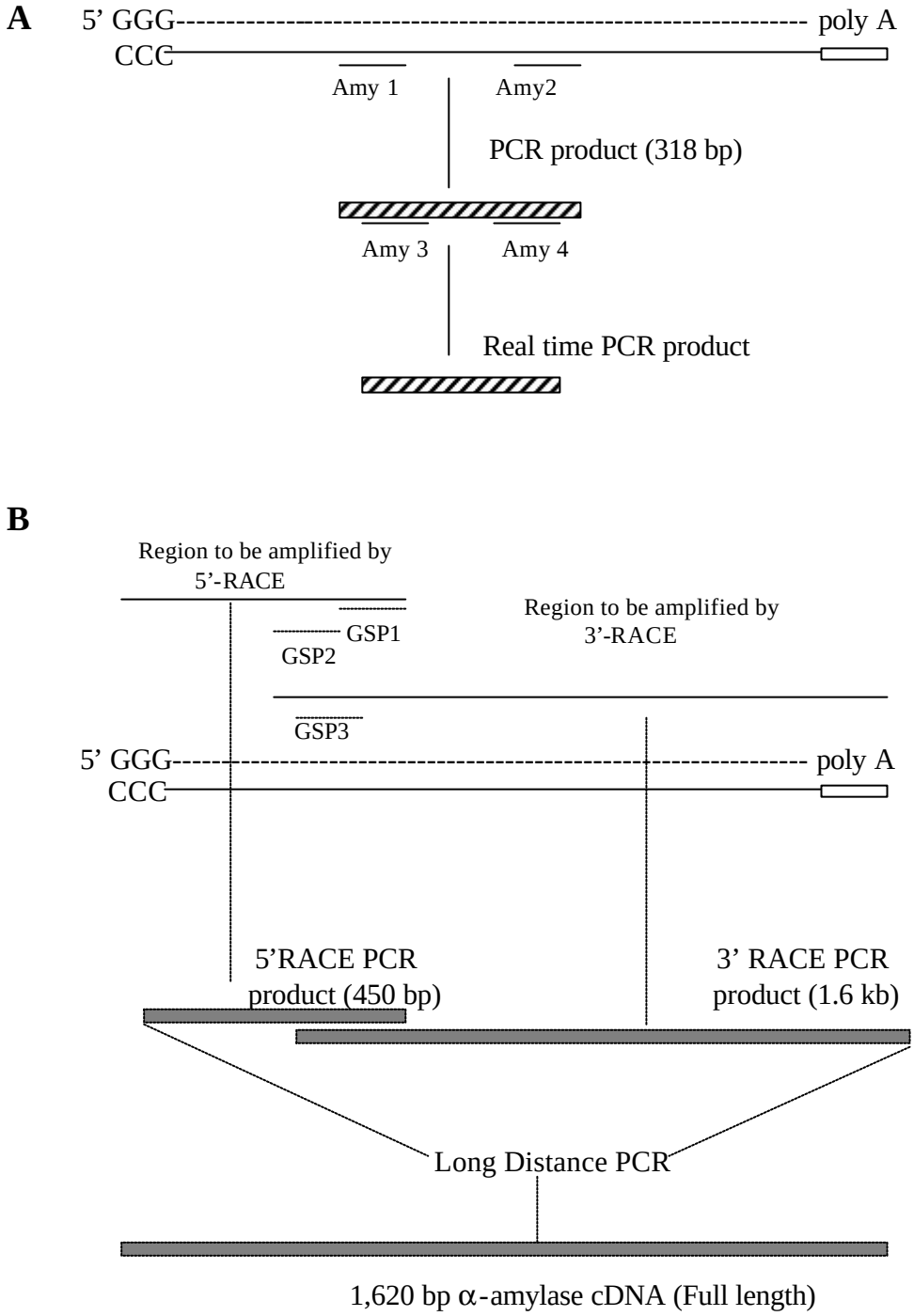


Fig. 3.8. Overview of RT-PCR amplification of seabass α-amylase 318 bp cDNA (A) and the SMART RACE procedure (B).

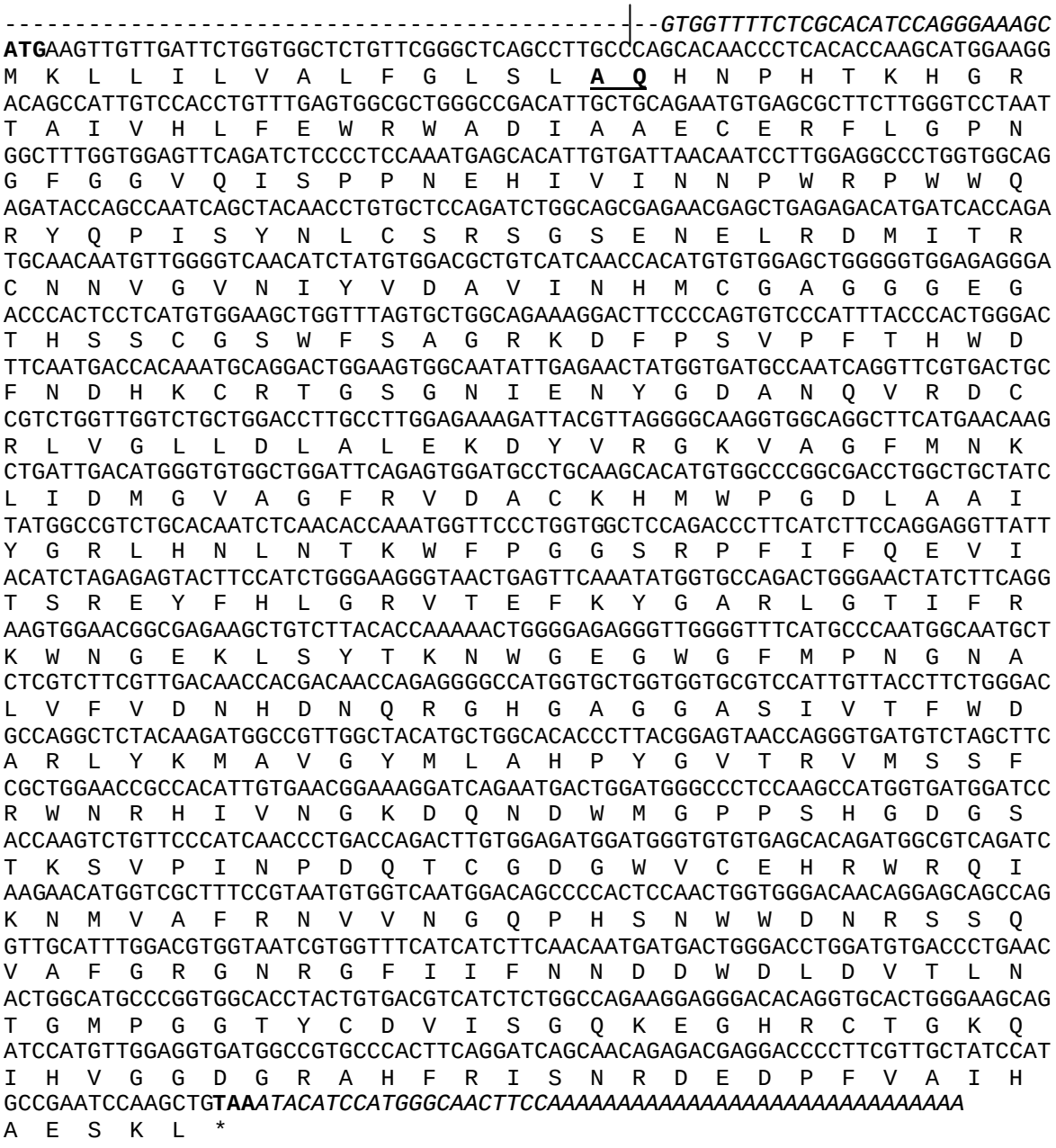


Fig. 3.9. The full length sequence of seabass α-amylase gene. The start and stop condons are in bold. The 5' UTR and 3' UTR are in italics. The signal peptide is indicated by arrow.

3.3.6. Seabass α-amylase protein

With the exception of the amino terminus, the rest of amino acid sequence of seabass amylase showed very high sequence identity to pancreatic amylase and pancreatic amylase precursor, followed by r salivary amylase, of other species. The predicted amylase polypeptide of seabass is identical in size to human and mouse amylase (Fig. 3.10). All ten cysteines involved in disulphide bond formation in mammalian amylase (Janacek, 1994) are well conserved in the seabass α-amylase. Amino acid residues involved in forming the calcium (Asn 115 and Arg 173) and chloride (Arg 210) binding sites, as well as active sites known to directly participate in the catalytic mechanism of α-amylase (Asp 182, Asp 212, Glu 248, Asp315) are well conserved in seabass α-amylase.

PCR of seabass amylase genomic DNA using the specific forward primer and the reverse primer based on extreme 5' and 3' end of cDNA gave a 4 kb product. Comparison of the cDNA and genomic sequences allowed the positions of 8 introns to be determined (Fig. 3.11), all of which possessed canonical GT/AG borders (Breathnach and Chambon, 1981). They vary in length from 95 bp to 826 bp. The transcribed region of amylase gene spans 4,090 bp, of which exons make up 1,620 bp and the intervening introns about 2,470 bp.

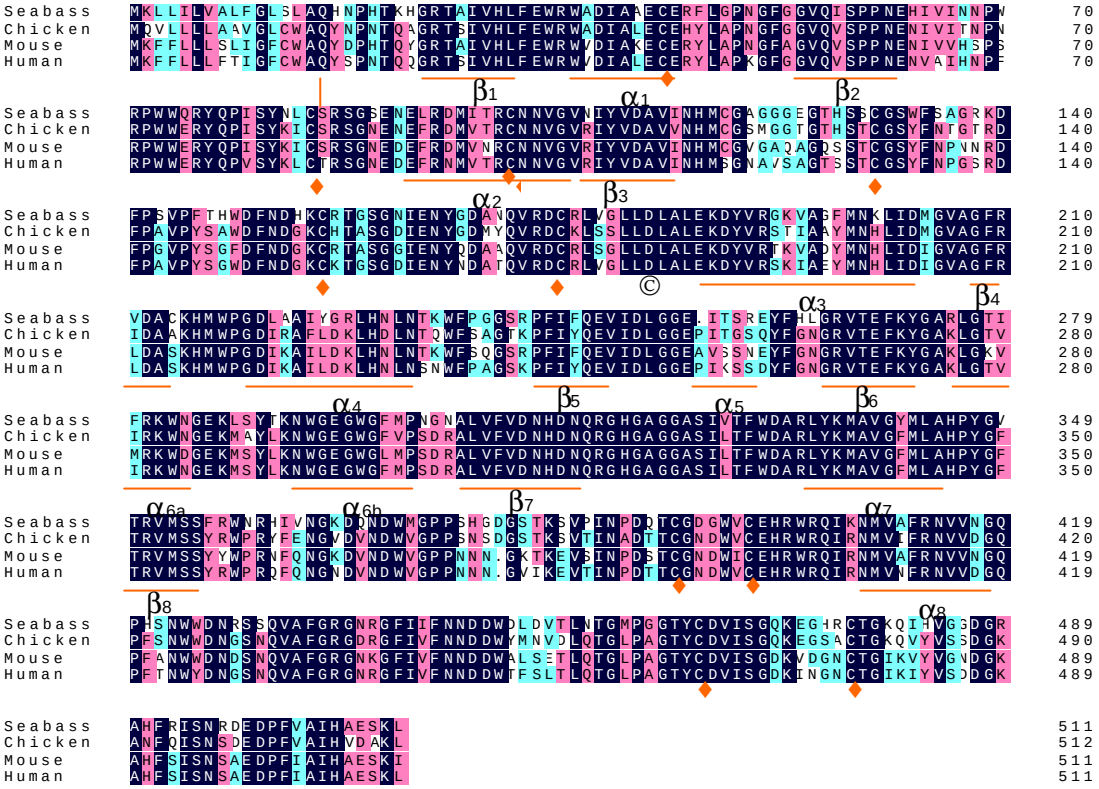


Fig. 3.10. Alignment of seabass α-amylase amino acid sequence with chicken, mouse and human pancreatic (Amy2A) amylases. The site of signal peptide cleavage is indicated by arrow. The eight α-helices and β-sheet are underlined. The 10 cysteines involved in disulphide bridges are indicated by ♦. The aspartic acid residue involved in calcium binding is indicated by ©.

```
ATG AAG TTG TTG ATT CTG GTG GCT CTG TTC GGG CTC AGC CTT GCC CAG CAC AAC
Met Lys Leu Leu Ile Leu Val Ala Leu Phe Gly Leu Ser Leu Ala Gln His Asn

CCT CAC ACC AAG CAT GGA AGG ACA GCC ATT GTC CAC CTG TTT GAG TGG CGC TGG
Pro His Thr Lys His Gly Arg Thr Ala Ile Val His Leu Phe Glu Trp Arg Trp

GCC GAC ATT GCT GCA GAA TGT GAG CGC TTC TTG GGT CCT AAT GGC TTT GGT GGA
Ala Asp Ile Ala Ala Glu Cys Glu Arg Phe Leu Gly Pro Asn Gly Phe Gly Gly

GTT CAG  GTACGTATGGCTGAACAC  intron A 175 bp        TTCCTCTGTGTTGCTCAG
Val Gln

ATC TCC CCT CCA AAT GAG CAC ATT GTG ATT AAC AAT CCT TGG AGG CCC TGG TGG
Ile Ser Pro Pro Asn Glu His Ile Val Ile Asn Asn Pro Trp Arg Pro Trp Trp

CAG AGA TAC CAG CCA ATC AGC TAC AAC CTG TGC TCC AGA TCT GGC AGC GAG AAC
Gln Arg Tyr Gln Pro Ile Ser Tyr Asn Leu Cys Ser Arg Ser Gly Ser Glu Asn

GAG CTG AGA GAC ATG ATC ACC AGA TGC AAC AAT GTT GGG GTAAACTACATTACAACGC
Glu Leu Arg Asp MET Ile Thr Arg Cys Asn Asn Val Gly

IntronB 267bp CAATCCTGTATCTATTCACAG GTC AAC ATC TAT GTG GAC GCT GTC ATC
                                    Val Asn Ile Tyr Val Asp Ala Val Ile

AAC CAC ATG TGT GGA GCT GGG GGT GGA GAG GGA ACC CAC TCC TCA TGT GGA AGC
Asn His MET Cys Gly Ala Gly Gly Gly Glu Gly Thr His Ser Ser Cys Gly Ser

TGG TTT AGT GCT GGC AGA AAG GAC TTC CCC AGT GTC CCA TTT ACC CAC TGG GAC
Trp Phe Ser Ala Gly Arg Lys Asp Phe Pro Ser Val Pro Phe Thr His Trp Asp
                                        481
TTC AAT GAC CAC AAA TGC AGG ACT GGA AGT GGC AAT ATT GAG AAC TAT GGT GAT
Phe Asn Asp His Lys Cys Arg Thr Gly Ser Gly Asn Ile Glu Asn Tyr Gly Asp

GCC AAT CAG GTAATGAACAGCTAAT intronC 826bp TAATTTACTGCCCCAG GTT CGT GAC
Ala Asn Gln                                                 Val Arg Asp

TGC CGT CTG GTT GGT CTG CTG GAC CTT GCC TTG GAG AAA GAT TAC GTT AGG GGC
Cys Arg Leu Val Gly Leu Leu Asp Leu Ala Leu Glu Lys Asp Tyr Val Arg Gly

AAG GTG GCA GGC TTC ATG AAC AAG CTG ATT GAC ATG GGT GTG GCT GGA TTC AGA
Lys Val Ala Gly Phe MET Asn Lys Leu Ile Asp MET Gly Val Ala Gly Phe Arg

GTG GAT GCC TGC AAG CAC ATG TGG CCC GGC GAC CTG GCT GCT ATC TAT GGC CGT
Val Asp Ala Cys Lys His MET Trp Pro Gly Asp Leu Ala Ala Ile Tyr Gly Arg

CTG CAC AAT CTC AAC ACC AAA TGG TTC CCT GGT GGC TCC AGA CCC TTC ATC TTC
Leu His Asn Leu Asn Thr Lys Trp Phe Pro Gly Gly Ser Arg Pro Phe Ile Phe

CAG GAG GTACTCCGCACACAGAA intron D 413 bp GCTGTTTTCCCTCTAG  GTT ATT GAT
Gln Glu                                                     Val Ile Asp

CTG GGA GGT GAG TCC ATT ACA TCT AGA GAG TAC TTC CAT CTG GGA AGG GTA ACT
Leu Gly Gly Glu Ser Ile Thr Ser Arg Glu Tyr Phe His Leu Gly Arg Val Thr
                                        840
GAG TTC AAA TAT GGT GCC AGA CTG GGA ACT ATC TTC AGG AAG TGG AAC GGC GAG
Glu Phe Lys Tyr Gly Ala Arg Leu Gly Thr Ile Phe Arg Lys Trp Asn Gly Glu
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AAG CTG TCT TAC ACC AA GTATGAGTGTTTCTGC intron E 196 bp TATTCTTGTCTCCAG
Lys Leu Ser Tyr Thr Ly

A AAC TGG GGA GAG GGT TGG GGT TTC ATG CCC AAT GGC AAT GCT CTC GTC TTC
S Asn Trp Gly Glu Gly Trp Gly Phe MET Pro Asn Gly Asn Ala Leu Val Phe

GTT GAC AAC CAC GAC AAC CAG AGG GGC CAT GGT GCT GGT GGT GCG TCC ATT GTT
Val Asp Asn His Asp Asn Gln Arg Gly His Gly Ala Gly Gly Ala Ser Ile Val

ACC TTC TGG GAC GCC AGG CTC TAC AAG ATG GCC GTT GGC TAC ATG CTG GCA CAC
Thr Phe Trp Asp Ala Arg Leu Tyr Lys MET Ala Val Gly Tyr MET Leu Ala His
                                                      1080
CCT TAC GGA GTA ACC AGG GTG ATG TCT AGC TTC CGC TGG AAC CGC CAC ATT GTG
Pro Tyr Gly Val Thr Arg Val MET Ser Ser Phe Arg Trp Asn Arg His Ile Val

AAC GGA AAG GTACATACTTTTCT intron F 95 bp ACCATCATTGTAG GAT CAG AAT GAC
Asn Gly Lys                                          Asp Gln Asn Asp
                              1140
TGG ATG GGC CCT CCA AGC CAT GGT GAT GGA TCC ACC AAG TCT GTT CCC ATC AAC
Trp MET Gly Pro Pro Ser His Gly Asp Gly Ser Thr Lys Ser Val Pro Ile Asn
                                                      1211
CCT GAC CAG ACT TGT GGA GAT GGA TGG GTG TGT GAG CAC AGA TGG CGT CAG ATC
Pro Asp Gln Thr Cys Gly Asp Gly Trp Val Cys Glu His Arg Trp Arg Gln Ile

AA GTCGATCAAGTGAGTTC intron G 120bp   CTATCTCCCCTTTAG G AAC ATG GTC GCT
Ly                                                  s Asn MET Val Ala

TTC CGT AAT GTG GTC AAT GGA CAG CCC CAC TCC AAC TGG TGG GAC AAC AGG AGC
Phe Arg Asn Val Val Asn Gly Gln Pro His Ser Asn Trp Trp Asp Asn Arg Ser
                                                            1344
AGC CAG GTT GCA TTT GGA CGT GGT AAT CGT GGT TTC ATC ATC TTC AAC AAT GAT
Ser Gln Val Ala Phe Gly Arg Gly Asn Arg Gly Phe Ile Ile Phe Asn Asn Asp

GAC TG GTAAGATCTGAGTA intron H 378 bp TCTTCCCCCTCAG G   GAC CTG GAT GTG
Asp Tr                                              p   Asp Leu Asp Val
               1380
CTG AAC ACT GGC ATG CCC GGT GGC ACC TAC TGT GAC GTC ATC TCT GGC CAG AAG
Leu Asn Thr Gly MET Pro Gly Gly Thr Tyr Cys Asp Val Ile Ser Gly Gln Lys

GAG GGA CAC AGG TGC ACT GGG AAG CAG ATC CAT GTT GGA GGT GAT GGC CGT GCC
Glu Gly His Arg Cys Thr Gly Lys Gln Ile His Val Gly Gly Asp Gly Arg Ala

CAC TTC AGG ATC AGC AAC AGA GAC GAG GAC CCC TTC GTT GCT ATC CAT GCC GAA
His Phe Arg Ile Ser Asn Arg Asp Glu Asp Pro Phe Val Ala Ile His Ala Glu

TCC AAG CTG TAA ATACATCCATGGGCAACTTCCAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
Ser Lys Leu ***
```

IntronA

GTACGTATGGCTGAACACAGGTGGATAGCTTCTAAGTCAGATGGACTTAGAAGAAAGAGGATA
TTTCCCCTTTAATAATTCTTAGACTCACTCTGACACTATGATCTTCTGTATACTGACAGCTCCTG
CTGTGTAAGTGGTAATTATTTTAATATGTTTCCTCTGTGTTGCTCAG

Intron B

GTAAACTACATTACAACGCTGATATAAATGAATGCCTCTGTCATGAGGAACCATCATTCACAAT
TTTATTAGAGGCATTTCCTGTAGCTGTTGCAGCTACATTTGAGTTTCTGGACCACCAACATTGCA
GCAAACCAACAGTGATCAGTGCTGCTGACATATTCCTCTGACCTGAGAATGCACTTAATGTGTG
AAGAAAAGGAGTGAAAATGACAAGGGAGGTGTTGATTAGTTGTGTTGCACTTACAATCCTGTA
TCTATTCACAG

Intron C

GTAATGAACAGCTAATATTATCTGTTCTAACCGGCTGTTTTGATAAATGTGTATAAGACATTTG
CTACGGTGGCTGGCACTAGTTCTGAAAAGACTGAGTATATCTGCATTAATGGCTCATTAGCTGT
CTTCCACAGATGAGCCATGAAATTAGCTAGCTTTGTAAAAGTGAAAAAATTGGAAAGATAGAT
AAACTGAAAATGATAGCGCTGTTGTTCATTTACTTGTGAGAACTGCCACACTACCTATTCCAAG
AATACACATCAATACAAAATCAAGTTCTTCAACAGGCTGTAGCCTACATTCCTCAATTACTACA
GCTTTCAGCAACAAAACCAGGCATGGCTGACATGACTACTACCAGGCTTAATTCTCTATAGGTT
ATACAAAAGGAGAAATGATGGTTTATTGTCATCCAAGACATGTATTACATATGAAAATAAGCA
CAAACGTAGACTAAATTCTGCTCTGCAGTGGTCAATTGAGCTGTGATGCGTTTGGTGACCTATT
ATATTGCCAGCAGTGAAGTCACAGCGCTCTCTGGTGGCCAGACTACACAATCACGACACTGGT
ATAAAAATAACTTACGCACCTATCTTTTACCAATTTATCTGCAAGTAGCTTATATCGATAAAAC
CAGTCAGCGGATCTATTGGTCAGGCTGTAGTCTGTTCATTTAAATGGTATGGCCAGTTAGCATT
ACACAAATTATGTTATGAAAAGCCTACACACAAATATGCGCATTGAAATAGATCATTATTAGT
GTGAAGTGTTATAAATCATGAATGCTATGAACTGTGATGTAAATGGTAATTTACTGCCCCAG

Intron D

GTACTCCGCACACAGAAGTACCAGTTCTTTTGTCTGTTCATATTTGGCCAACACTAGATGACAA
ACACGTGCAGATTGGGAAACGACTCCTAATATCTAAAATATGACTATTAGTTTACAACCGCCTG
TATATACAGGCAATACTGATGCTATAAGAGGTTGAAGTCAAAGGTGCTCAACTCAAAAATCAA
GTAAGCAACCTACAAGAGCAGCCTGTGATCTAAAAAAATTAATTCGCTTTGTCTCCACTACAAA
CATGAATATATAACAACAGAAATATGGTTTCTGTTAGTGCTGGATCAAAATTTGTTGTATCATT
TTTTGTCTATTCACAAATCACTGATATGGTACAGTTAATATAAACCATTCCTACGTTACATAGAT
TTATTAATTATGTGCTGTTTTCCCTCTAG

Intron E

GTATGAGTGTTTCTGCCTAAATCATATTTCAAAACTTGATATATTAGTAGAGCTTATTTAAACAT
ATCATTGCTACAATCCCATTCGCTGCNTAAATTGCTGGATTGAACAAATTATTACTTGCAAAGG
TCTCTAGGGATCATTGACTTGAACTGGTTCCTCCTCCTCATTAGTGAAATTGTATTCTTGTCTCC
AG

Intron F

GTACATACTTTTCTTAATTAGGGAAAATGATCAAATACAACATATAGAGTGTATTTGGAACATT
TTTTGTTAAGCCCATTAAACCATCATTGTAG

Intron G

GTCGATCAAGTGAGTTCTCAGGAAGCAAGACAGCACATACCATCTGTTGACTTTACGCATGGA
ATATTATTAACTTCTACATACACAAGATGATTTTAATTTGTCCTATCTCCCCTTTAG

Intron H

GTAAGATCTGAGTACTCTAACTGGTACTGACAAAAACTAATCAATCAGTCTAATAATAACCAG
ATAAAACAAAATGTGTTGGACAAGATGCAGATGCATGTCAGCATCTAACAAATTGTTTGGGCT
TACTAAAATATAACTTACAAACAAACTAACTAGAGCAGTCAGCCATGAGTTACAACAGTGCAT
TGGAAGAATTTCTGTTATAAAACTGGTACTTGTACTTTTAATATTTCTTTTTTTTGGTTTCTCTAT
CCATAATCAACATCTTATTAGTTAGCACATTCATTTGTATTCTGTCTCAAATACAACAAAATTAT
AAAGCAAGATCCTTTTATTGGGTTTTAAAAACAACATGTACCTTGTCTTCCCCCTCAG

Fig. 3.11. Exon/Intron organization of the seabass amylase gene.

3.3.7. Southern blotanalysis

To determine the copy number of the amylase gene in seabass genome, three probes, the full length cDNA, a 1 kb fragment spanning over exon III and a 0.6 kb probe spanning over exon VIII and IX were used in Southern blot analysis. When the genomic DNA was digested with *BamH* I and *EcoR* I, there was one *BamH* I site on exon VII and no *EcoR* I sites in cDNA and genomic DNA. *BamH* I digestion generated four bands with 1.4 kb probe (probe I), but *EcoR* I digestion showed two bands. After *BamH* I digestion, genomic DNA hybridized with probe 2 and 3, and the 1 kb probe 2 effectively visualized 2 larger bands (around 6 kb); similarly probe 3 detected two smaller fragments (Fig. 12). From the pattern observed in the Southern blot, two amylase genes were indicated in seabass genome.

