Molecular Characterization of the Insulin Genes in Nile Tilapia (*Oreochromis niloticus*)

by

Olga Hrytsenko

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Table of Contents

List of Tables

List of Figures

Abstract

The Nile tilapia (*Oreochromis niloticus*) is one of the most important fish species for the aquaculture industry worldwide. It is also a popular model/organism in different areas of endocrinology, genetics, and physiology. Recently, it became a source of pancreatic islet tissue for xenotransplantation research and potentially for the treatment of insulin-dependent diabetes mellitus. To estimate the usefulness of the tilapia pancreatic islets in diabetic research it is important to understand both the differences and similarities in regulation of energy homeostasis between mammals and fish. This study focuses on characterization of the tilapia insulin, a pancreatic islet hormone that plays a central role in maintaining glucose homeostasis. Using different molecular techniques, we examined expression, genomic localization, and allelic and non-allelic polymorphisms of the insulin gene in tilapia.

In contrast to the genomes of most mammalian species, the tilapia genome contains two insulin genes. The insulin 1 gene is located on the long arm of chromosome 1 (sex chromosome) and exists in three allelic variations. Two of the insulin 1 alleles were found in the genome of both male and female fish. The third one was preferentially observed in the male genome. The differences amongst insulin 1 alleles are comprised of sequences 2.3 kb upstream of the insulin 1 gene.

Analysis of the tissue distribution of the insulin 1 and 2 mRNA revealed that expressions of both insulin genes are not strictly restricted to pancreatic β-cells, as it is traditionally accepted for adult mammalian species. The insulin 1 gene is expressed predominantly in β-cells of pancreatic islets but also in some other extrapancreatic tissues including pituitary gland, brain, and gonads. A low level of insulin 2 gene expression was detected in almost all tissues tested.

Unlike short mammalian insulin promoters (400 bp) that are sufficient to activate high levels of β-cell-specific transcription, the high level of the tilapia insulin 1 expression in the β-cells required cooperative interactions between regulatory elements within the proximal and distal promoter regions. Moreover, it is likely that the gender-related sequences located 2.3 kb upstream of the insulin 1 gene are also involved in regulation of its expression and induce different levels of insulin 1 transcription in tilapia gonads and in tilapia fry prior to sex differentiation. The former finding together with detections of low levels of insulin 1 expression in the gonadotropin-producing cells of the tilapia pituitary gland, and with localization of the insulin 1 gene on the sex chromosome, indicate that insulin 1 might have acquired sex-related functions in tilapia.

Similar to mammalian β-cells, tilapia β-cells, when exposed to stimulatory concentrations of nutrients, were able to evoke insulin exocytosis and to maintain intracellular insulin content at a constant level. However, in contrast to the elevation of insulin mRNA levels in mammalian β-cells in response to glucose stimulations, intracellular insulin homeostasis in tilapia β-cells generally does not require transcriptional activation of the insulin 1 gene and most likely relies on an enlarged intracellular insulin 1 mRNA pool.

List of Abbreviations and Symbols Used

aa	amino acid/s
ADP	adenosine-5'-diphosphate
AFLP	amplified fragment-length polymorphism
ATP	adenosine 5'-triphosphate
BAC	bacterial artificial chromosomes
BB	Brockmann bodies
BCIP	nitro-blue tetrazolium chloride
B-HLH	basic helix-loop-helix
bp	base pairs
B-ZIP	basic leucine zipper
C-	carboxy
^{o}C	degree Celsius
CaMK	calcium/calmodulin-dependent protein kinase
cAMP	adenosine 3'5'-cyclic monophosphate
cDNA	complimentary DNA
C/EBP	CAAT/enhancer binding proteins
CNS	central nervous system
CoA	coenzyme A
COS-1	green monkey kidney fibroblast-like cell line
CPH	carboxypeptidase H
CRE	cAMP-responsive elements

CREM	cAMP response element modulator
dCTP	2'-deoxycytidine 5'-triphosphate
DIG	digoxigenin
DNA	deoxyribose nucleic acid
dNTPs	2'-deoxyribonucleotide 5'-triphosphates
dpf	days post fertilization
dph	days post hatching
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EF	translation elongation factor
eIF	eukaryotic translation initiation factor
ER	endoplasmic reticulum
ERK/MAPK	extracelular signal-regulated kinase / mitogen-activated protein kinases
FISH	fluorescence *in situ* hybridization
FSH	follicle-stimulating hormone
GH	growth hormone
GLP-1	glucagon-like peptide
GLUT	glucose transporter
GMP	guanosine monophosphate
G-protein	guanine nucleotide binding protein
h	hour/s
HIT-T15	Syrian hamster insulinoma β-cell line
HNF	hepatocyte nuclear factors
HRP	horseradish peroxidase
ICER-1	inducible cAMP early repressor
IEF-1	islet-specific protein complex
IGF	insulin-like growth factor
K	lysine
K_{ATP}	ATP-dependent K^+ channels
kb	kilobase pairs
kDa	kilo Dalton/s

KID	consensus phosphorylation site
Km	Michaelis constant
LH	luteinizing hormone
ml	milliliter
μl	microliter
mM	millimole
mRNA	messenger RNA
MW	molecular weight
N-	amine-
NBT	5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
NFAT	nuclear factor of activated T cells
NH	neurohypophysis
NIT-1	NOD mouse insulinoma β-cell line
NTins1	Nile tilapia insulin 1
NTins2	Nile tilapia insulin 2
PAH	phenylalanine hydroxylase
PBS	phosphate-buffered saline
PC1/3	prohormone convertase 1/3
PC2	prohormone convertase 2
PCR	polymerase chain reaction/s
PDX-1	pancreatic duodenal homeobox 1
PI	pars intermedia
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
PPD	proximal pars distalis
PTB	polypyrimidine tract-binding protein
PVDF	polyvinylidene fluoride
Q domain	glutamine-rich activation domain
qPCR	quantitative PCR
qRT-PCR	quantitative RT-PCR
R	arginine

RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
RPD	rostral pars distalis
rRNA	ribosomal RNA
RT-PCR	reverse transcription-PCR
s	second/s
SDS	sodium dodecyl sulfate
±SE	standard error
SRP	signal recognition particle
SSC	standard sodium chloride/ sodium citrate buffer
SSPE	standard sodium chloride/ sodium hydrogen phosphate/ EDTA buffer
SV40	Simian vacuolating virus 40
TF	transcription factor
TH	tyrosine hydroxylase
TSS	transcription start site
U	unit
UTR	untranslated region

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Chapter 1

Introduction

In mammals, the blood glucose level is maintained within extremely narrow limits through the combined actions of insulin and its antagonists. Insulin is the only hormone that can decrease blood glucose concentration and therefore plays a critical role in control of glucose homeostasis [10].

Insulin is a small peptide hormone (MW 6 kDa), produced almost exclusively in β-cells of the islets of Langerhans. Loss or dysfunction of β-cells is the main cause of development of insulin-dependent diabetes mellitus (type 1 diabetes) [46]. In Canada, more than 200,000 people have insulin-dependent diabetes, which accounts for the third highest occurrence rate of type 1 diabetes amongst children under 14 years in the world [87]. One possible treatment is transplantation of pancreatic islets from a donor organism to a diabetic recipient. However, the lack of an abundant human donor source of pancreatic tissue makes this approach impractical [263].

During the last several years, a new source of pancreatic islets — teleost fish, Nile tilapia (*Oreochromis niloticus*), has been investigated in our laboratory. Nile tilapia is one

of an approximately 3,000 members of the most species-rich Cichlids Family of vertebrates [104]. Originally tilapia was indigenous to warm fresh-water rivers and lakes of Africa, India and Middle East. Now, it is widespread throughout the world. Tilapia is one of the most important species for aquaculture, and is commercially farmed in 85 countries including countries of Africa, Asia, Oceania and America. In nature tilapia ingests a very broad variety of food including phyto- and zoo- plankton, algae, some aquatic macrophytes and invertebrates, fish larva and eggs, and decomposing organic matter. Tilapia is a mouthbreeder, breeds easily in laboratory conditions, and can produce 100-1000 eggs approximately every 2 weeks. It grows rapidly reaching maximum size of 75 cm and weight of 3 kg, and is sexually mature at 6 months. It is tolerant to variations in water temperature and oxygen levels. Tilapia endocrine islets (analogues of the mammalian pancreatic islets) are called Brockmann bodies and are localized separately from pancreatic exocrine tissue, which allows them to be easily identified and isolated [263]. Tilapia fasting (>48 h) and non-fasting plasma glucose levels (75.4± 3.0 mg/dl and 91.9± 3.3 mg/dl) are similar to those in humans (63 mg/dl and 90 mg/dl) [263]

However, the isolated tilapia insulin differs from human insulin by 17 amino acids, and is likely only 30-50% active in humans [138, 165]. Therefore, we have developed transgenic tilapia, which, in addition to their native insulin, produce "humanized" insulin under control of the tilapia insulin regulatory machinery [196]. Since tilapia insulin may be immunogenic for humans it may be necessary to generate transgenic fish, which synthesize only "humanized" insulin. In order to use these animals in medical research, it is critical to understand the differences and similarities in the regulation of insulin biosynthesis between mammals and fish. Moreover, since two non-allelic insulin genes have recently been found in fugu fish (*Takifugu rubripes*), and in zebrafish (*Danio rerio*) genomes [85], it is also important to know allelic and non-allelic variations of the insulin gene in tilapia.

This thesis summarizes our work on the characterization of the insulin genes in tilapia. The study is divided into seven main chapters. The second chapter provides a brief overview of regulation of the insulin biosynthesis in mammals as well as in fish. The third chapter describes our results on regulation of insulin 1 (named insulin in Chapters 2-4) biosynthesis and secretion in tilapia. The fourth chapter focuses on tissue-

specific expression of the insulin 1 gene in tilapia and describes our finding insulin 1 synthesis in the brain and pituitary of adult fish. The fifth chapter discusses the chromosomal localization of the insulin 1 gene and its allelic polymorphism in male and female fish. The sixth chapter presents our recent results of the cloning and preliminary characterization of the second non-allelic insulin gene in the tilapia genome. Finally, the last chapter outlines the main conclusions and future direction of the presented work.

Chapter 2

Regulation of Insulin Gene Transcription and Translation

2.1. Regulation of Tissue-Specific Insulin Gene Transcription

In mammals, the insulin gene is expressed predominantly in the β-cells of the islets of Langerhans. This restriction of insulin gene expression to the β-cells is regulated at the level of the transcription of the insulin gene [246]. Moreover, pancreatic β-cells regulate the rate of insulin gene transcription in response to a number of nutrients, the most important of which is glucose [60, 166]. Therefore, the mechanisms that control transcription of the insulin gene are essentially important for the regulation of both β-cell-type-specific insulin expression and energy metabolism.

2.1.1. Transcription of an Eukaryotic Gene

Transcription is the process of RNA synthesis from DNA. It consists of three steps: initiation, elongation and termination. β-cell-specific and glucose-stimulated transcription of the insulin gene is known to be regulated during the initiation step. This

step involves formation of the initiation complex on DNA upstream of the transcription start site (TSS) (promoter and enhancer regions). At least three classes of proteins must interact with each other and/or DNA to activate transcription of the insulin gene. These are: (1) RNA polymerase, (2) general transcription factors, and (3) gene specific transcription factors.

General transcription factors attach RNA II polymerase to the specific region on the promoter. Association of the general transcription factors with RNA polymerase II and promoter DNA is sufficient to activate transcription, but only at a basal level and without cellular control.

The third class of proteins involved in the initiation of insulin transcription are the gene specific transcription factors. Binding of the gene specific transcription factors to the enhancer region, their interactions with each other and with the general transcription factors, regulate the high level of β-cell-specific and glucose-stimulated insulin transcription (for review see [125, 251]).

2.1.2. Structure of the Insulin Promoter/Enhancer Region

Most of the information concerning regulation of the insulin gene transcription results from studies conducted on mammalian insulin genes. It was shown that the sequence approximately 400 bp upstream of the TSS is sufficient for the regulation of tissue-specific expression of the insulin gene [93, 246]. Despite its critical role, this region is not as well conserved among species, as is the translation part of the insulin gene. However, it contains several discrete sequence elements (*cis*-elements), which are highly similar in different species [52]. It is therefore assumed that the regulation of insulin gene transcription in mammalian species is controlled by a similar mechanism. This mechanism involves complex interactions among the promoter *cis*-elements and transcription factors (for review see [27, 170, 210]).

Many of these *cis*- and *trans*-acting factors have been identified and characterized in different mammalian species. These include several E elements, A elements, C elements, and G elements [19, 52].

Although most studies concentrate on characterization of the proximal promoter region (first 400 bp), it is also known that the distal region (up to 2 kb upstream) can be involved in the regulation of insulin transcription [28, 172].

The highly polymorphic (in length) repeat region is described in the insulin promoter in primates. This region contains a variable number of tandem repeats with consensus ACAGGGGTCTGGGG [100]. The G-rich consensus sequence can form an unusual tetrastrand structure that can affect insulin transcription [10, 100]. It is known that short numbers of repeats in this region correlate with the presence of insulin-dependent diabetes mellitus [100].

2.1.3. Transcription Factors of the Insulin Minienhancer

Mutation analysis of the insulin gene promoter/enhancer region reveals that no single element is absolutely required for the β-cell and glucose stimulated expression of the insulin gene [54, 93]. However, mutations in three sequences within the enhancer region decrease insulin expression by 5–10 fold. Simultaneous mutations in two of them leads to an extremely low (0.3% of the wild type) level of insulin transcription [93]. These two elements bind transcription factors known as pancreatic duodenal homeobox 1 (PDX-1) [190] and islet-specific protein complex (IEF-1) [181] that play a critical role in the tissue-specific and glucose-responsive regulation of the insulin gene.

2.1.3.1. Pancreatic Duodenal Homeobox 1

The homeodomain-containing transcription factor pancreatic duodenal homeobox 1 (PDX-1) is one of the best characterized insulin transcription factors [149, 221]. In adults, PDX-1 is predominantly expressed in β-cells, but it is also detected in δ-cells and cells of the duodenum [151, 221].

PDX-1 consists of three main domains: antennapedia-like homeodomain and two terminal domains (Fig.2.1). As is typical for transcription factors, DNA binding and transactivation domains are located separately. The binding domain of PDX-1 is contained within the homeodomain and the transactivation domain is located within the N-terminal domain [127, 188]. The homeodomain also contains a nuclear location signal, (RRMKWKK motif) that is required for the PDX-1 import to the nucleus from the

cytoplasm where it is produced [153]. The transactivation domain contains three evolutionary conserved motifs, essential for activation [187-189].

The first fish PDX-1 cDNA has been cloned and characterized by Milewski et al. [150]. It has 98% homology with mammalian PDX-1 in the homeobox region. Similarity in the N-terminal transactivation domain is low; however, three activation motifs have been identified, with two being very conserved. Under transfection into mammalian cells, zebrafish PDX-1 can recognize and activate the rat insulin promoter as effectively as mouse PDX-1 [150].

PDX-1 recognizes and binds to the A-boxes (TAAT motive) in the insulin promoter. There are three A-boxes in the human insulin promoter, with the A2 box having the highest affinity to PDX-1 and contributing the most to β-cell-specific and glucose-induced regulation of the insulin gene. Mutations in the A-box that prevent PDX-1 binding decrease promoter activity by 65% [190].

However, PDX-1 alone failed to activate transcription of the construct where the A-box was linked to a heteralogeous promoter in both insulin and non-insulin producing cells [171, 200]. Synergetic interactions with other transcription factors are required for PDX-1 action. Therefore, the presence of PDX-1 is essential but not sufficient for the activation of insulin transcription in mammalian β-cells [190, 221].

2.1.3.2. Islet-Specific Protein Complex

β-cell-specific and glucose-stimulated expression of the insulin promoter in mammalian cells is also regulated by the islet-specific protein complex (IEF1) that binds to the E-boxes (CANNTG) within the promoter [181, 223]. This complex is formed by the heterodimerizations between two members of the family of the basic helix-loop-helix (B-HLH) transcription factors — the islet-enriched BETA2/NeuroD [25, 162, 181] and the ubiquitously expressed E47/E12 [164, 181, 191].

Members of the B-HLH family of transcription factors participate in the regulation of developmental- and tissue-specific gene expression in muscle, brain, pancreas, and lymphoid tissue. The B-HLH motif is defined by the basic region that is involved in DNA binding, and the two amphipathic helices that are required for dimerization and are separated by the variable loop region [27, 234, 251] (Fig. 2.1). The

B-HLH family is divided into several classes.

Class A consists of ubiquitously expressed proteins, such as E12/E47, which function as homo- or heterodimers [164].

Class B of the B-HLH proteins contains tissue-enriched proteins, such as BETA2/ NeuroD. These proteins are dimerized with class A proteins and activate transcription of tissue-specific genes [25, 162]. Heterodimerization increases both the DNA binding capacity and activation potential. Dimerization between BETA2/NeuroD and E47/E12 proteins is required because neither E12/E47 nor BETA2/NeuroD separately are capable of activation of the transcription of the reporter construct contained in the E-box, but when present together they greatly stimulate the expression [162, 200].

Zebrafish BETA2/NeuroD cDNA has recently been cloned and characterized. A deduced amino acid sequence revealed 73.5% identity with the mouse BETA2/NeuroD sequence. It is highly conserved in the B-HLH region. In fact, only three substitutions are found in the B-HLH domain between zebrafish and mouse BETA2/NeuroD [109]. The function of BETA2/NeuroD in the regulation of zebrafish insulin gene transcription has not been elucidated.

The presence of the IEF-1 heterodimers in β-cells cannot explain all of the β-cell specific expression of the insulin gene. First of all, although BETA2/NeuroD is highly expressed in β-cells, it is also found in α-cells, intestinal and neuronal cells [162]. Secondly, insulin expression in homozygous BETA2/NeuroD-/- mice was severely reduced, but still detectable [161].

2.1.4. Formation of the Minienhancer Complex

Although both PDX-1 and IEF-1 are required for β-specific and glucose-stimulated insulin transcription, neither of them alone can stimulate insulin transcription significantly. However, when present together, they can raise transcriptional activation dramatically. Such a boost in transcription activation is a result of the synergetic interaction between PDX-1 and IEF-1 factors [200]. Homeodomain of the PDX-1 and B-HLH domains of BETA2/NeuroD and E47 that are involved in DNA binding, are also involved in the protein/protein interactions between PDX-1, BETA2/NeuroD, and E47 (Fig. 2.1). As a result of such interactions, PDX-1, BETA2/NeuroD, and E47 form a

stable ternary complex on the insulin enhancer region. [171]. This complex can activate expression of the reporter construct containing the insulin enhancer (E and A box regions) linked to a heterologous promoter in the insulin and noninsulin producing cell lines (designed to produce PDX-1, BETA2/NeuroD1, and E47) [31, 200]

In addition to the cooperative binding of PDX-1 and IEF-1 to the insulin enhancer, there are other proteins that can interact with and stabilize the PDX1/IEF-1 complex. One of these proteins is a non-DNA–binding p300 coactivator [200, 201] (Fig 2.1).

p300 promotes the synergetic activation of the insulin transcription by PDX-1/IEF-1 by at least 2 fold [200]. The exact mechanism of such a coactivation is unclear. However, p300 can function through multiple pathways:

1) Stabilization of the transcription initiation complex: it has been shown that p300 can bind directly to proteins that form the basal transcription complex [231]. Therefore, it creates a protein/protein bridge between the transcription factors of the insulin minienhancer (PDX1/IEF-1) and factors of the general transcriptional machinery [200, 201].

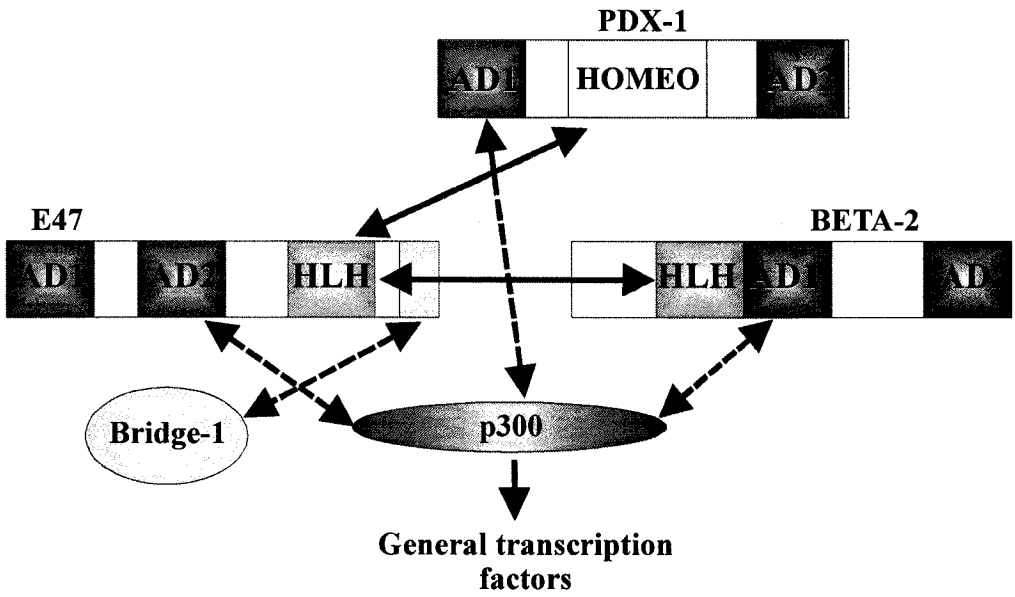

Figure 2.1. Schematic diagram of the protein/protein interactions in the insulin minienhancer complex
(AD) activation domain, (*HLH*) helix-loop-helix domain, (*HOMEO*) homeodomain

2) Acetylation of the nucleosomes: acetylation of lysine residues at the N-terminal tails of the nucleosomal histones leads to the destabilization of the histone-DNA complexes in nucleosomes and therefore to chromatin unfolding. This creates the necessary open space for the binding of the transcription factors to the promoter DNA [58, 231]. It has been shown that p300 containing an acetyltransferase domain catalyzes hyperacetylation of histone H3 in the proximal insulin promoter. This effect is specific for β-cells and has not been found in non-insulin producing cells [23]. Furthermore, p300 acetyltransferase also mediates hyperacetylation of histone H4 in the insulin promoter in a response to high concentrations of glucose [158]. Therefore, hyperacetylation of histones in the proximal insulin promoter plays an important role in β-cell-specific and glucose-stimulated regulation of insulin transcription.