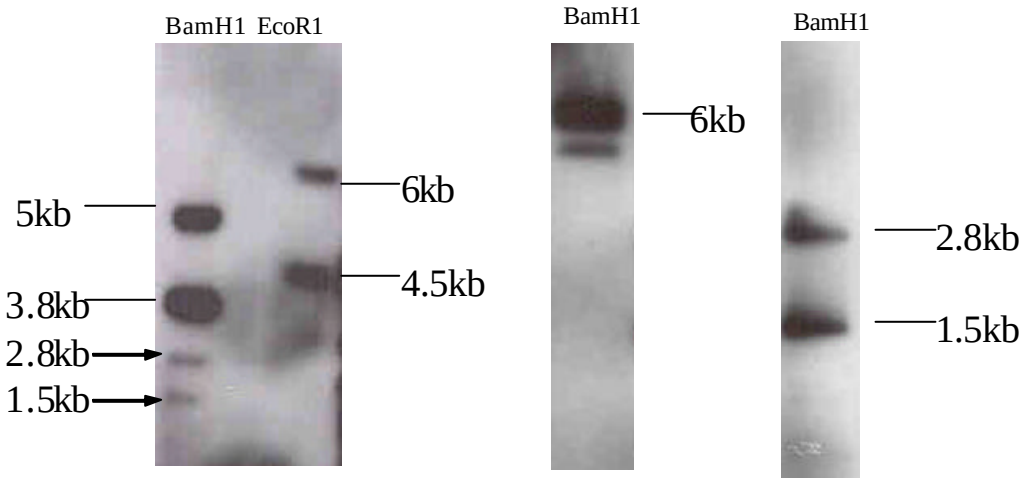

Fig. 3.12. Autoradiograms of genomic DNA hybridized with probe 1, 2 and 3 respectively. Three DNA fragments were used as probes for Southern hybridization. Probe 1 is full length cDNA, probe 2 is 1kb probe spanning over exon III, and probe 3 is 0.6 kb probe spanning over exon VIII and IX.

3.4 Discussion

3.4.1 Ontogeny of seabass α-amylase gene

There are two sources of enzymes for the midgut, the pancreas and the secretory cells in the gut wall with the pancreas perhaps secreting the greater variety and quantities of enzymes in fish. Carbohydrates have excited the most interest of all the enzymes, particularly since seasbass do not handle the large carbohydrate molecules very well (Walford and Lam, 1993), and many researchers have wanted to determine the reason. Further, because there are several carbohydrases, the possibility that different enzyme combinations might show adaptations to different diets also intrigued some investigators. Amylase is a widespread starch-digesting enzyme which occurs in saliva and pancreatic secretions into the small intestine, and the ability of animals to assimilate starch depends on their ability to elaborate amylase. Fish have been shown to secrete α-amylase, and in herbivorous fish its activity has been shown to be the greatest. Different authors have reported a close relation between herbivorous feeding habits and a high activity of amylase (Hidalgo *et al.*, 1999; Fernandez *et al.*, 2001). In carnivores such as the rainbow trout and sea perch, amylase is primarily of pancreatic origin whereas in herbivores, the enzyme is widespread throughout the entire digestive tract. In tilapia, the pancreas has been shown to be the site of greatest amylase activity followed by the intestine. Although the digestion of starch and dextrin by the carnivorous rainbow trout was shown to decrease progressively as levels of carbohydrates were increased beyond the 20 percent level, the fish could effectively utilize up to 60 percent glucose, sucrose or lactose in the diet (Chow and Halver, 1980). This demonstrates that, contrary to earlier belief,

carnivorous fish are capable of efficiently utilizing simple carbohydrates as a primary energy source.

Fish do not have a salivary gland. Most fish species do not possess a distinct pancreas but rather the pancreatic cells are diffused around the gut and /or in the liver (Yardley, 1988). α-amylase activity has been detected biochemically in the digestive tract of numerous marine fish. In the present study, the amylase activity peaked after the onset of feeding in larval fish. Similar findings have also been reported in other developing larvae of marine fish, such as Walleye pollock (*Theragra chalcogramma*) (Oozeki *et al.*, 1995), European seabass (*D. labrax*) (Peres *et al.*, 1998) and Senegal sole (*S. senegalensis*) (Martinez *et al.*, 1999). However, amylase expression has been reported to be higher during adult stage in fresh water mosquito fish while juveniles and embryonic stages had low levels (Yardely, 1988). This could be related to the omnivorous feeding habits of this species, since it is generally considered that α-amylase activity is higher in omnivorous fish than in carnivorous fish (Hidalgo *et al.*, 1999).

In all these studies including the present data for seabass, the α-amylase enzyme activity was measured in crude extracts using a biochemical method which is based on the ability of amylase to hydrolyze the corresponding substrate. However, these approaches might provide nonspecific results since the measured amylase activity may also reflect changes in other carbohydrate-metabolizing enzymes (e.g. maltase and glucoamylase), as well as amylase activity present in the ingested prey or in the gut flora (Oozeki, *et al.*, 1995). The calculated contribution of *Artemia* amylase activity was more than 50% of the total amylase activity measured in the Atlantic halibut larvae (Gawlicka *et al.*, 2000). In addition, the presence of other amylase isozymes could also cause the observed correlation

(Yardley, 1988). Therefore, it has been difficult to quantify the absolute amount of specific activity of amylase associated with larval gut.

In order to evaluate how well seabass larvae may be able to digest carbohydrate and to gain insight into mechanisms that govern tissue-specific gene expression, we have developed a specific real-time PCR assay for the measurement of mRNA levels of amylase. As it is a PCR-based technique, it is more sensitive than Northern blotting and requires much less RNA templates (Medhurst et al., 2000). This method is also more specific than an enzymatic assay, since primers specific only to seabass larvae α-amylase were used. The primers Amy1 and Amy2 do not amplify the α-amylase from ingested live food since RNA used for RT-PCR was obtained from 3 dph larvae which have not yet been fed with either rotifers or *Artemia* nauplii. This would mean that rotifer and *Artemia* RNA was not present to contaminate the seabass larvae RNA sample. Under the conditions used in this study, the primers Amy3 and Amy4 used in real time PCR were not able to bind to either the rotifer or *Artemia* first strand cDNA for amplification (data not shown). This also shows that the gene specific primers were able to amplify only seabass larvae α-amylase. In addition, comparisons between samples using Lightcycler were made during the exponential phase of PCR. Therefore, the real time RT-PCR assay developed in this study allowed a sensitive and accurate estimation of seabass α-amylase gene expression at early stages of development.

Douglas *et al.*, (2000) first isolated cDNAs for fish amylase gene and studied its expression during development in winter flounder. However, there are very few reports on the early developmental expression of amylase in fish. Our study is the first study to analyze the level of expression right from hatching and compare it with corresponding

amylase enzyme activity. The present study shows that the amylase enzyme and its mRNA levels are quite high around the time of the first feeding; the highest level was around 5 dph. Amylase enzyme activity could be detected even before the onset of feeding (2 dph) and this enzyme synthesis before the food signal can be explained by high levels of mRNAs before first feeding.

It is interesting to note that although amylase mRNA level was high on 1 dph, the enzyme level could hardly be detected in 1 dph larvae. There are two possibilities; (1) delayed translation of mRNA and/or (2) maternal origin of the mRNA in 1 dph larvae. The proteins necessary for early embryonic development in animals are translated from messenger ribonucleic acid (mRNA) molecules of maternal origin and the embryonic genome (Dworkin and Dworkin-Rastl, 1990); therefore it is possible that the mRNA in 1dph larvae was of the maternal origin. Nagler (2000) examined the importance of maternal mRNA during early embryogenesis in the rainbow trout (*Oncorhyncus*. *mykiss*), and the eggs were exposed to the translational inhibitor cycloheximide (CHX) and transcriptional inhibitor actinomycin D (AMD) to address the significance of maternal mRNA. Whatever the origin of the mRNA, it is clear that there was transcriptional control of amylase enzyme activities during early development of seabass larvae.

Although lipid and free amino acids have generally been considered to be the major substrates for energy metabolism in marine eggs and the early stages of larval development (Ostrowski and Divakaran 1991; Rønnestad and Fyhn 1993; Sivaloganathan *et al.*, 1998), the high levels of α-amylase mRNA and enzyme around the time of first feeding suggest a natural predisposition of seabass larvae to use carbohydrates as a nutritional substrate during this period. This indicates that the inclusion of a source of

carbohydrate in the formulated diets may be beneficial for the larvae during the early development period.

3.4.2 Seabass α-amylase gene

Among animals, cDNAs for amylase have been isolated from rat (Rutter, 1980), pig (Genebank accession No AF064742), mouse (Hagenbuchle *et al.*, 1980), human (Nakamura *et al.*, 1984), insects (Boer and Hickey, 1986; Grossman and James, 1993), shrimp (van Wormhoudt and Sellos, 1996), scallop (Le Moine *et. al.*, 1997), and, recently, several fishes (Douglas *et al.*, 2000; Bouneau *et al.*, 2003). Seabass α-amylase cDNA is 1620 bp in length and the deduced polypeptide has 522 amino acids. The seabass amylase sequence shows the highest similarity to that of puffer fish (*Tetraodon nigroviridis*) amylase protein-3 (90%) followed by those of winter flounder (*Pseudopleuronectes americanus*) (87%), *Tetraodon nigroviridis* amylase protein- 2 and 1(86%), mouse (74%) and human (71%). A phylogenetic tree constructed from the aligned amino acids sequences of the diverse animals species using phylogenic analysis using parsimony (PAUP) revealed that the amylases were divided into two major groups (Fig. 3.13). One group was vertebrates except *drosophila*, while the other group consisted of invertebrates: shrimp and oyster. All the fish species studied form one sub-group, whereas the higher vertebrates form another sub-group. Seabass amylase was clearly clustered with the other fish amylases.

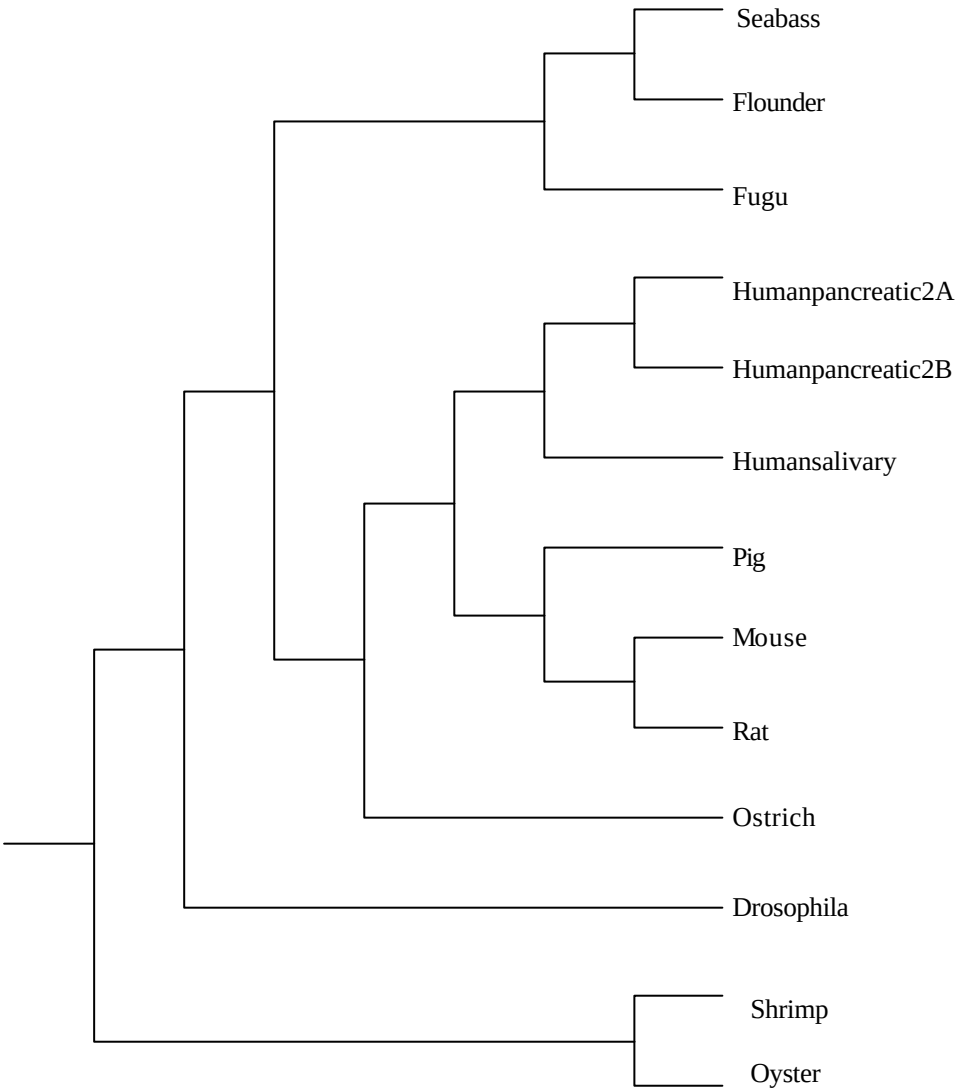

Fig. 3.13 Phylogenetic tree of selected α-amylases using PAUP (phylogenic analysis using parsimony). The following species α-amylases were used: seabass (*Lates calcarifer*) (AAL84163), flounder (*Pseudopleuronectes americanus*) (AAF65827), Fugu (*Tetraodon nigroviridis*) (CAC87127), human pancreatic amylase 2A (*Homo sapiens*) (NP_000690), human pancreatic amylase (NP_066188), human salivary (P04745), pig (*Sus scrofa*) (P00690), mouse (*Mus musculus*) (NP_031472), rat (*Rattus norvegicus*) (BAB39466), ostrich (*Struthio camelus*) (P83053), drosophila (*pseudoobscura*) (CAA53820), shrimp (*Litopenaeus vannamei*) (CAB65553), and oyster (*Crassostrea gigas*) (AAL37183).

Human Amy2, Amy1 and chicken amylase genes contain 10 exons, and human Amy1 from salivary gland contains a nontralated exon S near the 5' end of the gene (Horri *et al.,* 1987). The size of transcripts from these amylase genes is around 1.6 kb; however, there are only nine exons in Fugu and seabass amylases (Fig. 3.14). The positions of the introns within the coding regions of the chicken, fugu and seabass are identical to human amylases genes, except chicken, fugu and seabass contains shorter introns, resulting in shorter locus that spans roughly 4 kb; whereas the human counterparts measure about 10 kb. Comparing seabass with mammalian amylase protein sequences in the GeneBank gives a high percentage of conservation (more than 70 %), and it is not possible to identify genes homologous to pancreatic and salivary amylases by comparison with human sequences.

The positions of the introns within the coding regions of seabass amylase gene are almost identical to those found in mammals. In human and mouse, the pancreatic α-amylase genes contain 10 exons (Horri *et al.,* 1987), whereas in seabass, the loss of intron 6 resulted in only 9 exons (Fig. 3.15). Douglas *et al.,* (2000) isolated a partial portion of winter flounder amylase gene which also showed the loss of intron 6. Analysis of genomic DNA of a puffer fish species, *Tetraodon nigroviridis,* revealed that the amylase gene family (Amylase-1, -2 and -3) which is clustered on a 148 kb fragment (Bouneau *et al.,* 2003) has all lost intron 6. Based on a large-scale study of intron positions in human, mouse and an out-group *Fugu,* Roy *et al.* (2003) proposed that introns are lost through gene conversion with intronless copies of the gene, and the mechanism of intron loss may favour short introns. In each case, the intron loss was exact, without change in the surrounding coding sequence, and involved introns that are extremely short. In ancestral fish species, intron losses are completely akin to the intron loss in mammals; the intron 6

in human is the shortest one (94bp). Our results also favor the model Roy *et al* (2003) have proposed, and one explanation of intron loss in lower vertebrates may be the short generation time.

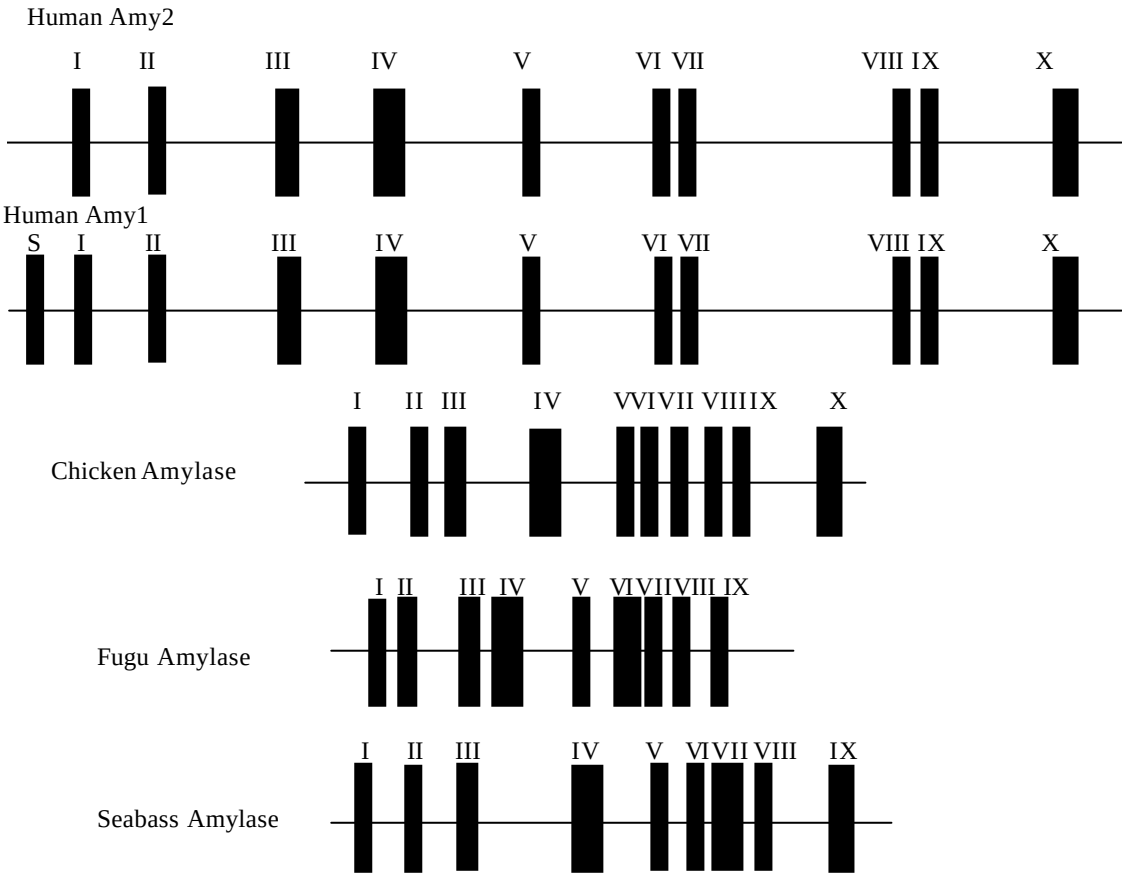

Fig. 3.14 Genomic organization of human Amy2, Amy1, chicken, Fugu and seabass amylase

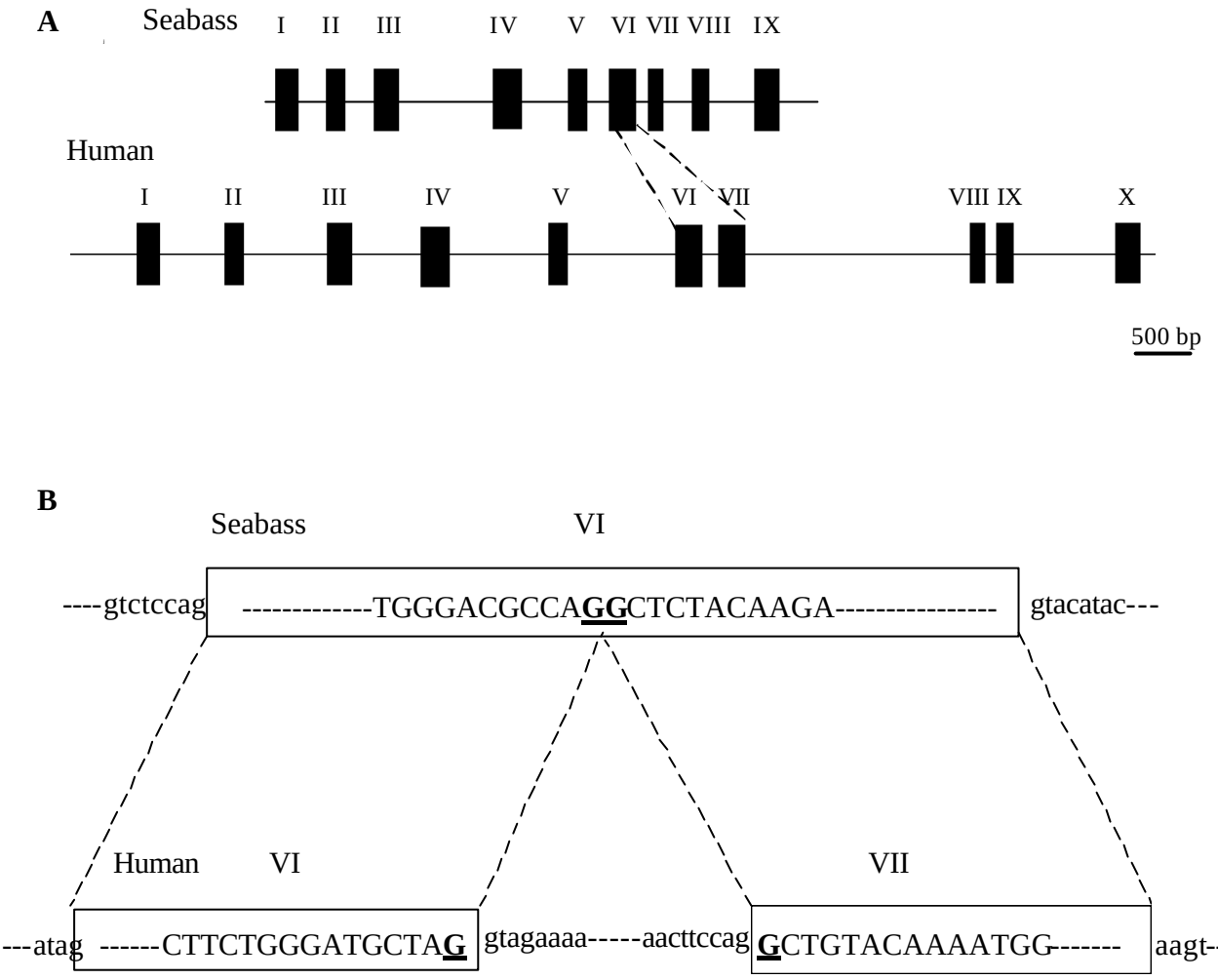

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Fig. 3.15. (**A)** Schematic diagram showing the relative position of exons and introns of human and seabass α-amylase genomic organizations. Roman numerals denote exon number. (**B**) Alignment with seabass exon VI and human α-amylase exon VI and VII to show the lost intron position in seabass

The seabass α-amylase shows more than 70% identity to mammalian amylase in terms of amino acids composition, and structure-based comparison indicates that seabass amylase and its mammalian counterpart are functio nally related. The molecular weight of the seabass amylase protein is 56,848 Da, similar to that of other animals. The predicted isoelectric point is 8.40, 1.5 unit higher than that of human, *Drosophila*, scallop and winter flounder.

Mammalian pancreatic amylases are known to fold into three domains, A, B and C (Rydberg *et al*., 1999). Domain A is an eight barrel (α/β) that binds an allosteric chloride ion. Domain B binds a calcium ion which is necessary to maintain the structural stability of an active site loop. Domain C is an eight stranded β-sheet domain at the C-terminal end of the amylase and is the putative starch binding domain (Fig. 3.16). The primary structure of seabass amylase amino acids showed features typical of the mammalian amylase family. Studies have shown that only four short segments of the polypeptide chain demonstrate reasonably good homology among animal and fungal α-amylases (Brayer *et al*, 1995). They include amino acid residues 111-116, 208-216, 248-251 and 309-316 (Fig. 3.9). The first of the four segments is involved in binding a calcium ion, while the rest contain each a putative active site residue.

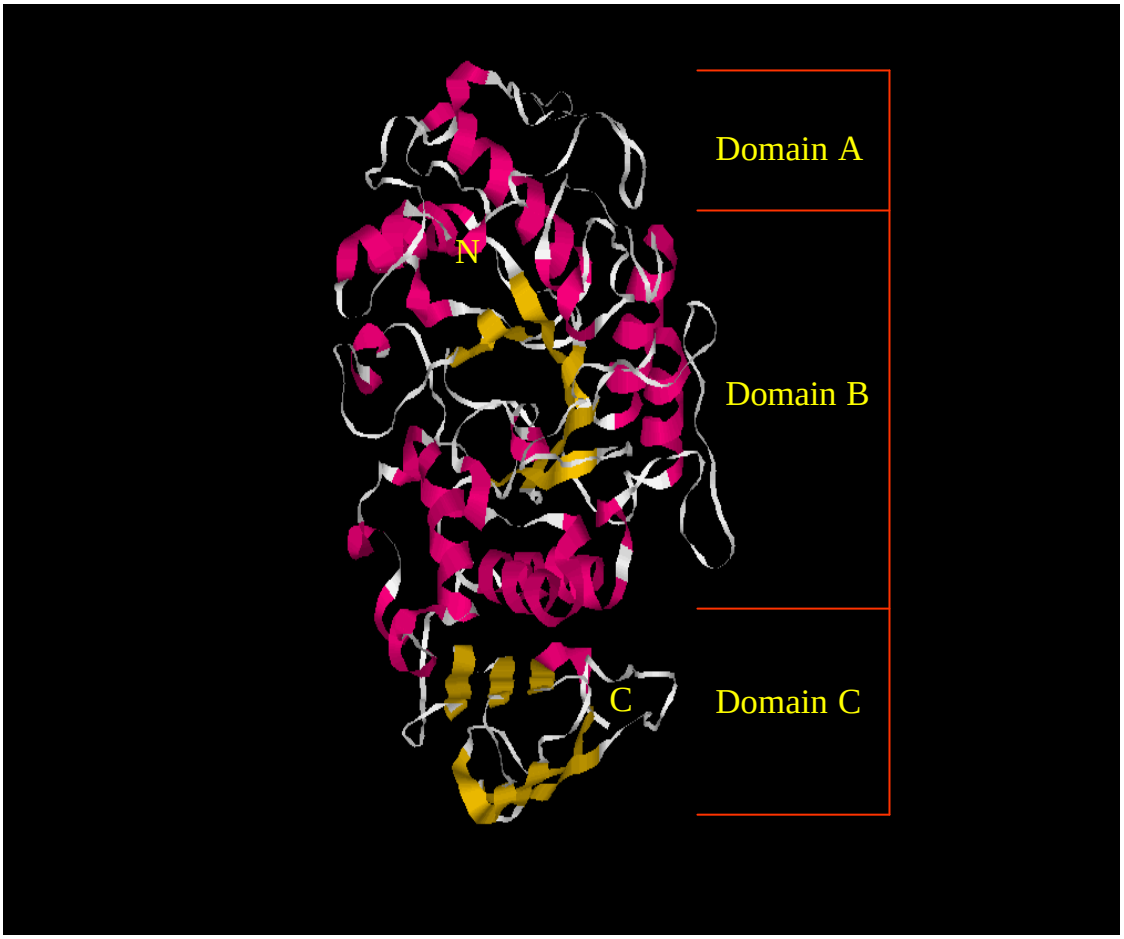

Fig 3.16 Human Pancreatic -Amylase from *Pichia pastoris*, Glycosylated Protein. N stands for N terminal; C stands of C terminal (From Rydberg *et al.*, 1999).

In human, amylase gene is encoded by a multigene family located on chromosome 1 (Gumucio *et al.*, 1988), and there are two types of genes which are expressed in a tissue-specific manner either in salivary gland (Amy-1) or in pancreas (Amy-2). The sequences of the pancreatic and salivary α-amylases share a 97% homology; only 10 amino acids

differ between the sequences of these two enzymes (Brayer *et al*., 1995). Five out of the ten amino acid differences occur in the segment 340-404 that link domain A and C (Fig. 3.17). It is interesting to note that these 5 amino acids are also different in the seabass amylase. They are His 347, Val 349, Lys 352, His 363, and Thr 367. Except for Thr 367 which is the same as salivary Thr 367, the other four residues are distinct in seabass amylase. Leu 196 is replaced by isoleucine in the middle of a highly conserved region where the direct left vicinity is Arg 195 and the right side is Asp 197. Arg 195 binds to chloride ion; Asp 197 binds to calcium ion. Changes occurring in such strategic region could have some effect on enzymatic function (Brayer *et al*, 1995). This may reflect the fact that fish do not have salivary glands, and most fish do not even have a distinct pancreas. Further study in this area may help us have a better understanding of amylase evolution.

Fig 3.17. Sequence alignment of human pancreatic amylase 2A, 2B and salivary amylase, the ten different amino acids between pancreatic and salivary amylase are indicated, their positions are labeled, and the corresponding amino acid in seabass amylase are indicated at the bottom.

In mammals, seven α-amylase genes are clustered in the human genome with two pancreatic amylase genes, three salivary amylase genes and two truncated pseudogenes (Gumucio *et al.,* 1988). In human, three tandem genes coding for amylase are present in 169 kb of chromosome 1p21.1 (Tricoli and shows, 1984). They are closely related in sequence, but present a distinct pattern of tissue-specific expression: the Amy2A and Amy2B are pancreatic, and the Amy1A is salivary. Analysis of the genomic DNA of *Tetraodon nigroviridis* revealed that an amylase gene family (Amylase -1, -2, -3) was clustered on a 148 kb fragment. Tetradontidae is a teleost group whose species have the smallest genome in vertebrates, eight times smaller than the human genome (Bouneau *et al,* 2003). Comparisons with the human protein sequences give a high percentage of conservation (between 68 and 71%), and it is not possible to identify genes homologous to pancreatic and salivary amylases by comparing T. *nigroviridis* amylases with human sequences. In winter flounder, two copies of amylase genes were also detected. Southern analysis of the seabass genome indicates that there are two α-amylase genes. In this study, the seabass genome was hybridized with three different probes. Probe I using the full length cDNA detected four fragments with *BamH* I digestion, and larger fragment appeared to be visualized at the same intensity. Similarly, *EcoR* I digestion also generated two bands. The pattern indicates that the two copies of amylase genes are homologous in the coding region, consistent with *T. nigroviridis* amylase gene 3 and 2 sharing more than 90% identity. Further sequencing of the proximal 3' end of the seabass amylase gene may permit us to trace the presence of the second copy of amylase sequence. Furthermore, it would be of interest to determine the tissue organization and whether these two copies of amylase genes carry salivary resource.

CHAPTER FOUR

CHARACTERIZATION OF THE SEABASS a-AMYLASE PROMOTER

4.1 Introduction

Transcriptional activation of eukaryotic genes throughout organogenesis is mediated by a regulated assembly of protein complexes at promoters. The activation of genes encoding tissue-specific proteins in terminally differentiated cells is the final result of this process. Therefore, the transcription factor complexes which activate the genes in terminally differentiated cells may be expected to contain components that are specific for a particular cell lineage.

In pancreas, several cis-acting elements have been shown to be involved in the control of amylase gene expression, such as pancreas transcription factor (PTF) and exocrine pancreas transcription factor (XPF) (Slater *et al*. 1993; Rose *et al*., 2001). In addition to the regulation by tissue-specific transcription factors, mammalian amylase gene expression is regulated at the transcriptional level by glucocorticoids (Logsdon *et al*., 1987; Swarovsky *et al.*, 1988). The glucocorticoid-response elements (GREs) are palindrome and bind glucocorticoid receptor as a homodimer (Logson *et al*. 1987, Johnson *et al*. 1993; Bamberger *et al*., 1996). These regulatory elements lie within 200 bp upstream from the cap site in the 5'-flanking region of the mammalian amylase promoter. The promoters of human and mouse pancreatic amylase gene are highly homologous between nucleotide -160 and the cap site and they are 65 % conserved in this region. However, similarity in the 5' flanking sequences of the salivary amylase gene is limited to several short sequence elements whose positions differ in the two species. The

glucocorticoid response element (GRE) and a general enhancer (PTF-1) are closely linked in this region (Gumucio *et al.* 1988, Slater *et al*. 1993).

Linking molecular mechanisms with physiological functions is helpful for understanding the exact nature of the hormonal control of amylase gene expression. The beneficial effects of cortisol on the mammalian gastrointestinal system are well documented; glucocorticoids exert multiple and phenotypic effects on the pancreas (Majumdar & Nielsen 1985). In the developing rat exocrine pancreas, exogenous glucocorticoids induce premature differentiation characterized by increased pancreatic weight, DNA, RNA and protein content, as well as increased amylase enzyme concentration and amylase gene expression (Kaiser *et al*. 1996).

Several recent studies have shown that marine fish larvae have a higher level of α-amylase mRNA levels around the time of first feeding (Douglas *et al.* 2000; Ma *et al*. 2001; Zambonino Infante & Cahu 2001; Chapter 3). We previously reported a strong correlation between enzymatic activity and mRNA level of α-amylase during the early development of seabass larvae (Ma *et al.* 2001; Chapter 3). These and the above mammalian studies suggest that the changes in α-amylase are controlled at the transcriptional level during early larval development of seabass, and cortisol may be an important regulator of its gene expression.

Fish do not have salivary glands, and most species do not have a distinct pancreas, but rather, the pancreatic cells are diffused around the gut and/or in the liver (Yardley 1988, Youson & Al-Mahrouki, 1999, Wallace & Pack 2003). It is conceivable that amylase gene regulation mechanisms have been retained during evolution. Thus it would

be of interest to study a fish amylase gene and its transcription regulatory mechanisms and compare them to those of higher vertebrates.

Nothing is known about the transcription factors that are involved in α-amylase gene expression in fish. Thus, a study of the seabass α-amylase promoter was undertaken.

4.2 Materials and Methods

4.2.1 Genomic DNA isolation

The genomic DNA was isolated using DNeasy™ Tissue kit (Qiagen). 25 mg seabass tissue was cut into small pieces and 180 µl of ATL buffer added. Proteinase K (20 µg) was added into the tube, and the mixture was incubated at 55 °C for 3 hours. Then 200 µl buffer AL was added, followed by incubation at 70 °C for 10 minutes. After addition of 200 µl ethanol, the precipitate was transferred into DNeasy spin column, and centrifuged for 1 minute. The DNeasy column was placed in a new 2 ml collection tube and 500 µl AW1 buffer added. After centrifugation, the DNeasy column was transferred to a new collection tube and 500 µl of AW2 buffer added. Finally, the genomic DNA was eluted with 200 µl buffer AE.

4.2.2 Promoter isolation

The upstream region of seabass amylase gene was isolated using a Universal Genome Walker Kit (Clontech). The principle of Genome Walker is shown in Figure 4.1. High molecular weight genomic DNA was isolated using DNeasy™ Tissue kit (Qiagen). The quality of genomic DNA was checked by DraI digestion, the smear band indicated

that DNA could be digested by restriction enzyme. The genomic DNA (2.5µg) was digested at 37°C overnight with one of four different restriction enzymes of *Dra*I, *Eco*RV, *Pvu*II or *Stu*I. The digested blunt end DNAs were purified by phenol extraction and ethanol precipitation. Each library construction was then ligated separately to the Genome Walker adaptor at 16°C overnight. The reactions was stopped by incubating each sample at 70°C for 5 min and a 1/10 volume of TE (pH 7.4) was added.

After the four libraries were constructed, two PCR (Primary and Secondary PCR) amplifications per library were conducted. The primary PCR was performed in PTC-100 thermal cycler (Peltier-effect; MJ Reseach) using the following two-step cycle program, which had been optimized with Advantage Genomic Polymerase Mix (Clontech). Each genomic library was amplified for seven cycles at 94°C for 2 seconds and 72°C for 3 minutes, and 32 cycles at 94°C for 2 seconds and 67°C for 3 minutes in the presence of an outer adaptor primer (AP1, 5'-GTAATACGACTCACTATAGGGC-3') and the outer, gene-specific primers (GSP1, 5'-CAGCGCCACTCAAACAGGTGGACAATGG-3'), followed by additional treatment at 67°C for 4 min. The primary PCR mixture was then diluted 50 times with deionized water and used as a template for a secondary or nested PCR with the nested adaptor primer (AP2, 5'-ACTATAGGGCACGCGTGGT-3') and the nested gene-specific primers (GSP2,5'-GCTGGGCAAGGCTGAGCCCGAACAGAG-3'). The secondary PCR was performed for five cycles at 94°C for 2 seconds and 72°C for 3 minutes, and for 20 cycles at 94°C for 2 seconds and 67°C for 3 minutes, followed by additional treatment at 67°C for 4 minutes. The final PCR products were analyzed on a 0.8 % agarose gel. The correct PCR fragments were cloned into a pGEM-T easy vector (Promega) and the insert was sequenced.

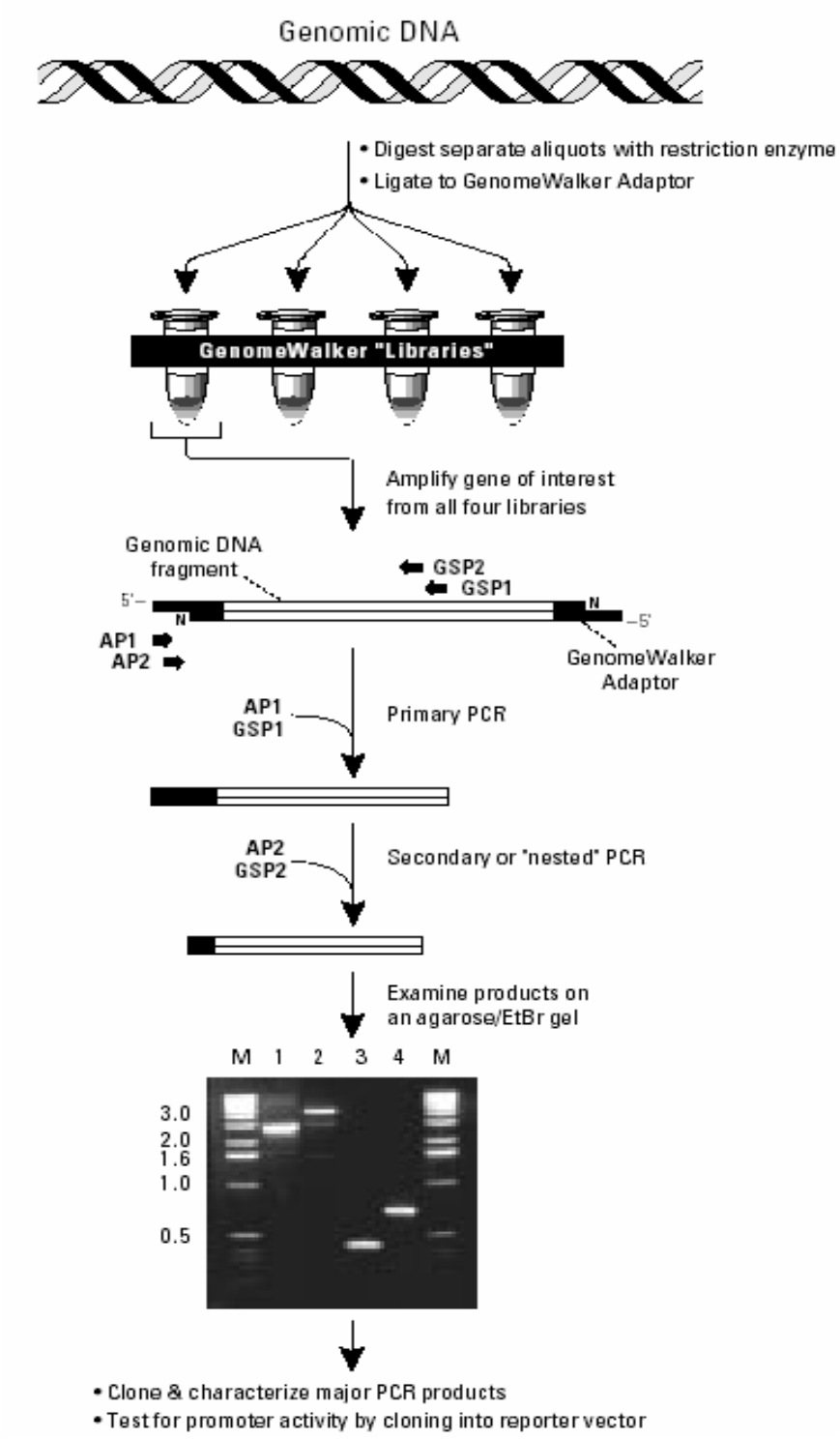

Fig.4.1 Flow chart of the GenomeWalker protocol (Clontech). N: Amine group that blocks extension of the 3' end of the adaptor-ligated genomic fragments. AP1: Adaptor primers. GSP: Gene-specific primers.

4.2.3 Construction of reporter plasmids

4.2.3.1 Vector preparation and ligation

To prevent self-ligation of the vector, Calf Intestinal Phosphatase (Promega) was used to dephosphorylate the vector 5' ends. Vector and insert DNA fragments were mixed in a ration of 1:3. Ligation reactions were usually carried out in a final volume of 10 ~20 µl with T_4 DNA ligase and the ligation buffer provided by the manufacturer (New England Biolabs).

4.2.3.2 Construction of pGL3-2291

PCR was used to amplify the DNA fragments for the construction of the plasmids. Mlu1 restriction enzyme sites were added to the 5' ends of both the forward and reverse primers (Forward primer: 5' GGGATTACTATAGGGTACGCGTGGTCGACG3'; reverse primer: 5' CGACGCGTGCTTTCCCTGGATGTGCGA 3'). The 2291 bp Mlu-*Mlu* PCR amplification product was digested by *Mlu* 1, and then cloned into the pGL3-Basic vector (Promega), which was pre-digested with *Mlu* 1. Cohesive end ligation was carried out at 16°C for 4-6 hours and chimeric construct (pGL3-2291) was obtained (Fig. 4.2).

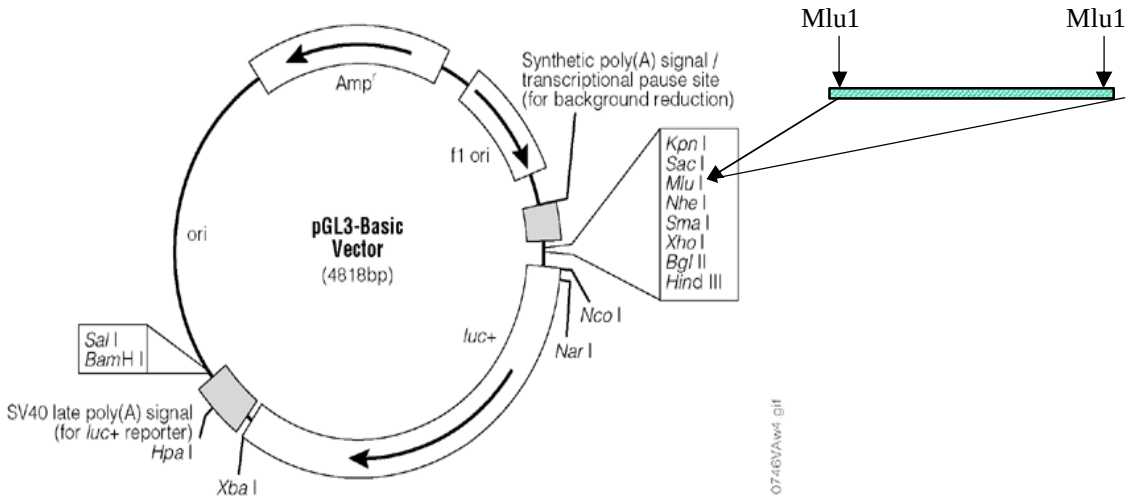

Fig.4.2. pGL3-Basic Vector circle map (Promega). Seabass amylase promoter was inserted *Mlu*1sites.

4.2.3.3. Construction of pEGFP-2291

Sac I and *BamH* 1restriction enzyme sites were added to the 5' ends of the forward and reverse primers respectively (Forward primer: 5' GCCGAGCTCCGCGATTTACTAGGGATTA3'; reverse primer: 5' GCGGATCCGCTTTCCCTGGATGTG3'). The *Sac* I/*BamH* 1 PCR amplification product was digested by *Sac* I and *BamH* 1 simultaneously, and then cloned into the pEGFP-1 vector (Clontech), which was pre-digested with *Sac* I and *BamH* 1. Ligation was carried out at 16℃ for 8 hours and the chimeric construct was obtained (Fig. 4.3).

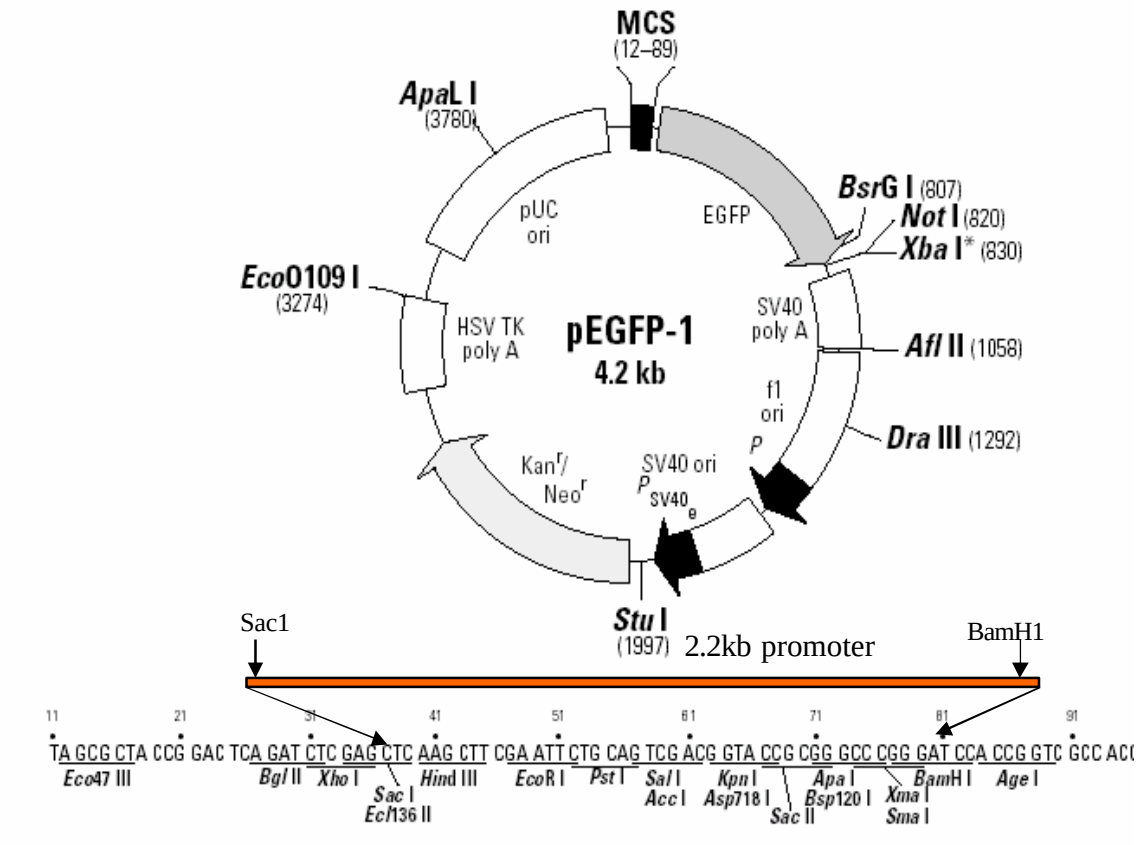

Fig.4.3. Diagram of chimeric construct pEGFP-2291. The 2,291 bp amylase promoter was inserted into pEGFP-1 vector (Clontech).

4.2.3.4 Generation amylase promoter deletion constructs

Nested deletion fragments of the α-amylase promoter were generated by PCR using pGL3-2291 as the template. All the primers contained *Sac* I and *Xho* I restriction sites (Table 4.1). PCR fragments were generated using different forward primers (-1461 *Sac* I, -1176 *Sac* I, -947 *Sac* I, -776 *Sac* I, -593 *Sac* I, -335 *Sac* I, and -149 *Sac* I) and the same reverse primer (Rev*Xho* I). All the fragments varied in their 5'-ends, but all their 3' ends terminated just before ATG starting codon (Fig. 4.4). The PCR products were digested with *Sac* I and *Xho* I to create the cohesive ends, subsequently purified and ligated into the

SacI/ *Xho* I site of the pGL3-basic vector. All constructs were confirmed by DNA sequencing.

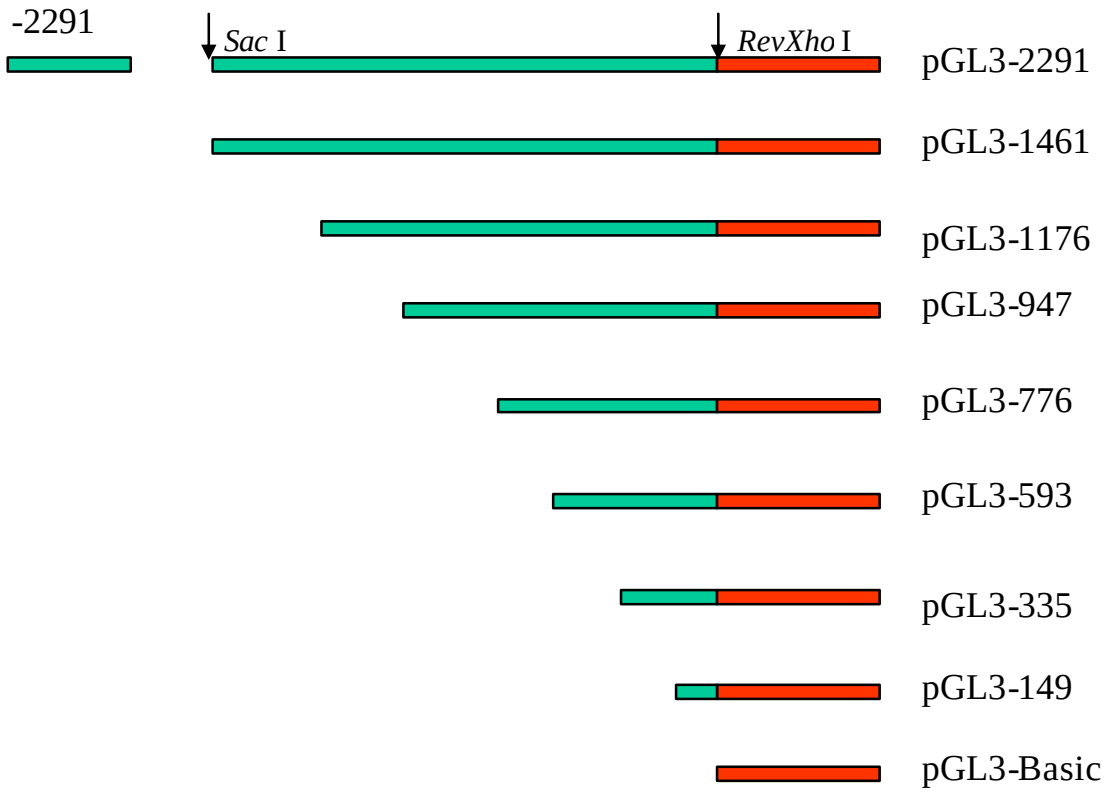

Fig 4.4. Schematic representation of the structure of the 2,239 bp amylase promoter and its sequentially deleted fragments inserted upstream from coding region in the pGL3-Basic vector (Promega). The numbers indicate the positions of the 5' ends of the deletion constructs relative to the first base in the translation initiation codon (ATG). The name of the plasmid denotes the 5'-upstream extension of the insert DNA.