3) Acetylation of transcription factors: it has been found that p300 and its associated factor PCAF acetylates a loop region within the B-HLH domain and C-terminal activation domain in BETA2/NeuroD. Acetylation of the BETA2/NeuroD stimulates heterodimerization of BETA2/NeuroD with its partner E47, and increases DNA binding and the activation potential of the BETA2/NeuroD/E47 complex [199].

The second coactivator of the PDX1/IEF1 complex is a Bridge-1 protein, described recently by Thomas et al. [241]. Bridge-1 contains a PDZ domain that is usually involved in protein-protein interactions. Bridge-1 is predominantly located in the nucleus of pancreatic cells. The PDZ domain of Bridge-1 is involved in the interaction with the C-terminus of E47 (521-649 aa), and functions as its coactivator by increasing E47-mediated activation of the insulin minienhancer [241].

The third coactivator that can stabilize the configuration of the insulin minienhancer by changing the DNA structure to a more favourable for the cooperative binding of PDX1, BETA2/NeuroD, and E47 factors has been described by Ohneda et al. [171]. This coactivator is HMG I (Y), a nonhistone, chromatin-associated nuclear protein that binds to the minor DNA groove at the A-box enhancer region. The DNA binding of PDX-1 and its synergetic association with BETA2/NeuroD/E47 is highly increased in the presence of HMG I (Y) [171].

2.1.5. Regulation of Fish Insulin Gene Transcription

Whether or not regulation of the fish insulin gene transcription has a mechanism similar to that found in mammalian species and whether the insulin gene can be at least partially activated through cooperative binding of PDX-1 and IEF-1 to the promoter/enhancer region has not been studied yet.

Recently, a trout insulin promoter has been characterized [8]. Although the proximal 5'-flanking region shows no significant sequence similarities to that of mammalian insulin genes, it activates the transcription of the reporter gene in a β-cell-specific fashion upon transfection into mammalian cell lines [8]. Also, the sequence of the trout insulin promoter contains several A elements and can be activated by the binding of the mammalian PDX-1 in non-insulin producing cells and also in most tissue of zebrafish embryos [8]. In addition, Huang et al. [77] have reported that the 903 bp sequence upstream of the ATG initiation codon is essential and sufficient to restrict expression of the zebrafish insulin gene to β-cells. This indicates that some functional parallels in insulin regulation probably exist between fish and mammalian species. However, some discrepancies are also present: for example, no E-box-like sequences were found within 280 bp upstream of the trout insulin gene transcriptional start site and the mammalian IEF-1 factor does not influence the promoter activity [8]. These data suggest that although the BETA2/NeuroD sequence is highly conserved among species, it is not essential for regulation of the trout insulin gene. However, it is possible that E-boxes are located further upstream in the trout insulin promoter, and have not been detected and characterized by Argenton et al. [8].

The tilapia insulin promoter contains several potential E-box-like sequences, and therefore, could possibly be activated via EIF-1 binding. From this, we can speculate that the regulation of the tilapia insulin gene may be different from that of the trout insulin gene and may share more similarity with the regulation of the mammalian insulin genes.

2.1.6. Additional Transcription Factors of the Insulin Gene Promoter/Enhancer

The E/A minienhancer of the insulin gene alone can activate transcription of a reporter gene when linked to a ubiquitous promoter in a β-cell-specific fashion when inserted into tumor cell lines. However, this region fails to show any activity in *in vivo*

experiments with transgenic mice [31]. Under the same conditions, the region that contains a whole insulin enhancer (-346 to -103 bp region upstream of the rat insulin I TSS) activates expression of a reporter gene in pancreas and, at lower levels, in brain [31, 32]. However, in a construct where the insulin enhancer was reassociated with the insulin promoter region (-109 to +9 bp region), insulin expression was restricted exclusively to the pancreas [31]. Therefore, both promoter and enhancer regions are required for the β-cell-specific expression of the insulin gene. In addition to the transcription factors that bind to the insulin minienhancer, these regions also bind to other β-cell enriched factors that synergistically regulate insulin transcription.

2.1.6.1. CRE Binding Proteins

Some of these additional factors that modulate transcription of the insulin gene belong to the family of cAMP-responsive elements binding proteins (CRE binding proteins). As indicated by their name, CRE binding proteins regulate transcription of insulin in a response to the change in cAMP concentrations via the cAMP-dependent pathway [243]. CRE binding proteins function as insulin transcription activators or repressors through the binding to CRE (5'-TGACGTCA-3') sequences in the promoter region. This family consists of basic leucine zipper (B-ZIP) proteins that homo- or hetero-dimerized in order to bind to CRE sites. The basic domain of the CRE binding protein is involved in DNA binding but also contains the nuclear translocation signal that directs localization of these factors to cell nuclei. Another common element found within the N-termini of these proteins is the consensus phosphorylation site (KID), which is required for transactivational activity. Phosphorylation of the KID domain can be mediated by several kinases (PKA, CaMK, CREBK), and therefore it is a common target for several signal transduction pathways [243, 247].

Some CRE binding proteins (for example CREB or CREM activators) can enhance transcription of the insulin gene in the presence of high glucose [80, 81, 192]. Positive effects of activators depend on the presence of glutamine-rich activation domain/s (Q) that flank the KID domain. CREM binds to the CRE element and also directly interacts with the TFIID factor and therefore stabilizes formation of the basal promoter complex. These interactions are mediated through the Q domain of CREM and

one of the TFIID associated factors. However, it has also been shown that CRE activators can interact with the basal transcription machinery indirectly, through the associations of Q domains with the p300/CBP (CREB binding protein) coactivator that also participates in the formation of the insulin E/A minienhancer (see above). p300/CBP can recognize CRE activators only after phosphorylation of the KID domain of CRE activators [80, 243].

On the other hand, chronic exposure of β-cells to supraphysiological concentrations of glucose and fatty acids down-regulate the transcription of the insulin gene. This effect is partially modulated via overexpression of several other members of CRE binding proteins. Two of these are the cAMP response element modulator (CREM-17X) and the inducible cAMP early repressor (ICER-1), both of which lack two Q domains [80, 275]. Usually, affinity of the repressors to the promoter is higher than that of the activators. Therefore, CRE sites in the promoter are completely blocked by the repressors. In addition, due to the lack of Q domains in their structure, repressors fail to interact with the basal promoter complex and therefore can not stabilize it [80].

2.1.6.2. CAAT/Enhancer Binding Proteins

Similar to the CRE binding factors, CAAT/enhancer binding proteins (C/EBPs) are a family of B-ZIP transcription factors with intrinsic cAMP-inducible activity. In addition to the B-ZIP domain, C/EBPs contain constitutive and cAMP-inducible activational domains [65]. However, they lack a well-determined KID phosphorylation activational domain, and cannot be phosphorylated directly. [243, 259] Therefore, C/EBPs mediate cAMP responses indirectly by several mechanisms: (1) through the increase in expression of the C/EBPs genes, (2) through the increase in translocation from the cytoplasm into the nucleus, (3) through the phosphorylation of the C/EBPs coactivator that leads to the binding and activation of C/EBPs [258, 259].

It has been shown both *in vivo* (in ZDF diabetic mice) [222] and *in vitro* (in insulinoma cells) [128] that prolonged exposure of β-cells to supraphysiological concentrations of glucose decreases insulin gene expression at least partially through the activation of expression of C/EBPβ [128].

C/EBPβ binds to the CEB box (T(T/G)NNGNAA(T/G)) in the insulin promoter, but also interacts with E47 (transcription factor of the insulin minienhancer), possibly via formation of a zipper between the ZIP domain of C/EBPβ and a leucine zipper repeat in AD2 of E47. The AD2 of E47 is involved in interaction with the p300 coactivator. Therefore, heterodimerization with C/EBPβ prevents p300 binding and, consequently, activation of the insulin minienhancer [128].

2.1.6.3. Pur-1

The transcription factor Pur-1 contains a zinc-finger domain and binds to the G–box (GAGA) in the insulin promoter region. A purine-reach G-box is located in the proximal insulin promoter, close to the TATA box and is well conserved amongst species. Mutation of the G-box decreases insulin promoter activity by 60% in insulinoma cells. Pur-1 is widely expressed in different tissues but its binding to and activation of the insulin promoter is β-cell- specific [101]. Both the trout and the tilapia insulin promoters contain conservative G-boxes in the same position as in the mammalian insulin promoters. Whether or not Pur-1 is capable of activating fish insulin promoters remains to be determined.

2.1.6.4. Maf Family

It has been known for a long time that at least three main elements in the insulin enhancer contribute to β-cell-specific and glucose-stimulated insulin transcription: A- E- and C-boxes [54, 93, 190, 223, 224]. Transcription factors that recognize two of them (A- and E-boxes) have been well characterized and known as the insulin minienhancer proteins. The third β–specific transcription factor is a C-box activator which has been isolated and characterized recently as the MfaA transcription factor, belonging to the large Maf family [91, 174]. MfaA strongly activates the insulin enhancer in insulin cell lines and in non-insulin producing cell lines designed to express MfaA. Furthermore, it also increases insulin gene activation in response to the elevation in glucose concentrations [91, 96, 142, 143].

MfaA is a 46 kDa protein that contains a B-ZIP domain at the C-terminus, and an acidic amino acid (serine/threonine/proline)-rich activation domain at the N-terminus.

The middle part of the MfaA contains clusters of histidine and glycine that are conserved in all members of the Mfa family. The basic domain is involved in DNA binding, and the leucine zipper domain is responsible for dimerization [91, 96, 143, 174]. It is likely that MfaA regulates insulin gene expression as a homodimer, however, it is also possible that it can dimerize with the other members of Mfa family, such as c-Mfa or MfaB [143].

MfaA is known to be a β-cell-enriched factor. Its expression is detected only in β-cells and in the eye [96, 143]. MfaA expression increases in a response to higher glucose concentrations in β-cells [91, 96]. Furthermore, glucose may also regulate post-translational modifications of MfaA, such as tyrosine/serine phosphorylation in the activation domain. It has been shown that such modifications increase the activation potential and DNA binding capacity of MfaA [96].

SMaf1 is a zebrafish homologue of mammalian MfaA that has been isolated by Kajihara M et al. [91]. Its sequence is highly conserved amongst species. In fact, the zebrafish SMaf1 cDNA and the mouse MfaA cDNA have 85% identity in acidic domains, 92% identity in basic regions and 86% identity in leucine zipper domains [91].

MfaA recognizes 13- and 14-bp palindromic sequences named MAREs elements [96, 142]. Analysis of the tilapia insulin promoter did not localize any MAREs-like motifs within its 1.5 kb region upstream of the transcription start site. Therefore, despite evolutionary conserved sequence and structure, zebrafish SMaf1 may not perform the same function and may not be involved in regulation of the tilapia insulin transcription.

2.1.6.5. PAX6

PAX6 is a member of the PAX family of transcription factors that contains a conserved paired domain. This domain is involved in DNA binding to the C2-box (GGTGGAA) in the rat insulin promoter [212]. In addition to the paired domain, PAX6 contains a homeodomain that has an activational function. PAX-6 is an important regulator of the pancreatic islet cells development. However, it is also involved in the regulation of insulin transcription [26]. The insulin gene transcription is reduced by 8% in PAX6 mutant homozygous mice [212]. Similarly to the MAREs-like elements, we did not detect any C2-like sequences in the tilapia insulin promoter.

2.1.6.6. LIM Family

Insulin expression have been detected in mice with a homozygous null mutation of PDX-1 and in insulin producing cells where PDX-1 expression is highly suppressed [2, 92, 188]. One of the possible explanations is that PDX-1 is not the only transcription factor that can bind to the A-box and activate the insulin promoter. It has been shown that several other factors can replace PDX-1. Two of them are Lmx1.1 and Lmx1.2 [89, 171]. Lmx1.1 and Lmx1.2 are two closely related members of the LIM family of transcription factors. They contain two activating zinc-binding LIM domains that are located at the amino-termini, and one DNA-binding homeodomain [89, 209]. The second LIM domain cooperatively interacts with the HLH domain of E47, activating the insulin minienhancer [89]. Furthermore, transcription activation initiated by Lmx1.2/E47 is higher than that initiated by PDX-1/E47 [171]. In contrast, Lmx1.1/Lmx1.2 does not interact with BETA2/NeuroD or minienhancer coactivators, which are important for the full activation of insulin transcription [171]. Therefore, it is apparent that activation of the insulin gene by PDX-1 is higher than that by Lmx1.1 or Lmx1.2.

Expression of the zebrafish homologue of the mammalian LIM containing proteins — the zebrafish Isl-1 is detected in the primary nervous system during early development therefore it is identified as the marker of neuronal differentiation [108]. Whether or not zebrafish Isl-1 is also involved in regulation of insulin transcription remains to be elucidated.

2.1.6.7. The Hepatocyte Nuclear Factors (HNFs)

HNFs initially had been described as the important regulators of development and function in the liver. They are also known to be widely acting transcription activators in pancreatic islets. It has been shown that HNF 4α and HNF1α are bound to 11% (1087) and 0.8% (106) of the gene promoters in islets cells, respectively. Both HNF4α and HNF1α are thought to be direct activators of the insulin promoter [110, 169].

HNF1α is a homeodomain transcription factor that also contains an additional loop within the homeodomain and can dimerize through its N-terminal region. It binds to and activates the E/A minienhancer that is linked to a heterologous promoter in experiments performed with cultured tumor cell lines [45]. HNF1α can interact with the

p300 coactivator and therefore increases insulin expression via histone hyperacetylation [182]. However, lack of HNF1α does not decrease insulin transcription in HNF1α null-mutant mice, and therefore may not be essential for insulin transcription *in vivo* [182].

HNF4α is a member of the nuclear receptor superfamily. It can activate insulin transcription indirectly via the well-known activation of HNT1α, and directly, through the binding to its site in the proximal insulin promoter and also by synergetic interactions with PDX-1 [13, 152].

2.1.6.8. NK Family of Transcription Factors

Homeodomain transcription factors of the NK family contain a conservative NK decapeptide at the N-terminus. At least two members of the NK family, Nkx2.2 and Nkx 6.1, play an important role in the growth and differentiation of β-cells and insulin expression [74, 209, 213].

It is known that insulin is a highly suppressed in knockout mouse lacking Nkx 2.2 and Nkx 6.1 [213, 236]. However, expression of other β-cell-specific markers, such as PDX-1 and islet amyloid polypeptide, remains unaffected.

In mammals, Nkx2.2 and Nkx 6.1 are expressed in the pancreas and in the ventral CNS. In the pancreas, Nkx2.2 expression is found in α, β, and PP-cells, whereas Nkx 6.1 expression is completely restricted to β-cells [88, 209].

Nkx 2.2 and Nkx 6.1 have weak similarity between each other, except for the region of the homeodomain and the NK-domain [209]. The exact mechanism of Nkx 6.1- and Nkx2.2-dependent activation of insulin expression is unclear. The direct binding of Nkx2.2 to the insulin promoter has been described by Cissell et al. [26]. However, in this report, Nkx2.2 functions as a weak negative regulator of insulin expression in β-cell lines [26].

The zebrafish nk2.2 is the highly conserved homologue of the mammalian Nkx 2.2. In fact, it has 100% identity with the mouse Nkx 2.2 within its NK- and homeodomains. It has been shown that nk2.2 is involved in the neuronal differentiation of zebrafish embryo [12]. Whether or not it also participates in regulation of transcription of the fish insulin gene has not been studied yet.

2.1.6.9. Z-Element

Z-element is a cell-specific and glucose-responsive activator that has been described by Sander et al. in the human insulin promoter [211]. Z-element binds several proteins that cooperate with each other to activate insulin gene transcription, but work independently from the known E/A minienhancer and therefore can be considered to be a second insulin minienhancer. Transcription factors of Z-element have not yet been identified, but one of them is specific to the primary β-cells. Furthermore, Z-element can dramatically increase transcription only in primary β-cells, but not in insulinoma cells or non-insulin producing cells [211].

2.2. Glucose Regulation of Insulin Gene Transcription

2.2.1. Mechanism of Insulin Secretion

Release of insulin from β-cells plays a key role in maintaining blood glucose homeostasis. β-cells sense elevation in the circulating glucose concentrations and respond immediately by activation of a cascade of reactions that leads to the exocytosis of stored insulin. This complex process starts with non rate-limited glucose uptake into β-cells through the low-affinity high Km glucose transporter (GLUT-2). As a result, glucose concentration inside β-cells rapidly equilibrates with the increased blood glucose concentration. After entering cells, glucose is phosphorylated to glucose-6-phosphate by glucokinase. Glucokinase exhibits a high Km (11-12 mM) and therefore works as a glucose sensor. It adjusts the rate of glucose metabolic flux through glycolysis and the Krebs cycle related to the increase in extracellular glucose levels. Subsequent elevations in glycolysis and mitochondrial oxidation result in the rise of the ATP/ADP ratio. This leads to the closure of ATP-dependent K^+ channels (K_{ATP}), membrane depolarization, and opening of voltage-dependent Ca^{2+} channels, and finally to the influx of the extracellular Ca^{2+} into cells. An increase in the cytoplasmic free Ca^{2+} is a trigger for the insulin exocytosis (for reviews see [10, 46]).

In addition to glucose, other nutrients (leucine and arginine), and hormones (glucagon, glucagon-like peptide-1 (GLP-1), and somatostatin) can modify insulin secretion [10, 40].

As it is in mammals, glucose also induces secretion of insulin from β-cells in fish [154, 206, 207]. In addition to glucose, amino acids (arginine, lysine, leucine) and carbohydrates (galactose [50] and mannose [207]) have positive effects on stimulation of insulin secretion. It has been described [82-84] that amino acids (lysine and arginine) are much stronger secretagogues than glucose and can stimulate a higher range of insulin exocytosis in fish. However, the underlying molecular mechanism of such strong stimulation is unknown. It has been discussed, that in mammals arginine can induce insulin secretion via several potential mechanisms.

First, and most likely, arginine can trigger insulin release and can potentiate glucose-induce insulin secretion by rapid but limited membrane depolarization resulting from transport-mediated accumulations of this cationic amino acid within β-cells [17, 72, 240].

Another putative mechanism is the nitric oxide synthase mediated formation of nitric oxide from arginine in β-cells and consequent activation of soluble guanylate cyclase resulting in the elevation of the cyclic GMP level [144, 219].

Finally the insulinotropic effect of arginine could be mediated by glucagon that is secreted from α-cells in response to arginine stimulations and potentiate insulin release from β-cells [239]. At least this last mechanism likely does not play an important physiologically role in arginine-stimulated insulin secretion in fish since arginine-induced insulin secretion was not abolished by addition of glucagon antibodies in some fish species [154].

2.2.2. Timeframes of the Glucose Effect on Insulin Transcription

In addition to its effect on insulin secretion, glucose stimulates insulin biosynthesis by increasing insulin translation and transcription rates and by stabilizing insulin mRNA. Therefore, β-cells can replenish insulin stores, which enables them to respond rapidly to changes in blood glucose concentrations over long periods of time [166, 252, 253].

It is commonly accepted that the effect of glucose on insulin transcription occurs after a relatively long time of exposures to high glucose concentrations [42, 86]. This long-term regulation plays an essential role in the adaptation of β-cells to dietary

changes, such as periods of fasting or intake of carbohydrate-enriched food. On the other hand, several recent reports describe short-term effects of glucose on insulin transcription [116, 117]. In these studies, glucose-induced transcription of insulin was detected as early as 10-15 min after the start of glucose stimulation, reaching maximal level after 30 min and then decreasing after 2h. A decline in the level of insulin transcription was observed even when cells were continued to be exposed to elevated glucose concentrations. This may indicate the presence of an "exhaustion" mechanism in the regulation of glucose-stimulated insulin transcription. The short-term regulation may be explained as an adaptation of β-cells to short increases in blood glucose levels after regular feeding, when cells are exposed to elevated glucose concentrations over minutes rather than over hours [116, 117].

2.2.3. Phosphoinositide 3-Kinase (PI3K) Pathway

Glucose elevates transcription of the insulin gene via activation of several signalling pathways that are designed to increase activation of several insulin transcription factors (Fig. 2.2). At least three glucose-sensitive elements have been identified within the insulin promoter. These are E- and A-elements that form the insulin minienhancer and bind PDX-1, E-47, and Beta2/NeuroD transcription factors, and the C-element that binds the MafA protein [54, 190, 223, 224].

The exact mechanism of glucose-induced activation of these factors is unclear. Most likely, complex interactions between several pathways are involved.

It has been shown by many [120, 202, 266], that glucose induces transcription of insulin via the phosphoinositide 3-kinase (PI3K) pathway. Downstream targets of PI3K remain undefined. Two possible signal transduction pathways might be activated. The first involved is the p38/SAPK2a pathway [132, 266] and the other is mediated by p70S6 kinase activation [117, 120].

p38/SAPK2a is a stress-activated protein kinase. High concentrations of glucose can be considered the stress stimulus that activates the p38/SAPK2a pathway and leads to stimulation of PDX-1 [132].