Table 4.1. List of Primers used in constructing sequential promoter deletion

ForSacI1	5’-CGCGAGCTCGTGCAAAATCCACTGTACTG-3’ *SacI*
ForSacI2	5’-CGCGAGCTCGACCTATAACCTTTCTCACTCG-3’ *SacI*
ForSacI3	5’-GCGCGAGCTCGCATGTGATAACATCAGTA-3’ *SacI*
ForSacI4	5’-CGCGAGCTCGGAAGGAATCCAAGGTACAC-3’ *SacI*
ForSacI5	5’-GCGCGAGCTCGAATTTCATCTTACTGCCAC-3’ *SacI*
ForSacI6	5’-CGCGAGCTCCACAGAGACCCTCCCTAACAG-3’ *SacI*
ForSacI7	5’-CGCGAGCTCCAAGGTAGGAAGCCATGAGA-3’ *SacI*
RevXho1	5’-CTTAGATCGCAGATCTCGAGCCCGGGCTAG-3’ *XhoI*

4.2.4. Site-directed mutagensis

Plasmids carrying specific point-mutations were generated using the QuickChange site-directed mutagenesis kit (Stratagene). A pair of complementary PCR primer was designed such that the desired mutations were located in the middle, and the flanking regions contained another 15 to 20 nucleotides that were identical to the template sequence. The primers were PAGE (Poly Acrylamide Gel Electrophoresis) purified with predicted T_m of not less than 78 °C. The sequences were as follows: Mut1: 5’-

TGGCACATTTTTGTCCGCACGCGCGTTGATACTTGAATAAACC-3', Mut2: GGTTTATTCAAGTATCAACGCGCGTGCGGACAAAAATGTGCC-3'. These primers were used to amplify the entire plasmid DNA by PCR using Pfu DNA polymerase (Statagene). The total volume of PCR reaction was 50 µl, and contained 50 ng template, 125 ng forward and reverse primers, 0.8 mM of each dNTPs and 2.5 U Pfu DNA polymerase. The PCR parameters usually consisted of an initial 95 °C denaturation for 30 seconds, followed by 25 cycles at 95 °C for 30 seconds, 55 °C for 1 minute and 68 °C for 6 minutes. After PCR reaction, the mixture was treated with *Dpn* I which digested only the hemimethylated template DNA, and the PCR products carrying the desired point mutations were transformed into competent E.coli DH 10B cells. Plasmids were sequenced to assure correct mutations were obtained.

Table 4.2. List of plasmid constructs used in this study.

Construct	Brief Description of plasmid Construction	Reference
pGL3-Basic	Promega	This study
pCMV-RL	Kind gift from Dr. Liu Yiwen (IMA, NUS)	Unpublished
pGL3-2291	2,291bp amylase promoter was amplified and inserted into the pGL3-Basic as a *Mlu*/*Mlu* fragment	This study
GRE/Mut	pRL3-2291with mutated GRE binding site (-891/-876)	This study
pEGFP-1	Clontech	This study
pEGFP-2291	2,291bp amylase promoter was amplified and inserted into the pEGFP-1 as a *Sac* I/*BamH* 1 fragment	This study

4.2.5. Transient transfection of cells

4.2.5.1 Maintenance of cells

4.2.5.1.1 Maintenance of Medaka embryonic stem cells, Medaka testis cells, Hela cells and CHO cells

Medaka (*Oryzias Iatipes*) embryonic stem cells (ES) and testis cells (TES) were kind gifts from Dr. Hong Yunhan (DBS, NUS). They were maintained as described by Hong and Schartl (1996). Medaka ES and TES cells were cultured in DMEM containing 4.5 g/L glucose supplemented with 20 mM HEPES, antibiotics (penicillin, 50 U/ml; streptomycin, 50 µg/ml; ampicillin, 50 µg/ml), glutamine (2 mM), sodium-pyruvate (1mM), sodium-selenite (2 nM), nonessential amino acids (1 mM), 2-mercaptoethanol (50 µM), human recombinant bFGF (10 ng/ml), human recombinant LIF(10 ng/ml), fish embryo extract (1 embryo/ml), FBS (15%) and common carp (*Cyprinus carpio*) serum (1 %).

Fish serum was prepared from common carp and rainbow trout (*Oncorhynchus mykiss*) purchased from local fish dealers. The blood was collected from the caudal vein, the blood cells were removed by centrifugation, and the plasma was allowed to clot overnight at 4°C. Serum was separated from the clotted material by centrifugation, sterilized through a 0.2 µm filter, heat-inactivated at 56°C for 20 minutes.

HeLa and CHO cells were kind gifts from Dr. Low Boon Chuan's lab (DBS, NUS). HeLa cells were maintained as described by Freshney (1992). HeLa cells were cultured in Eagle's Minimal Essential Medium (MEM) supplemented with 10 % fetal calf serum (FCS), gentamicin (10 µg/ml), glutamine (2 mM), HEPES (N-2-hydroxyethylpiperazine-

N'-2-ethane sulfonic acid) buffer (10 mM) and $NaHCO_3$ (2 mg/ml). CHO cells were cultured in Kaighn's modification of Ham's F12K medium (F12 K) supplemented with 10 % fetal calf serum (FCS), glutamine (2 mM), and sodium bicarbonate (1.5 mg/ml).

4.2.5.1.2 Maintenance of AR42J cell line

The AR42J rat pancreatic acinar cell line was obtained from American Type Culture Collection (ATCC). The AR42J cells were maintained in Kaighn's modification of Ham's F12 medium (F12K) supplemented with 2 mM glutamine, 1.5g/L sodium bicarbonate, 20% fetal bovine serum and antibiotics (penicillin/streptomycin) (GibcoBRL, Life Technologies). The AR42J cells were recovered by rapid thawing in a 37 °C water bath. To remove the dimethyl sulfoxide (DMSO) that was added as a cryoprotectant, the cells were transferred into a 14 ml falcon tube containing 9 ml pre-warmed F12K medium and pelleted by centrifugation at 125g for 7 minutes, and the supernatant was discarded. Cells were next resuspended in 7 ml pre-warmed fresh F12K, and transferred into a 25 cm^2 flask (NUNC). The cells were incubated at 37 °C, 5% CO_2, and 4 ml fresh medium was added on day 6; then the first medium change was carried out on day 8. After two weeks of culture, a subculture at a 1:3 ratio was done. The AR42J cells grew as colonies, pile up and only grew to 50% confluence and required subculture once every 6 to 8 days (Fig. 4.5). Because Dulbecco's modified Eagle medium (DMEM) works best for Lipofectamine Plus™ reagent (Life technologies) used in the transfection, the culture medium for AR42J cells was changed to Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and cultured for two or three passages before starting transfection.

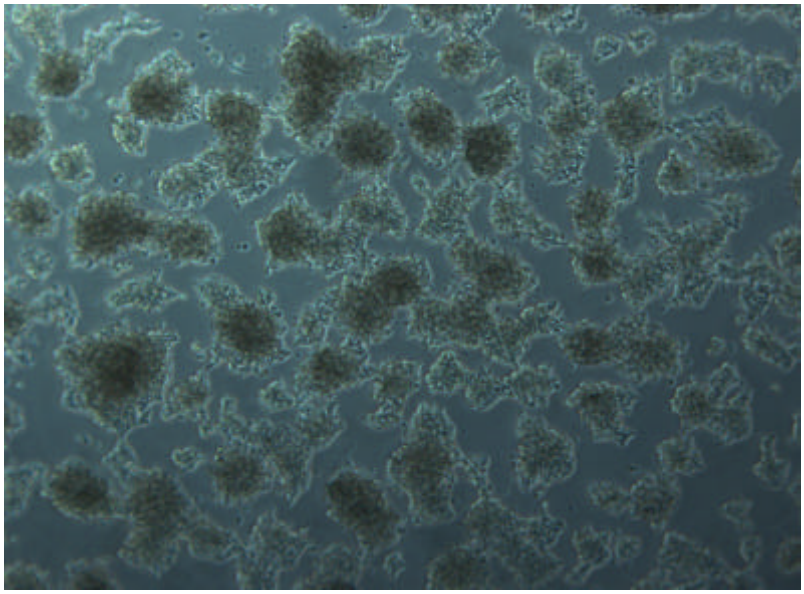

Fig.4.5. Morphology of AR42J cells from a 7 days culture.

4.2.5.2 Preparation of fetal bovine serum stripped of cortisol

Cortisol was removed from the FBS using the protocol of Leake et al. (1987) with slight modifications. The FBS was mixed with 2% dextran-coated charcoal (W/V) overnight on a shaker at 4 °C. The charcoal was removed by centrifugation at 2,000g for 15 minutes. The top layer was carefully aspirated and stored at -20 °C.

4.2.5.3. Preparation of Dexthamethasone

Dexthamethasone (Dex) is purchased from Sigma. The Dex stock solution was prepared by dissolving 25 mg of powder in 1 ml ethanol (95%). The stock solution was diluted with appropriate amount of sterile culture media and stored at -20 °C.

4.2.5.4 Transfection

4.2.5.4.1 Transient Transfection in AR2J cells

For transfection, AR42J cells were seeded at 1.0×10^5 in 24-well plates in 0.5 ml DMEM and transfection was performed using Lipofectamine Plus™ reagent (Life technologies) according to the instructions of the manufacturer. After 24 hours, the cells should reach 50% confluence and were ready for the transfection procedure. A single transfection pre-complexed DNA was prepared for each treatment or construct to be tested (n=3). For each transfection, 0.4 µg of the firefly luciferase reporter construct, 25 ng of the pCMV-RL, which encodes a *Renilla* luciferase gene and was used as an internal control, and 25 µl DMEM (no serum and antibiotics) were mixed with 4 µl Plus™ reagent at room temperature for 15 minutes. In the meantime, 1 µl Lipofectamine reagent was diluted with 25 µl DMEM (no serum and antibiotics) in a second tube. Pre-complexed DNA was combined with 25 µl diluted Lipofectamine reagent and incubated for 15 minutes at room temperature. While the complexes were forming, the medium of the cells was replaced with 0.2 ml DMEM (no serum and antibiotics). The DNA- Plus™-Lipofectamine reagent was added to each well of cells; the complexes were mixed gently and incubated at 37 ℃ at 5% CO_2 for 3 hours. After 3 hours incubation, DMEM with serum (charcoal-treated FBS) was added to bring the final composition and volume to that of normal growth medium. The medium was replaced by fresh DMEM with cortisol-stripped FBS the day after transfection. After 8 hours, the medium was changed to fresh cortisol-stripped DMEM supplemented with different concentrations of Dexamethasone (Dex), and the tranfected cells were incubated for another 24-48 hours. Finally, cells were lysed using 100 µl lysis buffer (Promega) and used immediately or stored at -20 ℃ for luciferase reporter assay.

4.2.5.4. Transient Transfection in HeLa, CHO, Medaka embryonic stem cells and Medaka testis cells

Cell seeding density, plate, transfection master mix and transfection times in these four kinds of cells were the same as AR42J cells transfection as described above. For HeLa cells, SuperFect Reagent (Qiagen) was added into the cells at 2.5 µl per transfection reaction, and mixed by vortex. The mixture was incubated at room temperature for about 10 minutes to allow the formation of liposome-DNA transfection complexes. Culture medium was removed from HeLa cells and the cells were washed once with 1.5 ml of PBS and PBS buffer was then discarded. Meanwhile, the liposome-DNA transfection complexes were mixed with 350 µl of MEM supplemented with 10 % of FBS, and the whole mixture was immediately added into the cells after removal of the PBS. The cells were then incubated at 37 °C for a further 3 hours. After the transfection, the cells were washed with PBS, normal growth medium containing serum added, and the cells were then incubated for another 24-48 hours until they were ready for assay.

For CHO cells, Lipofectamine 2000 (LF2000) reagent (Life technologies) was used according to the instructions of the manufacturer. Plasmid DNA was diluted using OPTI-MEM I medium (Life technologies). Diluted DNA was combined with diluted LF2000 reagent and incubated at room temperature for 20 minutes to allow DNA-LF2000 complexes to form. Each well of cells was added 100 µl complexes and incubated at 37 ℃ for a total of 24-48 hours until they are ready for assay.

For Medaka embryonic stem cells and Medaka testis cells, GeneJuice Transfection reagent (Novagen) was used according to the instructions of the manufacturer. Three µl of GeneJuice was added dropwise directly to 100 µl OPTI-MEM I medium (Life

technologies) and incubated for 5 minutes. DAN was added to the GeneJuice/serum free medium and incubated for 15 minutes. The entire volume of DNA/GeneJuice mixture was added dropwise to the cells in complete medium, and incubated at 37 °C for a total of 24-48 hours until the cells were ready for assay.

4.2.6. Dual-luciferase reporter assay

Dual-luciferase reporter assay was carried out following the manufacturer's protocol (Promega). The activity of firefly luciferase was measured in a 12 × 75 mm glass tube (Fisher Scientific) for 5 second with a Lumat LB 9501/16 Berthold luminometer (EG&G Berthold). The background luminescence was first established by adding 100 µl of luciferase reagent II (LR II) into the glass tube, and it was usually very low. The firefly luciferase reaction was initiated by the addition of 20 µl of AR42J cell extracts. Finally, the Renilla luciferase activity of the internal pCMV-RL control was measured by adding 100 µl of the Stop&Glow reagent into the same tube, and measured for 10 seconds.

4.2.7 Electrophoretic mobility shift assay (EMSA)

The electrophoretic mobility shift assay (EMSA*)* using the native PAGE gel is a popular tool for studying protein-DNA interactions. This assay is based on the electrophoretic resolution of protein-DNA complexes from each other and from free DNA. Transcription factors, in particular, have been studied extensively by using this assay to determine their binding specificity with regards to their cognate binding sites. Therefore, it is a simple and sensitive method for detection of sequence-specific DNA-binding proteins in crude extracts. Usually, a radioactive DNA fragment containing the

known or assumed protein binding site is added to a crude cell extract along with non-specific DNA such as poly dI.dC and calf thymus DNA to bind other DNA-binding proteins. The presence of a retarded moving band is indicative of a specific DNA-protein interaction (Ceglarek and Revzin, 1989).

4.2.7.1 Extraction of nuclear protein from AR42J cells

Nuclear extracts from AR42J cells were obtained by the protocol developed by Dignam *et al* (1983) with minor modifications. The monolayer cells ($5\text{-}8 \times 10^8$) were washed with 1×PBS, and pelleted by centrifugation for 10 min at 400 × g. All the remaining steps were performed at 4 °C. The cell pellets were resuspended in 4 °C 1×PBS to a final volume of 5 times that of the packed cells. The cells were precipitated by centrifugation for 10 min at 400 × g, resuspended in hypotonic buffer A (20 mM HEPES-KOH, pH 7.9, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF), to a final volume of 3 times that of the original packed cells, and allowed to swell for 10 min on ice. The swollen cells were homogenized with 20 strokes of a loose fitting Dounce homogenizer (Wheaton, USA). The nuclei were collected by centrifugation for 10 minutes at 3,000 × g, resuspended in 5 volumes of Buffer A, and washed once by centrifugation. Proteins were extracted from washed nuclei by an equal volume of extraction buffer C (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, pH 8.0, 0.5 mM DTT, 1 mM PMSF, 2μM pepstatin A), stirred gently for 1 hour, and then precipitated by centrifugation at 25,000 × g for 30 minutes. The supernatant was collected as the nuclear extract and dialyzed against buffer D (20 mM HEPES-KOH, pH 7.9, 0.1 mM EDTA, 100 mM KCl,

20% glycerol, 0.5 mM DTT, and 0.5 mM PMSF) for 8-12 hours. After the pellets were removed by centrifugation at 12,000 × g for 20 minutes, the supernatant was collected as the nuclear extract. Protein concentration was determined by the Bradford assay and the nuclear extract was stored at -80 °C.

4.2.7.2. γ-^{32}p ATP labeling of the oligonucleotides

The sequences of the commercially synthesized oligonucleotides are listed in Table 4.2. Before labeling, the single-stranded oligonucleotides were annealed in the annealing buffer (10 mM Tris-HCl pH 7.9, 2 mM $MgCl_2$, 50 mM NaCl and 1mM EDTA) with corresponding DNA strands at 85 °C for 15 minutes. After incubation, the reaction was cooled down slowly to 25 °C and incubated for another 30 minutes. Subsequently, the annealed double-stained oligonucleotides were end-labeled with fresh γ-^{32}p ATP with T4 polynucleotide kinase (NEB). The radioactivity and labeling efficiency of the labeled probes were measured by liquid scintillation counting.

4.2.7.3 DNA-protein interaction

In each binding assay, 1×10^5 cpm/pmol probe and 10 µg nuclear extract containing 10 µg of poly dI.dC were incubated at room temperature for 30 minutes in a 30 µl binding buffer before being subjected to a native 4% PAGE gel. The binding reaction buffer contained 10 mM HEPES, 50 mM KCl, 1 mM EDTA, 2.5 mM DTT, 10% glycerol and 0.05% NP-40.

Table 4.3. Probes used in EMSA

Probe Names	Probe Sequences
GRE/WT	5’-GCACATTTTTGTACAcacTGTGATTGATACTT -3’ 5’-AAGTATCAATCACAgtgTGTACAAAAATGTGC-3’
GRE/MUT	5’-GCACATTTTTGTACAcacGCGCGTTGATACTT-3’ 5’-AAGTATCAACGCGCgtgTGTACAAAAATGTGC-3’
PTF1/WT	5’-GGTCAACTGAAGCCCCTCGGGGGAAAAAGCTTGTGTTTTG-3’ 5’-CAAAACACAAGCTTTTTCCCCCGAGGGGCTTCAGTTGACC-3’
PTF2/WT	5’-CAAGTGTGATCCTCTTCAGCAGAAGTTTCAGTTTG-3’ 5’-CAAACTGAAACTTCTGCTGAAGAGGATCACACTTG-3’
HNF/WT	5’-GAAGTTTCAGTTTGTTTTGACCCCCACAGAGAC-3’ 5’-GTCTCTGTGGGGGTCAAAACAAACTGAAACTTC-3’
HNF/MUT	5’-GAAGTTTCAGAGCGTCGAGACCCCCACAGAGAC-3’ 5’-GTCTCTGTGGGGGTCTCGACGCTCTGGAAACTTC-3’

Note: The name of GRE/WT denotes pRL3-2291 as a control; GRE/MUT denotes pRL3-2291 with GRE mutated binding site; PTF1/WT denotes pRL3-2291 with putative PTF binding site (-1210 to -1203); PTF2/WT denotes pRL3-2291 with putative PTF binding site (-344 to -329); HNF/WT denotes pRL3-2291 with putative HNF binding site; HNF/MUT denotes pRL3-2291 with mutated binding site.

4.2.7.4 Binding specificity of glucocorticoid receptor to glucocorticoid receptor element

To rule out the possibility of non-specific binding between the DNA sequences with background proteins, the competition assay was performed. The principle of competition assay is to use unlabeled DNA probe (cold probes) to compete against the complex formed between the labeled probes with proteins. If the binding is specific, the intensity of DNA/protein complex will appear reduced with the unlabelled probes bearing the same sequence after autoradiography, while the intensity of DNA-protein will remain unchanged with an unlabelled probe of mutated sequence. For competition assay, 5×, 10×, 100×, 200× excess of consensus probes and 200× excess mutated probes were added in the binding reaction.

4.2.7.5 Autoradiography of the PAGE gel

Each binding assay was subjected to a 4 % native PAGE after incubation. After 3 hours of electrophoresis at 130 V at room temperature, the gel was fixed in 7% acetic acid for 5 minutes. The gel was then transferred to a same size 3 mm Whatman covered by a saran wrap to be dried in the gel drier (Biorad, model 583) for 2 hours. The dried gel was exposed to an X-ray film (Biomax, Kodak). After overnight autoradiography, the film was developed in an automated developer (Kodak X-Omat MP-4).

4.2.8 Statistical Analysis

Data from luciferase assay are presented as means ± SD after normalization by the *Renilla* luciferase activity. Each transfection reaction was carried out in triplicate and the experiment was repeated twice and shown to be reproducible. P values for comparison of

samples were obtained by using the paired t-test with two tailed distribution using the Microsoft Excel program.

4.3 Results

4.3.1 Isolation and characterization of seabass α-amylase promoter

4.3.1.1 Isolation of seabass amylase promoter

Seabass amylase promoter was isolated using Universal Genome Walker method as described in section 4.2.1. The basic procedure for the amplification consists of two rounds of PCR. The outer adaptor primer (AP1) provided in the kit and the gene-specific primer (GSP1) derived from the first exon of amylase cDNA was used in the primary PCR. Multiple fragments ranging in size from 500 bp to 2.5 kb were observed with three of the four libraries (Fig 4.6A), but there was no major band observed in *EcoR* V library. The primary PCR mixture was then diluted 50 times with deionized water and used as a template for a secondary PCR using AP2 and GSP2 primers. Major bands were observed with each of the four libraries, and the bands were gel-purified followed by cloning into pGEMT Easy vector (Promega). Subsequently, a total 2.3 kb in length of the seabass α-amylase promoter from *Pvu* II library was obtained and sequenced.

4.3.1.2 Characterization of seabass α-amylase promoter

The 2.3 kb promoter was sequenced completely. In comparison with the 5' flanking sequence of human and rat amylase genes, the upstream region of fish and mammalian α-amylase is distinctively divergent.

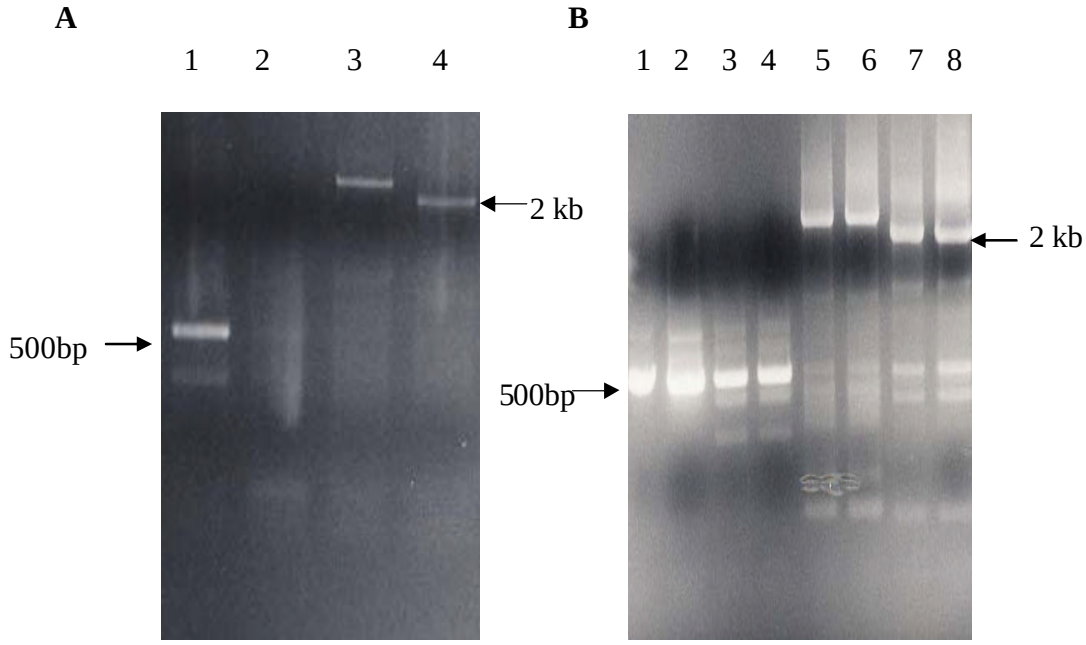

Fig. 4.6 Results of primary and secondary Genome Walker PCR. (A) The gel showed the primary PCR products generated by walking with seabass Genome Walker libraries and gene-specific primer 1 (GSP1) derived from the first exon of seabass cDNA. Lane 1: *Dra* I library. Lane 2: *EcoR* V library. Lane 3: *Pvu* II library. Lane 4: *Stu* I library. (B) The secondary (nested) PCR products generated with AP2 and gene-specific primer 2 (GSP2). Lane 1 and 2 (duplicate): *Dra* I library. Lane 3 and 4 (duplicate): *EcoR* V library. Lane 5 and 6 (duplicate): *Pvu* II library. Lane 7 and 8 (duplicate): *Stu* I library.