Some data suggest that p38/SAPK2a increases the binding and activation capacity of PDX-1 via its phosphorylation by an unknown kinase [132, 189, 266]. Shifts in MW of

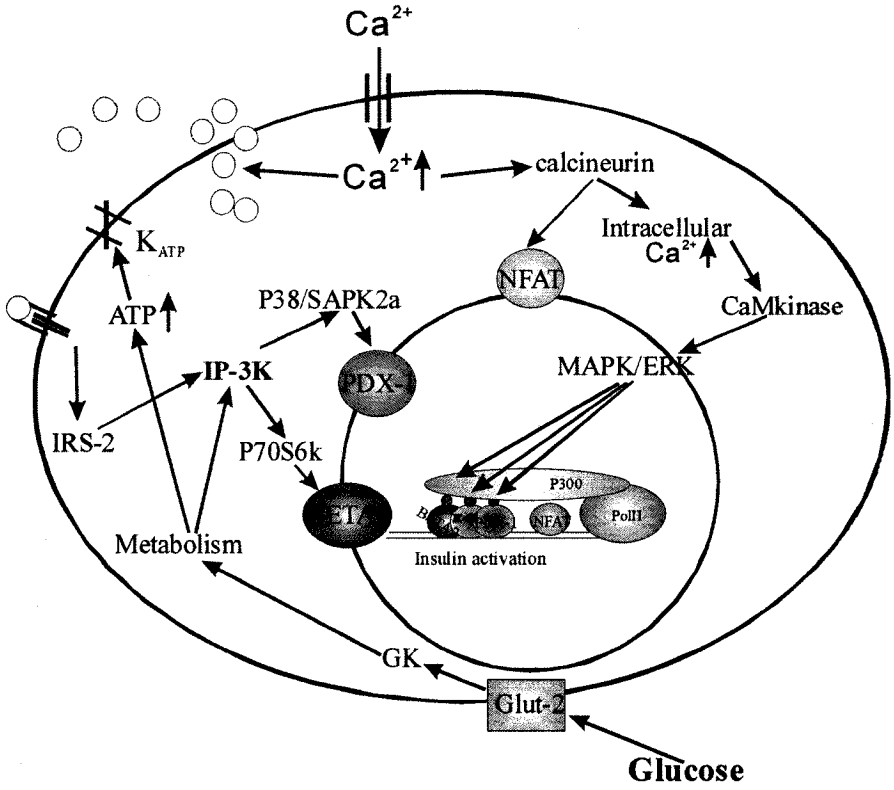

Figure 2.2. Signaling pathways involved in regulation of glucose-stimulated insulin gene transcription

PDX-1 from 31 kDa to 46 kDa due to phosphorylation in the presence of the high concentrations of glucose has been described by Macfarlane et al. [130, 132].

In contrast, Rafiq et al. and Wang et al. [202, 250] did not detect any change in MW of PDX-1 in response to glucose. Moreover, Moede et al. [153] reported that point mutations of several possible phosphorylation sites did not alter the glucose stimulation effect on PDX-1. Therefore, a different mechanism may be involved. For example, glucose can stimulate translocation of PDX-1 from the cytoplasm to the nucleus through the activation of the PDX-1 nuclear exportin [202]. An increase in insulin expression due to translocation of PDX-1 has been observed after only 10 min of glucose stimulation, becoming maximal after 15-30 min (2.2-2.5-fold increase in transcription activity), and then declining after 2 h [250]. These data support the hypothesis of short-term regulation of glucose-stimulated insulin transcription.

Rafiq et al. [202] did not detect any involvement of p38/SAPK2 in the glucose stimulation of insulin gene transcription. Instead, PI3K can activate p70S6 kinase as its

downstream target. It is possible that p70S6 kinase stimulates activation of insulin transcription via E-box binding transcription factors (BETA2/NeuroD or E47) [120, 266].

What mechanism links elevation in blood glucose concentration to the activation of PI3K? At least two main models have been described. One of them suggests that glucose metabolism generates signals sufficient to induce insulin transcription [60]. The other proposes that insulin transcription can be triggered by insulin exocytosis and therefore insulin regulates its transcription in an autocrine manner [117, 120].

In agreement with the first model, data obtained by Goodison et al. [60] describes that, in addition to glucose, other metabolized nutrients (such as mannose, leucine, and glutamine) can stimulate transcription. Non-metabolized nutrients, such as galactose and arginine (potent secretagogues of insulin), as well as increased concentrations of intracellular Ca^{2+} had no effect on insulin transcription [60]. Furthermore, β-cells that lacked K_{ATP} channels and, therefore, failed to activate voltage-dependent Ca^{2+} channels and subsequent insulin exocytosis, maintain normal glucose-sensitive insulin transcription via activation of PDX-1 [131].

In contrast to Goodison et al., [60] and in agreement with the second autocrine model of glucose regulation of insulin transcription data were obtained by Leibiger et al. [120] who showed that inhibition of insulin secretion caused by blocking Ca^{2+} channels, decreases glucose-activated insulin transcription. Moreover, insulin transcription can be triggered by KCl or components of K_{ATP} channels, without increase in glucose concentrations [120]. According to Leibiger et al., insulin that is secreted from β-cells in response to augmented glucose concentrations binds to and activates its receptors in β-cells. There are two different isoforms of the insulin receptor in β-cells (A and B) that arise from alternative splicing of the same gene. Autocrine stimulation of insulin transcription is mediated via activation of the A-type insulin receptor, leading to receptor autophosphorylation and subsequent activation via tyrosine phosphorylation of its downstream target, IRS-2, and further activation of IP-3K [117, 120].

2.2.4. Ca^{2+}-Dependent Pathways

In addition to the IP-3K pathway, other pathways may be involved in the regulation of glucose-stimulated insulin transcription. One of them is activated by the

elevation of intracellular Ca^{2+} concentrations that lead to exocytosis [102]. Increases in Ca^{2+} concentration triggers activation of Ca^{2+}-dependent protein kinases (such as Ca^{2+}/calmodulin-dependent protein kinase II [CaM kinase II]) and Ca^{2+}/camodulin-dependent protein phosphatase (calcineurin) [117].

Activation of insulin transcription via the calcineurin transduction pathway has been described by Lawrence et al. and Redmon et al. [114, 203]. It involves activation of the nuclear factor of activated T cells (NFAT). Expression of NFAT is detected in β-cells. Furthermore, the rat insulin promoter contains three potential NFAT binding sites. Two-bp mutations in one of them result in a 68% decrease in glucose-stimulated insulin promoter activation. Ca^{2+}-dependent activation of calcineurin leads to the dephosphorylation of NFAT serines and results in the opening of the nuclear localization signal, and the consequent translocation of NFAT to the nucleus where it binds to the *cis*-element and activates the insulin promoter [114, 203].

The mechanism of the glucose-activation of insulin transcription via CaM kinase II pathway is less clear. It has been shown that an elevation in intracellular Ca^{2+} concentrations leads to activation of calcineurin, CaM kinase II and the ERK/MAPK kinase pathway [9, 102, 103]. The possible mechanism includes: a rise in intracellular Ca^{2+} due to an influx of Ca^{2+} through L-type Ca^{2+} channels, activation of calcineurin that leads to Ca^{2+} release from intracellular stores, and the consequent activation of CaM kinase II. Afterwards, CaM kinase II activates an ERK/MAPK cascade, possibly through the recruitment of another tyrosine kinase [9]. The final component of the ERK/MAPK pathway is ERK1/2, which phosphorylates transcription factors of the insulin minienhancer (Beta2/NeuroD, E47, PDX-1). Phosphorylation of these factors increases their transactivation capacities. In addition, phosphorylation of Beta2/NeuroD and E47 enhances heterodimerization between them. Most of the phosphorylation sites of Beta2/NeuroD, E47 and PDX-1 by ERK1/2 have been identified [103]. They are located in activation domains involved in the binding to the p300/CBP coactivator. It is known that phosphorylation of transcription factors such as CRE binding proteins, dramatically increases their binding to p300/CBP [243]. The same mechanism may be involved in glucose regulation of the insulin minienhancer. Thus, phosphorylation of Beta2/NeuroD,

E47 and PDX-1 can modulate gene expression via changes in their affinity to the p300/CBP coactivator [103].

2.2.5. Effect of Glucocorticoids, Glucagon-Like Peptide and Amino Acids on Insulin Transcription

Glucose is not the only modulator of insulin gene transcription. There are a number of studies showing that transcription of the insulin gene is down-regulated by glucocorticoids [49, 61, 225, 226]. Negative effects of glucocorticoids on insulin transcription most likely are the result of synergetic interactions between several possible mechanisms. First, glucocorticoids inhibit expression of the two main insulin transcription activators — PDX-1 and BETA2/NeuroD [226]. Second, glucocorticoids induce expression of the transcription factor C/EBPβ that downregulates transcription of the insulin gene through the binding to its *cis*- element in the insulin promoter [225]. A third, direct effect of glucocorticoids on insulin transcription has been described by Goodman et al. [61]. The region within –258 bp to -279 bp of the insulin promoter that inhibits insulin transcription in insulinoma cells can bind glucocorticoid receptors and act as a glucocorticoid response element [61].

Transcription of the insulin gene is also up-regulated by GLP-1. GLP-1 is a gut hormone, produced in intestinal L-type cells, that is secreted into the bloodstream in response to food uptake [48]. It binds to, and activates, G-protein coupled receptors on the surface of β-cells [99]. Subsequently, GLP-1 receptors activate several different pathways, including cAMP, PI3K, p38/SAPK, calcineurin, and MAPK/ERK pathways [9, 99, 113, 249, 250].

The stimulatory effect of GLP-1 on insulin transcription via the cAMP pathway leads to the activation of PKA, which triggers elevation of PDX-1 transcription, stabilization of PDX-1 and its translocation to the nucleus. As opposed to the short-term effects of glucose on translocation of PDX-1, the effect of GLP-1 is considered to be long-term, and has been detected only after 3h of incubation [250].

A rise in cAMP levels due to activation of GLP-1 receptors also activates calcineurin and ERK/MAPK pathways that have similar effects and the same targets as when they are activated by a rise in glucose [9, 113].

Surprisingly, GLP-1 activation of the p38/MAPK pathway has an inhibitory effect on insulin transcription [99]. This effect is directed by the targeting of the CRE elements on the insulin promoter. Therefore, it has been assumed that in addition to the well-known positive effect of GLP-1 on insulin transcription, there is also a negative effect that inhibits transcription of insulin via the p38/MAPK pathway. The stimulatory effect is larger, however, so that the net effect of GLP-1 on transcription of insulin is positive [99].

It has been shown, that metabolized amino acids such as L-leucine can act similarly to glucose and induces insulin transcription in transient transfection experiments [60], increases insulin mRNA level in isolated rat islets [254], and elevates the rate of insulin biosynthesis in isolated mouse islets [7]. On the other hand, low metabolized amino acid such as arginine had no effect (in presence of low glucose concentrations) or negative effect (in presence of higher glucose concentrations [5 mM]) on the rate of insulin biosynthesis in isolated mouse pancreatic cells [7]. In addition arginine (15 mM) in combination with low glucose (1 mM) suppressed insulin transcription in transient transfection experiments in HIT insulinoma cells [60]. However, high concentrations of arginine (19 mM) in combination with high concentrations of glucose (16.7 mM) substantially increase insulin mRNA levels in perfused rat pancreatic cells after short periods of stimulations (2 h). In the same experiment, stimulation with only 16.7 mM glucose or stimulations with lower glucose concentrations (5.5 mM) together with arginine (19 mM) had no effect on insulin mRNA level over the same time period [105]. This suggests, that arginine can potentiate the effect of glucose on insulin transcription in response to extremely high demand for insulin in mammalian β-cells [105].

2.3. Regulation of the Insulin mRNA Stability

Insulin mRNA is a highly abundant transcript in mammalian pancreatic β-cells under basal conditions. Each cell contains approximately 40,000-50,000 copies of the insulin transcript, which is 60-80 times more than the number of the β-actin mRNA molecules and constitute 30% of the total mRNA in the β-cell [59, 173, 242]. The basal level of insulin mRNA increases at least 5-fold in response to elevation of glucose levels [242]. This effect of glucose on insulin mRNA level is achieved by elevation of insulin

transcription (as described above) and by stabilization of insulin mRNA against degradation [242, 252]. The half-life of insulin mRNA is 29 h under basal conditions and increases up to 77 h when β-cells are stimulated by glucose for 24 h [242]. Short-term (15 min-1 h) glucose stimulation has no effect on insulin mRNA stability and can even initiate degradation of newly synthesized insulin mRNA [116].

The mechanism involved in stabilization of insulin mRNA remains to be elucidated. It has been reported by Tillmar et al. [242] that the 3'-untranslated region (3'-UTR) of the insulin RNA is important for glucose-stimulated insulin mRNA stability. This region contains a pyrimidine-rich segment downstream of the coding sequence that is highly conserved among species [242].

The pyrimidine-rich segment of the insulin mRNA can bind polypyrimidine tract-binding protein (PTB). Binding of PTB to the insulin 3'UTR increases in response to elevation in glucose concentrations. Furthermore, mutations in 3'UTR that prevent binding of PTB result in a marked increase in degradation of the insulin mRNA. Therefore, it may indicate that PTB participates in stabilization of the insulin mRNA [242].

2.4. Glucose Regulation of Insulin Translation

In order to rapidly replenish secreted insulin and to maintain insulin stores at a constant level, β-cells of islets of Langerhans prompt stimulation of insulin translation within a few minutes after glucose-induced insulin exocytosis. Elevation of glucose concentrations produces a 20-30-fold increase in translation of insulin in a 45 min to 1h time period [41, 59, 86, 90, 253].

It is commonly accepted that glucose-stimulated translation of insulin is independent from glucose-induced transcription, and can rely on pre-existing insulin mRNA [86, 90]. However, Leibiger et al. [119] have demonstrated that insulin secreted from β-cells in response to an elevation of glucose levels may stimulate insulin translation in an autocrine manner. This effect of insulin on its translation depends on newly synthesized insulin mRNA [118]. Therefore, the total increase in insulin biosynthesis in a response to the glucose elevation results from the equal contribution of two independent components: (1) the direct glucose stimulation of translation of the pre-

existent insulin RNA, and (2) the autocrine stimulation of insulin translation via activation of insulin transcription [118].

The molecular mechanism that is involved in the β-cell translational response to high-glucose stimuli is not clearly understood. The activation of insulin translation depends on the glucose metabolism via glycolysis and the Krebs cycle [230, 253]. In addition to glucose, other metabolites that can enter into intermediate stages of glycolysis and the Krebs cycle stimulate insulin translation. It has been shown by Skelly et al. [230] that products of pyruvate metabolism in mitochondria, such as oxaloacetate, citrate, malate, acetyl-CoA, and malonyl-CaA can be the second messengers of glucose metabolism and transmit "glucose signals" to activation of insulin translation via several possible mechanisms [230].

One of these mechanisms leads to stimulation of the general translation factors that are involved in the initiation and elongation of translation. For example, activation of the guanine nucleotide-exchange factor eIF-2B in a response to glucose in β-cells has been reported by Gilligan et al. [57]. The other glucose-induced translation factor is eIF-4E which can be activated via insulin exocytosis and the IP-3K pathway [268]. Both eIF-2B and eIF-4E are involved in initiation of translation. In addition, activation of EF-2 by glucose is mediated via protein phosphatase-2A and increases the rate of elongation [269].

The effect of glucose, which is transduced via the up-regulation of translation factors of the general translation machinery, can stimulate basal protein biosynthesis in β-cells by approximately 2-fold [65, 253]. However, glucose activates insulin translation in β-cells at a much higher rate. Therefore, other mechanisms specific for insulin translation must be involved.

One of them could be a glucose-stimulated co-translational translocation of the insulin from the cytosol to the endoplasmic reticulum (ER) [253]. This process involves the binding of signal recognition particle (SRP) to the signal peptide of the nascent insulin which is translated on the free ribosomes in cytosol. This arrests future elongation of insulin translation until the SRP- ribosome-insulin complex is translocated to the ER and SRP is associated with SRP receptor in the membrane of the ER. Glucose-stimulated translocation of the insulin is possibly mediated via modifications of SRP and/or SRP

receptor. The SRP-mediated mechanism stimulates translation of all proteins that contain signal peptides in their structure [65, 253]. In fact, Guest et al. have showed a 15-30 fold increase in activation of translation of 32 major constituents of the insulin secretory granule in response to glucose [65].

Finally, the effect of glucose on the specific regulation of insulin translation is mediated via regulatory elements in the insulin transcript. Wicksteed et al. [257] have found that 5' and 3' untranslated regions (UTRs) of the insulin mRNA can dramatically induce translation of the insulin in response to glucose elevations. This glucose-stimulated activation of insulin translation is specific to β-cells, and therefore may be mediated via binding of β-cell-specific factors to the sequences within 5' or 3'UTRs of the insulin mRNA. One of these potential sequences is a stem-loop structure that is located in 5' UTR of the insulin mRNA and is well-conserved amongst mammalian species [257].

2.4.1. Glucose Regulation of Translation in Fish

Effects of glucose on insulin biosynthesis have been studied in several fish species, including hagfish (*Myxine glutinosa*) [44], catfish (*Ictalurus punctatus*) [3], anglerfish (*Lophius americanus*) [15, 167], and carp (*Cyprinus carpio*) [78]. Glucose fails to increase the biosynthesis of hagfish insulin [44], and also has a negative or no effect on the biosynthesis of anglerfish and catfish insulin over short periods of time of incubation with high glucose [3, 167]. On the other hand, during long periods of incubation, glucose stimulates insulin synthesis in these species [3, 15, 78]. In mammals glucose has to be metabolized in order to activate translation of insulin. Since the rate of the fish metabolism is much lower than that of mammals [154], longer times are required for fish β-cells to metabolize glucose and to transduce the "glucose signal" into the translational and/or transcriptional responses. In addition, longer times are necessary for insulin biosyntheses itself and for the accumulation of newly synthesized insulin in β-cells. In fact, insulin biosynthesis and its processing took 20 h in the experiments with hagfish [44]. Thus, the negative effect of glucose on insulin biosynthesis over a short time of stimulation may reflect the glucose stimulation of a rapid insulin release from β-cells without simultaneous stimulation of insulin biosynthesis.

The positive effect of glucose over long stimulations was significantly lower than that found in mammalian species. In fact, Huth and Rapoport have reported only 0.4-fold increase in the total protein biosynthesis and no further stimulation of insulin specific biosynthesis in the response to high glucose in isolated carp Brockmann bodies [78].

In contrast, stimulatory effect of glucose was specific to insulin biosynthesis and the total rate of protein synthesis had not changed in experiments with anglerfish and catfish Brockmann bodies [15]. The specific increase in the insulin biosynthesis may be mediated via the regulatory sequences within the insulin mRNA transcript, as has been described for the regulation of the rat insulin [257]. Although the rat and the tilapia insulin 5'UTRs have no homology between each other, analysis of the tilapia insulin 5'UTR using GeneRuner software reveals a presence of the stem-loop structure similar to that found in 5'UTR of the rat insulin.

2.5. Summary

Insulin is a key hormone in the regulation of blood glucose homeostasis. It is commonly accepted that in adult mammals insulin expression is restricted to β-cells of the pancreatic islets of Langerhans. This β-cell-specific expression of insulin is regulated at the level of insulin transcription via interaction of the transcription factors with the insulin promoter/enhancer region. Despite the fact that some of the insulin transcription factors are predominantly expressed in β-cells, none of the currently identified insulin transcription factors is exclusively β-cell specific. Therefore, the β-cell-specific expression of the insulin gene does not depend on transcription activation by a single β-cell-specific factor, but is comprised of a complex interaction among several β-cell-enriched transcription factors.

Although many of the insulin transcription factors are able to change the level of insulin transcription themselves, the full activity of the insulin promoter depends mostly on complex interactions between transcriptional factors, DNA structural proteins, intra-cellular signalling molecules and the RNA polymerase complex.

In addition to the regulation of the cell-specific transcription of the insulin gene, β-cells "sense" elevation in the blood glucose concentrations and respond immediately by secreting stored insulin and by increasing the rate of insulin biosynthesis. It is commonly

accepted that over short periods of stimulation β–cells are able to compensate for any insulin loss by exocytosis and to maintain intracellular insulin homeostasis via augmentation in the translation of the pre-existing insulin mRNA from the cytosol storage pool and therefore without transcriptional activation of the insulin gene [184, 186, 253]. However, after long periods of stimulation activation of insulin biosynthesis strongly relies on elevation of the insulin mRNA level through a rise in the rate of insulin gene transcription and through an increase in the stabilization of the insulin mRNA against degradation [90, 121, 166, 185, 252].

Glucose-stimulated transcription of the insulin gene depends on interplay amongst several signalling pathways that transduce "glucose signals" into an increase in activation of insulin transcription factors.

The regulation of insulin translation depends on the combined effects of glucose at three distinct levels: (1) unspecific up-regulation of total protein biosynthesis in β-cells via activation of the initiation and elongation steps of translation; (2) glucose-induced translocation of nascent peptides from the cytosol to the ER, mainly specific to the proteins of secretory pathways; and (3) insulin specific elevation of translation via the involvement of regulatory sequences of the insulin mRNA.

In addition to the regulation of insulin transcription and translation glucose increases stability and therefore half-life of the insulin mRNA.

The coordinated regulation of insulin biosynthesis at the levels of translation, transcription, and mRNA stability allows β-cells to maintain a tight control over the intracellular insulin stores and to respond rapidly to elevations in blood glucose levels.