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-2258 CCGCGATTTACTAGGGATTACTATAGGGTACGCGTGGTCGACGGCCCGGG

-2208 CTGGTCCTTATTTACAAAATCAATACACACAAAACCACAGACAATGTGGC

-2158 ATTAACTGCCCTTTTTTATGCAGAGCACCCTTAGTGTCATAAGTACATTC

-2108 ATTTCCATGTTGTTAGGCATGTGTACTGAAGCCATATGGCTTTGCTGATG

-2058 AAATATCCTACTTGTTGAGAAATAATGTGGAATACAATCTAATACAATCA

-2008 ATGATACAGAGGATGTTATTTATAAAATTTGTAGTTTATGATTATTCCCT

-1958 CTCAATTCCACATATTAATAGCTAATTAATAAATGTGTCTATATTATGAA
GR

-1908 CAAAATAATAATATCTACATACATATATAGGCATTTTACTACAATTACTT

-1858 AAGGAAGAAATCTTGTTATAGAACATGAGGTGAACACAGAATGATGTATG
GR

-1808 TATATACTGTATATTTTTTAAGTGAAGCAGTTTCTCTCAAGGACCATTGG

-1758 AGGAGCTATTGTAATATAAAAGCACATCCCACCATAACTGTTATAACATC

-1708 CCAGGGCTATGACTGACATGACTTTATCACTGCTGTGGAGTTGTGCAGCA

-1658 CATTCAGGAGTAATGTAATTCCACATTTATACAGGATTTAATGAACTAAA
GR

-1608 TGAATTAAATTTGGAATATTTTTCTTTCCTTCTGACCTTATTAAAAAGAC

-1558 CTTAGAAAAGTGTTGGGGGAAAATTTGCAAAACAATTAGAAAAACAATGT

-1508 TAAGAATGTTTTTTGAGCTGTTAATGACAGAACTAAGCACACAATACTCT
GR

-1458 GTGCTCACTATATATACATTCATACTCTATGGCATGAATTCAGCTTTAAA

-1408 AAGAATGAGTTTGAATTTAGTAGTGCAAAATCCACTGTACTGTGTGGTTA
HNF-4

-1350 TAATTTTATTTAAGTAGTAAGTAATTTAAGTCATTTTGAGGATTAACCTT

-1308 ATGTAATACCACAAATTTCTCTTGAGAGCTGACACACTGGTGGCACGACC
GR

-1258 CTTGTCCCAAATACAGTACCTATAAGTGGAGGGTCAACTGAAGCCCCTCA
GR

-1208 TGGGAAAAAGCTTGTGTTTTGTCAGGGCTGGTTTAGTTAACTTGTTTTGC
PTF1(BoxA)

-1158 ATAACATATTTGACCTCTAACCTTTCTCACTCGAGTGTAGCTTATCTGTA

-1108 TTGGTTTTCCTTCGTGCAAAACGGATAGTAACACCGTGAAGTTATACAAG

-1058 GCATGTTTTGTAACCGCTGCACATCGAACCTTTTTTTTTTGCATGTGATA
GR

-1008 ACATCAGTAATGTAAAATGGACCACTGTTATGGTAATTTAATATAACAAG

-958 CCCAACTGATTGTAAGATCTGATAAATACAAAACCTCAGTTTGTTACTGG

-908 GAATTTTGGCACATTTTTGTACAcacTGTGATTGATACTTGAATAAACCT
GR

-858 ATACAGTTTTTGAAATGCCAATGAAACATAATACATTTTAAAATCTTGGA

-808 AGGAATCCAAGGTACACTTCAAATTTCTATACAAAAAAATTCAAAATTCA

-758 AGAAAAGCACAACACATCGAC**ATGAGA**TACATAAATAGTATTATTTCCCT
GR XPF-1

-708 CCTGGGCAGAGTTTGTTCCGTTATTCAAAATTTAATTAACTGCCAAATTC
HNF-1

-658 AGGACACAGAAAGTCACTTAAGGAGAAGAGTAGATTTAAGCTACTTGTTA
GR GR

-608 CAACATTATCTACTTATTGGACTATGTGAACAAATACTAAAACAGGATAA
GR

-558 AACTAAACTGTCATGTTCTGAGTGGAAAGAGCTTTAAAGTACAATTTCAT
GR

-508 GTCCTCATATCAGAATCAGTGCAAGATAAA**TCTCAT**TAATCGATGAAATG
GR XPF-1

-458 TAGTAATATTGTGAATGTTAAAAGCCAGATAATACTATTTTTGAGGGCTA

-408 GTGTTGCTGCTGAATCCCTGAATTTCATCTTACTGCCACATAGTGAGAGG

-358 CAAGTGTGATCCTC**TTCAGCAGAAGTTT**CAGTTTGTTTTGACCCCCACAG
GR PTF1 (Box A) HNF3

-308 AGACCCTCCCTAACAGATATGTCACTGACATTTAAGAAAATGCATACAAA

-258 AGCCTTAGGATATTTTTAGTCTCTTTAAGCCACTTCTTTATTTATTTTTT

-208 ATAATGGGGCAAAAACACTATGCACACCTCATGCTTCTAAGTTTCTACGT

-158 GCCTTGAAGCTATTTTTCCTTGTGCTGGTTTTGAAACAAGGTAGGAAGCC

-108 **ATGAGA**AAGAGG***CACCTGT***TTAACCTCCATGGTGATGTTTCTGAGCC**TCT**
XPF-1 EII/box B XPF-1

-58 **CAT**TGGTCCATCAGAGGGAGAAAATGTGTATAAATACAGAGGTAAAGCAG
+1

-8 CAGGAGAGTG**T**GGTTTTCTCGCACATCCAGGGAAAGCATG

Fig. 4.7. The 2,307 bp sequence upstream of the ATG start codon of seabass amylase gene. The transcription factor binding sites were predicted using Transcription Element Search System (TESS, http://www.cbil.upenn.edu/tess). Transcription start site was predicted using Neural Network Promoter Prediction software (http://www.fruitfly.org/seq_tools/promoter.html). The start site is marked as +1. The ATG start codon and potential TATA box are shown in red. All potential GR binding sites are underlined. PTF binding sites are shown in pink. HNF3 binding site is shown in green. EII is indicated in Italics.

Several cis-acting elements have been shown to be involved in the control of amylase gene expression in mammals, such as pancreas transcription factor (PTF), exocrine pancreas transcription factor (XPF), hepatocyte nuclear factor 3 β or 3γ (Slater *et al*. 1993; Cockell *et al*.1995). In addition, mammalian amylase gene expression is regulated at the transcriptional level by glucocorticoids.

A total of 14 glucocorticoid receptor (GR) half-binding sites and one imperfect palindrome GRE (TGTACAcacTGTGAT) have been identified within the 2,307 bp of 5' flanking region of amylase gene (Fig. 4.7). The palindrome GRE was located at position -891to -876 and may be a potential GR binding site. Several cis-acting elements which were involved in the control of mammalian amylase gene expression were also identified in seabass amylase promoter: two PTF (Box A) binding sites were located at position -344 to -330 and -1209 to -1204; four XPF-1 sequences were identified at position -61 to -56, -108 to -103, -478 to -473 and -737 to -732; HNF binding site was located at position -328 to -320; an Element II (EII) was located at -96 to -90, and EII is also a Box B. Organization of seabass amylase promoter is shown in Figure 4.8.

4.3.2. Tissue-specific expression of seabass pancreatic α-amylase promoter

So far, no normal acinar cell lines have been reported (Ulrich *et al*., 2002). However, cell lines derived from acinar cell tumors with acinar (AR42J) or ductal phenotype (AR4 IP) exist. The acinar cell line AR42J was generated from hyperplastic pancreatic nodules of azaserine-treated male rats and is used extensively for pancreatic exocrine cell studies (Christophe, 1994). Therefore we choose rat AR42J cells to test the seabass α-amylase promoter. The luciferase expression level in rat AR42J cells was dramatically higher than those obtained in HeLa, CHO, medaka ES and TES cells (Fig.

4.9). This indicated that the expression of the α-amylase promoter required pancreas transcription factors and the expression was tissue-specific.

4.3.3 Dexamethasone induction of amylase promoter activity in AR42J cells

To determine whether the seabass amylase promoter could be induced by dexamethasone (Dex), the pGL3-2291 construct was transfected into AR42 J cell line. Both the time-course and dose-dependence of the Dex induction were examined. The induction by 100 nM of Dex as measured by luciferase activity was detectable after two hours of incubation, reached one-half maximum after 24 hours, and peaked at 48 hours (Fig. 4.10 A). Subsequently, the amylase promoter construct was treated with increasing Dex concentrations (0 to 10^{-5} M) and maximal stimulation (5-fold) occured at 100 nM (Fig. 4.10 B). The Dex induction of the seabass α-amylase promoter was similar to that observed for the mammalian amylase promoter but smaller in magnitude than the 6~7 fold response in mouse and human (Logsdon *et al* 1987, Slater *et al* 1993).

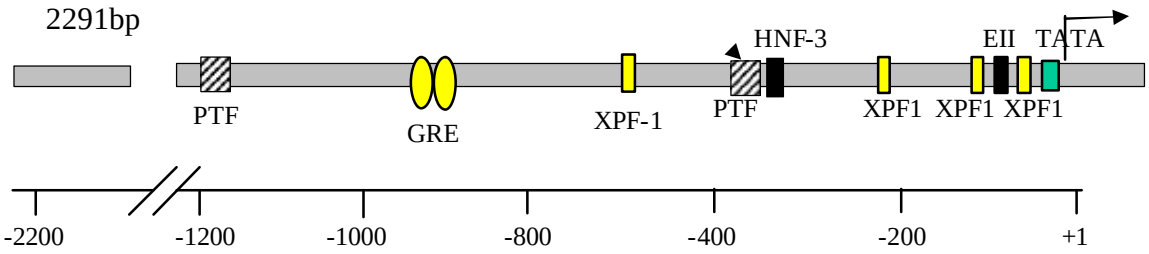


Fig. 4.8. Organization of seabass α-amylase promoter. The promoter contains the putative pancreatic transcription factor (PTF), glucocorticoid response element (GRE), hepatocyte nuclear factor 3 (HNF-3) binding site, exocrine transcription factor 1 (XPF-1), EII element and TATA box.

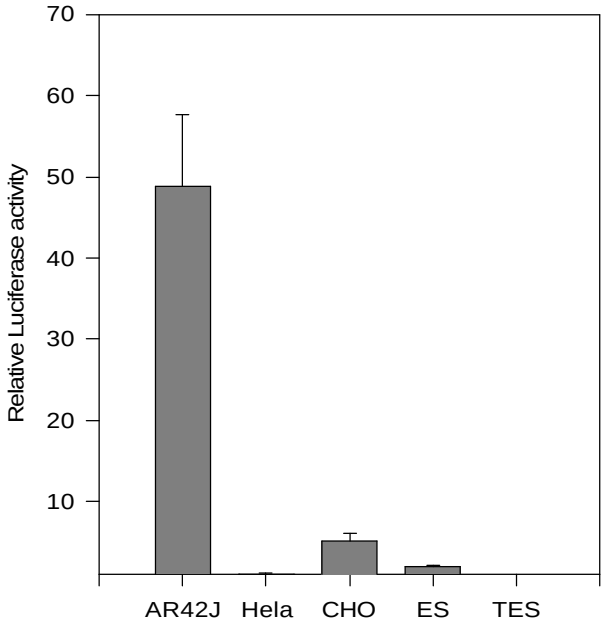
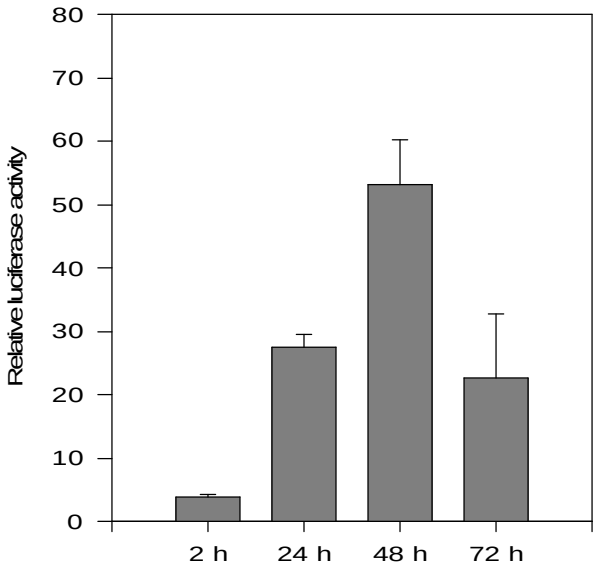


Fig.4.9. Experiment comparing seabass α-amylase construct (pGL3-2291) activities expressed in AR42J, Hela, CHO, Medaka ES and TES cells.

A



B

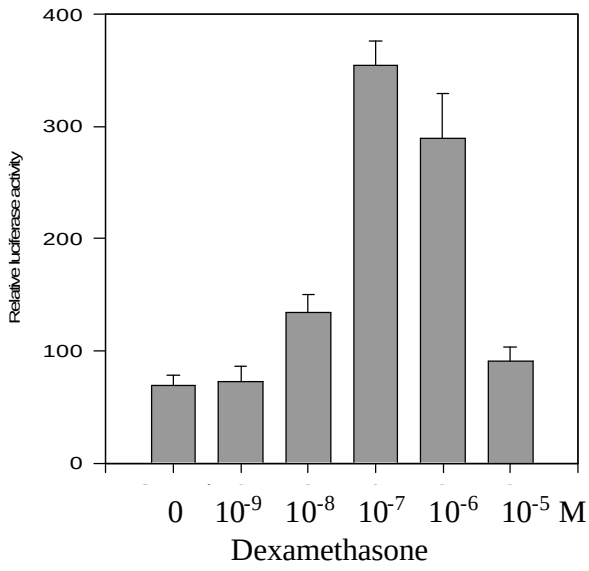


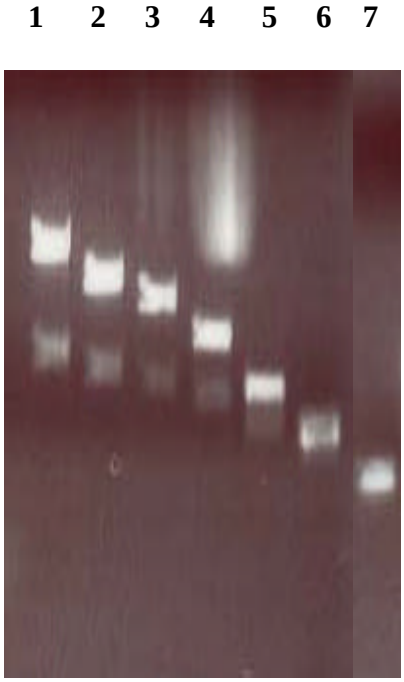
Fig 4.10 Amylase promoter luciferase activity in (A) increasing time course and (B) increasing dexamethasone concentrations (0 to 10^{-5} M). Each transfection reaction was carried out in triplicate and the experiment was repeated twice and shown to be reproducible. The firefly luciferase activity presented had been normalized by the *Renilla* luciferase activity. Data are presented as means± SD.

4.3.4 A palindromic glucocorticoid response element is essential for hormone induction

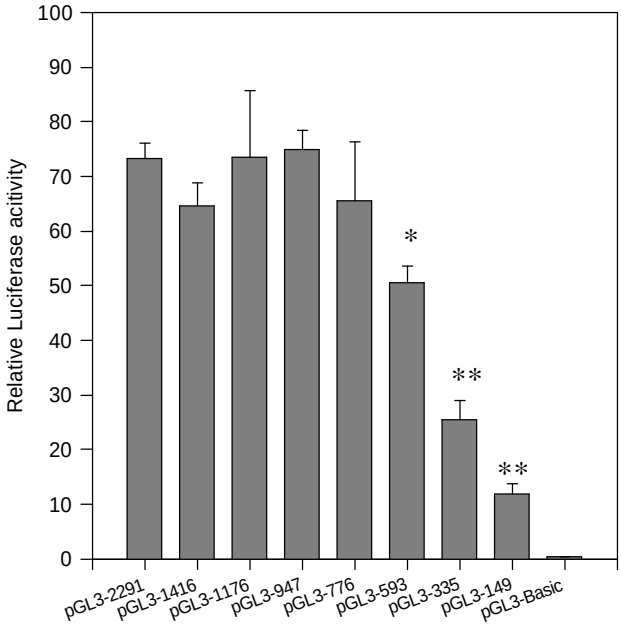
To localize the GRE in the seabass α-amylase promoter, a series of 5' deletion constructs was generated (Fig. 4.11A) and transfected into AR42J cells, and treated with or without Dex. Deletions extending to -947 bp of the 5'-flanking sequence showed no significant effect on the promoter activity. A further deletion to -776 bp slightly reduced the promoter activity by 8.9%. A successive deletion to -335 bp reduced its activity by 66%, while pGL3-149 construct resulted in loss of 85% of the promoter activity (Fig. 4.11B). Furthermore, the three longer constructs (pGL3-1416, pGL3-1176, pGL3-947) responded to Dex stimulation by ~5 fold (Fig 4.11C). However the shorter fragments downstream of -947 showed no significant response to Dex stimulation. These results suggested that the region between base 947 and 776 harbors a positive response element to Dex stimulation. To test whether the motif identified is actually functional and responsible for the glucocorticoid effect, the binding site (TGTACAcacTGTGAT) was mutagenzied to a sequence (TGTACAcacT**CGCG**T) that would not be recognized by the glucocorticoid receptor. Transfection experiments demonstrated that this site-directed mutagenesis almost eliminated the response to DEX stimulation, the induction having dropped from ~5 fold to 1. 6 fold (Fig. 4.12).

In mammalian amylase promoters, GRE is located very near to the transcription start site (Logsdon *et al* 1987, Slater *et al* 1993). However, a series of 5'-deletions in this study did not show a significant change in DEX stimulation at this region (-776 to the cap site), but there was a 5 fold induction by DEX stimulation between -947 to -776, which was shown to contain a palindrome GRE motif by sequencing analysis.

A


1 2 3 4 5 6 7

B


Relative Luciferase acitivity
100
90
80
70
60
50
40
30
20
10
0
*
**
**
pGL3-2291
pGL3-1416
pGL3-1176
pGL3-947
pGL3-776
pGL3-593
pGL3-335
pGL3-149
pGL3-Basic

C

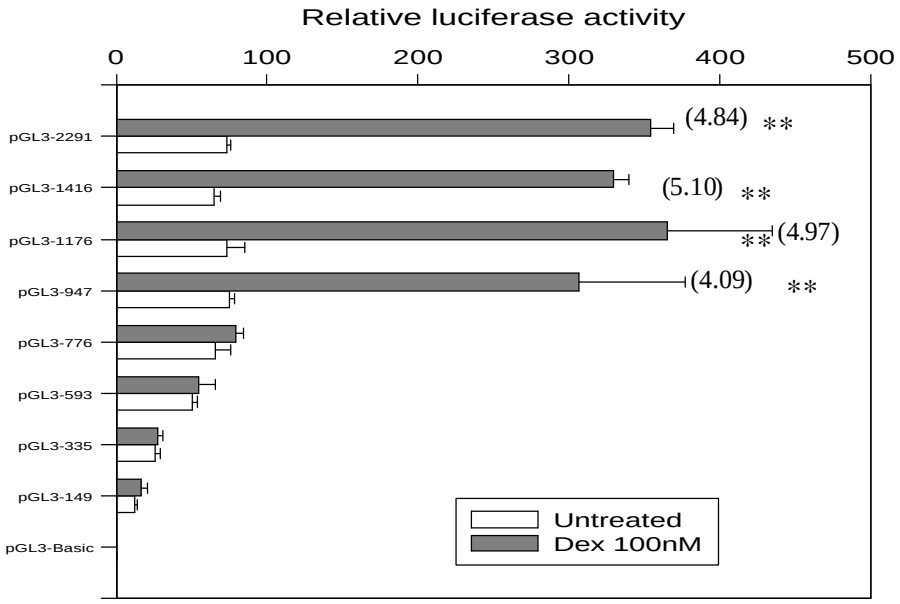


Fig. 4.11. (A) 5' deletion PCR products from -1416 to -149 are shown from lane 1 to lane 7. (B). Luciferase assays of promoter activity with deletion mutants transfected into AR42J cells; Data are presented as means± SD, Student's t-test is used to calculate statistical difference. * $p<0.05$; ** $p<0.01$. (C). Luciferase assays of promoter activity with deletion mutants transfected into AR42J cells, numbers in the bracket indicating Dex fold inductions. Data are presented as means± SD, * $p<0.01$ vs untreated; ** $p<0.001$ vs untreated. Each construct was treated with dexamethasone as indicated by dashed box. The result was normalized by Renilla luciferase PRL-CMV. Each transfection reaction was carried out in triplicate and the experiment was repeated twice.

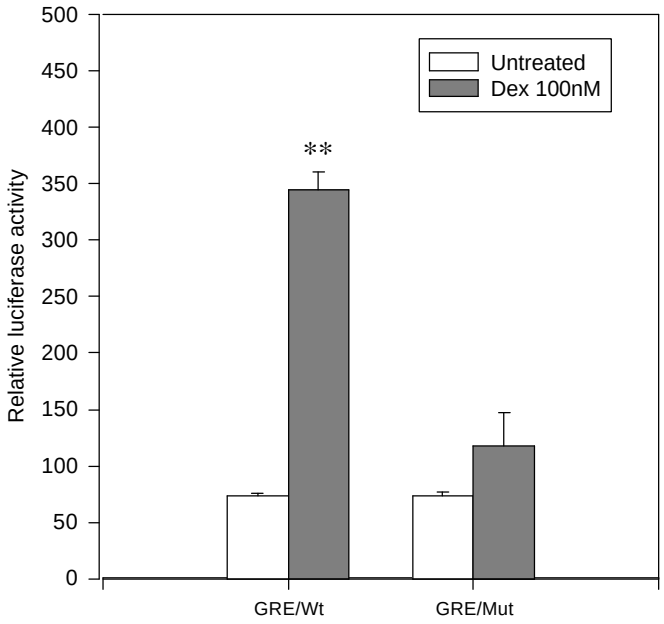


Fig. 4.12. Effect of site-directed mutagenesis in GRE site on luciferase activity. The name of GRE/Wt denotes pRL3-2291 as a control; GRE/Mut denotes pRL3-2291 with GRE mutated binding site. Data are presented as means± SD. Student's t-test is used to calculate statistical difference. ∗ $p<0.05$ vs. untreated; ∗∗ $p<0.01$ vs. untreated.

4.3.5 Confirmation of GRE by EMSA

To confirm that the glucocorticoid receptor can bind to canonical GRE that we have identified in seabass amylase promoter, EMSA was carried out using nuclear extracts prepared from AR42J cells. Incubation of the seabass α-amylase GRE with AR42J nuclear extract gave one major DNA-protein complex which was not observed with the mutated GRE oligonucleotide (Fig. 4.13). The complex formed by the seabass α-amylase

GRE and GR was progressively reduced by increasing amounts of excess cold GRE probe, while the mutant GRE oligonucleotide had no effect. The binding shift assay results further confirmed that the GRE (TGTACAcacTGTGAT) located at -891 to -876 mediates the response to glucocorticoid induction.

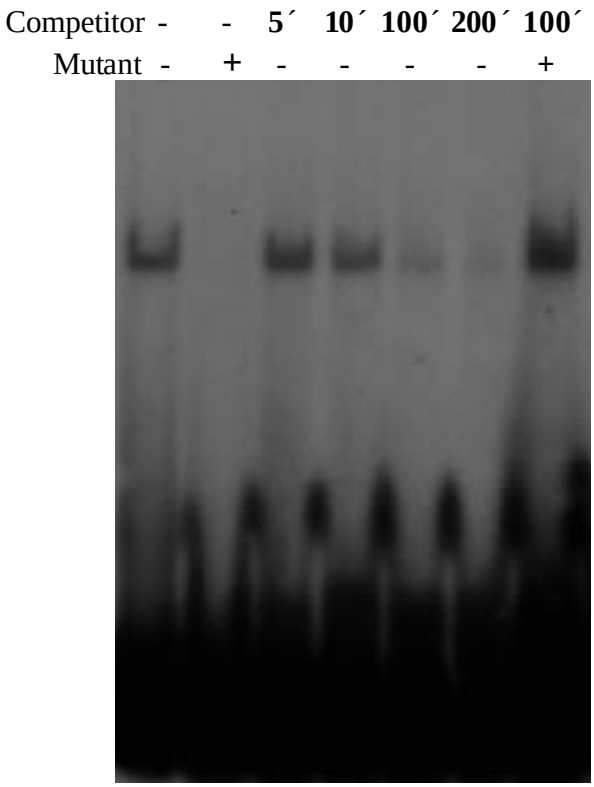


Fig. 4.13. Electrophoretic mobility shift assays on the glucocorticoid receptor binding to amylase promoter. Lane 1:DNA probe containing the wild-type GRE sequence (-891to -876). Lane 2: DNA probe containing the mutated GRE sequence. Lane 3-6: 5×, 10×, 50×,100× excess of the unlabeled consensus probes. Lane 7: 100× excess of the unlabelled mutated probe.

4.3.6 Regulatory elements of seabass amylase promoter

4.3.6.1. Putative Pancreas transcription factor (PTF) binding site in seabass amylase promoter

The cis-acting PTF has been shown to be involved in the control of the expression of pancreas-specific genes, such as α-amylase, elastase and trypsin (Cockell *et al*., 1989 and Yasuda *et al*., 1998). In human or mice, PTF-1 contains a bipartite binding site termed box A and box B. The box A and B are separated by one or two internal helical turns of B-form DNA (Petrucco *et al.*, 1990). The core sequence of box A is TTTCCC or TCCCAT and box B, CAC/GCTG, which is also within E box consensus sequence that binds to bHLH transcription factors.

Sequence analysis showed that -1209 to -1204 region contains a box A sequence (ATGGGA). However, the promoter deletion results indicated that this cis-element was not functional as pGL3-1176 activity (75 ± 3.50) was slightly but not significantly higher than pGL3-1416 (73.58 ± 12.16). Further promoter deletion studies revealed that the region from -593 to -335 and -335 to -149 contained regulatory elements of amylase promoter, as luciferase activity dropped dramatically by 49.6% and 52.9% respectively. Sequence analysis showed that there were two potential transcription elements in this region (Fig. 4.7). One was pancreas transcription factor (PTF1) and the other was hepatocyte nuclear factor 3 (HNF-3). Site-directed mutation of PTF region (-344 to -330) showed no effect on amylase promoter activity (Fig 4.15); the EMSA results further confirmed that no functional PTF binding sites were present in seabass amylase promoter (Fig 4.14). Thus results showed no evidence for an involvement of PTF cis-elements in

the regulation of the seabass amylase gene expression. It remains to be seen whether functional PTF elements are located elsewhere in seabass α-amylase promoter. Nevertheless, with PTF1 discounted, the other potential transcription element, HNF-3, comes to the fore.

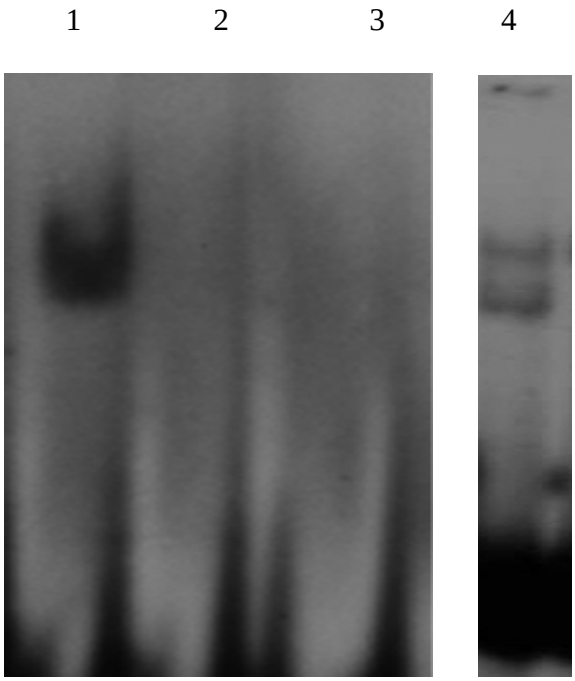


Fig. 4.14. Electrophoretic mobility shift assays on the pancreas transcription factor (PTF) and hepatocyte factor 3 (HNF-3) binding to amylase promoter. Lane 1: DNA probe containing consensus PTF sequence (ATGGAGTTCTGAAGAACCTTCAGCTG) used as a control. Lane 2: probe containing putative PTF sequence (-1210 to -1203) on amylase promoter. Lane 3: probe containing putative PTF sequence (-344 to -330) on amylase promoter. Lane 4: probe containing putative HNF-3 sequence (-328 to -320) on amylase promoter.

4.3.6.2. Hepatocyte nuclear factor 3 (HNF-3) required for expression of amylase promoter

Murine Hepatocyte nuclear factor 3 (HNF-3) is a family of developmentally regulated transcription factors that share homology with the winged helix/*fork head* DNA binding domain and participate in embryonic pattern formation. It is composed of HNF-3α, HNF-3β, and HNF-3γ proteins (Lai *et al*., 1991). HNF-3β also mediates cell-specific transcription of genes important for the function of hepatocytes, intestinal and bronchiolar epithelial cells, and pancreatic acinar cells (Rausa, *et al.,* 1997). Efficient expression of the α-amylase II gene requires binding of both PTF and hepatic nuclear factor (HNF)-3 β or (HNF)-3γ (Cockell *et al.*, 1995).