Chapter 3

Regulation of Insulin Biosynthesis and Secretion in the Pancreatic Islet Cells of Tilapia (*Oreochromis Niloticus*)*

3.1. Abstract

Compared to mammals, little is known about insulin gene expression in fish. In this study we demonstrate that transcription of the Nile tilapia (*Oreochromis niloticus*) insulin gene is regulated in a β-cell-specific manner when expressed transiently in mammalian insulinoma cell lines. Using deletion analysis we found that cooperative interactions between regulatory elements within the proximal (-1 to -396 bp) and the distal (-396 bp to -1575 bp) promoter regions were necessary for induction of the β-cell-specific transcription. In contrast to the regulation of mammalian insulin genes, transfection experiments revealed that transcription of the tilapia insulin gene is not up-regulated by glucose.

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* This chapter is a modified version of the manuscript "Regulation of insulin gene expression and insulin production in Nile tilapia *(Oreochromis niloticus)*" that is *in press* in the journal of "General and Comparative Endocrinology" (2007) by Olga Hrytsenko, James R. Wright Jr., and Bill Pohajdak

Effects of glucose and arginine on endogenous insulin secretion, translation, and transcription in isolated tilapia Brockmann bodies were determined using Northern hybridization, Western analysis, and quantitative RT-PCR. Similar to the regulation of mammalian insulin, we found that increases of glucose (1-70 mM) and arginine (0.4-25 mM) induced insulin secretion. However, transcription of the insulin gene was activated only by extremely high concentrations of glucose and arginine added simultaneously. When stimulated for 24 h with low concentrations of both inducers or with either of them added separately, tilapia β-cells were able to replenish secreted insulin and to maintain insulin stores at a constant level without elevations of the insulin mRNA levels. Since the basal level of insulin mRNA was approximately 3.7-fold higher in tilapia β-cells than it is in mammalian β-cells, insulin production in tilapia cells probably relies on an enlarged intracellular insulin mRNA pool and does not require the transcriptional activation of the insulin gene.

3.2. Introduction

Recently, teleost species have become an attractive model system in many different areas of hormonal and metabolic research [147], including studies of insulin-dependent [98] and non-insulin-dependent [260] diabetes mellitus. One of the main advantages of employing teleost fish in experimental pancreatic endocrinology is, that in some fish species [including Nile tilapia (*Oreochromis niloticus)*], the pancreatic endocrine cells are separate from the pancreatic exocrine tissue forming discrete islet organs (i.e., Brockmann bodies), which can be easily identified and harvested [271]. In this context, tilapia can also be used as an excellent donor organism for islets xenotransplantation research and possibly as a future treatment for insulin-dependent diabetes mellitus [196, 263, 264, 271].

Little is known about tissue-specific and nutrient-stimulated regulation of insulin biosynthesis and secretion in the fish islet cells. To date, only the trout insulin promoter has been partially characterized [8]. Although its sequence shows no significant similarity to that of mammalian insulin promoters, the trout promoter activates the transcription of the reporter gene in a β-cell-specific fashion upon transfection into mammalian cell lines [8]. To our knowledge, there are no data available regarding the effects of glucose or

other nutrients on the steady-state level of insulin mRNA or on the regulation of insulin gene transcription in fish.

As in mammals, β-cells of fish Brockmann bodies respond to increases in blood glucose levels by activation of insulin secretion [154]. However, most reports on fish indicate no effect of glucose on induction of insulin translation [154]. In addition to glucose, amino acids (arginine, lysine, leucine) stimulate insulin secretion. Moreover, in some fish species arginine is a more potent insulin secretagogue than glucose, and can stimulate a higher insulin release [84, 154, 206]. On the other hand, the effect of arginine on regulation of insulin biosynthesis in fish species has not yet been elucidated.

In this study, we further characterized mechanisms of fish insulin biosynthesis and secretion to better understand the usefulness and application of teleost species in diabetalogical and islet endocrinological studies. We have applied two different approaches: (1) the analysis of the β-cell-specific and glucose-stimulated transcription of the tilapia insulin gene in transient transfection experiments using commonly employed mammalian cell lines; and (2) *in situ* studies of isolated cultured tilapia Brockmann bodies to confirm the results obtained in mammalian insulinoma cells and to further analyze insulin regulation in the native environment. To our knowledge, this is the first report concerning the effect of nutrients on the regulation of insulin transcription in fish.

3.3. Materials and Methods

3.3.1. Plasmid Constructions

The p3.5INS construct containing 1575 bp of 5' insulin flanking region was cloned in our laboratory earlier [138, 196]. It was used to prepare reporter constructs for the promoter studies. In the first series of experiments, different fragments of the 5' insulin flanking region were inserted upstream of the firefly luciferase gene in the pGL2-Basic vector (Promega, Madison, WI). Inserts of all promoter constructs contained the first 14 bp of the transcribed region and various 5'flanking regions (Fig. 3.1 and 3.2 A).

To test the enhancer activity of the distal 5' flanking region, PCR products corresponding to the –535 to-396 bp, -784 to-396 bp, and –1575 to-396 bp sequences in the insulin promoter were generated using specific primers and p3.5INS DNA as a

template. PCR products were purified, cloned into the pCRII vector (Invitrogen, Burlington, ON), and then transferred into the pGL2-Promoter vector (Promega) (Fig. 3.3. A).

The phins construct, containing 378 bp of the human insulin promoter, was used as a positive control. The –378 bp to +7 bp region of the human insulin promoter was amplified using specific primers and human genomic DNA, extracted from YT cell line. PCR product was purified, cloned into the pCRII vector (Invitrogen, Burlington, ON), and then transferred into the pGL2- Basic vector (Promega).

The correct orientation and the nucleotide sequence of all constructs were confirmed by manual sequencing using the TermoSequenase Radiolabeled Terminator Cycle sequencing kit (Amersham Biosciences, Baie d'Urfe, QC).

3.3.2. Cell Culture and DNA Transfection

All cell culture media were supplemented with 10% fetal bovine serum (MEDIcorp, Montreal, QC), 2 mM glutamine (GIBCO-BRL, Burlington, ON), 100 units/ml penicillin and 100 μg/ml streptomycin (GIBCO-BRL). HIT-T15 (Syrian hamster insulinoma β-cell line), NIT-1 (NOD mouse insulinoma β-cell line) and COS-1 (Green monkey kidney fibroblast-like cell line) were obtained from the ATCC corporation (Manassas, VA). HIT-T15 and NIT-1 were maintained in Ham's F-12 medium (7 mM glucose) (Sigma, Oakville, ON). COS-1 was maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO-BRL). Twenty-four hours before transfection, cells were seeded in 60-mm cell culture dishes in RPMI 1640 medium (MEDIcorp) supplemented with different concentration of glucose. In the experiments with the β-cell-specific promoter activations (the first section of the results), we used a low glucose concentration (1 mM). For the studies of glucose effects on transcriptional activation (the second section of the results), the concentrations of glucose were adjusted from 3 mM, to 70 mM (3 mM, 7 mM, 20 mM, 28 mM and 70 mM).

All transfection experiments were performed using 62 to 80 passages of the HIT-T15 cells and 27 to 45 passages of the NIT-1 cell lines.

A mixture of 1 μg of one of the promoter constructs and 1 μg of the internal control plasmid [pSV-β-Galactosidase (Promega), which contains the β–galactosidase gene

under the control of the promoter] were used in each transfection. Transfections were performed using LipofectAmine 2000 transfection reagent (Invitrogen).

3.3.3. Luciferase and β-Galactosidase Assay

Forty-eight h after transfections, cells were harvested and resuspended in the Reporter Lysis Buffer (Promega). Luciferase activity was assayed using the Luciferase Assay System (Promega) and quantified on a PerkinElmer Wallac scintillation counter (PerkinElmer, Boston, MA). β-galactosidase concentrations were assayed using β-Galactosidase Enzyme Assay System (Promega) and quantified on a SPECTRAmaxR*PLUS*384 Microplate Spectrophotometer (Molecular Devices Corp, Sunnyvale, CA). The β-Galactosidase enzyme assay was used to determine the efficiency of transfections. Ratios of luciferase activity/ β-galactosidase activity were calculated for each transfection.

3.3.4. Tilapia Maintenance and Brockmann Body Isolation and Culturing

The Nile tilapia were purchased from Northern Tilapia INC (Lindsay, ON), and were reared in 40-gallon aquaria with circulating aerated fresh warm (approximately 27 ^{0}C) water in the Biology Department at Dalhousie University as described by [261]. Fish were fed twice a day with commercially available fish pellet food (7.5 Aqua Brood, Fredericton, NB) containing 45% of crude protein, 20% of crude fat, 1.7% of crude fibre, 2.1% of calcium, 1.5% of phosphorous, 0.6% of sodium, 6600 IU/kg of vitamine A, 4800 IU/kg of vitamine D3, and 500 IU/kg of vitamine E. The equal amounts of Brockmann bodies were isolated either from 3 to 4 large fish (approximately 1000-1500 g) or from 8-12 small fish (150-400 g) [39] after anesthetizing with 2-phenoxyethanol (Sigma) as described by [271]. Briefly, Brockmann body regions were excised from the tilapia, placed in Hanks balanced salt solution containing 27 mM HEPES, 200 U/ml penicillin, 200 μg/ml streptomycin sulfate, and 3 mg/ml collagenase type II (Sigma) and digested at 37 ^{0}C for 10-15 min. The white Brockmann body pellet was transferred to fresh Hanks balanced salt solution and the remains of connective tissues were removed by micro-dissection using microvascular scissors and jeweller's forceps under a dissecting microscope.

Thirty pieces of Brockmann bodies approximately 500 μm diameter were precultured in RPMI 1640 medium (GIBCO-BRL) or DMEM medium (MEDIcorp) containing low (1 mM) glucose. After 4 h, the medium was replaced with the experimental medium, which was either RPMI 1640 medium supplemented with various glucose concentrations or DMEM medium adjusted with various concentrations of glucose and/or arginine. Both these media were supplemented with 10% fetal bovine serum (MEDIcorp), 2 mM glutamine (GIBCO-BRL), 100 units/ml penicillin and 100 μg/ml streptomycin (GIBCO-BRL). Brockmann bodies were cultured at 37°C in a humidified atmosphere containing air: CO_2=95:5. Similar culturing conditions, and even harsher, have been used by us previously and are known not to cause changes in cell viability or in cell morphology at 24 h of incubation [262, 265].

In experiments without actinomycin D, the culture medium was collected and replaced with fresh experimental medium after 23 hours. The cells were then cultured for an additional hour, then both medium and cells were harvested. For experiments with actinomycin D, after 12 hours of incubation the medium was replaced with fresh experimental medium containing 1μg/ml of actinomycin D (Sigma). The medium and cells were then collected following another 12 h of incubation.

3.3.5. RNA and Protein Extractions

Total RNA and proteins were isolated from tilapia Brockmann bodies using TRIzol reagent (Invitrogen) according to the recommended protocol.

3.3.6. Northern Blot and Hybridization

Aliquots of the total RNA were loaded on a denaturing 1% agarose gel, electrophoresed and blotted onto a nylon membrane (Hybond-N+, Amersham Biosciences). A 210 bp insulin probe and a 168 bp 18S rRNA probe that represented parts of the insulin or 18S cDNA were DIG-labeled and used in two consecutive hybridizations. First, the membrane was hybridized with the insulin probe and then, after stripping, with the 18S rRNA probe. Labeling, hybridizations, and detection were performed using DIG High Prime Labeling and Detection Starter Kit II (Roche Applied Science, Laval, QC) according to the protocol provided. The hybridizations with the 18S

rRNA probe were performed in order to compare and to normalize the results of different experiments. The level of intensity of the obtained signals was quantified using Scion Image software for Windows.

3.3.7. Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

To remove contaminating DNA, 0.5 μg or 0.8 μg aliquots of isolated total RNA were treated with 1U of DNAseI (Invitrogen). For comparison of the insulin mRNA levels in Brockmann bodies stimulated with glucose and arginine, total RNA was reverse transcribed using a SuperScript III Platinum Two-Step qRT-PCR Kit with SYBR Green (Invitrogen). For the quantification of the insulin and actin mRNA ratios in β-cells of tilapia Brockmann bodies, the total RNA was reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen) with Oligo(dT) primer (Invitrogen). qRT-PCR primers were created with the OligoPerfect™ Designer program (www.invitrogen.com). The size of all PCR products and primer sequences are listed in Table 1 (in appendices). Quantitative amplifications of insulin 18S rRNA and β-actin products, and data analysis were performed as described by us [75]. Each cDNA sample was analyzed in four duplicated PCR representing four different template dilutions ranging from 1:10 to 1:100. In preliminary experiments linear correlations between the inputted cDNA template and the calculated transcript concentration were determined to occur within this range of dilutions. To compensate for any variance in the amount or quality of the input RNA, the levels of insulin mRNA are shown relative to the levels of 18S rRNA or β-actin mRNA that were used as internal controls.

3.3.8. Western Blot and Hybridization

Protein concentrations were determined using the Bicinchoninic acid protein assay kit (Sigma). Equal amounts of proteins or culturing medium were electrophoresed in a 4-12% SDS-polyacrylamide gel with glycine buffer or in 4-15% SDS-polyacrylamide gel with tricine buffer, and electroblotted onto PVDF transfer membrane (Hybond-P, Amersham Biosciences). For insulin or actin detection, filters were hybridized for 12 h with rabbit anti-tilapia insulin 1^{o} antibodies (1:100), developed by us [270], or with anti-actin 1^{o} antibodies (1:250) (Sigma). Membranes were then washed with PBS/0.1%

Tween and incubated for 1 h with HRP-conjugated goat anti-rabbit 2^{o} antibody (1:5,000) (Santa Cruz Biotechnology, Santa Cruz, CA). For the tubulin detection, filters were hybridized for 12 h with DM1A, mouse monoclonal anti-α-tubulin 1^{o} antibodies (1:500) (Sigma) followed by washes with PBS/0.1% Tween and by 1 h incubation with HRP-conjugated rabbit anti-mouse 2^{o} antibody (1:5,000) (Santa Cruz Biotechnology). After hybridization, filters were processed for chemiluminescent detection using a Western Blotting Luminol Reagent kit (Santa Cruz Biotechnology). Exposed film was analyzed using Scion Image software for Windows. The α-tubulin or actin signals were used for normalization and for comparison.

3.3.9. Negative Control for In Situ Experiments

Liver tissue was isolated from the same fish as were the Brockmann bodies, cultured under the same conditions and used as a negative control (n=1) for the *in situ* experiments described above. We did not detect insulin or insulin mRNA in any of the liver samples tested (results not shown).

3.3.10. Data Analysis and Statistics

All experiments were repeated at least three times. Results were analyzed using ORIGIN software for Windows. Data were considered significantly different at the level of $p<0.05$ using an unpaired Student's *t* test.

3.4. Results

3.4.1. Analysis of the β-Cell-Specific Transcription of the Tilapia Insulin Gene

The 1572 bp region upstream of the tilapia insulin transcription start site (TSS) has been cloned and sequenced in our laboratory [138, 196]. Alignments of this region to the corresponding sequences of the trout, human, both mouse and both rat insulin promoters revealed little sequence similarity. Computer analysis of the same region using the TESS database (www.cbil.upenn.edu) identified several potential general and insulin-specific transcription factor binding sites, including E-, and A-like elements, a TATA-box, and CEB and CRE boxes (Fig. 3.1). Like the human insulin promoter, this region also contains a tandem repeat region (CA_{17}) found at position –251 bp to –283 bp.

Figure 3.1. Nucleotide sequence of the tilapia insulin gene 5' flanking region
Locations of the potential regulatory elements are highlighted: -E-boxes, TAAT-A-like elements, GAGA-G-like sequence, -boxes. TATA-box is in red and the $\underline{CA_{17}}$ simple repeat sequence is underlined. Arrows indicate locations of the 5' ends of the deletion constructs. TSS-transcription start site.

To further localize the transcriptionally important sequences in the 5' flanking region of the tilapia insulin gene, we prepared a series of reporter constructs containing 5' deletions of the tilapia insulin promoter/enhancer region (from 67 bp to 1.5 kb upstream of the insulin TSS) linked to the firefly luciferase gene (Fig. 3.2 A). These were then transfected into two insulin-producing β-cell lines—HIT-T15 and NIT-1 and into the non-insulin producing COS-1 cell line. Forty-eight hours after transfections, the cells were harvested and the cell extracts were assayed for luciferase (LUC) activity (Fig. 3.2 B). We found that the region from +14 bp to -67 bp of the tilapia insulin promoter can activate transcription of the reporter gene at least 6-fold in both insulin and non-insulin producing cell lines. Further elongations of the promoter size up to -396 bp caused no

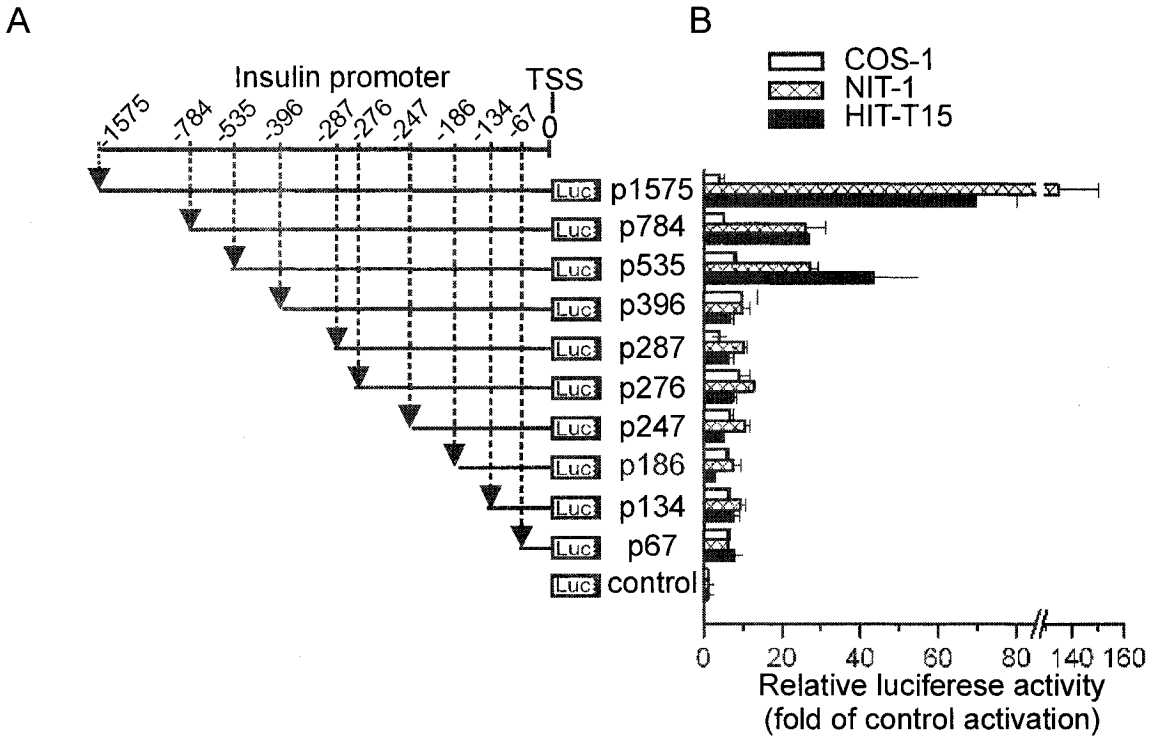

Figure 3.2. Functional analysis of the 5' flanking region of the tilapia insulin gene in different cell lines

(A) Schematic representation of the reporter luciferase constructs containing indicated regions of the tilapia insulin promoter/enhancer. *(B)* Luciferase activities were assayed 48 h after transfections of the promoter-LUC plasmids and promoter-less control into HIT-T15, NIT-1, and COS-1 cell lines. Results are presented as means ±SE of at least three independent experiments and are normalized against efficiency of transfections. Relative luciferase activities of the promoter-LUC constructs are expressed as fold of increase in activation over the control plasmid that was arbitrary set as 1.0.

significant changes in transcriptional activity in all constructs transfected into HIT-T15 cells and in the majority of the constructs transfected into COS-1. In fact, in COS-1 cells, only the LUC activity of p287 (containing the CA_{17} repeat) was significantly lower (37%) than that of p67, suggesting that a long CA repeat can be involved in suppression of promoter activity in noninsulin-producing cells. However, the expression level of the reporter gene was restored to the level of the p67, when the promoter size was elongated up to –369 bp (9.9-fold of activation for the p369). This may indicate that a regulatory element, which is located within the region –276 bp to-396 bp, can inactivate the negative influence of the long CA repeat.

In NIT-1 cells, LUC activities of p247, p276 (containing the CA_{13} repeat) and p287

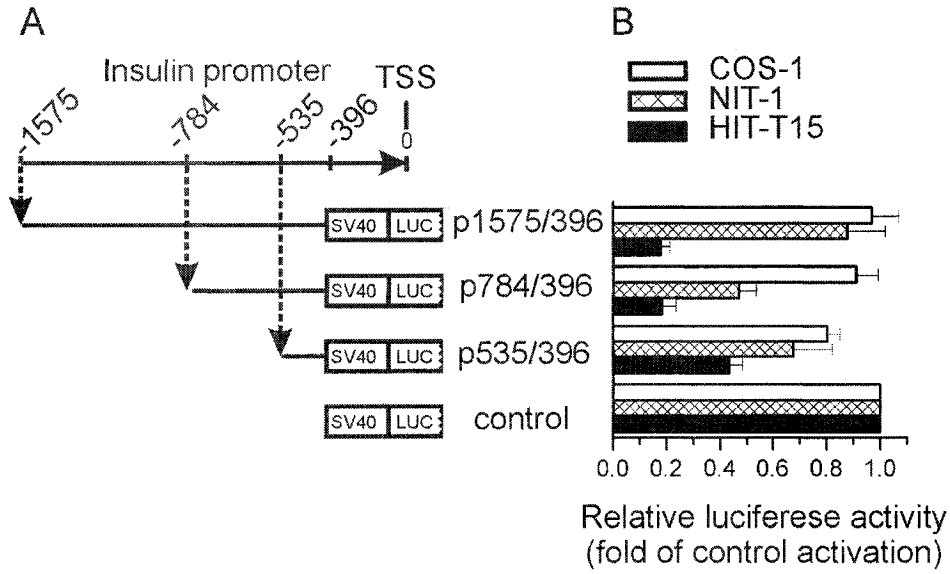

Figure 3.3. Functional characterizations of the distal insulin flanking region (–1575 bp to –396 bp)
(A) Schematic representation of the reporter luciferase constructs containing indicated regions of the distal tilapia insulin promoter/enhancer linked to the strong SV40 promoter. *(B)* Luciferese activities were assayed 48 h after transfections of indicated plasmids and of the control plasmid (SV40- promoter-luciferase vector) into HIT-T15, NIT-1 and COS-1 cell lines. Results are presented as means ±SE of at least three independent experiments and normalized against efficiency of transfections. Luciferese activity of the control plasmid was arbitrary set as 1.0 and luciferase activities of promoter-SV40-LUC constructs are expressed relative to it.

(containing the CA_{17} repeat) were modestly increased (approximately 2-fold) compared to the LUC activity of the p67. This might suggest that weak β-cell specific regulatory element(s) are located within -186 and –247 bp in the insulin promoter.

When promoter size was further increased up to –535 bp, we observed a strong rise in expression levels of the luciferase gene in both insulinoma cell lines (6-fold increase in HIT-T15 cells and 3-fold increase in NIT-1 cells compared to the LUC level of p396), but not in COS-1. Furthermore, elongation of the promoter sequence from –784 bp to –1575 bp caused an additional substantial increase of LUC activity in both insulinoma cell lines (5.2-fold in NIT-1 cells and 2.6-fold in HIT-T15 cells compared to the LUC level of p784). These results suggest that the distal regions (-396 bp to -535 bp and –784 bp to -1575 bp) of the tilapia insulin promoter contain strong β-cell-specific activators. Transfections of p535, p784, and p1575 into COS-1 cells revealed no significant changes

in the level of expression for p535 or p784 and a slight drop in LUC activity for p1575, suggesting that the –784 to -1575 bp region might also contain the negative element(s) capable of suppression of promoter activity in non-β-cells.

Next, we examined whether the distal part of the tilapia insulin flanking sequence (-396 bp to 1575 bp) may function as an independent β-cell-specific enhancer in association with any heterologous promoter, or if its activational potential relies on specific interactions with the proximal insulin promoter region. We prepared three additional constructs: p535/396, p784/396, and p1575/396, where the –396 to -535 bp, -396 to -784 bp, and –396 to -1575 bp regions of the tilapia insulin promoter/enhancer, respectively, were cloned upstream of the ubiquitous SV40 promoter in the luciferase vector (Fig. 3.3 A). The p535/396, p784/396, and p1575/396 together with the control plasmid (luciferase gene governed by the enhancerless SV-40 promoter) were transfected into HIT-T15, NIT-1, and COS-1 cell lines. As shown in Figure 3.3 B, the expression levels of all constructs in COS-1 cells remained at approximately the control level. The LUC activity of all constructs in HIT-T15 was 60%-83% lower than the activity of the control. When transfected into NIT-1 cells, only the p784/396 construct had a transcriptional activity significantly lower than that of the control. These data indicate that the regions between -396 bp and -1575 bp, when linked to a heterologous promoter, appeared to cause either no effect or a negative effect on transcriptional activation.

3.4.2. Analysis of Glucose-Stimulated Regulation of the Tilapia Insulin Promoter

To investigate the effect of increased glucose concentrations on the activation of the tilapia insulin promoter and to localize putative glucose sensitive elements in its sequence, we performed transient transfections of all ten promoter constructs into HIT-T15 and NIT-1 insulin-producing cell lines grown in three different glucose concentrations: 1 mM, 7 mM, and 70 mM. As shown in Figures 3.4 A and 3.4 B, we did not find any positive dose-dependent correlation between the three glucose concentrations and LUC activity in any construct tested in both cell lines.

Interestingly, when transfected into HIT-T15 cells cultured at 7 mM glucose LUC activities of p784, p287 and p276 were significantly suppressed compared to those at 1 mM and 70 mM glucose. A similar effect was observed in NIT-1 cells where the

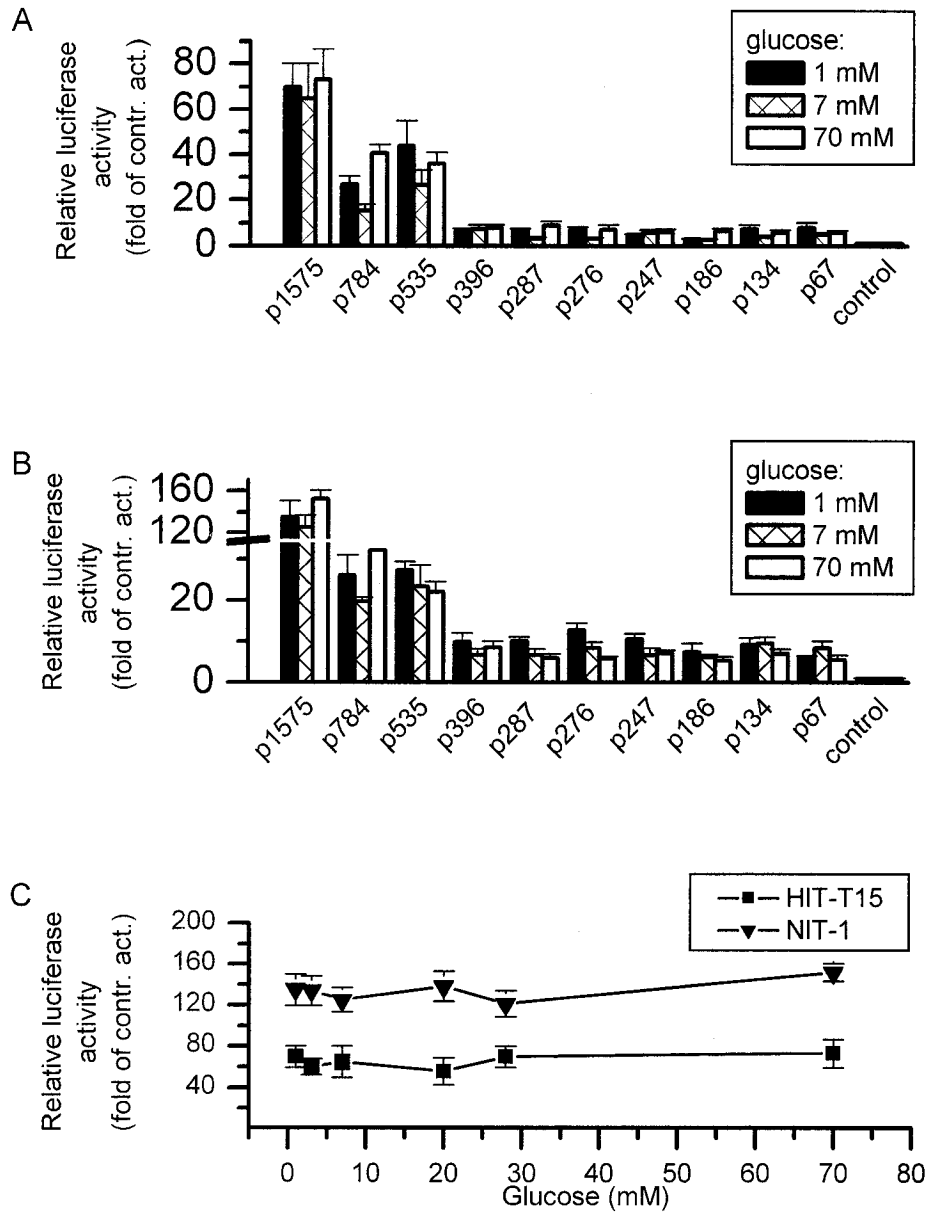

Figure 3.4. Effects of glucose concentrations on transcriptional activation of the tilapia insulin promoter/enhancer

Insulin-producing cell lines HIT-T15 *(A)*, and NIT-1 *(B)* were grown in 1 mM, 7 mM, and 70 mM glucose and transfected with the same promoter-LUC constructs and control plasmid as described in Fig. 3.2. A. *(C)* HIT-T15 *(rectangle)* and NIT-1 *(triangle)* were grown in 1 mM, 3 mM, 7 mM, 20 mM, 28 mM, and 70 mM glucose and transfected with the longest promoter construct containing 1575 bp of the tilapia insulin 5' flanking region and the control plasmid (not shown). All results (*A, B,* and *C*) are presented as means ±SE of at least three independent experiments and normalized against efficiency of transfections. Relative luciferase activities of the promoterLUC constructs are expressed as fold of increase in activation over the control plasmid that was arbitrary set as 1.0.

transcriptional activities of p287 and p276 were 1.7 and 2.2-fold lower at 70 mM glucose then at 1 mM glucose, suggesting that the region –247 bp to -276 bp may contain a negative glucose-regulated element sensitive to the specific glucose concentrations (supraphysiologically high in NIT-1 cells and relatively high in HIT-T15 cells).

Our results with the longest construct, p1575 (Fig. 3.4 A, B) indicated that neither 1 mM, 7 mM, nor 70 mM glucose had any effect on the transcriptional activation of the whole promoter region (n=4-6). However, in most studies [53, 54, 148, 168, 172], transcriptional activation of mammalian insulin genes is tested in response to glucose concentrations in the range of 2 mM to 20 mM. Thus, in order to further examine glucose-responsiveness of the tilapia insulin promoter we also tested the effects of 3 mM, 20 mM, and 28 mM glucose on transcriptional activation of the longest construct (p1575) transfected into HIT-T15 and NIT-1 cells. Again, we did not find any significant changes in the level of p1575 expression at any of the six concentrations tested in either cell line (Fig. 3.4 C). However, LUC activities were an approximately 1.7-2.2-fold higher in NIT-1 cells than in HIT-T15 cells.

Thus, our results indicate that, in contrast to the glucose-sensitive transcription of the mammalian insulin genes, transcription of the tilapia insulin gene is not stimulated by glucose, at least when tested using mammalian insulinoma cell lines.

3.4.3. Effects of Glucose and Arginine Added Separately on Transcription, Translation and Secretion of Tilapia Insulin in Isolated Tilapia Brockmann Bodies

To study the effects of glucose, isolated tilapia Brockmann bodies were incubated in RPMI 1640 medium with increasing concentrations of glucose (from 1 mM to 70 mM). To examine the effects of arginine, Brockmann bodies were incubated in DMEM medium containing low glucose concentrations (1 mM) and increasing concentrations of arginine (from 0.4 mM to 25 mM). After 23 hours, the medium was collected and replaced with fresh experimental medium, followed by another hour of incubation. The islet cells and medium were then harvested and the total RNA and proteins were isolated from the islet cells.

To test the effects of glucose and arginine on the secretion of the tilapia insulin, aliquots of the culture medium collected after 23 h and 1 h of incubation were screened

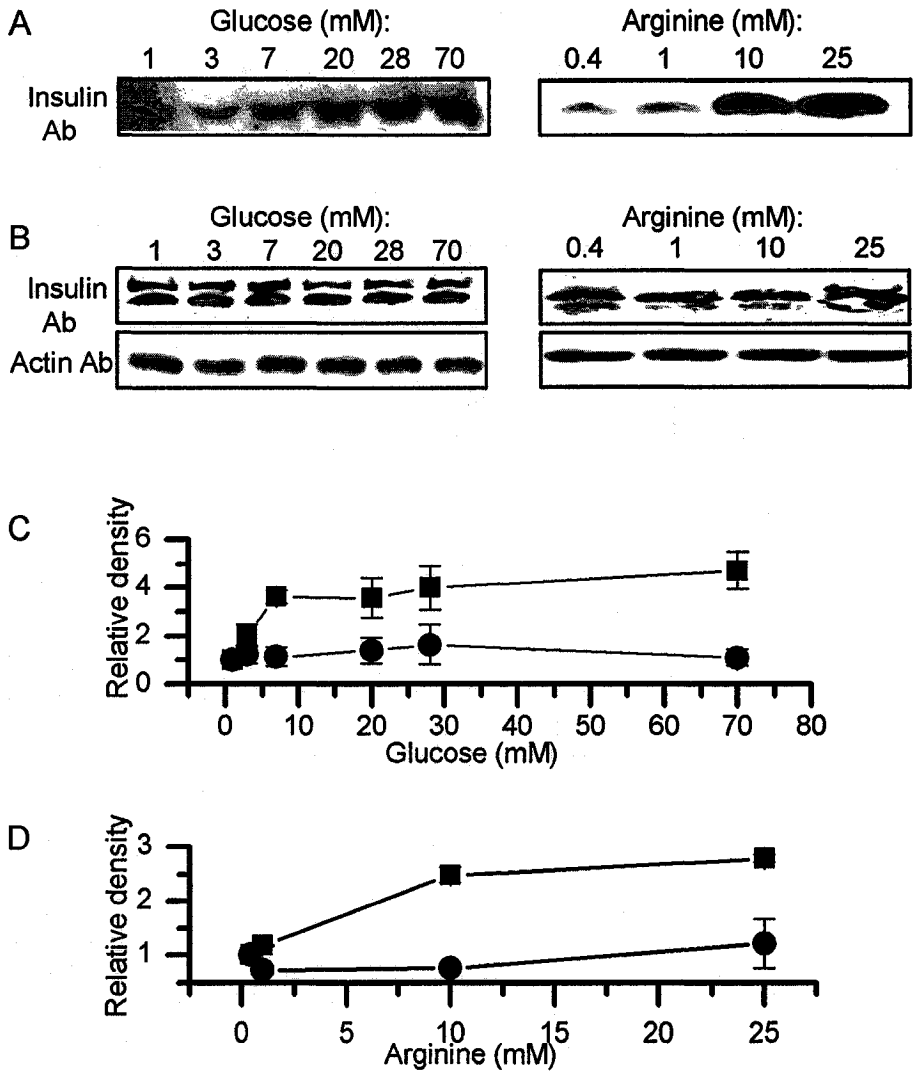

Figure 3.5. Effect of glucose or arginine concentrations on insulin content and insulin secretion in isolated tilapia Brockmann bodies

Equal amounts of tilapia Brockmann body tissue were exposed to the indicated glucose or arginine concentrations for 23 h. Typical results of the Western analysis of *(A)* aliquots of the culture medium hybridized with the tilapia insulin antibodies; *(B)* intracellular proteins hybridized with tilapia insulin antibodies *(top panel)* or actin antibodies *(bottom panel)* that were used as internal controls. Obtained signals were quantified using Scion Image densitometry analysis *(C and D)*, where *(Square)*- levels of the secreted insulin, and *(Dot)*- levels of the intracellular insulin shown as the insulin/actin or insulin/tubulin ratios. Data are expressed as fold increases in density relative to the density of the sample exposed to the lowest concentration of stimulator: 1 mM glucose *(C)*, or 0.4 mM of arginine *(D)* which are taken as 1.0. All data are presented as means ±SE of at least three independent experiments.

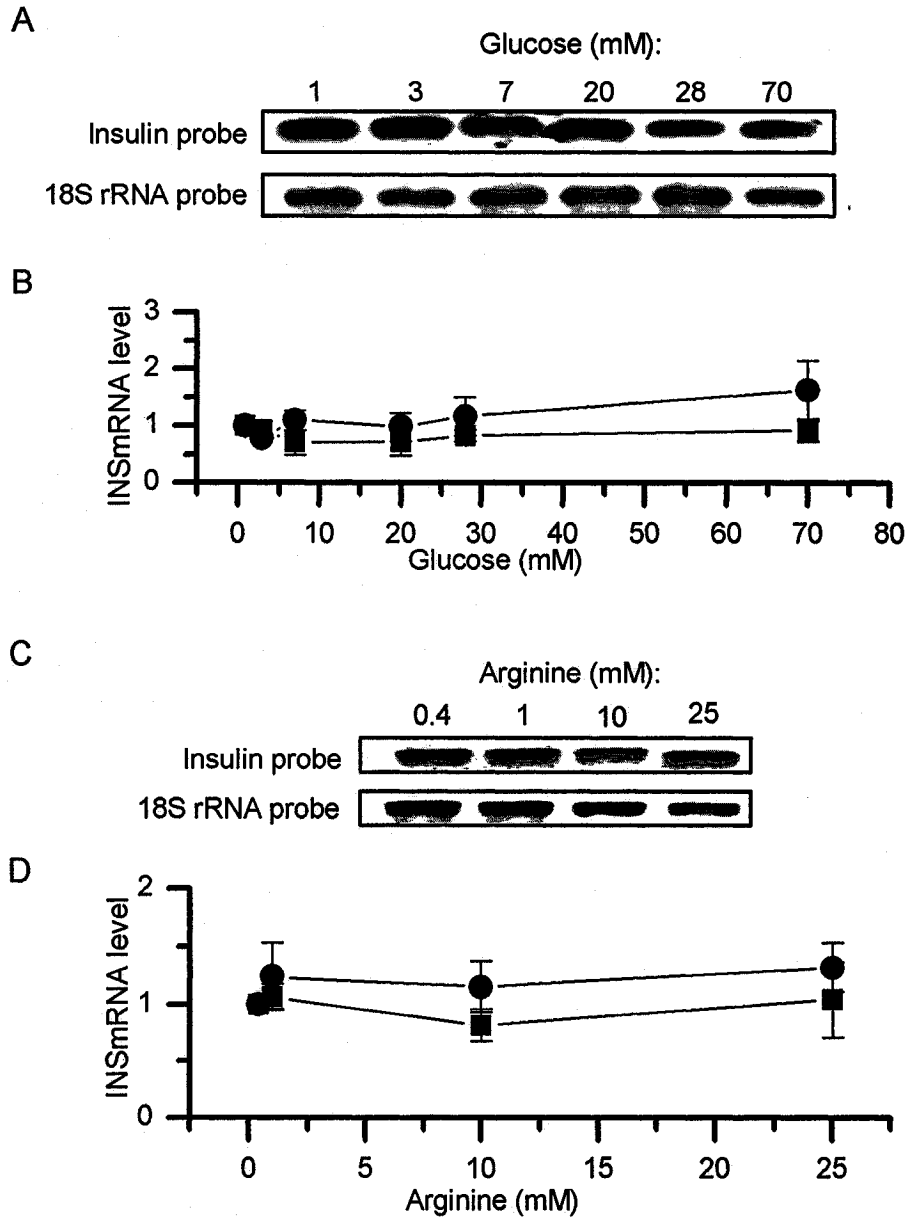

Figure 3.6. Effect of glucose or arginine concentrations on the insulin mRNA content in isolated tilapia Brockmann bodies

Tilapia Brockmann bodies were exposed to indicated glucose or arginine concentrations, total RNA was isolated and insulin mRNA levels were examined using Northern hybridizations and qRT-PCR. *(A and C):* typical results of Northern hybridization with insulin probe *(top panel)* and 18S rRNA probe *(bottom panel). (B and D):* the relative levels of insulin mRNA (presented as insulin mRNA/18S rRNA ratios) were quantified by Scion Image densitometry analysis of the signals obtained in Northern hybridizations *(Square)* and by qRT-PCR with insulin-specific and 18S rRNA-specific primers *(Dot).* The insulin mRNA/18S rRNA ratios obtained in samples induced with the lowest concentration stimulators (1 mM glucose or 0.4 mM arginine) are arbitrary set as 1.0 and all data are normalized against those. All results are expressed as means ±SE of at least three independent experiments.

using Western blots probed with tilapia insulin antibodies. A rise in glucose concentration from 1 mM to 70 mM and in arginine concentration from 0.4 mM to 25 mM caused strong dose-dependent increases in secreted insulin levels in the culture medium after 23 h of incubation (Fig. 3.5 A, C, D). The secretory response was most pronounced within glucose concentrations of 1-7 mM and arginine concentrations of 1-10 mM. Thus our results indicate that, as in mammalian β-cells, glucose and arginine stimulate insulin secretion in isolated cultured tilapia Brockmann bodies. Similar results were obtained using Western blots performed with 1 h medium aliquots, suggesting that at the time when the intracellular insulin and insulin mRNA were extracted (after 24 h of incubation) the cells continued to secret insulin and to maintain their glucose- and arginine-responsiveness.

Total proteins isolated from the Brockmann bodies were examined using a Western blot hybridized with tilapia insulin antibodies. We did not observe any significant differences in signal intensity among samples isolated from the Brockmann bodies induced with a range of concentrations of glucose (1-70 mM) or arginine (0.4-25 mM) (Fig. 3.5 B–D), suggesting that the intracellular insulin levels remained constant regardless of the increase in glucose or arginine concentrations in the medium.

To examine the effect of glucose and arginine on the level of the insulin mRNA, aliquots of total RNA were used for Northern hybridization screened with the insulin probe and for qRT-PCR performed with insulin-specific primers. We did not detect any statistically significant variations in the glucose-induced and arginine-induced steady-state level of the insulin mRNA in either assay (Fig. 3.6). However, the level of insulin mRNA determined by qRT-PCR was slightly but not significantly higher after stimulation with 70 mM glucose. This indicated that neither glucose (1-70 mM) nor arginine (0.4-25 mM) had any effect on the insulin transcription or on stabilization of the insulin mRNA in the isolated tilapia Brockmann bodies.