The -335 to -149 region contained regulatory elements of amylase promoter, as Luciferase activity dropped dramatically 52.9%. Sequence analysis revealed that -328 to -320 region contained a putative HNF-3 binding site and promoter deletion indicated that that HNF 3 binding site may be involved in regulating promoter activity. Furthermore, mutation of HNF site reduced the activity of seabass α-amylase promoter from 73.02±3.07 to 45.65±5.96 RLU (37.48 % loss of promoter activity) (Fig 4.15). Next we tested the ability of crude nuclear extracts from AR42J cells to retard an oligonucleotide containing the putative HNF-3 sequence (-328 to -320) on seabass α-amylase promoter; two differently migrating protein-DNA complexes were observed in EMSA (Fig. 4.14 lane 4). This indicated that HNF-3β and -3γ may play a role in seabass α-amylase promoter expression.

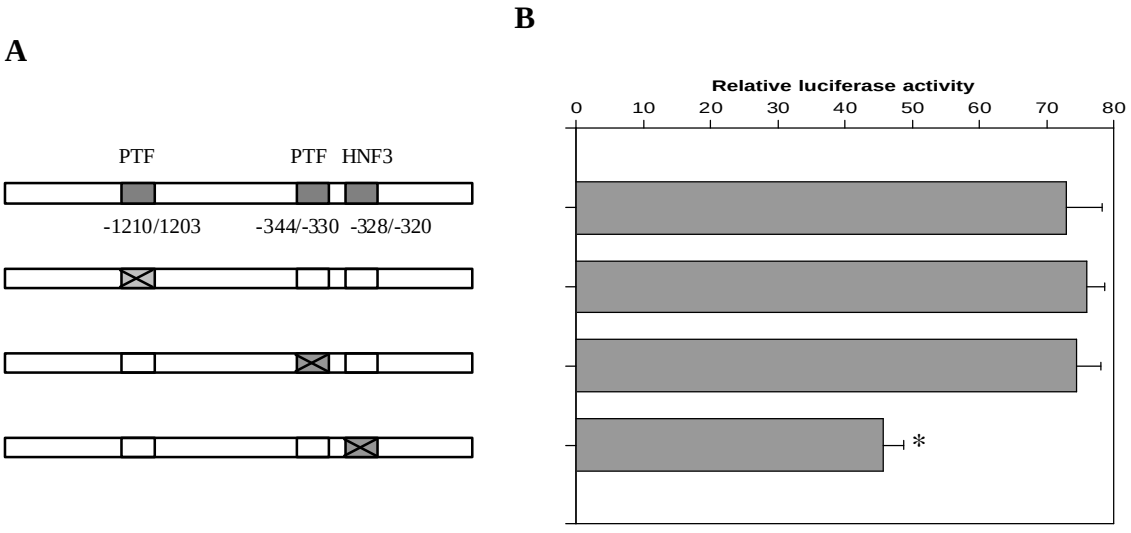


Fig. 4.15. Effect of site-directed mutagenesis in PTF and HNF sites. **(A)** Site-directed mutagenesis constructs of pGL3-2291 are shown. The location of PTF and HNF sites on promoter are indicated. **(B)** Transcriptional analysis of pGL3-2291 site-directed mutated constructs. Data are presented as means ± SD. Student's t-test to calculate statistical difference. ∗ $p<0.05$.

4.4. Discussion

4.4.1. Tissue specificity of amylase promoter

Even though pancreatic cells are believed to originate from the same ancestral cell type (Slack 1995), the terminally differentiated cells of the endocrine and exocrine glands

strictly express different sets of specific genes. Whereas exocrine cells synthesize and secrete digestive enzymes, endocrine cells synthesize and secrete different peptide hormones. The pancreas is present only in vertebrates and the fishes are lowest vertebrates. Thus, the fishes are excellent models to investigate phylogenetic development of the pancreas. The morphology diverges greatly among bony, cartilaginous and agnathan fishes and the standard pancreas morphology presented in higher vertebrates cannot be observed in some fishes.

Expression of the genes encoding digestive enzymes in the acinar pancreas has been used as a model for tissue-specific gene expression. Studies performed over the past years from a number of laboratories have resulted in the description of nucleotide sequences within the exocrine–specific genes which bind tissue-specific factors, including pancreas-specific factor 1 (PTF-1) (Cockell *et al*., 1989), pancreas-specific factor (Pan) (Nelson et al., 1990), exocrine pancreas transcription factor 1 (XPF-1) (Weinrich et al., 1991), and Hepatocyte nuclear factor 3β (HNF-3β) (Cockell *et al*., 1995). In this study, the luciferase expression level in rat pancreatic AR42J cells was dramatically higher than those obtained in non-pancreatic cell lines (Fig. 4.9). This indicated that AR42J cells provided required pancreatic transcription factors for expression of the amylase promoter, and seabass α-amylase promoter expression was tissue-specific. However, little is known about how these transcription factors are involved in exocrine pancreas gene expression and about the factors that are involved in exocrine cell differentiation (Kannius-Janson *et al*. 2000).

4.4.2. Functional characterization of the seabass α-amylase promoter

4.4.2.1. Identification of a functional glucocorticoid response element in the amylase promoter

Glucocorticoids are steroid hormones produced predominantly in the adrenal cortex in mammals. They play important roles in metabolism, immune responses, cell proliferation and differentiation. Glucocorticoid regulates the gene expression of many digestive enzymes in the exocrine pancreas. For example glucocorticoid increases amylase, trypsin and chymotrypsin gene expression and decreases kallikrein gene expression in rat pancreas (Kaiser *et al*. 1996). These effects are mediated by the glucocorticoid receptor (GR) which binds with high affinity in the presence of glucocorticoids to specific regulatory DNA sequences and regulates transcription of gene expression in either a stimulatory or inhibitory manner (Kaiser *et al*., 1996). When bound to its ligand, GR homodimerizes and translocates to the nucleus where it interacts with the specific DNA sequences, known as glucocorticoid response elements, in target genes. Thereby, transcription from these genes is increased or decreased (Beato *et al.* 1995). GRE is usually located in the 5' region of the promoter of glucocorticoid-sensitive gene. A consensus palindrome sequence for the GRE has been defined as GGTACAnnnTGTTCT, with the downstream half TGTTCT being the most important since initial binding by the GR monomer occurs at this site (Dahlman-Wright *et al*., 1991). However, the view that full GRE is required for GR binding is changing, since some half GRE sites are also functional, and glucocorticoid is effective in the presence of other transcription factors (Karin 1998, Morin *et al.* 2000, Tseng *et al*. 2001). In addition, more recent results have demonstrated that the GR can stimulate the expression of target genes without directly contacting a GRE (Rüdiger *et al.* 2002, Subramaniam *et al.* 2003). The direct protein-

protein interaction of the GR with other transcription factors such as AP-1, NF-κB, Stat3, Stat 5, and Stat6, was demonstrated to inhibit their transcription activity. This also seems to be the case for other nuclear receptors. For instance, the progesterone receptor has been shown to transduce its effect through Sp1 in the p21Cip1 gene (Owen *et al.* 1998).

The consensus GRE in human pancreatic and salivary amylase genes, and a 224-bp sequence of the upstream promoter region of human and mouse amylase were sufficient to direct amylase reporter gene expression and was responsive to glucocorticoid induction in rat or mouse acinar cell line (Slater *et a.l* 1993, Cockell *et al*. 1995). Two domains in this region are designated as elements I and II which function as general enhancers for pancreatic expression. The element I binds the pancreatic acinar transcription factor PTF-1, and element II present in both human and rat pancreatic amylase promoter is common to several immunoglobin genes and may be a binding site for a general transcription factor. In mouse α-amylase 2 gene, the DNA binding domain of the GR binds to a half site in the promoter as a monomer, and participation of PTF-1 is required for glucocorticoid induction (Slater *et al.* 1993).

We choose the pancreatic acinar cell line AR42J as a model system because it has been demonstrated that AR42J cells express functionally active GR (Rosewicz *et al*., 1989) and retain a variety of features characteristic of pancreatic acinar cells, e.g. expression of digestive enzymes, zymogen granules, expression of membrane-bound receptors (e.g. for bombesin, cholecystokininm, insulin), and a regulated secretory pathway (after stimulation with dexamethasone). In this study, a 2,291-bp 5' flanking sequence of seabass amylase gene was linked to the luciferase reporter gene and transfected into rat acinar AR42J cell line. Dexamethasone (Dex) stimulated amylase

promoter activity 5-fold; the degree of stimulation was comparable to that of mammalian amylase. Sequence analysis revealed that there are 14 potential half GRE and one imperfect palindrome GRE (TGTACAcacTGTGAT) located at region -947 to -776 (Fig. 4.7). Comparing the cis-elements for pancreas-specific expression between mammals and seabass, it is interesting to note that the putative homologues of PTF-1 binding site (element 1), half GRE and element II sites can also be found in the seabass amylase promoter within 360 bp upstream of transcription start site. However, further dissections of the promoter by 5' series deletion experiments and Dex treatment showed that the half GRE and the closely associated PTF1 were neither functional nor DEX-responsive. In contrast to the mammalian counterpart, we found that the sequence mediating glucocorticoid response was the imperfect full GRE located far upstream of the promoter region (-947 to -776). EMSA showed that there was a DNA-protein complex formed between the full GRE and the glucocorticoid receptor. The cold consensus probe could only compete with the DNA-protein complex at more than 100 × times excess; therefore the binding between DNA and GR protein is strong (Fig. 4.13). Site-directed mutagenesis of GRE severely impaired the response to DEX, showing its importance in mediating glucocorticoid stimulation.

Despite the lack of extensive sequence similarity between mammals and seabass amylase promoters, our results demonstrate that they share a common GRE although its position is not conserved. During vertebrate evolution, the conservation of GRE appears critical for glucocorticoid functions. For example, the main neuroendocrine control mechanisms of the integrated stress response of fish are comparable to those of mammals and thus conform to a general vertebrate pattern (Wendelaar 1997). Glucocorticoids play a dominant role in stress response, and an animal tries to cope with a stressor by reallocation

of energy via hydrolysis of glycogen. In the stressed state, glucocorticoid secretion is elevated, and we hypothesize that this activates the GR-transcription system, thereby upregulating amylase gene expression and increasing glycogen hydrolysis.

4.4.2.2 Cis-elements for exocrine pancreas-specific expression

The α-amylase enzyme is synthesized specifically in pancreatic acinar cells and is an excellent exocrine pancreas-specific marker. A 224-bp sequence of the upstream promoter region of human and mouse amylase was sufficient to direct amylase reporter gene expression; this indicates that the 224-bp region contains much of the regulatory information for directing gene expression in mammalian pancreatic acinar cells. Several cis-acting elements have been identified to be involved in the control of amylase gene expression, such as pancreas transcription factor-1 (PTF1), exocrine pancreas transcription factor (XPF), and hepatocyte nuclear factor 3 β or 3γ (Slater *et al.* 1993; Cockell *et al*.1995).

The PTF1 has three distinct subunits, which are all members of the family of basic helix –loop-helix proteins (Krapp *et al*., 1998). p75 does not contact DNA directly but is required for import of the factor into the cell nucleus (Sommer 1991). The two DNA-binding subunits (p64 and p48) recognize the cis-element as a heterodimer, a bipartite cognate site that contains two distinct sequence motifs. p64 contacts a TGGGA sequence whereas p48 binds to CANNTG, the canonical binding site for bHLH proteins (Cockell *et al*., 1989; Roux *et al.*1989). The conserved structure of bipartite PTF-1 binding site has been identified in 300 bp of 5' flanking region of pancreas-specific genes in mammals (Table 4.4). For example, mouse amylase II promoter contains a PTF-1 binding site

(ATGGGAGTTTCTGAAGAACCTTCAGCTGTGG), which is comprised of box A (ATGGGA) and box B (CAGCTGTGC) motifs separated by two DNA turns. The TTTCCC motif has also been found in the 5' flanking region of elastase *2*, chymotrypsinogen, and trypsin gene while the ATGGGA motif has been found in 5' flanking sequence of carboxypeptidase A gene (Cockell et al., 1989). The conserved structure for PTF-1 binding site consisting of box A (TTTCCC or ATGGGA) and box B ($CA^{C}/_{G}CTG$) motifs separated by one or two DNA turns is present in the 5' flanking regions of all pancreatic acinar genes in mammals. However, there are still large gaps in our understanding of the role of PTF1in pancreas development. A recent study showed that PTF1-p48 is expressed at early stages in the progenitors of pancreatic ducts, and exocrine and endocrine cells, rather than being an exocrine-specific gene as previously described (Kawaguchi *et al*., 2002). These experiments provide evidence that PTF1-p48 expression is specifically connected to the acquisition of pancreatic fate by undifferentiated foregut endoderm.

Table 4.4 Conserved PTF elements (Box A and B) associated with amylase gene

Element	Gene	5'-Terminus	Sequence
Box A	Rat Amy2	-154	T T C C A T G A G A G T T T C
	Mouse Amy2.1	-158	C T C C A T G A G G G T T T C
	Mouse Amy2.2	-158	T T G C G T G A G A G T T T C
	Mouse Amy2A	-164	T T C C A T G A G A G A C T T
	Mouse Amy2B	-164	T T C C A T G A G A G A C T T
	Seabass Amy	-354	T T C A G C A G A A G T T T C
Box B	Rat Amy2	-125	C A G C T G T
	Mouse Amy2.1	-131	C A G C T G T
	Mouse Amy2.2	-131	C A C C T G T
	Mouse Amy2A	-137	T A C C T G T
	Mouse Amy2B	-137	C T C C T G T
	Seabass Amy	-96	C A C C T G T

However, it appears unlikely that PTF1 alone will be sufficient to modulate the expression of individual genes of the pancreas in response to different physiological stimuli or different nutritional needs (Slater *et al.*, 1993; Cockell *et al.*, 1995; Kannius-Janson *et* al., 2000). For example, efficient expression of the α-amylase II gene requires binding of both PTF1 and hepatic nuclear factor (HNF)-3β or HNF-3γ (Cockell *et al.*, 1995). HNF3 family of transcription factors have been implicated to play a role in determining the fate of embryonic cell populations undergoing commitment to specific lineages (Clevidence *et al.*, 1993; Sasaki and Hogan 1993). HNF3α and HNF3β have a role in specifying the formation of definitive endoderm and gut tissue (Sasaki and Hogan 1993). Less is known about the early developmental role of HNF3γ. HNF-3β is important not only for hepatocyte-specific gene expression, but also participates in gene regulation in epithelial cells of the esophagus, trachea, lung, stomach, intestine and pancreas (Rausa *et al.*, 1999). HNF-3β regulates the transcription of the α-amylase gene in the pancreatic acinar cells (Cockell *et al.*, 1995). Furthermore, HNF-6, a liver enriched transcription factor, is required for HNF-3β promoter activity and also recognizes the regulatory region of numerous hepatocyte-specific genes (Rausa *et al.*, 1997). More detailed analysis of HNF-6 and HNF-3β's developmental expression patterns provides evidence of co-localization in hepatocytes, intestinal epithelial, and in the pancreatic ductal epithelial and exocrine acinar cells. The authors proposed that maintenance of the HNF-3β promoter expression in embryonic and adult hepatocytes is due to collaboration between the cut-homeodomain HNF-6 protein, bZIP (C/EBPa and C/EBPb), and PAR bZip (VBP) family members, and the orphan receptor family member fetoprotein transcription factor (FTF), which recognizes a critical HNF-3β promoter binding site (UF2-H3). Activation of the

HNF-3β promoter also involves autoregulation and cross-regulation by the HNF-3α protein (Fig. 4.16). They also proposed that HNF-6 regulates pancreatic expression of HNF-3β in the embryonic and adult exocrine acinar cells. Furthermore, HNF-3β regulates the transcription of the *pdx*-1 gene, a homeobox transcription factor that is necessary for the formation of the pancreas. In fish, an HNF-3 family member from Atlantic salmon was characterized (Stenson *et al*., 2000). Expression of HNF-3 protein in adult salmon tissues was not exclusive to liver but was also present in the pancreas and intestine. Furthermore, from studies of the rat elastase I promoter, an exocrine-specific enhancer including three different elements (A, B and C) was identified. The factor binding to the A element is PTF1. To the B element a complex binds containing pancreatic and duodenal homebox transcription factor 1 (PDX1), PBX1B and MRG1 (the product of a melanocyte-specific-gene) (Swift *et al*., 1998), while the factor that binds to the C element is still not identified (Kruse *et al*., 1993).

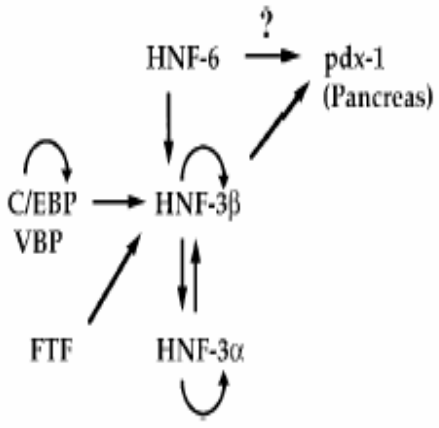


Fig. 4.16**.** Model for regulation of HNF-3β expression by cell-restricted transcription factors in liver and pancreas. Schematically shown is the regulation of HNF-3β in which arrows indicate positive stimulation and curved arrows indicate autoregulation (From Rausa *et al.*, 1997).

Comparing the cis-elements for pancreas-specific expression between mammals and seabass, it is interesting to note that the homologues of PTF-1 binding site (box A), GRE half-site and element II sites are also found in the seabass amylase promoter within 360 bp upstream of the transcription start site. In contrast to the mammalian counterpart, we found in the seabass α-amylase gene that the imperfect full GRE located far upstream of the promoter region (-947 to -776) is important, and not the half-site. Further mapping of the upstream region of full GRE revealed a PTF1 (Box A), but deletion studies suggested that the putative PTF was not involved in DEX stimulation (Fig. 4.11).

The upstream of fish and mammalian α-amylase promoter is distinctively divergent. The region -169 to transcription start site of mammalian amylase promoter is sufficient for tissue-specific expression and hormone regulation, in contrast, the corresponding part of seabass amylase promoter was not induced by Dex treatment, and the palindrome GRE was located at far upstream region (-947 to -776). The cis-acting PTF has been shown to play an essential role in the transcriptional control of pancreas-specific genes, such as α-amylase, elastase and trypsin (Cockell *et al.* 1995, Yasuda *et al.* 1998). Strikingly, no such sequence-function correlation was found in seabass α-amylase promoter. Sequence analysis of promoter revealed only three partial PTF-1 binding sites and our EMSA results further confirmed that no functional PTF binding sites were present in seabass amylase promoter (Fig. 4.14). However, site-directed mutation experiment revealed that HNF-3 may be a positive regulatory element in seabass amylase promoter. In mammals, HNF-3 is not only important for hepatocyte-specific gene expression, but also mediates cell-specific transcription of genes in the exocrine acinar cells (Rausa *et al.* 1997). In this study, efficient expression of the α-amylase promoter required binding of HNF 3β or 3γ, as the

promoter lost 37.48% activity when the HNF-3 binding site was site-directed mutated and EMSA also revealed two differently migrating DNA-protein complexes. Furthermore, when amylase constructs were transfected into Hela, CHO and medaka testis cell lines, the expression level was very low (data not shown). This is consistent with the requirement of HNF-3 because Hela, CHO and medaka testis cell lines have neither PTF1 nor HNF3 factors. This indicated that sufficient expression of α-amylase promoter required pancreas transcription factors and the expression was tissue-specific. Introduction of p48 and p64 into Hela cells allows the formation of a bHLH complex and activates transcription of an ELA1 minienhancer, which is a simple model for the control of pancreatic acinar cell-specific transcription (Rose *et al*., 2001). This study suggests that non-pancreatic cells can be reconstituted of acinar cell-specific activity when some pancreas transcription factors are introduced.

Numerous transcription factors involved in pancreas development have been characterized (Edlund, 1998; Kim and Hebrok, 2001; Assouline *et al*., 2002). Pdx1 (pancreatic duodenal transcription factor 1), a homeobox gene, was identified as the earliest pancreas-specific marker (Fig. 4.17). Although Pdx1 is the earliest marker for pancreatic cells and is expressed both in the endocrine and exocrine pancreatic cells in the early development of the pancreas, its expression can be detected only in beta cells in adult pancreas. Islet-1, a LIM homeodomain protein gene, is initiated soon after the islet cells have left the cell cycle (Ahlgren *et al*., 1997). It is expressed in all islet cells in adult pancreas. The islet-1 knock-out mice fail to form the dorsal pancreas completely with loss of differentiated islet cells in the ventral pancreas though the exocrine cells in ventral pancreas develop normally. Furthermore, the dorsal exocrine cells differentiation can be rescued by the provision of mesenchyme of wide type embryos while endocrine cells

differentiation is still deficient (Ahlgren *et al*., 1997). These results suggest that islet-1 is required for generation of all endocrine islet cells as well as for the development of the dorsal exocrine pancreas. There are other identified transcription factor genes which are involved in differentiation of early endocrine pancreas, including Pax6, NeuroD, Pax4, Nkx2.2 and Nkx6.1. In comparing with endocrine pancreas, the mechanism controlling exocrine pancreas is less understood (Fig. 4.17). Although Mist1 and HNF-6 are expressed in exocrine cell lineages, their functions have not yet been assessed by inactivation experiments (Edlund, 1988; Grapin-Botton and Melton, 2000). One of the interesting features observed in this study is that the liver-enriched transcription factors binding sites, including HNF-3, HNF-1 and HNF-4, are all present in seabass amylase promoter (Fig. 4.7). A recent study showed that HNF4α, together with HNF1α, played a crucial role in the function of pancreatic β-cells (Eeckhoute *et al*., 2003). However, it is not clear whether HNF-4 and HNF-1 are involved in exocrine cells functions or exocrine specific gene expression. Further studies on seabass α-amylase gene expression and the relevant transcription factors may significantly increase our understanding of exocrine pancreas development and exocrine specific gene expression.

4.4.3 Evolution of α-amylase gene

The human and mouse pancreatic promoter (Amy-2) are 65 % conserved in the region -160 to +1, but there is no evidence of conservation of the salivary amylase genes (Amy-1) upstream of the first coding exon (Fig. 4.18). Since both human genes resemble mouse Amy-2 more than they do mouse Amy-1, it appears that the homogenization process changed the human salivary amylase sequence into a sequence

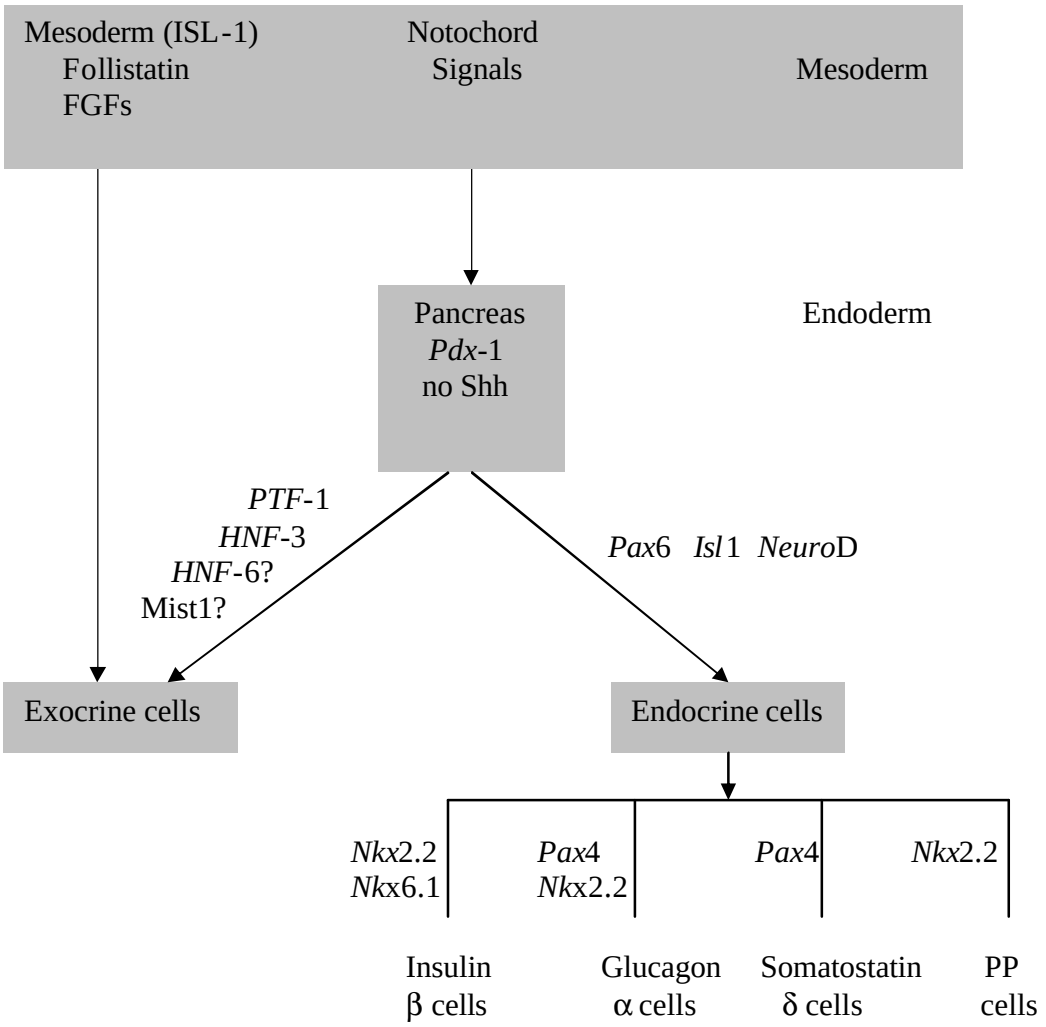


Fig. 4.17. Molecular pathway of cell fate choice in early pancreas development.
*Pax*6 and *Isl*1are expressed early and their disruption leads to the reduction or absence of endocrine differentiation. In *Isl*1mutant mice, *Pax*6is not expressed, which suggests that *Pax*6is downstream of Isl1. Although *Pax*4, *Neuro*D, *Nkx*2.2and *Nkx*6.1are expressed as early as *Pax*6and *Isl*1, they affect the differentiation of only a subset of lineages. *Pax*4is required for glucagon-producing and somtatostatin producing cell differentiation, *Nkx*2.2is expressed in all islet cells, except somatostatin-producing cells, and its inactivation leads to the absence of the cell types where it is expressed. *Nkx*6.1is itself required for insulin-cell differentiation (modified from Grapin-Botton and Melton, 2000).

derived from the pancreatic amylase gene (Gumucio *et al*., 1988). Furthermore, a nonhomologous, unequal crossover between a pancreatic amylase gene and a salivary amylase gene could have generated a human "fusion gene" containing the salivary-type promoter and non-translated exon (NTE) adjacent to the pancreatic-type promoter and structural sequences. Therefore, the authors proposed that all five of the human amylase genes may be recently derived from a pancreatic amylase precursor.