3.4.4. Simultaneous Effects of Glucose and Arginine on Transcription, Translation and Secretion of Tilapia Insulin in Isolated Tilapia Brockmann Bodies

Next, we studied the effects of the combined elevations in glucose and arginine concentrations on insulin biosynthesis and secretion. We also compared these to the

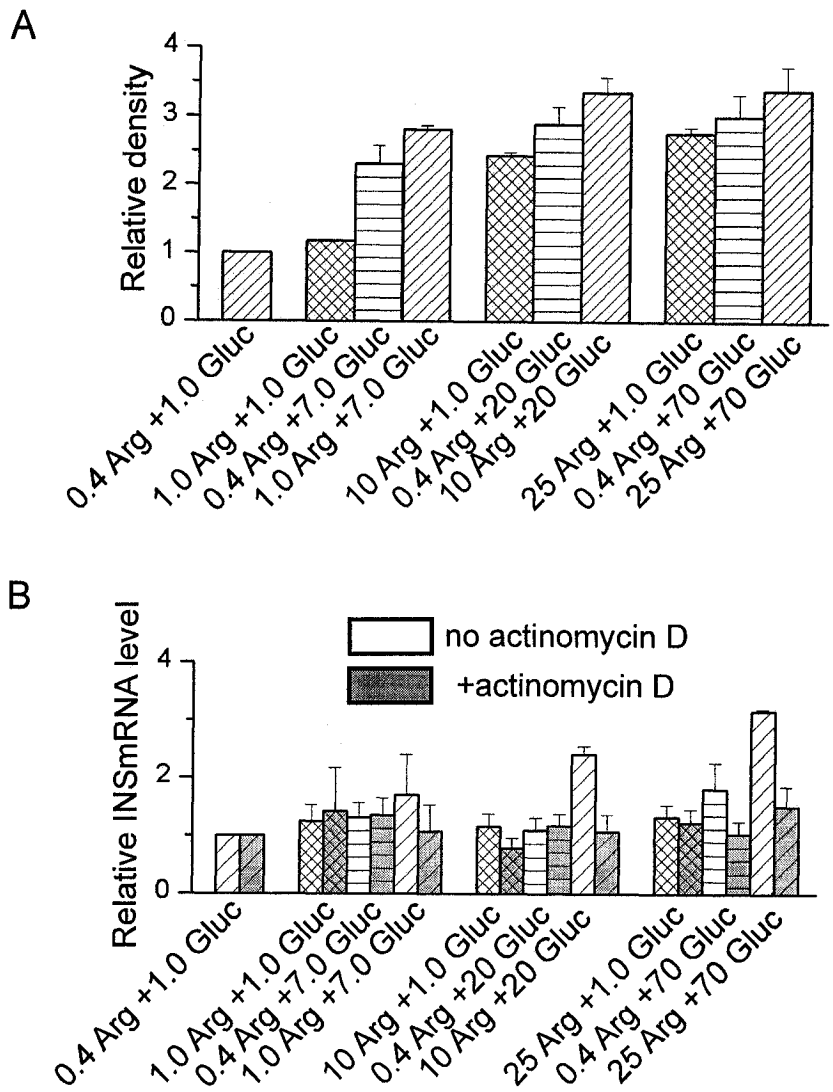

Figure 3.7. Effects of different concentrations of glucose and arginine added alone or together on insulin secretion, transcription and insulin mRNA level

Brockmann bodies were exposed to three different combinations of various increasing concentrations of glucose (mM) *(Gluc)* and arginine (mM) *(Arg)*. *(0.4 Arg+ 1.0 Gluc)* basal concentrations of glucose and arginine; (⊠) increasing concentrations of arginine at basal glucose; (▤) increasing concentrations of glucose at basal arginine ,(▨) combined increases of glucose and arginine. *(A)* Levels of the secreted insulin were determined in Western analysis of culture medium aliquots followed by densitometry analysis. Data are expressed as fold increases in density relative to the density of the sample exposed to basal glucose and arginine, which is taken as 1.0 *(B)* Relative levels of the insulin mRNA in samples treated *(grey bars)* and non-treated *(white bars)* with actinomycin D measured by qRT-PCR. Results are presented as the insulin mRNA/18S rRNA ratios and expressed relatively to the insulin mRNA/18S rRNA ratio of the sample exposed to basal glucose and arginine, which is taken as 1.0. All data are presented as means±SE of at least three independent experiments.

effects of elevations in the level of glucose only at a basal arginine level (0.4 mM) and to elevations in the level of arginine only at a basal glucose level (1 mM). Isolated tilapia Brockmann bodies were incubated in DMEM medium containing different concentrations of glucose and arginine as shown in Figure 3.7. After 23 h, the culture medium was replaced with fresh medium, followed by an additional hour of incubation. The culture medium and the islet cells were then collected and total RNA and proteins were isolated from the islet cells.

To examine the rates of insulin secretion induced by simultaneous elevations of arginine and glucose concentrations and to compare them with the rates of insulin secretion induced by either arginine or glucose alone, aliquots of medium collected after 23 h of incubation were used in Western analysis with tilapia insulin antibody (Fig. 3.7 A). We found that a combined increases in arginine and glucose concentrations induced insulin secretion in a dose-dependent manner. The most marked induction (2.8-fold) was detected between 0.4 mM arginine / 1 mM glucose and 1 mM arginine / 7 mM glucose concentrations. The higher elevations of glucose (up to 70 mM) and arginine (up to 25 mM) caused only small and not statistically significant increases in insulin secretion. Moreover, levels of insulin secretion induced by combined concentrations of glucose and arginine were significantly greater than those induced by arginine alone but only slightly higher than those induced by glucose alone. In general, our results suggest that whereas glucose and arginine can potentiate the effects of each other on insulin release, the rate of insulin secretion is saturated at a level approximately three-fold above that found under basal conditions (0.4 mM arginine / 1 mM glucose) and cannot be up-regulated much higher, at least under our current experimental conditions.

Next, we tested the effects of glucose and arginine added separately or in combination with each other on the level of insulin biosynthesis. Glucose- and/or arginine-induced levels of intracellular insulin and insulin mRNA were assayed using qRT-PCR and Western blot analysis as described above. No detectable differences in the levels of intracellular insulin were observed in any of the samples tested after 24 hours of stimulation using Western blot analysis with insulin antibodies (results are not shown). However, the steady state level of insulin mRNA determined by qRT-PCR was 2.4-fold higher after inductions with 10 mM arginine / 20 mM glucose and 3.2–fold higher after

inductions with 25 mM arginine / 70 mM glucose, compared to levels after induction with basal glucose and arginine or with glucose (20 mM or 70 mM) or arginine (10 mM or 25 mM) added separately (Fig. 3.7 B white bars).

3.4.5. Effects of Actinomycin D on Insulin mRNA Level in Isolated Tilapia Brockmann Bodies

In this study we also examined whether the synergetic effect of glucose and arginine on the level of insulin mRNA is mediated via transcriptional activation and/or via stabilization of the insulin mRNA. To test this, isolated tilapia Brockmann bodies were incubated for 12 h in DMEM medium with adjusted glucose and/or arginine concentrations (Fig. 3.7), and then the culture medium was replaced with the fresh medium that, in addition to glucose and arginine, contained actinomycin D (1μg/ml) (transcriptional inhibitor). After 12 h of incubation with actinomycin D, Brockmann bodies were harvested and total RNA was extracted and used in qRT-PCR. We did not detect any significant differences in the level of insulin mRNA amongst all samples tested (Fig. 3.7 B grey bars). Thus, our results indicate that increases in the insulin mRNA levels in response to simultaneous stimulation with high concentrations of glucose and arginine occur via transcriptional activation of the tilapia insulin gene.

3.4.6. Level of Insulin mRNA in Tilapia Brockmann Bodies

It has been determined that in human β–cells there are 60-80 molecules of insulin mRNA per molecule of actin mRNA [242]. In this study, we compared the levels of insulin and actin mRNA in β–cells of the tilapia Brockmann bodies. Brockmann bodies were isolated from 6 adult fish (2 fish per sample), then total RNA was extracted and used in qRT-PCR with insulin- and actin-specific primers. Considering that in tilapia Brockmann bodies 33% of the cells are insulin positive [270], our results indicate that the insulin / actin mRNA ratio in these cells is 259±18.9, which is about 3.7-fold higher than it is in human β–cells.

3.5. Discussion

Our results demonstrate that, similar to transcription of the mammalian insulin genes, transcription of the tilapia insulin gene is regulated in a β-cell-specific manner.

It has been shown for both mammalian (rat, mouse and human) and trout insulin promoters that regions approximately 400 bp upstream of the insulin TSS are sufficient to restrict the expression of insulin gene to β-cells [8, 93, 246, 256]. In contrast, we have found that the region up to 396 bp upstream of the tilapia insulin gene, when linked to the reporter gene, has no β-cell-specific expression upon transfection into mammalian cells. This region is approximately equally active in both insulin and non-insulin producing cells. Moreover, in contrast to the mammalian insulin promoters where the proximal -90 bp to -350 bp region contains strong positive and negative regulatory elements [18, 93, 256] we did not detect any dramatic difference (neither positive nor negative) in expression of the five deletion constructs containing sequences from -67 bp to -396 bp of the tilapia insulin promoter. The shortest region tested (67 bp upstream of the TSS) is sufficient to generate a basal level of transcriptional activation in both insulin and non-insulin producing cell lines. This region contains the classical TATA-box, which is involved in binding of general transcription factors. We also found that a weak β-cell specific activator may be located within –186 to –247 bp in the tilapia promoter. Analysis of this region revealed presence of a putative E-box–like sequence (GC**CA**TC**T<u>C</u>**) at position -233 bp to –240 bp. The E elements, which are found in all mammalian insulin promoters, are required for the high level of the tissue-specific activation of the mammalian insulin genes [210]. However, the E-box-like site in the tilapia insulin promoter has one G/C substitution (underlined) in the core sequence (bold). This may reduce binding of the regulatory proteins to the E-box and, therefore, could decrease its activation potential, resulting in only a weak elevation of transcriptional activation of the reporter constructs.

Interestingly, we found a CA_{17} simple repeat sequence in the tilapia insulin promoter (positions –251 bp to –283 bp). The guanine-rich repeat region, which is highly polymorphic in length, is located in approximately the same position in the insulin promoters of primate species, where it is known to be involved in the regulation of insulin transcription. The short repeat regions induce a lower rate of insulin transcription

than the long repeats do, and, therefore, are associated with the presence of insulin-dependent diabetes mellitus [100]. We did not detect any significant differences in the LUC level of the two constructs containing CA_{17} or CA_{13}, in both insulinoma cell lines. This suggest that in contrast to regulation of human insulin transcription, variations in the repeat number in the tilapia insulin promoter do not correlate with the transcriptional level of the insulin gene in pancreatic cells. However, in non-β-cells transcriptional activation of the construct containing the CA_{17} repeat was slightly reduced (37%), indicating a possible role of the long CA repeat in suppression of insulin transcription in non-insulin-producing cells. Similarly, it has been reported by Takeda et al., [238] that the guanine-rich repeat region suppressed the activation of the human insulin promoter in non-pancreatic cells.

Further elongations of the promoter size up to 1.5 kb revealed the presence of at least two strong tissue-specific activators within the (a) –535 bp to –396 bp and (b) –1575 bp to –784 bp regions of the tilapia insulin promoter. Similarly, it has been reported that the distal region of the human insulin promoter (–2 kb to –400 bp upstream of the TSS) contains several regulatory elements that can affect expression of a fused reporter gene in tumor cell lines [28, 172] as well as in primary cultured cells [168].

The β-cell-specific pattern of expression was completely lost in the three longest promoter constructs when the proximal promoter regions (-1 bp to -392 bp) were substituted with the ubiquitously active SV-40 promoter. Moreover, when transfected into HIT-T15 cells, LUC activity of these constructs was significantly lower than the activity of the control (SV-40 promoter alone). This strongly suggests that neither the distal promoter region nor the proximal promoter region is solely sufficient to confer β-cell-specific type of expression. Cooperative interactions of both regions of the insulin promoter are required for the activation of the β-cell-specific transcription of the tilapia insulin gene. Perhaps, the transcription factors that bind to the distal promoter region must synergize with the specific transcription factors that bind to the proximal promoter region to activate β-cell-specific transcription of the tilapia insulin gene. It is unclear if the same distal region of the tilapia insulin promoter can down-regulate transcription when fused to any unspecific promoter, or whether the negative effect was observed only in association with the SV-40 promoter. One possible explanation could be that the

transcription factors of the distal insulin promoter separated from the specific factors of the proximal insulin promoter form a secondary structure that decreases the accessibility of the SV-40 promoter to the transcription machinery and therefore suppress transcriptional activation of the SV-40 promoter.

Future experiments will involve detailed analysis of the distal and proximal promoter regions using DNase footprinting assay and cotransfection experiments and will enable us to define specific interactions of the transcription factors with each other and with *cis*-elements in the insulin promoter.

In the present study we did not find any strong glucose responsive element in the tilapia insulin promoter. Only some promoter constructs exhibited weak up- or down-regulation at certain glucose concentrations. We found that activational potential of the –247 bp to –275 bp region could be suppressed by 7 mM glucose in HIT-T15 cells and by 70 mM glucose in NIT-1 cells. No differences in activation of transcription by 1 mM, 7 mM or 70 mM glucose were found after elongation of the promoter up to -396 bp in both insulinoma cell lines. The similar compensatory effect produced by –287 bp to –396 bp region was observed in COS–1 cells where the LUC activity of the p287 was significantly suppressed; however, after promoter elongation up to –396 bp (p396) it was restored to the level of that shown by p67. Sequence analysis of the –287 bp to –396 bp region localized several potential binding sites for β–cell-specific and ubiquitously-expressed transcription factors, including E-box, A-box, CRE-box, Nkx-box, and Isl-box. Thus, we can speculate that occupation of one or several of these sites by transcription factors may block access for the CA-specific or C/EBP factors to the downstream region (–287 bp -247 bp), and as a result inactivate their negative effect on transcription.

In our experiments, a stepwise increase in glucose concentrations (1-70 mM) did not change expression of the reporter gene fused to the longest promoter region. This indicates that, in contrast to the regulation of the mammalian insulin genes, the tilapia insulin gene is not sensitive to the glucose stimulation, at least at the level of transcription initiation. One possible explanation for these results is that glucose is not the most effective inducer of insulin biosynthesis in tilapia. Indeed, in nature the fish diet does not contain high levels of simple carbohydrates, and glucose is not a major energy source for

fish [154]. Instead, amino acids such as arginine are considered stronger insulin secretagogues than glucose in some fish species [84, 154, 206].

Alternatively, the results of transcriptional insensitivity to glucose may be attributed to the artefacts of the experimental system used. In the absence of a fish β-cell line we used mammalian insulinoma cells to study regulation of transcription of the tilapia insulin gene. Therefore, results of the transient transfections may be affected by differences in the sequence or in expression of the transcription factors among species. This drawback can be avoided once tilapia (or fish) β-cell lines become available. Moreover, glucose responsiveness of the immortalized cell lines may be different from that of the primary cells and can be effected by many factors, such as cell passage number, culturing or stimulatory conditions [66, 175, 205, 273]. However, the last assumption seems unlikely since the stimulatory effect of glucose on insulin transcriptional activation at least in HIT-T15 cells in conditions similar to our experimental conditions is well established [60, 67, 172]. Nevertheless, *in situ* experiments using tilapia primary cultured pancreatic islets cells should eliminate most of the difficulties related to application of mammalian tumor cell lines. Therefore, to verify results previously obtained using mammalian β-cell lines, we set out to examine the effects of different concentrations of glucose and arginine on the regulation of tilapia insulin biosynthesis and secretion in isolated cultured tilapia Brockmann bodies.

Our results indicate that both glucose and arginine can induce insulin secretion in a dose-dependent manner in the isolated tilapia Brockmann bodies. Furthermore, when added together, glucose and arginine may slightly potentiate the stimulatory effects of each other on insulin secretion. Similarly, the stimulatory effects of glucose, arginine and the synergetic effect of both of them on insulin secretion have been well documented in mammals [17, 72, 105, 145], as well as in some fish species [84, 206].

Mammalian β-cells not only secrete insulin but, in order to replenish emptied intracellular stores, react immediately by activating the rate of insulin biosynthesis via stimulation of insulin translation, transcription, and by stabilization of insulin mRNA (for review see [40, 42]). In contrast, most studies of insulin regulation in fish show no stimulatory effects of glucose on the rate of insulin biosynthesis [154], at least over short periods of incubation (6-8 h) [3, 50]. However, during longer periods of incubation (24

h), glucose can induce a small increase in insulin synthesis in several fish species [3, 15]. In the present study, we did not monitor the rate of insulin biosynthesis directly. However, our results indicate that neither elevation of glucose concentrations nor elevations of arginine concentrations added separately or in combination with each other caused any significant change in the intracellular insulin content in the isolated tilapia Brockmann bodies. Therefore, similar to the mammalian β–cells [30], the tilapia β-cells were able to compensate for the amounts of secreted insulin and somehow maintained the insulin stores at constant levels over longer periods of stimulation.

On the other hand, our results indicate that neither glucose nor arginine alone, nor low concentrations of both of them added together had any significant effect on the steady state level of the insulin mRNA over long periods of stimulation. The insulin mRNA level was elevated only in response to stimulation with extremely high concentrations of glucose and arginine added simultaneously. Moreover, in experiments with actinomycin D we determined that such increase occurred via transcriptional activation of the insulin gene and not via stabilization of the insulin mRNA against degradation.

Therefore, with the exception of superphysiological stimulations, tilapia β–cells managed to constantly compensate for the secreted insulin without significant elevations of the insulin mRNA pool. This is different from long-term insulin regulation in mammals, where elevations in the level of insulin mRNA (at least 5-fold) tightly correlate with the cell's capability to increase the rate of insulin biosynthesis [56, 90, 121, 184]. But, it appears to be similar to the effects of short stimulation, when mammalian β–cells are able to compensate for the secreted insulin by transferring pre-existing insulin mRNA from the inactive cytoplasmic pool to ribosomes and by activation of insulin translation [253]. Perhaps a similar regulatory mechanism exists in tilapia β–cells. However, since the insulin mRNA storage pool is larger in tilapia than in mammalian β–cells, it is likely that insulin mRNA is not a rate-limiting factor for the translational activation of insulin in tilapia β–cells even after long stimulatory periods, unless there are extremely high demands for newly synthesized insulin. Thus, threshold concentrations of stimulators for the activation of the tilapia insulin gene transcription are much higher than those for the activation of insulin secretion. Possibly, the basal level of the insulin gene

transcription in tilapia β–cells is initially high and could be further stimulated only by superphysiological concentrations of stimulators. This may explain the augmented size of the insulin mRNA cytoplasmic pool and the results of our transfection experiments where the 1.5 kb region of the tilapia insulin promoter stimulated expression of the reporter gene up to 70-fold in the HIT-T15 cell line and up to 135-fold in the NIT-1 cell line. In comparison, the porcine insulin promoter induced expression of the reporter construct only by 40-fold when examined in the NIT-1 cell line under similar conditions [68]. Interestingly, although we did not detect any significant synergetic effect of high concentrations of stimulators on insulin secretion, insulin gene transcription was up-regulated only in the presence of both inductors together. This indicates that there may be differences in the regulatory pathways that trigger activations of insulin secretion and insulin transcription.

An alternative explanation for the obtained results is that an enlarged insulin mRNA pool leads to an increase in the basal rate of insulin translation and therefore to the elevation in the size of the intracellular insulin stores. The last hypothesis may also explain the data of others that show no significant effect of glucose on the rate of insulin translation in fish [3, 44, 50, 62]. Perhaps a constant high rate of insulin translation cannot be up-regulated much further. In this case, secretion might cause only modest leakage of the large intracellular insulin content that would be below sensitivity of the Western blot analysis. Currently, we are trying to develop more sensitive and specific methods for the quantification of the tilapia insulin concentrations. It will be one of our future goals to test the absolute amounts of the intracellular and exogenous insulin and to examine translational rates of the tilapia insulin.

Finally, since two non-allelic insulin genes are present in the genome of teleost fish, it is also possible that intracellular insulin homeostasis depends on nutrient-stimulated transcriptional activation of the second insulin gene. However, since the level of insulin 2 mRNA in adult tilapia Brockmann bodies is at least several thousand fold lower than that of insulin 1 mRNA [76], this last hypothesis seems unlikely.

In conclusion, in this study we demonstrate that the β-cell-specific pattern of expression of the tilapia insulin gene is regulated at the level of transcription initiation via cooperative interactions between regulatory elements within both the proximal (from -1

to -396 bp) and the distal (from -396 bp to -1575 bp) promoter regions. Furthermore, our results indicate that tilapia insulin secretion is strongly stimulated by glucose and arginine. At the same time, tilapia β–cells manage to compensate for the secreted insulin and maintain insulin stores at a constant level. From both tilapia insulin promoter analysis in transient transfection experiments and *in situ* incubations of the isolated tilapia Brockmann bodies, we can conclude that activation of tilapia insulin transcription is not sensitive to stimulation by glucose or arginine added separately or to stimulation by low concentrations of both of them added together. It can be significantly potentiated only via the synergetic effect of high concentrations of both inducers. Therefore, in general, insulin biosynthesis in tilapia β–cells does not rely on transcriptional activation of the tilapia insulin gene even over long-term stimulation, but likely depends on a large insulin mRNA storage pool. To our knowledge, our studies provide the first results regarding glucose and arginine regulation of fish insulin gene transcription.