Despite the lack of extensive sequence similarity between mammalian and seabass promoter, several regulatory elements of mammalian pancreatic amylase promoter are present in seabass amylase promoter. This may suggest that teleost and higher vertebrate amylases share a common ancestor. The difference in regulatory region between the seabass and mammalian amylase genes may arise from the structural change for better adaptation to the function. This may also reflect a difference in GR in fish. In mammals, there are two classes of steroids, glucocorticoid and mineralocorticoid, each with its own receptor. Glucocorticoid receptor (GR) has a high affinity for dexamethasone, but low affinity for aldosterone; *vice versa* for mineralocorticoid receptor (MR) (Knoebl *et al.*, 1996). In contrast, cortisol is the major corticosteroid in teleosts where it plays a dual role in contributing to the regulation of both carbohydrate and salt/water balance (Bern & Madsen 1992, Wendelaar 1997). It has been suggested that fish use glucocorticoid for both metabolic and salt control through a single class of glucocorticoid receptor (GR) which may be the evolutionary precursor to mammalian GR and MR (Ducouret *et al*. 1995).

Yardley (1988) proposed that fish may utilize a "mixed" pancreatic-salivary α-amylase expression pattern. However seabass does not appear to use this strategy as seabass amylase promoter does not show homology with mammalian salivary promoter. Despite the lack of extensive sequence similarity between mammalian and seabass amylase promoter, several cis-elements of mammalian pancreatic amylase promoter are present in seabass amylase promoter. Our results showed no evidence for an involvement of PTF in seabass amylase promoter activity. Similar study on zebrafish elastase (elaA-gfp) has also shown that no functional PTF was identified in its promoter (H Wan and Z Gong, unpublished data). Furthermore, characterization of the trout insulin promoter shows no evidence for an involvement of bHLH factors (Argenton *et al.,* 1997). To date, there are only these three fish pancreas-specific gene promoters available, and in each case a functional PTF was not identified. It remains to be seen whether functional PTF elements are located elsewhere in seabass amylase gene. Therefore it will be interesting to study PTF in fish and show whether PTF is absent as a gene. Further studies on fish amylase gene expression and regulation mechanisms may reveal fascinating evolutionary differences and offer some clues for exocrine pancreas origin and evolution.

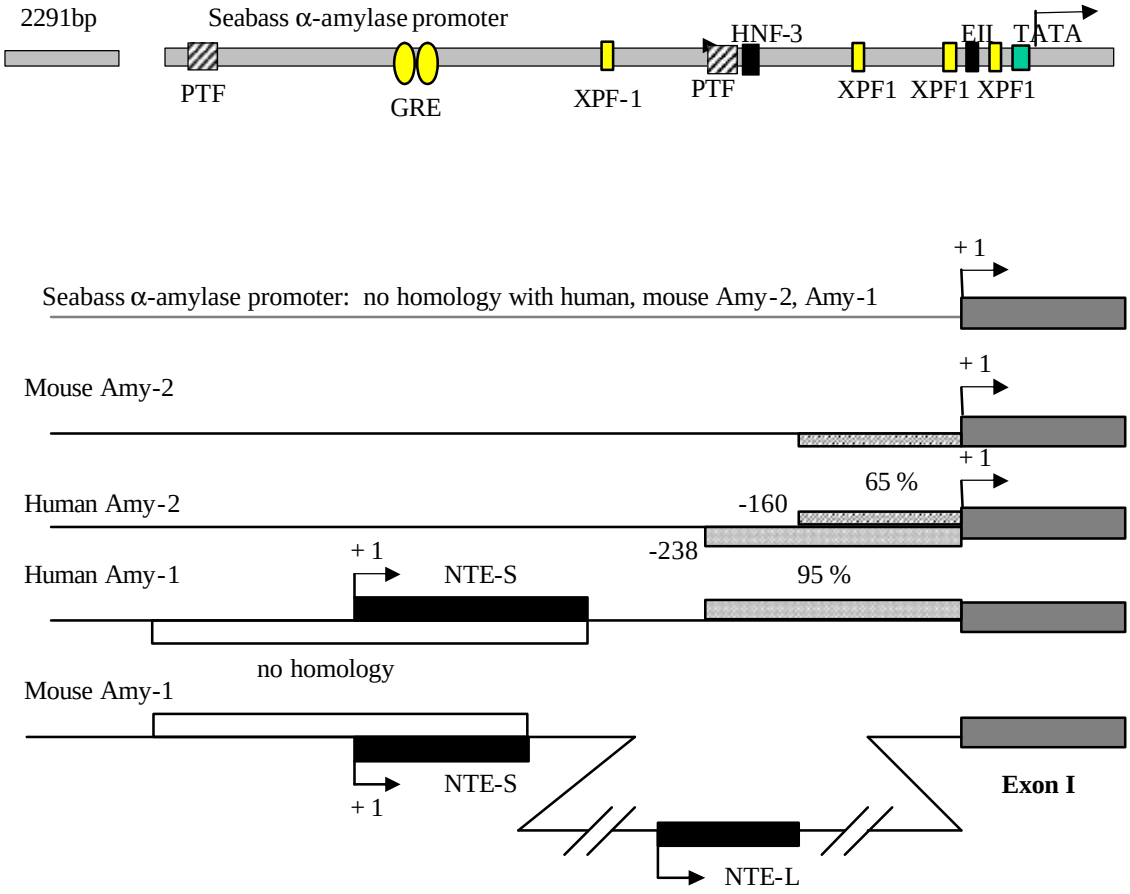


Fig. 4.18. Relationships among the 5' regions of the human, mouse and seabass amylase genes. The boxed sequences were compared for homology (modified from Gumucio *et al.*, 1988). NTE: non-translated exon.

CHAPTER FIVE

HORMONAL INFLUENCE ON AMYLASE GENE EXPRESSION

5.1 Introduction

The beneficial potential of hormonal application in aquaculture has been widely explored. Exogenous treatment with cortisol and thyroid hormones has been shown to enhance larval growth /development in various fishes (Lam 1994; Brown and Kim, 1995; Mathiyalagan *et al*; 1996; Kim and Brown, 1997). The profiles of cortisol and thyroid hormones during the ontogeny of the Asian seabass have been measured and shown to follow a similar pattern (Dayanti *et a*l; 1994; Sampath-Kumar *et a*l, 1995). Fertilized eggs contained varying levels of cortisol presumably originated from the mother. Cortisol levels declined as embryogenesis proceeded. The rate of decline was fairly constant and the decline continued up to about 4 hours post-hatching when cortisol reached minimum levels. Then the levels started rising steadily, and the rise is presumed to be the result of onset of endogenous cortisol production by the larvae. The onset of this rise is much earlier than that reported for other species. As for thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3) levels declined in newly hatched larvae and then increased in 24-hour old larvae. The levels increase before complete yolk sac resorption, and this suggests that the onset of thyroxinogenesis occurs within the first day after hatching in seabass larvae. Following endogenous thyroxinogenesis, the T_4 level in larvae increased dramatically around 6 dph, peaked around 12 days, and the level decreased and remained steady for up to 30 dph. However the T_3 concentration in larvae did not show a similar surge as T_4 but remained at a lower level than T_4 throughout the early ontogeny. The T_3 decreased within the first 24 hours after hatching and began to increase on the second day

after hatching, similar to the whole body T_4 concentration. In Japanese flounder cortisol and thyroid hormone levels have also been found to follow similar patterns throughout early development; both were present in eggs and peaked simultaneously during metamorphic climax (De Jesus *et al*. 1991).

It is probable that hormones interact with one another in the regulation of development. Cortisol was shown to increase the effectiveness of thyroid hormones in the regulation of larval development, and increase the conversion of T_4 to the active T_3 in the toad larvae *Bufo boreas* (Hayes and Wu 1995). Brown and Kim (Brown and Kim 1995; Kim and Brown 1997) treated Pacific threadfin larvae with T_3 and/or cortisol and observed acceleration in development and improvement in larval survival, at least in part by advancing the timing of initial intestinal absorptive function. The activities of amylase and serine protease were increased throughout the experimental period.

The hormone-dependent increase in digestive capacity coincident with first feeding will improve nutrient utilization; however the underlying mechanisms are far less understood. In this chapter, we present studies undertaken to investigate the effects of cortisol and T_3 on α-amylase gene expression during early developmental stages of seabass larvae. At the same time, the effect of food restriction and deprivation in seabass larvae on α-amylase gene expression was also studied.

In the preceding Chapter, dexamethasone, a potent glucocorticoid, was shown to induce α-amylase promoter activity *in vitro.* Whether cortisol, another glucocorticoid, would similarly stimulate α-amylase promoter activity thereby promoting α-amylase gene expression *in vivo* was therefore of interest. Combining molecular and cellular mechanisms with effects at organismal level will allow us to have a better understanding

of physiological roles of cortisol and thyroid hormone, and the effects of food restriction and deprivation on amylase gene expression.

5.2 Material and Methods

All general procedures, such as rearing and sampling larvae, RNA extraction and DNA preparation, ligation and restriction enzyme digestion, transformation and sequencing, and real time PCR assay, are described in Chapter 2. Cell culture, transfection and luciferase assay are described in Chapter 4.2.

5.2.1. Preparation of fetal bovine serum

Thyroid hormones (TH) were removed from the fetal bovine serum (FBS) by the protocol of Samuels *et al.* (1979). For each ml of FBS, 50 mg of AG1-X8 resin with mesh size of 100 to 200 μm (Biorad) was used. The AG1-X8 resin was washed three times with sterile ddH_2O to remove the fine particles before use. FBS was mixed with the resin in 50 ml Falcon tube and continuously shaken for 5 hours at room temperature on a rotary platform. The AG1-X8 was then pelleted by centrifugation at 1,000 g, 4 °C for 10 minutes. The FBS was transferred into new 50 ml Falcon tubes and the same volume of fresh resin was added. The mixture was again shaken at room temperature for a further 15-18 hours. Finally the FBS was cleared by centrifugation at 1,000 g at 4 °C for 10 minutes to remove the large resin particles and 30,000 g at 4 °C for 10 minutes to remove the fine resin particles. Finally, the TH-stripped FBS was filter sterilized, aliquoted and stored at -20 °C.

5.2.2. Hormone treatment of seabass larvae

T_3 and cortisol are purchased from Sigma. The T_3 stock solution was prepared fresh just before use. The powder was mixed with 5 μl of 1M NaOH before appropriate amount of ddH_2O was added to give the final concentration. The cortisol stock solution was also prepared fresh and dissolved in 100 % ethanol as quickly as possible. The appropriate amount of ddH_2O was added to give the final concentration. A control medium was included in which the same amount of ethanol was added. The media were renewed daily. The larvae were divided into 15 groups and treated by immersion in T_3 (5nM, 10nM) or cortisol (100nM, 200nM) with triplicates for each treatment. Sampling was carried out in the morning before feeding. Subsequently, the levels of α-amylase mRNA in the larval samples were analyzed by real-time RT-PCR method. The experiment was terminated on the 30^{th} day.

5.2.3. Diet restriction

12 dph seabass larvae were divided into four groups with triplicates tanks for each group. In the control group, larvae were fed with one-day-old *Artemia* nauplii (2 individuals ml^{-1}) daily until the end of study. The second group was fed with one-half of the *Artemia* ration. The third group was fed with one-fourth of the *Artemia* ration. The fourth group was fed one-eighth of the *Artemia* ration.

5.2.4. Food deprivation

In the control group (3 groups), 3 dph larvae were fed once a day with rotifers (2 individuals ml^{-1}), while in a second group, food was deprived and all the larvae died on 8 dph.

5.2.5. Enzymatic determination of glycogen

After amyloglucosidase hydrolysis the glycogen content of the whole body was measured according to Keppler and Decker (1974) with minor modifications. The frozen samples were homogenized in 5 ml of cold (0 °C) 0.6M perchloric acid (PCA) using a homogenizer. After centrifugation (10 min, 5000 ×g, 4 °C), 3 ml of the supernatants were neutralized with 1.5 ml 1.2 M KH_2CO_3. The neutralized extracts were carefully mixed to allow CO_2 to degas and potassium perchlorate to precipitate before centrifugation. Each supernatant was filtered (0.45 µm) and was frozen immediately at -20 °C for glycogen determination.

Glycogen was hydrolyzed by adding 600 µl of neutralized PCA extract to 1000 µl glycoamylase solution (10 kU/L, 0.2 M acetate buffer, pH =4.8). After which the mixture was incubated at 40 °C for 2 hours, the reaction was stopped by adding 500 µl 0.6 M PCA. The suspension was neutralized by the addition of 365 µl 1.2 M KH_2CO_3. After centrifugation, the supernatant was used for the determination of glucose. Aliquots of samples were transferred to separate cuvettes and 1000 µl reaction buffer (1 mM ATP, 0.9 mM β-NADP, G6P-Dh in triethanolamine/magnesiumsulfate buffer) was added. When absorbance (339 nM) was stable (around 5 minutes), the reading was taken (A1). Then 40 µl hexokinase suspension was added and mixed thoroughly. After 5 minutes, the reaction

was stopped and the absorbance was taken again (A2). All absorbances were corrected for volume differences. Under the given conditions the reaction proceeds stoichiometrically , therefore the glucose content in the homogenate, after subtraction of the free glucose present before hydrolysis, corresponds to the glycogen content of the homogenate. The glycogen content is expressed in µmol units per gram wet tissue.

5.2.6 Statistical analyses

Data from luciferase assay are presented as means ± SD after normalization by the *Renilla* luciferase activity. Each transfection reaction was carried out in triplicate and the experiment was repeated twice and shown to be reproducible. P values for comparison of samples were obtained by using the paired t-test with two tailed distribution using the Microsoft Excel program.

5.3. Results

5.3.1. Quantification of mRNA level of trypsinogen using Real-time PCR

Using a series of diluted trypsinogen plasmid DNA as templates, a standard curve for trypsinogen was obtained using Lightcycler real-time PCR. The primers for trypsinogen amplification are based on seabass trypsinogen gene sequence provided by Tan *et al.,* (2000) and underlined in Fig. 5.2. The quantitative changes in mRNA level of amylase and trypsinogen in developing seabass larvae are shown in Figure. 5.1. The real-time PCR conditions for amplification of these two genes are the same except for the primers. Primers for amylase are used as described in Chapter 3.

The trypsinogen mRNA could be detected on 1 dph. There was a significant increase at 3 dph, followed by a marked increase at 5 dph and remained high on 7 dph. However, the amylase mRNA could be detected as early as 0 dph (just after hatching); this was followed by an increase at 1 dph, and decreased on 3 dph. Amylase peaked on 5 dph; subsequently the mRNA levels decreased at 7 dph. Digestive enzyme genes in seabass, including trypsinogenogen, pepsinogen and aminopeptidase were cloned in our laboratory, and their expressions during larval development were studied (Tan *et al.*, 2001). Pepsinogen and aminopeptidase expression could be detected at 2 dph during larval development. Thus, it appeared that the ontogeny of digestive enzyme gene expression in seabass larvae follows a pattern in which amylase is the first to be expressed, followed by proteases. The timing of amylase gene expression suggests an importance of carbohydrates during the critical period of first feeding.

5.3.2. Induction of amylase promoter by cortisol and triiodothyronine (T_3)

The induction of amylase promoter activity in rat AR42J cells by Dexamethasone was presented in Chapter Four. In brief, amylase promoter activity was increased 5 fold in response to Dex treatment (100nM). Subsequently, triiodothyronine (T_3) induction of amylase promoter was also studied in rat AR 42J cells. T_3 induction of amylase promoter was tested by increasing the T_3 concentration (0-10^{-5} M). In the absence of T_3, the luciferase control was 48.9 ±8.79 RLU. With increasing concentration of T_3, the luciferase activity was not greatly affected with luciferase activity ranging from 56.8 ±17.65 to 44.57 ±7.21 RLU (Fig 5.3). After statistical analysis, the above results showed that there was no significant T_3 induction of the amylase promoter activity.

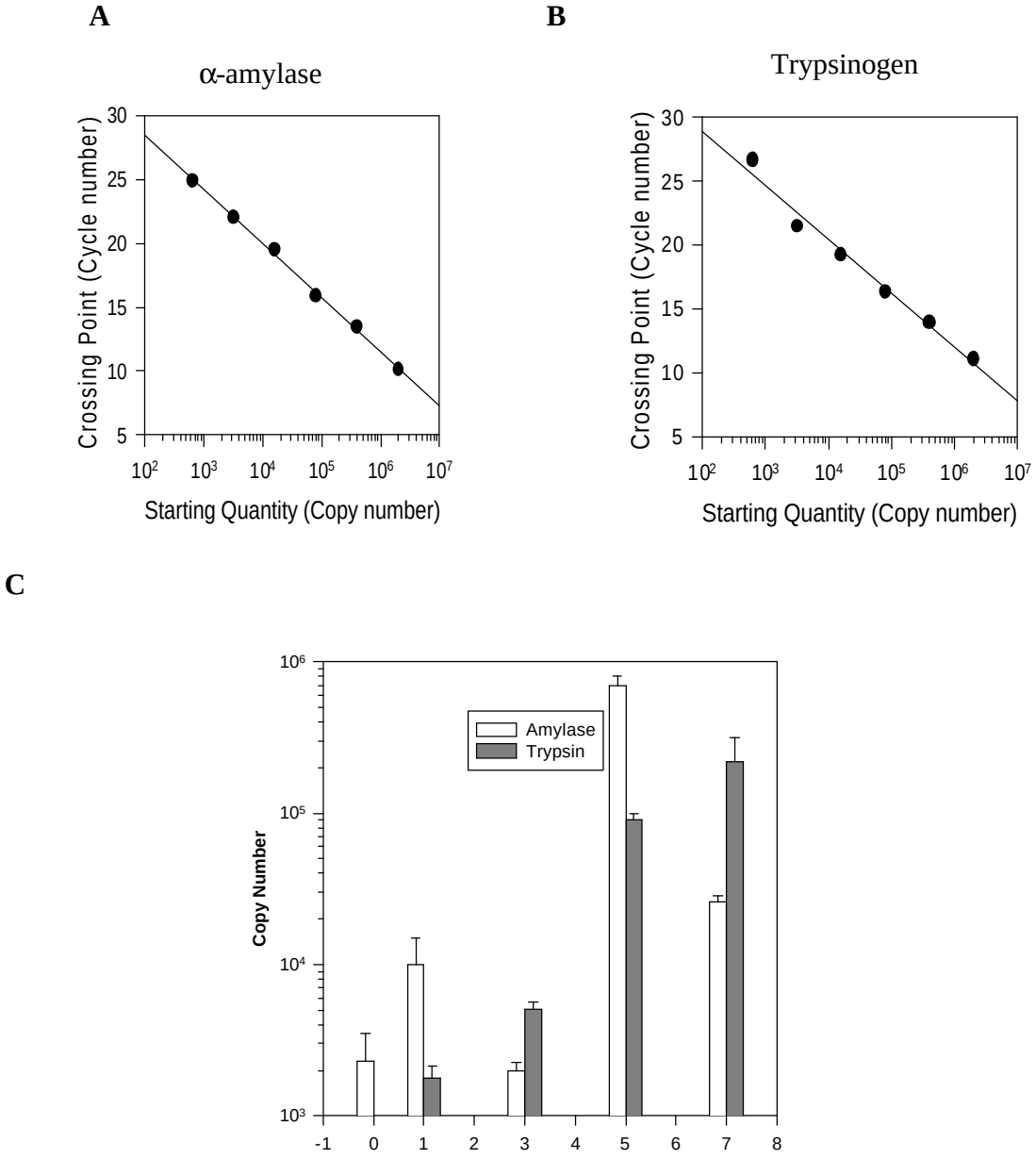


Fig. 5.1. Real-time PCR analysis of α-amylase and trypsinogen mRNA expression during seabass larval development. (A): Real-time PCR standard curves using a cloned amylase plasmid DNA as a template. (B): Real-time PCR standard curves using a cloned trypsinogen plasmid DNA as a template (a kind gift from Tan Siok Hwee and Konda Reddy, TMSI, NUS) and calibration curves obtained by correlating crossing point and plasmid copy number. (C). Data analysis of α-amylase and trypsinogen mRNA expression during larval ontogeny was based on the crossing point of each sample and the data were converted into copy numbers according to the standard curves obtained in (A) and (B).

```
1          GGCTACCACTTCTGCGGCGGCTCCCTGGTCAGCGAGAACTGGGTTGTGTCTGCTGCTCAC
1           G  Y  H  F  C  G  G  S  L  V  S  E  N  W  V  V  S  A  A  H

61         TGCTACAAGTCCCGTGTGGAGGTGCGTCTGGGCGAGCACAACATCAGGGTCACTGAGGGA
21          C  Y  K  S  R  V  E  V  R  L  G  E  H  N  I  R  V  T  E  G
                      Trypsinogen-F
121        ACCGAACAGTTCATCAGCTCCTCCCGTGTCATCCGCCACCCCAACTACAGCTCCTACAAC
41          T  E  Q  F  I  S  S  S  R  V  I  R  H  P  N  Y  S  S  Y  N

181        ATCAACAATGACATCATGCTGATCAAGCTGAGCAAGCCCGCCACCCTCAACCAGTACGTG
61          I  N  N  D  I  M  L  I  K  L  S  K  P  A  T  L  N  Q  Y  V

241        CAGCCCGTGGCTCTGCCCACCAGCTGTGCCCCCGCTGGCACCATGTGCAAAGTCTCTGGC
81          Q  P  V  A  L  P  T  S  C  A  P  A  G  T  M  C  K  V  S  G

301        TGGGGCAACACCATGAGCTCCACTGCTGACAGGAACAAGCTGCAGTGCCTGGACATCCCC
101         W  G  N  T  M  S  S  T  A  D  R  N  K  L  Q  C  L  D  I  P

361        ATCCTGTCCGACAGGGACTGTGATAACTCCTACCCTGGAATGATCACCCAGGCCATGTTC
121         I  L  S  D  R  D  C  D  N  S  Y  P  G  M  I  T  Q  A  M  F
              Trypsinogen-R
421        TGCGCTGGATACCTGGAGGGAGGCAAGGACTCTTGCCAGGGTGACTCTGGTGGTCCCGTT
141         C  A  G  Y  L  E  G  G  K  D  S  C  Q  G  D  S  G  G  P  V

481        GTGTGCAATGGTGAGCTGCAGGGTGTTGTGTCCTGGGGTCACAGATGTGC
161         V  C  N  G  E  L  Q  G  V  V  S  W  G  H  R  C
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Fig 5.2. The sequence of trypsinogen gene of seabass (Tan *et al*., 2001). The primers used in real time PCR are underlined.

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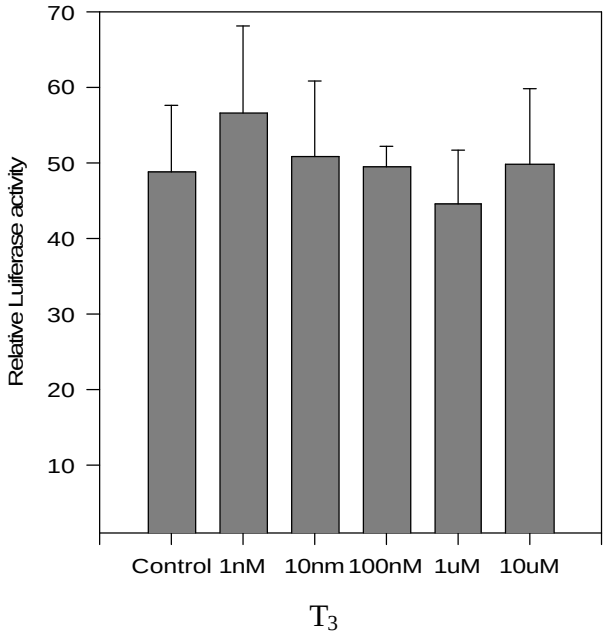


Fig. 5.3. Amylase promoter luciferase activity in increasing T_3 concentrations (0 to 10^{-5} M). The firefly luciferase activity presented was normalized by the *Renilla* luciferase activity. Data are presented as means± SD. Each transfection reaction was carried out in triplicate and the experiment was repeated twice. Student's t-test is used to calculate statistical difference. The means in increasing T_3 concentrations are not significantly differnet.

5.3.3. Treatment of seabass larvae with cortisol and T_3

Seabass larvae (2 dph) were treated separately with cortisol (100 nM and 200 nM) and T_3 (5nM and 10 nM) by immersion. At 3 dph, upregulation of α-amylase gene expression by cortisol treatment was observed (Fig 5.4), and 100nM cortisol seemed to be more effective that 200nM cortisol. No differences from the control were observed when the larvae were treated with 10 nM T_3. However amylase gene expression was upregulated with 5 nM T_3. At 5 dph, a similar pattern was observed in larvae treated with 100 nM cortisol and 5 nM T_3 increasing transcripts more effectively than 200nM cortisol and 10

nM T_3 respectively. A slight elevation of amylase gene expression at 7 dph was observed in larvae treated with 100 nM cortisol and 5 nM T_3, while 200 nM cortisol and 10 nM T_3 had no effects.

An elevation of amylase gene expression from 3 to 7 dph was observed in the controls in the absence of hormones. When larvae were treated with 100nm cortisol and 5nm T_3, there was an up-regulation in amylase mRNA expression compared with untreated individuals (Fig 5.4), suggesting that endocrine factors may regulate α-amylase at the transcriptional level. In summary, the hormone treatment appeared to increase transcripts more effectively in 100 nM cortisol and 5 nM T_3 than 200 nM cortisol and 10 nM T_3 respectively.

5.3.4. Larvae fed different *Artemia* rations and their effect on amylase gene expression

Artemia nauplii are usually given to satiation in normal conditions, and ½ ration could still be assumed to meet the requirement of seabass larvae; however one fourth and one eight rations represent a restricted diet condition for the larvae (J. Walford, personal communication).

Amylase gene expression in these four groups followed a similar pattern in which amylase mRNA decreased from 12 dph until the end of the experiment (Fig 5.5). This decrease was more marked and appeared earlier in the less restricted group (1/2) and the control group. In contrast, this decline was less pronounced in 1/4 and 1/8 groups and the α-amylase gene expression was relatively high in these groups compared to the control and 1/2 fed groups.

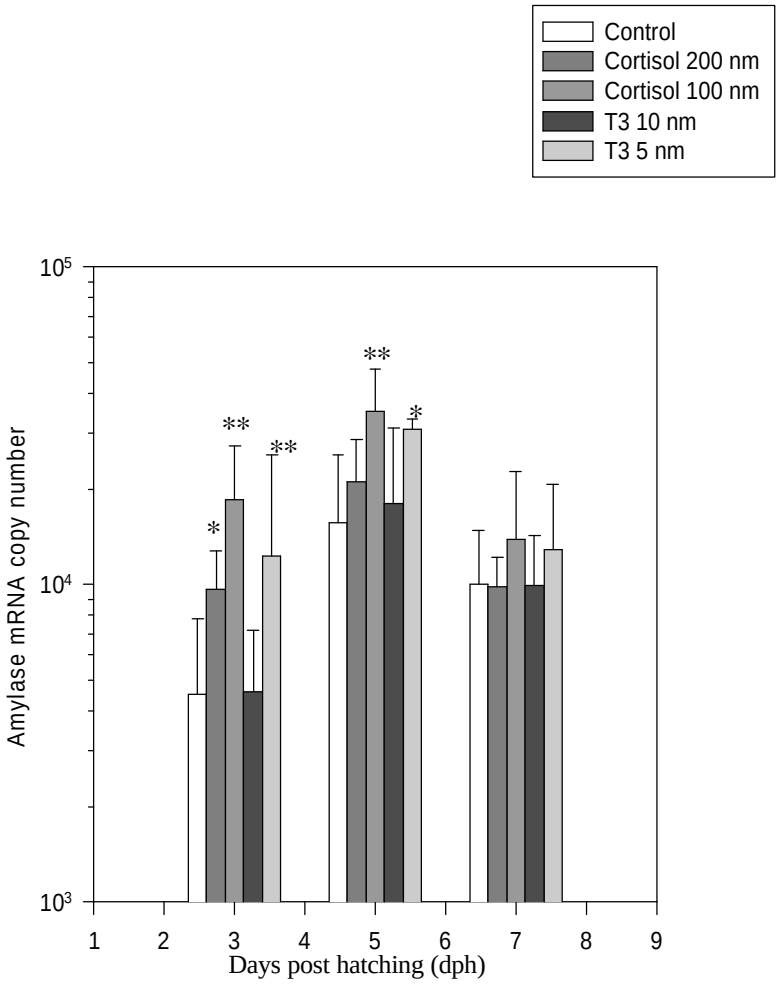


Fig. 5.4. Effects of cortisol and T_3 on seabass amylase gene expression at 3, 5, 7 dph. Cortisol treatments were done at concentrations of 100 nM and 200 nM, while T_3 treatments were performed at concentrations of 5 nM and 10 nM. Student's t-test is used to calculate statistical difference. 3 dph, ∗ $p<0.01$ vs. untreated; ∗∗ $p<0.001$ vs. untreated; 5 dph, ∗ $p<0.05$ vs. untreated; ∗∗ $p<0.01$ vs. untreated; 7 dph, ∗ $p<0.05$ vs. untreated.

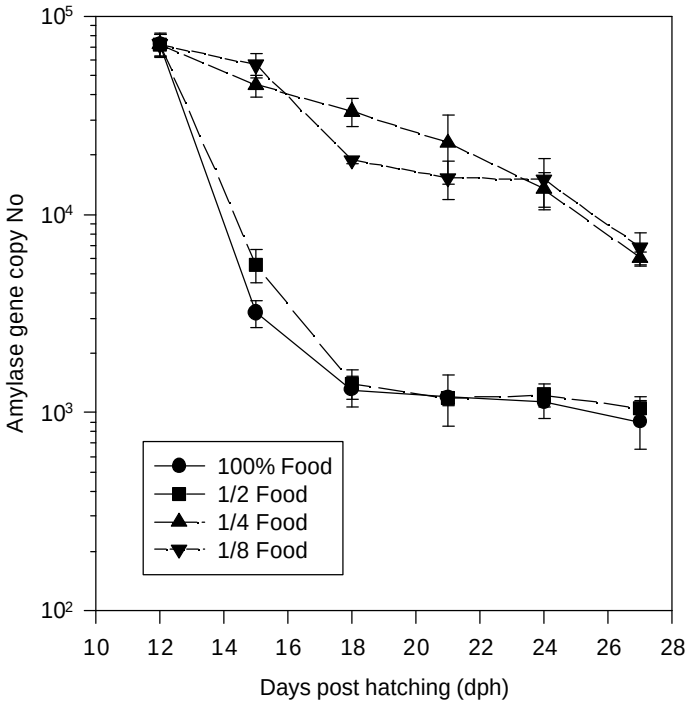


Fig. 5.5. Amylase gene expression in seabass larvae in four dietary groups. One group was fed to satiation (2 individuals/ml), and the other groups were fed one-half, one-quarter and one eighth ration respectively. Data are present as means±SD.

5.3.5 Amylase gene response to food deprivation in seabass larvae

To study food deprivation effect on amylase gene expression, the larvae were deprived of food right from 3 dph. For the control group, the larvae were fed once a day

with rotifers (2 individuals ml^{-1}); the experimental group was not given food. All the unfed larvae died on day 8. The amylase gene expression in unfed larvae was 5 fold higher that that in fed larvae on 5 dph, while on the other days the difference between fed and unfed group was not apparent (Fig. 5.6). Due to mass death on 7 dph, unfed group sampling was not enough for RNA extraction.

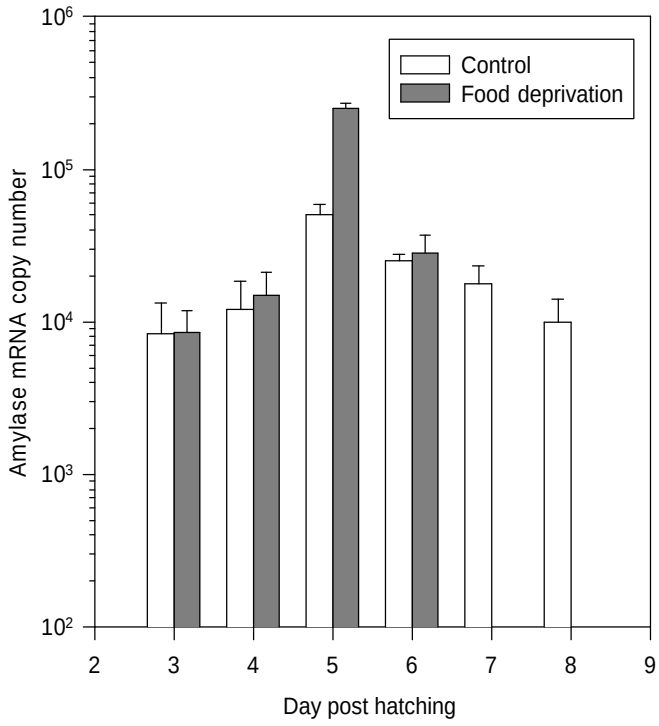


Fig. 5.6. Fasting effect on amylase gene expression.

5.3.6. Glycogen levels in fasting (food-deprived) larvae

The influence of food deprivation on glycogen was measured during early larval development. Compared with fed larvae, whole-body glycogen concentration was significantly lower in the fasting larvae from 5 dph (Fig. 5.7). The first feeding of larvae occurred on 3 dph in the control group. Glycogen level in food-deprived fish on 4 dph showed no significant difference compared with fed fish. However, there was a sharp decrease in glycogen concentration in food-deprived fish on 5 dph, and the level was almost undetectable on 6 dph. The glycogen concentration in fed larvae was maintained at a stable level. Most of the larvae died on 7 dph.

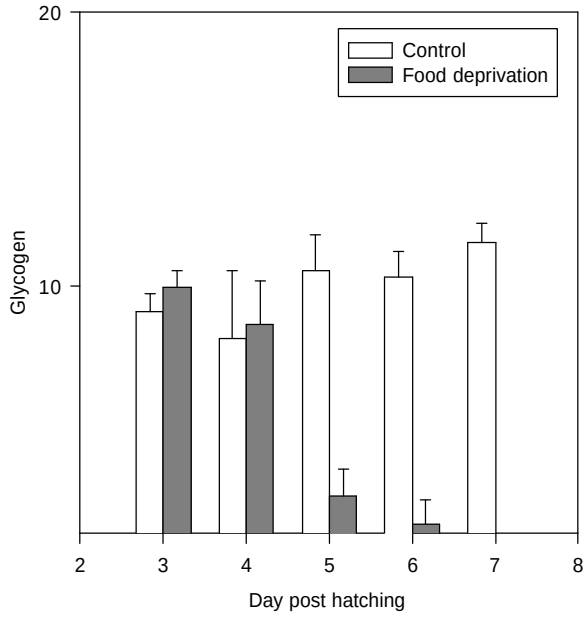


Fig. 5.7. Seabass whole body glycogen content in fed and unfed seabass larvae during early larval development.

5.4 Discussion

5.4.1 Onset of digestive enzymes

In seabass larvae, the differentiation of exocrine pancreas cells and the appearance of the excretory duct occur before mouth opening (~3 dph). Amylase and trypsinogen are produced by exocrine pancreatic cells. Amylase is responsible for the hydrolysis of α-amylase glycoside bonds in starch and glycogen. Trypsin has been shown to be an important indicator of nutritional status, and for detection of food limitation in the early stages of exogenous feeding of larval herring (Pederson *et al*. 1987, 1990). Trypsin is also useful to evaluate digestive processes and the nutritional condition of field-caught marine fish larvae that have been observed to have a significant correlation with food conversion efficiency in the Atlantic cod (*Gadus morhua*) (Lemieux *et al*., 1999).

Table 5.1 summarizes the different pancreatic enzymes detected in Asiatic seabass (*Lates calcarifer*), European seabass (*Dicentrarchus labrax*), sole, red drum and threadfin. Amylase and trypsinogen activities were detected before mouth opening in these species (Zambonino and Cahu, 1994; Ribeiro *et al*., 1999; Lazo *et al*., 2000; Brown *et al*., 2001; Ma *et al*., 2001). The presence of enzymatic capacity before first feeding suggests that those activities were not induced by food.

In recent years, there has been increasing interest to study the development of digestive enzymes in marine fish larvae in an attempt to facilitate the choice of feeding strategy. However, enzyme assay methods are inaccurate as the initial grinding of samples often give rise to undesirable reactions. In addition, enzyme assays are often performed under conditions that are different from those in the gastrointestinal tract. On the other hand, measurement of gene transcripts will be more accurate and specific. To achieve this

goal, genes for pepsinogen, trypsinogen, aminopeptidase, α-amylase were cloned in our laboratory and their expression pattern have been studied (Tan *et al*., 2001; Ma *et al.*, 2001).

Table 5.1 Detection of amylase and trypsin enzyme activities in five marine fish larval species

Fish	Amylase (dph)	Trypsin (dph)	References
Asian Seabass	1	1	Ma *et al*., 2001; Walford and Lam, 1993
Europe Seabass	4	4	Zambonino and Cahu, 1994
Sole	3	2	Ribeiro *et al*.,1999
Red drum	1	1	Lazo *et al*., 2000
Threadfin	1	—	Kim *et al*., 2001

In seabass, all three proteolytic enzymes were found to exhibit low levels of gene expression just after hatching and increase around the first feeding (Tan *et al*., 2001). In contrast, we have reported previously (Chapter 2) that amylase gene expression was detected as early as 0 dph, and amylase gene exhibited a higher expression level at the early stage compared to proteolytic enzymes genes. Thus the ontogeny of digestive enzymes gene expression in seabass exhibited a pattern in which α-amylase was the first to be expressed, followed by proteases later in early larval development. The timing of digestive enzymes gene expression in seabass indicates a greater importance of

carbohydrates during the period around first feeding than might be expected for carnivorous fishes.

Similarly in Pacific threadfin (*Polydactylus sexfilis*) and bluefin trevally (*Caranx melampygus*), amylase enzyme activity increased prior to first feeding, peaking in the middle of the larval period, and becoming nearly undetectable by the time of larvae-to-juvenile metamorphosis (Kim *et al.*, 2001); however protease and lipase activities increased during the second half of larval development. Peres *et al.*, (1998) demonstrated that higher amylase mRNA levels are found in young European seabass larvae (*Dicentrarchus labrax*) than in older larvae, and suggested that the coordinated decrease between specific activity and mRNA levels of amylase is transcriptionally regulated during larval development. The decline in amylase-specific activity assayed in dissected pancreatic segment in European seabass is linked to a decrease in mRNA coding for amylase. A correlation between enzyme activity and mRNA level of α-amylase has been more extensively demonstrated in early Asiatic seabass larvae in our study (Chapter 2; Ma *et al.*, 2001). In the winter flounder (*Pleuronectes americanus*), amylase transcripts peaked at approximately day 20, then decreased during metamorphosis (Douglas *et al.*, 2000). Therefore, amylase represents a good example for studying an ontogenetic change in enzyme expression during larval development.

5.4.2 Hormonal manipulation of seabass amylase gene expression

In recent years, the potential use of hormones in marine fish aquaculture to accelerate gastrointestinal maturation and increase feeding efficiency in marine fish larvae has been emphasized (Kim and Brown, 2000). Thyroid hormones and cortisol are particularly featured (Lam 1994; Kim and Brown, 2000). However, the way in which

hormones bring about their actions on digestive enzymes gene expression and the underlying mechanisms remain to be studied. Furthermore, there are inconsistencies and confusion in the literature regarding the action of the hormones towards their physiological effects. Much of the confusion is caused by differences in species, sampling procedures, seasonal and diet changes, photoperiod, nutritional conditions and sexual maturation of the fish (Audet *et al*., 1986; Vijayan and Moon, 1994; Reddy *et al*., 1995; Pickering *et al*., 1987; Mommsen *et al*., 1999). Due to the extreme sensitivity of endogenous hormones, the slightest experimental variation in parameters is likely to alter the hormone set-point and make it a difficult task to study the mechanisms of actions of hormones. However, linking molecular mechanisms to its functional physiology would lead to a better understanding of the multiple faces of hormones.

In our previous study (Chapter 4), the 2.4 kb amylase promoter was isolated and several important features were found in the promoter, including one imperfect palindrome GR element, 14 half GRE sites and several pancreas transcription factor elements.. The induction of amylase promoter by 100 nM Dex was 5 fold after 48 hours of treatment. However, when the binding site (**TGTACACACTGTGAT)** was mutagenized to a sequence (**TGTCCGCACTCGCGT**) that would not be recognized by the glucocorticoid receptor, the induction of amylase promoter by Dex was almost eliminated. Site-directed mutagenesis of the putative GRE and EMSA confirmed that this region was responsible for Dex induction.

Identifying a functional GRE-like motif on seabass amylase promoter suggests that cortisol could act directly on amylase gene transcription by binding to GR and then to GRE. This is supported by the *in vivo* studies in which seabass larvae were treated with cortisol; the results showed that cortisol increased amylase gene expression.

On the other hand, sequence analysis showed that there were no thyroid hormone response elements (TRE) on seabass amylase promoter. Furthermore, when amylase promoter was transfected into the rat AR42J cells, the amylase promoter construct activity was not significantly affected by the presence of varying concentrations of T_3 (0-10^{-5} M). This indicated that a functional binding site is required for T_3 induction. This suggests that thyroid hormone may not have a direct effect on amylase gene expression. However when seabass larvae were treated with T_3, amylase gene expression was promoted at least at 5 nM T_3. This suggests that T_3 may stimulate amylase gene expression *in vivo* indirectly. Because thyroid hormones can increase epidermal mitotic rate by controlling the synthesis of specialized proteins during cell differentiation within the digestive system (Hourdy, 1993), T_3 stimulation of amylase gene expression may be secondary to its promotion of one or more developmental processes.

5.4.3 Effects of food rationing and deprivation on amylase gene expression

Fish larvae can show a different level of digestive enzyme activity when fed with a compound diet compared to live prey (Zambonino Infante and Cahu, 1994a; Zambonino Infante and Cahu, 1994b). This response may be the result of enzymatic adaptation to a specific nutritional substrate contained in the compound diet or may reflect malnutrition induced by insufficient diet utilization leading to poor growth as observed in larvae fed compound diets. It is difficult to evaluate the relative importance of each phenomenon on the digestive enzymes changes because studies on the assessment of the effects of under-nutrition are very few. When larvae were fed with ½ ration of food in this study, the amylase gene expression maintained a similar pattern of rapid decline as the control group

which was fed with full ration of food (Fig.5.5). However, the amylase gene showed a significantly slower decline in groups fed more restricted rations (1/4 and 1/8). As expected, growth was directly related to food ration as growth was significantly slower in 1/4 and 1/8 groups. Survival was not affected by ration for the duration of this experiment (12-27 dph). Similar study was performed in European seabass (*D. labrax*). In this case, amylase and leucine-alanine peptidase enzyme levels were measured instead of mRNA levels. The enzyme levels were maintained at a higher level at day 38 in highly food-restricted larvae (1/4 and 1/8 of the satiation ration) compared to the satiated larvae (Zambonino Infante *et al.,* 1996). Thus the overall amylase picture for food restrition was similar as in our study.

It has been shown that the dietary starch content can modulate the decline in amylase-specific activity in late larval development. This decline was slower in European sea bass larvae fed a diet containing 25% glucides than in larvae fed a diet with only 5% glucides (Peres *et al*., 1998). Amylase adaptation to the starch level of the diet has been extensively described in mammals (Sheele, 1993) and fish (Kawai and Ikeda, 1973). Red drum larvae exhibited an efficient modulation of amylase synthesis in response to incorporation of starch in the diet (Buchet *et al.,* 2000). In European seabass larvae, modulation in amylase activity by dietary starch content is also efficient from day 18 after hatching, but levels of mRNA transcripts of amylase remain unchanged, suggesting that the change of amylase enzyme activity may be the result of a post-transcriptional regulation of amylase synthesis (Peres *et al*., 1998).

This seems to contradict the above discussion of the relationship of food restriction and amylase specific activity or gene expression. A lower ration of the same food should reflect a lower starch content and yet the decline in amylase specific activity or gene

expression was slower, contrary to the effect of a reduced dietary starch content (Peres *et al*., 1998). The difference may lie in the fact that in the former the total amount of food was reduced while in the latter the total amount of the food remained the same; only the starch content was reduced. It is conceivable that the former constitutes a much greater stress than the latter. It is possible cortisol was secreted as a stress response in the former thereby upregulating amylase gene expression as discussed above.

Fasting (food deprivation) represents a drastic change in condition. It has been reported in mammals that fasting can induce conflicting enzymatic responses as a result of stress (Raul *et al*., 1982). In the case of fish larvae, death occurs very quickly. In unfed larval threadfins, amylase activity was relatively lower in the fed larvae, yet the amylase activity increased followed by a sharp decrease before die-off (Kim *et al*., 2001). Food deprivation in this study resulted in a significant increase in amylase gene expression (5 fold higher than control at 5 dph), whereas the glycogen level at 5 dph was dropped to nearly undetectable level in unfed larvae. The findings are interpreted as a stress response whereby cortisol secretion was elevated which activated the GR-transcription system and upregulated amylase gene expression.

Sustaining the higher glucose production during stress is due to two pathways: 1) gluconeogenesis; and 2) glycogenolysis. Vijavan *et al*., (2003) reported that higher phosphenolpyruvate carboxykinase (PEPCK) mRNA abundance, a glucocorticoid responsive gene, correlated with elevated plasma glucose concentration, supporting an increase in liver gluconeogenic capacity by cortisol in fish. Therefore, they proposed that this higher glucose production during stress is due to hepatic gluconeogenesis mediated by glucocorticoids. However, the production of glucose through glycogenolysis pathway cannot be excluded, particularly as glucocorticoid-inducible α-amylase plays a role in

glycogen metabolism (Koyama *et al*., 2001). Furthermore, α-amylase has been shown to have a high affinity for glycogen in rat liver (Koyama *et al*., 2001). Our studies clearly showed transcriptional regulation of amylase gene expression in response to glucocorticoids. Our results taken together, suggest that amylase participated in glycogen metabolism to cope with the increased energy demand associated with stress of food deprivation or restriction. Although it is not clear how amylase would stimuclate glycogenolysis if indeed the case.

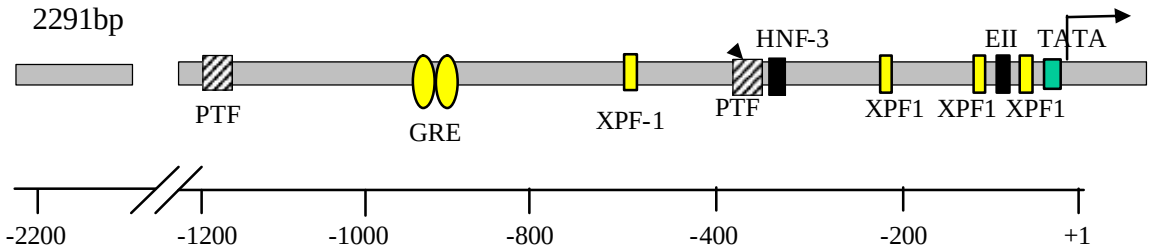
CHAPTER SIX
CONCLUDING REMARKS

6.1 Conclusions

One of the problems in marine fish aquaculture is the inability of larvae to digest ingested food properly because of their simple digestive system. This has resulted in reduced survival rates and low growth. It would be desirable to accelerate digestive system development by stimulating digestive enzymes synthesis. Studies in digestive enzymes gene expression and regulation will be helpful to achieve the goal. The enzymatic activities of α-amylase and their corresponding messenger RNA levels in developing seabass (*Lates calcarifer*) larvae were studied from hatching until 27 days post hatching (dph). An increasing activity of amylase enzyme was measured until 5 dph, from then the activity gradually decreased reaching a constant level by 12 dph. Based on a 318-bp fragment of α-amylase cDNA sequence, a real-time reverse transcriptase polymerase chain reaction technique was developed to monitor the changes in the mRNA levels in the larvae. A correlation between enzymatic activity and mRNA level of α-amylase was demonstrated during the early development of seabass larvae. This suggests that the changes in α-amylase are controlled at least at the transcriptional level during the early larval development of sea bass.

After cloning and characterizing the full length cDNA, I subsequently focused on the molecular mechanisms of amylase gene and transcriptional regulation by investigating amylase promoter region. The expression of mammalian α-amylase gene is controlled at the transcriptional level, through several well defined cis-elements located in the proximal 5'-flanking region. Several of the transcription factors involved in cell-specific transcription have been defined: they include PTF1 and XPF1, and GRE is also located in

this region. However, the promoter region is distinctively divergent between the mammalian and seabass α-amylase (Fig. 6.1). The 5' promoter deletion analysis revealed that the GRE of the seabass amylase promoter was located far upstream, (-947 to -776 bp), of the promoter, and no functional PTF-1 binding site, which is responsible for pancreas-specific transcription in higher vertebrates, was identified. Instead a Hepatocyte Nuclear Factor 3 binding site was found to modulate the seabass amylase promoter expression.



	PTF	GRE	PTF	HNF	XPF
Dex induction	No induction	5 fold induction	No induction	No induction	No induction
Deletion	No detectable effect	Positive regulatory element	No detectable effect	Positive regulatory element	Future study
Site-directed mutagenesis	No effect	Dex induction eliminated	No effect	Amylase promoter activity reduced by 37.5%	Future study
EMSA (DNA-protein interaction)	No interaction	Interaction between GRE and GR	No interaction	Two DNA-protein complexes detected	Future study
Remarks	Non-functional	Functional	Non-functional	Functional	Future study

Fig. 6.1 Summary of the organization of cis-elements in seabass α-amylase promoter.

Our results concur with mammalian studies clearly showing transcriptional regulation of seabass α-amylase gene expression in response to glucocorticoids. Dexamethasone induced amylase promoter activity *in vitro* and cortisol treatment of seabass larvae upregulated amylase gene expression. Thyroid hormone (T_3) treatment also upregulated the gene expression and no T_3 response element was identified on the promoter, suggesting indirect action of T_3. Food restriction and deprivation upregulated amylase gene expression. The findings are interpreted as a stress response whereby cortisol secretion was elevated which activated the GR-transcription system and upregulated amylase gene expression. The higher amylase gene expression correlated with elevated glycogen breakdown in food restricted or deprived seabass larvae supporting a glycogenolytic activity of cortisol.

6.2. Suggestions for future work

In some higher vertebrate species, α-amylase production is limited primarily to the pancreas. In other species, such as the mouse, where both the salivary glands and the pancreas are the major sites of α-amylase production, a different set of amylase genes is expressed in each of the two tissues (Benkel *et al*., 1997). Fish do not have a salivary gland; a strong expression of α-amylase gene is detected in the pancreas. Southern hybridization indicated there were two amylase gene copies in the Asiatic seabass genome. Analysis of the genomic DNA of *Tetraodon nigroviridis* revealed that an amylase gene family (Amylase -1, -2, -3) was clustered on a 148 kb fragment (Bouneau *et al*, 2003). Further sequencing of the proximal 3' end of the seabass amylase gene may permit us to sequence the second copy of amylase gene detected by the Southern blot. It

would be of interest to determine the tissue organization and whether the second copy of amylase gene carries pancreatic resource.

Seabass α-amylase promoter have been characterized and several transcription factors binding sites were defined. This study reported that functional PTF was not identified in the seabass α-amylase promoter region. It remains to be determined whether functional PTF elements are located elsewhere in the seabass amylase gene. Therefore it would be interesting to study PTF in fish and see if PTF exists. Further studies on fish amylase gene expression and regulation mechanisms may reveal some fascinating evolutionary differences and offer some insights into the possible origin and evolution of exocrine pancreas.

With the exception of a small number of species, teleosts have a diffuse pancreas. It would be interesting to study pancreas development in fish. Since seabass α-amylase gene expression is exocrine-specific and expressed just after hatching, it could be used as a good marker to study the development of pancreas in fish. Generation of GFP-amylase transgenic seabass will be especially useful not only in developmental biology but also for aquaculture.

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