Chapter 4

Insulin Expression in the Brain and Pituitary Cells of Tilapia *(Oreochromis Niloticus)*[*]

4.1. Abstract

While the presence of immunoreactive insulin in the central nervous system of many vertebrate species is well known, the origin of brain insulin is still debated. In this study, we applied RT-PCR, quantitative RT-PCR (qRT-PCR), and Northern hybridization to examine expression of the insulin gene in different tissues of an adult teleost fish, the Nile tilapia (*Oreochromis niloticus*). We found that the insulin gene is transcribed at a high level in Brockmann bodies (pancreatic islet organs) and at a low level in the brain and pituitary gland. In the brain, insulin transcripts were detected in all areas by qRT-PCR and *in situ* hybridization. The highest level of insulin mRNA was found in the hypothalamus. The level of insulin transcription in the pituitary gland was 6-

[*] This chapter is a modified version of the manuscript "Insulin expression in the brain and pituitary cells of tilapia *(Oreochromis niloticus)*" published in "Brain Research" (2007) 1135, 31-40 by Olga Hrytsenko, James R. Wright Jr., Carol M. Morrison and Bill Pohajdak

fold higher than that in the brain and 4.6-fold higher than that in the hypothalamus. Furthermore, insulin mRNA and immunoreactive insulin-like protein was detected in the pituitary gland using in situ hybridization, immunohistochemistry, and Western blot analysis. Our results indicate that in adult tilapia insulin expression is not restricted to the endocrine pancreatic cells, but also occurs in endocrine cells of the pituitary gland and in the neuronal cells of the brain, suggesting that the brain/pituitary gland might represent extrapancreatic origin of insulin production.

4.2. Introduction

Insulin is a small peptide hormone that plays a major role in the regulation of glucose homeostasis. It is traditionally accepted that in adult mammals, insulin transcription is restricted to β-cells of the pancreatic islets of Langerhans. However, existence of extrapancreatic insulin-like production has been widely discussed in higher vertebrates (mainly during early stages of development), in lower vertebrates, and in non-vertebrates that have no developed pancreases (for review see [123]). In most cases, extrapancreatic insulin-related immunoreactivity has been detected in the neuronal tissue [123]. Insulin in the brain could either have pancreatic origin and be delivered to the brain through the blood-brain barrier, or synthesised in the brain. While active insulin transport to the brain has been unequivocally confirmed, the presence of *de novo* insulin synthesis in the brain still remains controversial (for review see [55, 220]). Insulin mRNA has been detected by RT-PCR and by *in situ* hybridization in fetal and postnatal mammalian brains, in cultured brain cells and in adult rat brain [35, 36, 215, 216, 218, 272, 274]. In addition, insulin-like mRNA and protein have been detected in a small portion of cells located in the anterior pituitary of several rodent species by *in situ* hybridization [21]. However, none of these findings show indisputably that transcription of the insulin gene in brain and pituitary tissue is a common phenomenon for vertebrates. For example, insulin expression found in these studies may only result from certain culturing conditions, could only occur at early stages of development, may depend on the specificity of hybridization probes, or, in the case of insulin expression in rodent brain/ pituitary, it may be affected by the presence of two non-allelic insulin genes in the genome of this group. Thus, it is well established that insulin transfer can occur through

the blood-brain barrier, but the lack of strong evidence for its expression in intact adult brain cells suggests that brain insulin is of pancreatic origin [180, 220].

In this report, we demonstrate that the tilapia insulin gene is transcribed in the brain and pituitary gland, as well as in the endocrine pancreatic tissues [known as Brockmann bodies (BB) in some fish species]. This suggests that in contrast to the general point of view, there is extrapancreatic (brain and pituitary) production of insulin in fish.

4.3. Materials and Methods

4.3.1. Fish Maintenance and Tissue Isolation

Tilapia (*Oreochromis niloticus*) were produced in the Biology Department at Dalhousie University. Tissues, including kidney, BB region, liver, brain, adipose tissue, small intestine, pituitary gland and heart, were extracted from adult tilapia after anesthetizing with 2- phenoxyethanol (Sigma). Purified BB were isolated as described earlier [271]. For the studies of insulin expression in different brain areas: hypothalamus, olfactory cortex, proximal spinal cord and brainstem, visual cortex, cerebellum, and thalamus were separated from the tilapia brain using a razor blade.

4.3.2. RNA Extractions

Total RNA was isolated from tilapia tissues using TRIzol reagent (Invitrogen) according to the recommended protocol.

4.3.3. Northern Blot and Hybridization

Aliquots of the total RNA were loaded on a denaturing 1% agarose gel, electrophoresed and blotted onto a nylon membrane (Hybond-N+, Amersham / Pharmacia). A 210 bp insulin probe and a 200 bp β-actin probe that represented the parts of the tilapia insulin and tilapia β-actin cDNA were labeled with [^{32}P] dCTP (Amersham/ Pharmacia) and then used in two consecutive hybridizations. First, the membrane was hybridized with the insulin probe and then, after stripping, with the β-actin probe. Hybridizations were performed overnight at 42°C in a hybridization mixture composed of

50% formamide, 200 μg/ml carrier RNA, 5X SSPE, 0.1% SDS, 1% Denhardts, and 10% dextran sulfate. Hybridizations were followed by washes with 5X SSPE for 5 min at room temperature, 2X SSPE and 0.1% SDS for 10 min at room temperature, and 1X SSPE and 0.1% SDS for 1 h at 52°C.

4.3.4. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Aliquots of the isolated total RNA were reverse transcribed using reverse transcriptase kits (Invitrogen): SuperScript II (for tissue expression studies) or SuperScript III (for detection of the insulin, PC1, PC2, CPH mRNA in brain regions and in pituitary gland) and oligo(dT) primers (Invitrogen). For the amplification of the insulin transcripts, two microliters of the obtained cDNA were used as template in each of two PCR- one with the insulin primers (In49 / In16) and another with the β-actin primers (Act1/Act2 or actFOR/actREV). Sequences of all primers are listed in Table 1 (in appendices). Both PCR mixtures were composed of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM of each of dNTPs, 2 μl of cDNA, 200 nM of each of the primers and 1u of Taq polymerase. PCR were performed in a UNOII Thermocycler (BiometraLtd.) using the following conditions: 50 cycles of denaturing (94^0C) for 20 s, annealing (56^0C) for 30 s, and elongation (72^0C) for 1 min. PCR products were resolved on a 6% polyacrylamide gels, visualized by staining with ethidium bromide and photographed.

4.3.5. Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

To remove contaminating DNA, 1μg aliquots of isolated total RNA were treated with 1U of DNAseI (Invitrogen). The total RNA were reverse transcribed using SuperScript III reverse transcriptase kit (Invitrogen) and oligo(dT) primers (Invitrogen). Amplifications of transcripts were performed on a Rotor Gene 2000 real-time PCR machine (Corbett Research, Sydney, Australia) using Platinum SYBRGreen qPCR SuperMix-UDG Kit (Invitrogen) in accord with the provided protocol. qRT-PCR primers (insREV and insFOR for the insulin amplification and actFOR and actREV for the actin amplification) were created with OligoPerfect™ Designer program (www.invitrogen.com). Primer sequences are listed in Table 1 (in appendices). 10 μl

PCR contained 2 μl of the diluted cDNA template and were performed in 0.1 ml tubes using the following parameters: hot start (50^0C) for 2 min; initial denaturing (95^0C) for 2 min; and 40 cycles of denaturing (95^0C) for 15 s, annealing (55^0C) for 30 s, elongation (72^0C) for 30 s (acquired) and second acquisition at 82^0C after 15 s. Subsequent melting curve measurements were obtained within 72^0C and 95^0C. Each cDNA sample was analyzed in three or four duplicated PCR representing three or four different template dilutions ranging from 1:5 to 1:100. In preliminary experiments a linear correlation between the inputted cDNA template and calculated transcript concentration were determined to occur within this range of dilutions.

All data were analyzed according to the mathematical model developed by Liu and Saint [126] using ORIGIN software for Windows (MicroCal Software Inc., Northampton, MA, USA). The concentration of the transcript in the sample is expressed as R_{mean}, where $R=R_{max}/[1+\exp(n_{1/2}/k)]$[126]. To compensate for any variance in the amount or quality of the input RNA, the presented levels of the insulin mRNA are shown relative to the levels of β-actin mRNA that was used as an internal control.

All results are expressed as mean ±SE of two to four independent experiments. For quantification of insulin mRNA levels in BB and in brain, total RNA was isolated from three tilapia brains and BB of six fish (two for each sample). For quantification of insulin mRNA levels in different parts of brain, total RNA was extracted from different brain areas from 6—12 fish (three for each sample). For quantification of insulin mRNA level in pituitary gland, total RNA was isolated from six pituitaries (two per sample).

4.3.6. Cloning and Sequencing of the Tilapia Brain and Pituitary Prohormone Convertase 1/3 (PC1/3), Prohormone Convertase 2 (PC2), Carboxypeptidase H (CPH) cDNA and Brain Insulin cDNA

The 410 bp PCR product that represents the full coding region as well as 12 bp the of 5' and 62 bp of the 3' –UTRs of the tilapia insulin cDNA was amplified using tilapia brain cDNA as a template under the same conditions as described above except In49 primer was substituted with In17 primer (Table 1 in appendices). The PC1, PC2 and CPH partial transcripts were amplified using brain and pituitary cDNA as a template and degenerative primers listed in Table 1 (in appendices). PCR products were resolved on a 1% agarose gel, extracted and purified using QIAquick gel extraction kit (Qiagen),

cloned into the pCRII (Invitrogen), pDRIVE (Qiagen) or pJET (Fermentas) vectors and commercially sequenced. Obtained insulin sequence was compared with the known sequence for the tilapia insulin gene [138] and with BB preproinsulin cDNA using the Blast2 program (www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi). Obtained PC1, PC2 and CPH sequences were analyzed using Blastn (www.ncbi.nlm.nih.gov/BLAST/ Blast.cgi), and submitted to GenBank.

4.3.7. Western Blot and Hybridization

Adult tilapia whole brain, hypothalamus, pituitary glands and BB tissues were homogenized in lysis buffer (150 mM NaCl, 1% Triton X-100, 50mM Tris-HCl pH 7.5, 2 mM EDTA, 10% Glycerol) supplemented with protease inhibitors (1 tablet per 10 ml of buffer) (Roche), and then centrifuged. Supernatant aliquots were electrophoresed in a 4-15% SDS-polyacrylamide gel, and electroblotted onto PVDF transfer membrane (Hybond-P, Amersham/ Pharmacia). Filters were hybridized for 12 h with a rabbit anti-tilapia insulin 1^{o} antibodies, developed by us [270], washed with PBS/0.1% Tween and then incubated for 30 min with HRP-conjugated goat anti-rabbit 2^{o} antibody (1:10,000) (Santa Cruz Biotechnology). After hybridizations filters were processed for chemiluminescent detection using Western Blotting Luminol Reagent kit (Santa Cruz Biotechnology).

4.3.8. Section Preparation

For the paraffin embedded sections three tilapia pituitary glands and brains were fixed in Bouin's solution for 4 h, transferred to buffered formalin for 24 h, and then processed to paraffin blocks. For the frozen sections brain and pituitary were fixed in 4% paraformaldehyde/ PBS overnight at $4^{o}C$, cryoprotected in 30% sucrose/PBS for 24 h at $4^{o}C$, equilibrated in solution containing 15% sucrose, 50% OCT (TissuTeek4583), 1X PBS for 1 h at 4 ^{o}C, and quickly frozen in the same solution. Sections were cut at 10 μm and either stored at $-70^{o}C$ or used immediately.

4.3.9. Immunohistochemical Analysis

Histological sections were stained for insulin using either mouse anti-tilapia monoclonal or rabbit anti-tilapia polyclonal insulin 1^{o} antibody and the avidin-biotin complex immunoperoxidase technique as described by us earlier [270]. For the detection of the insulin immunoreactivity in pituitary and brain, both paraffin and frozen sections were used. However, no signal was found when brain paraffin sections were stained with either mono- or polyclonal antibodies, so, these results are not included.

4.3.10. In Situ Hybridization

4.3.10.1. Probe Preparation

Plasmid containing 410 bp insulin cDNA in pDRIVE vector (Qiagen) was digested either with Bam HI and Pvu II or with Xho I and Pvu II following gel electrophoresis. Insulin containing fragments were extracted as describe above and dioxigenin (DIG)- labeled using DIG RNA labeling kit (Roche), SP6 (for antisense probe) and T7 (for sense probe) RNA polymerases, accordingly to the provided protocol. Labeling efficiencies and probe integrities were confirmed by dot blot hybridization and RNA gel electrophoresis.

4.3.10.2. Hybridization

Frozen brain and pituitary sections were air dried for 30 min and used in hybridizations. Hybridizations were performed in humified chamber, overnight at $65^{o}C$ in a hybridization mixture composed of 50% formamide, 1 mg/ml rRNA, 1% Denhardts, 10% dextran sulfate, 195 mM NaCl, 10 mM Tris-HCl (pH 7.5), 50 mM EDTA, 40 mM Na_2HPO_4, 50 mM NaH_2PO_4, and either sense or antisense insulin probe. Hybridizations were followed by five 30 min washes in 50% formamide, 0.1% Tween-20, 1X SSC at $65^{o}C$.

After hybridization, slides were washed in 1X Washing buffer (Roche) two times for 15 min at room temperature. Then sections were incubated in blocking solution containing 2% Blocking buffer (Roche), 20% horse serum (GIBCO-BRL), 1X Maleic acid (Roche) for 1 h at room temperature and in the same solution containing 1: 500

alkaline phosphatase-coupled antibody against DIG (Roche) overnight at 4°C. After five 20 min washes in 1X Washing buffer (Roche) sections were stained for colour development in solution containing 100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris-HCl (pH 9.5), 0.1 % Tween-20, 10% polyvinyl alcohol, 4.5 µl/ml NBT (Roche), and 3.5 µl/ml BCIP (Roche) in the dark for 6 to 18 h. Each brain area was examined with sense and antisense probes. No signal was observed with the sense probe after 6 h of colour development and only a weak background was found after 18 h. The nuclei were visualized using neutral red counterstaining.

4.4. Results

4.4.1. Tissue-Specific Expression of the Insulin Gene

To determine the tissue distribution of tilapia insulin mRNA, we performed RT-PCR and Northern analysis using total RNA isolated from different tilapia tissues including kidney, BB, liver, brain, adipose tissue, small intestine, and heart. Northern hybridization with an insulin probe revealed a single strong signal in the BB but not in any of the other tissues tested (Fig. 4.1 A). However, by RT-PCR, the insulin transcript was detected not only in the BB but also in the brain (Fig. 4.1 B). To verify our results, and to eliminate any possibility of tissue cross-contamination, we extracted brain RNA samples separately from any other tissues and repeated the RT-PCR. As shown in Figure 4.1 C, an insulin transcript was observed in all samples tested. Furthermore, in order to amplify the product containing a full insulin coding sequence, two brain RNA samples were also used in RT-PCR with a different primer set (Fig. 4.1 D). The obtained product (410 bp) was cloned and its identity to the known tilapia preproinsulin transcript was confirmed by sequencing. Thus, our results show that in tilapia insulin expression is not completely restricted to the BB, but also occurs at low levels in brain tissue. When measured by qRT-PCR, the concentration of insulin transcript was 9.6×10^4 fold lower in brain than in the β–cells of the BB (Fig. 4.2 A).

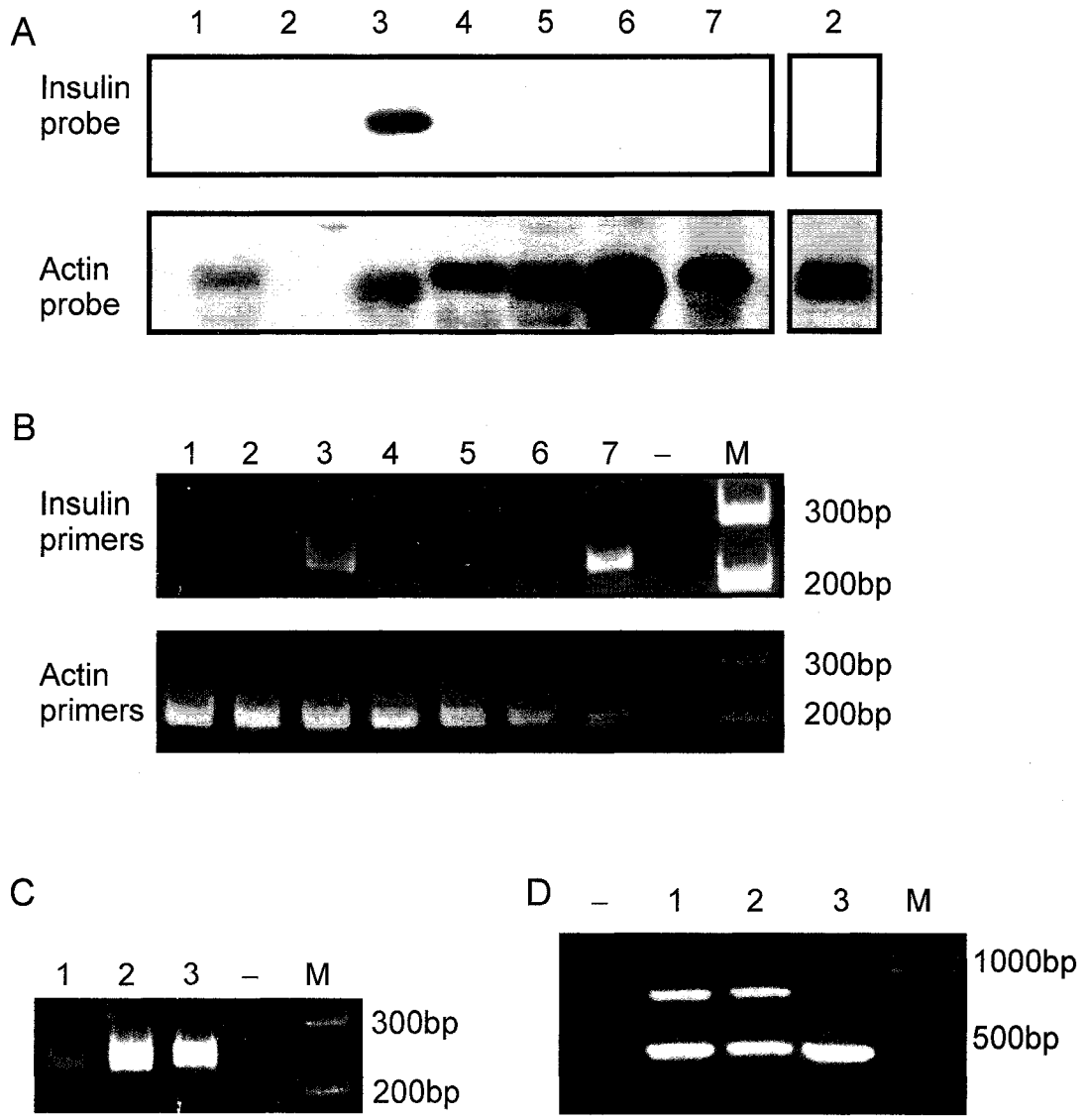

Figure 4.1. Transcription of the tilapia insulin gene in BB and in brain tissues *(A* and *B)* Total RNA was isolated from several tilapia tissues: kidney *(1)*, liver *(2)*, Brockmann body region *(3)*, heart *(4)*, small intestine *(5)*, adipose tissue *(6)*, and brain *(7)* and was used *(A)* in Northern blot hybridized with insulin probe *(top panel)* and actin probe *(bottom panel)* and *(B)* in RT-PCR with insulin primers *(top panel)* and actin primers *(bottom panel)*. *(C* and *D)* Several individual RNA samples were isolated from tilapia brains and used in RT-PCR to amplify *(C)* 210 bp product containing partial coding region of the insulin transcript *(1-3)* and *(D)* 410 bp product containing full coding region of the insulin transcript *(1, 2), (3)* is insulin transcript amplified with BB cDNA and used for a positive control. Note: top bands in lanes *(1* and *2)* are due to genomic DNA contaminations. *(-)* negative control, *(M)* molecular weight marker.

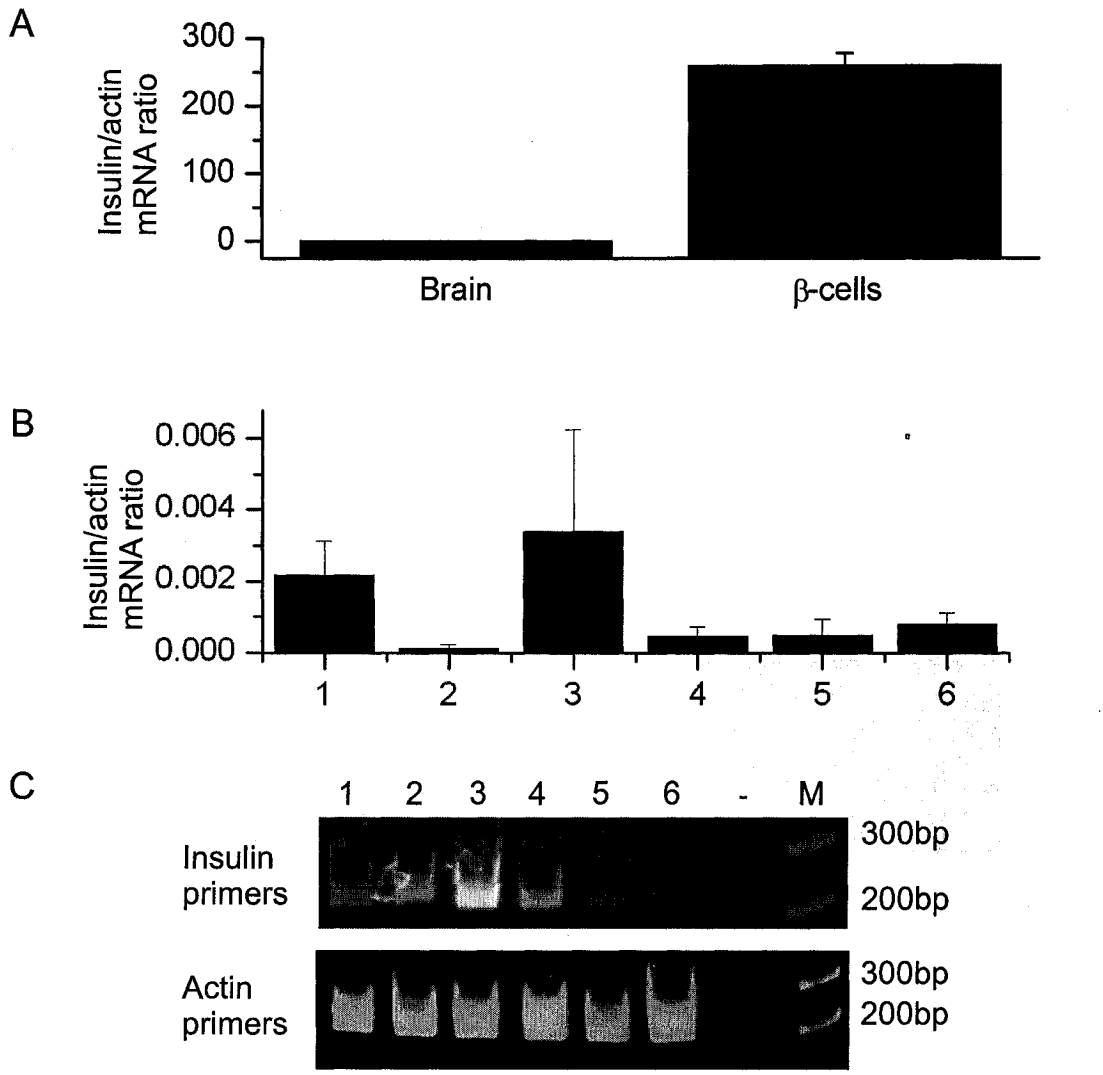

Figure 4.2. Levels of insulin expression in BB, whole brain and in different brain areas

(A, B) Graphs represent comparative levels of insulin transcription measured by qRT-PCR *(A)* in β-cells of BB and in whole brain and *(B)* in six brain areas: proximal spinal cord and brainstem area *(1)*, cerebellum *(2)*, hypothalamus *(3)*, visual cortex *(4)*, thalamus *(5)*, olfactory cortex *(6)*. Results are presented as mean±SE of insulin /actin ratio of all samples tested. *(C)* Insulin transcript was detected in all brain areas using RT-PCR: insulin primers *(top panel)* and actin primers *(bottom panel)*. *(1-6)* are the same as in *(B)*, *(-)* negative control, *(M)* molecular weight marker.

4.4.2. Localization of the Insulin mRNA in the Brain

A low insulin/actin mRNA ratio in whole brain tissue may be affected by accumulation of the insulin transcript only in specific anatomical brain areas. Therefore, we examined insulin expression in several brain regions using RT-PCR, qRT-PCR, and *in situ* hybridization. For RT-PCR and qRT-PCR, total RNA was extracted separately from the hypothalamus, telencephalon, thalamus, proximal spinal cord and brainstem, visual cortex, and cerebellum. As shown in Figure 4.2 C, insulin mRNA was detected in all regions using RT-PCR. The qRT-PCR revealed that, on average, 45% of brain insulin mRNA is concentrated in the hypothalamus and 29% of it is located in the proximal spinal cord and brainstem area (Fig. 4.2 B). In the other brain areas the levels of insulin transcription were 4- to 25-fold lower than in the hypothalamus. However, in some cases, the levels of insulin mRNA varied dramatically amongst different RNA samples extracted from the same brain area.

Consistent with RT-PCR results, *in situ* hybridization identified insulin mRNA-containing cells in the hypothalamus within the nucleus recessi lateralis (Fig. 4.3 G, H, O), and in groups of large cells located in the medulla oblongata and near the base of the cerebellum. Based on the location, shape and using hematoxylin and eosin staining, we identified them as neuron cell bodies. (Fig. 4.3 M, N, L). The hybridization signals were located in the perinuclear areas of the positive cells in the nucleus recessi lateralis (Fig. 4.3 O), and were more disperse in the cytoplasm in neuron cell bodies of the medulla oblongata (Fig. 4.3 M, N). However, after colour had developed for a longer time, weak hybridization signals were observed in all brain areas, and were associated with neuronal body accumulations, such as the granular layers in the optic tectum and cerebellum (Fig. 4.3 D, E).

4.4.3. Biosynthesis of Insulin in the Brain

To determine if insulin mRNA is further translated and processed by the brain cells, we performed immunohistochemical staining of the brain sections with tilapia insulin mono- and polyclonal antibodies and Western blot analysis using protein extracts

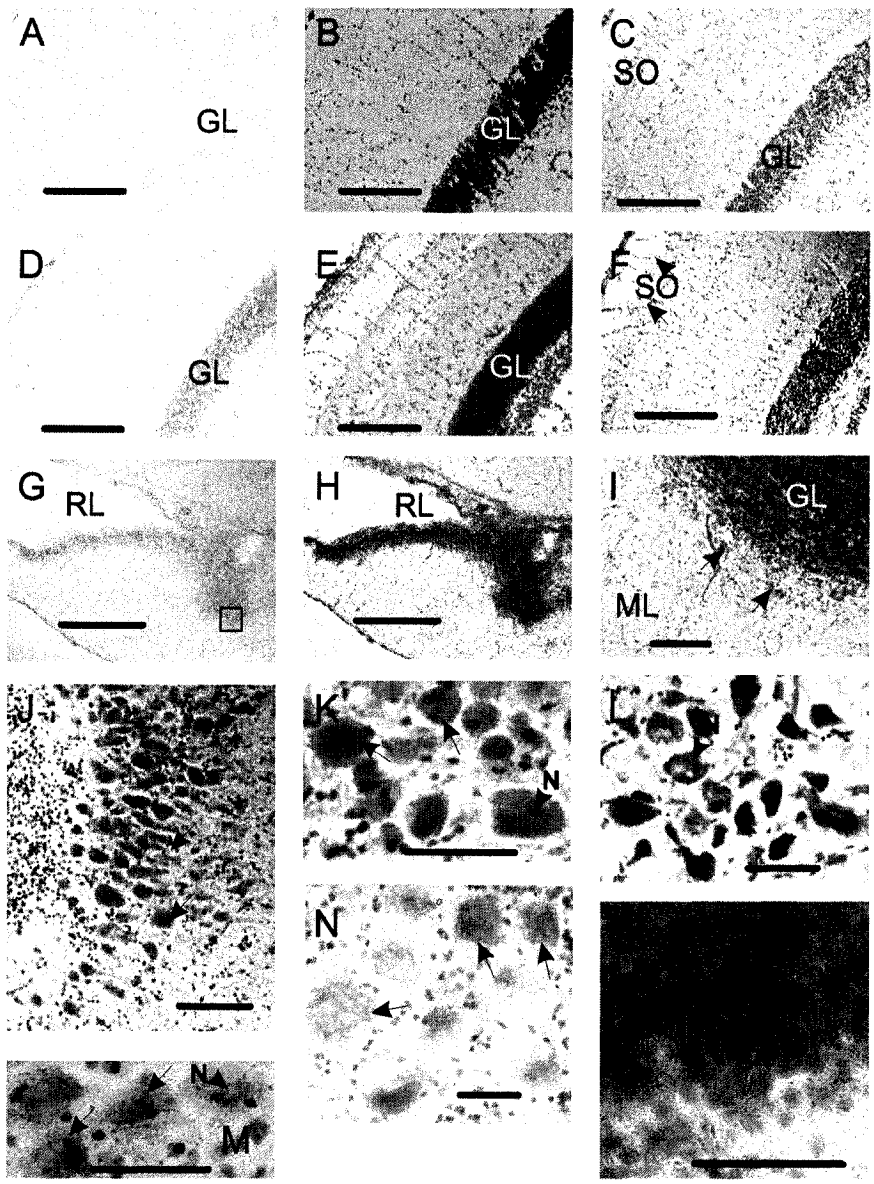

Figure 4.3. Localization of the insulin mRNA and immunoreactive insulin in longitudinal sections of tilapia brain

(A-F) optic tectum, *(G, H, O)* nucleus recessi lateralis in the hypothalamus, *(I)* cerebellum, *(J-N)* proximal spinal cord and brainstem area.

(A and *B) in situ* hybridization:using insulin sense probe. *(D, E, G, H, M, N, O) in situ* hybridization using insulin antisense probe. Note: *(A, D, G)* show the hybridization signal only, and *(B, E, H)* show nuclei only; *(M, N, O)* show both the hybridization signal and nuclear neutral red staining. *(O)* represents the marked area of *(G)* at higher magnification. *(F, I, J, K)* immunoperoxidase staining for tilapia insulin. *(K)* shows insulin immunoreactive cells in the spinal cord at a higher magnification.

(C and *L)* hematoxylin and eosin staining.

Arrows indicate hybridization signal. *(GL)* granular layer, *(ML)* molecular layer, *(RL)* recessus lateralis, *(SO)* stratum opticum, *(n)* nucleus.

Scale bars *(A-J)* 200 μm, *(K, L, N, O)* 50 μm, *(M)* 35 μm.

from the whole brain and from the hypothalamic area. In addition, using degenerative primers designed to two neighbouring exons we cloned and sequenced partial brain transcripts encoded for enzymes involved in insulin processing (prohormone convertase 1/3 (PC1/3) (GenBank accession nos. EF081461 and EF081462), prohormone convertase 2 (PC2) (GenBank accession nos. EF081463 and EF081464) and carboxypeptidase H (CPH) (GenBank accession nos. EF081466 and EF081468). Immunohistochemical staining using polyclonal antibodies resulted in a high general background and no specific granular staining in any areas tested. However, staining with monoclonal antibodies revealed weak immunoreactivity in groups of neuron cell bodies in the medulla oblongata (Fig. 4.3 J, K) and in cerebellar Purkinje neurons (Fig. 4.3 I). These results were consistent with our finding of insulin mRNA in the same cells by *in situ* hybridization. In addition, diffuse signal was observed in stratum opticum of optic tectum. Western analysis failed to detect any insulin-like immunoreactivity in extracts from whole brain (results are not shown). However, faint signals corresponding to proinsulin, but not to processed insulin, were observed in hypothalamic samples after long exposure (Fig. 4.4 A).

4.4.4. Localization of the Insulin mRNA and Insulin in the Pituitary

It is well known that hypothalamic neurohormones that are synthesized in preoptic nuclei are later transferred via the preoptico-hypophysial tract to the neurohypophysis where they are stored and secreted by axon endings. Accordingly, we

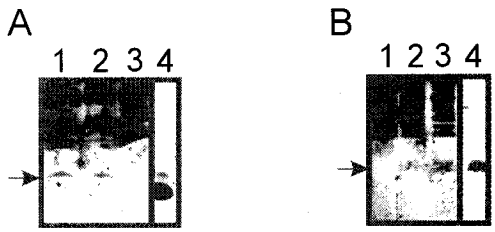

Figure 4.4. Insulin-like immunoreactivity in protein extracts from the tilapia hypothalamus and pituitary gland tissues
Western analysis of proteins extracted from *(A)* two tilapia hypothalamus *(1,2)* olfactory cortex (negative control) *(3)* and BB (positive control) *(4)*; and from *(B)* pituitary glands *(1-3)*, and BB (positive control) *(4)*; *(1,2,4)* loading buffer had no β-mercaptoethanol or DTT added; *(3)* protein sample same as in *(1)* run in the presence of β-mercaptoethanol/ DTT. Note: Radioautographs of BB samples are shown after shorter exposure.

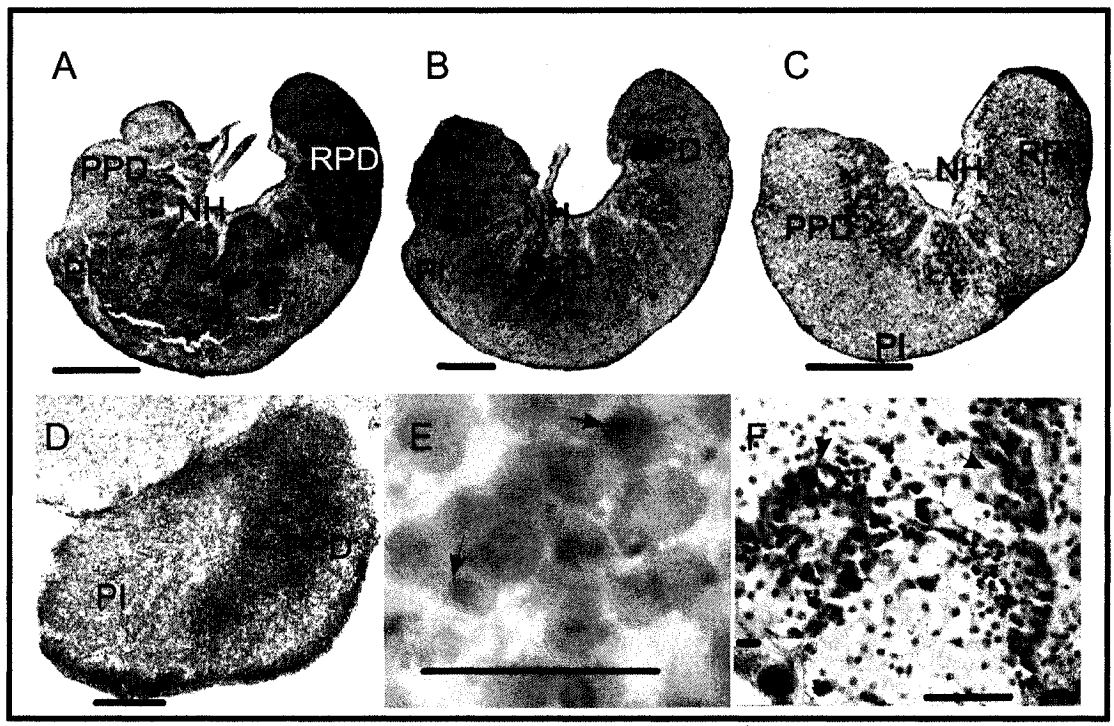

Figure 4.5. Localization of immunoreactive insulin and insulin mRNA in the tilapia pituitary gland

Longitudinal sections of the tilapia pituitary. *(A-C)* lateral to mid-line; *(D)* sagittal section, *(A, F)* immunoperoxidase staining for tilapia insulin: *(A)* using insulin monoclonal antibody and frozen section, *(F)* using insulin polyclonal antibody and paraffin embedded section, showing details of the insulin immunoreactive cells in the PPD. A single cell is shown in the insert.

(B-E) in situ hybridization, *(C)* using insulin sense probe and *(B, D, E)* using insulin antisense probe. *(E)* shows detailed localization of the hybridization signals in nuclear and perinuclear areas, as indicated by arrows. *(PPD)* proximal pars distalis, *(RPD)* rostral pars distalis, *(PI)* pars intermedia, *(NH)* neurohypophysis.

Scale bars *(A-D)* 400 μm, *(E-F)* 50 μm, *(F)* (insert) 4.5 μm.

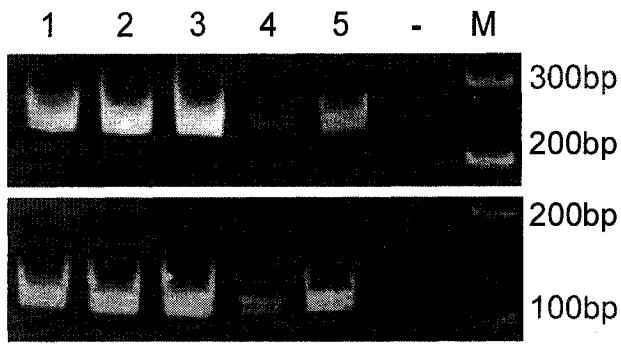

Figure 4.6. Insulin transcription in the tilapia pituitary gland
Total RNA was isolated from six tilapia pituitary glands (two per sample) *(1-3)*, brain *(4)*, and BB *(5)* and used in RT-PCR with insulin primers *(top panel)* and actin primers *(bottom panel)*. *(-)* negative control, *(M)* molecular weight marker. Note: diluted sample of BB cDNA *(5)* was used in PCR.

also examined the presence of the mature insulin in protein extracts from the tilapia pituitary gland using Western blot analysis. As shown in Figure 4.4 B, weak insulin-like signals (6 kDa) were detected in the pituitary gland samples. Moreover, as expected for insulin, the 6 kDa signal was not observed when the same pituitary protein sample was denatured with β-mercaptoethanol/ DTT. To localize insulin positive cells in the tilapia pituitary tissue, we performed immunohistochemical staining with tilapia insulin antibodies (mono- and polyclonal). Surprisingly, using both antibodies, insulin-like immunoreactivity was observed in the adenohypophysis but not in axon endings in the neurohypophysis (Fig. 4.5 A, F), suggesting that adult tilapia endocrine cells of the pituitary gland are enrolled in insulin biosynthesis. In the adenohypophysis, the insulin-positive cells were typically localized in the proximal pars distalis (PPD) in clusters and cords of cells scattered ventral to growth hormone (GH) producing cells, and also in small clusters of cells in the pars intermedia (PI).

Further evidence of insulin expression in the tilapia adenohypophysis was obtained by RT-PCR, qRT-PCR, and by *in situ* hybridization. Total RNA was extracted from the pituitary glands of six adult fish (two per sample) and analyzed by RT-PCR and qRT-PCR with insulin- and actin-specific primers. Using RT-PCR, we were able to detect insulin transcripts in all RNA samples from the tilapia pituitary glands (Fig. 4.6). Pituitary insulin RT-PCR products had similar size and identical melting characteristics

to those of BB and brain insulin. The qRT-PCR revealed that the level of insulin transcription in the pituitary gland (0.01601±0.00325 insulin/actin mRNA ratio) was 1.6×10^4-fold lower than that found in BB. However, it was 6-fold higher than the brain insulin mRNA level, and 4.6-fold higher than the hypothalamus insulin mRNA level.

The distribution of insulin mRNA containing cells in the tilapia pituitary correlated with the localization of the insulin immunoreactive cells there (Fig. 4.5 A, B). In the longitudinal sections, insulin mRNA containing cells were observed in cords along the nerve fibres in central and ventral PPD (Fig. 4.5 B), ventrally from GH containing cells [with exception of the anterior part of PPD on the border with rostral pars distalis (RPD)]. In sagittal sections of the pituitary, insulin mRNA expressing cells were detected in the external rim of the PI and occasionally in small clusters around neuronal branches in the PI in addition to the cells of PPD (Fig. 4.5 D). Hybridization signals were generally nuclear and perinuclear (Fig. 4.5 E), consistent with the sites of insulin translation in the endoplasmic reticulum. The distribution patterns for insulin and insulin mRNA expressing cells in the tilapia pituitary corresponds to the previously described localization of gonadotropin producing cells that are usually organized in cords or clusters and dispersed in all parts of the PPD, except for the regions of the GH cells (located in dorsal part of PPD) and thyrotropin cells (found on the border with RPD), and in the periphery of the PI [94, 177, 179].

Furthermore, to confirm that pituitary proinsulin can be correctly processed to mature insulin, we cloned and sequenced partial transcripts encoded for PC1/3, PC2 and CPH using pituitary RNA as had been described above for the brain enzymes (see the same GenBank accession numbers).

Thus, our results indicate that in adult tilapia, the insulin gene is expressed in the pituitary gland and brain tissues as well as in the BB. Furthermore, both tilapia pituitary and brain tissues have the capacity to process proinsulin and can produce detectable amounts of mature insulin.

4.5. Discussion

This report demonstrates that insulin transcription in adult tilapia is not completely restricted to pancreatic β-cells (as is traditionally accepted for mammalian

insulin) but also occurs at low levels in the brain and pituitary gland. To our knowledge, this is the first time that an insulin transcript has been extracted and sequenced from the intact brain of a lower vertebrate species. In contrast to our data, Plesetskaya et al., [194] did not detect the presence of any insulin mRNA in adult salmon brain tissue using the same technique. This discrepancy is likely due to the significantly higher sensitivity of the RT-PCR enzymes that are available now. However, a different transcription pattern of the insulin gene between the two teleost species cannot be totally excluded.

Concerning insulin gene transcription in brain/pituitary cells, it should be addressed whether the same gene or two different insulin genes encode BB and brain transcripts. Two different nonallelic insulin genes have been described in the genome of salmon [97], zebrafish, and fugu fish [85]. Moreover, expression of the second insulin gene was detected in brain and pancreas in zebrafish embryos at 24 and 48 hours post fertilization, while the expression of the first insulin gene was strictly restricted to the pancreatic area at that time. However, the partial sequence of the tilapia brain insulin cDNA containing coding region, 12 bp of 5' and 62 bp of 3' –untranslated regions (UTRs) was identical to the corresponding regions in the tilapia insulin gene [138] and to the BB insulin cDNA. We did not sequence the pituitary insulin transcript, but its size and melting curve were identical to those of the insulin transcripts from BB and the brain. These results suggest that brain, pituitary, and BB insulin mRNA are the products of the same insulin gene, isolated by us earlier [138], that is transcribed at high level in BB and at lower levels in the brain and pituitary gland.

Our results indicate that the levels of insulin transcription in the brain and pituitary gland are substantially lower than those in the β-cells of BB. However, even the lowest concentration of the insulin transcripts found in the whole brain samples (0.0027±0.0015 insulin /actin mRNA ratio) was well within the known concentrations for rare transcripts in the cells (from 0.01 to 0.001 mRNA species per 1 actin mRNA), indicating a potential physiological importance of insulin expression in brain. There is a growing evidence that in mammalian brain insulin plays an important role in regulation of feeding behavior, body weight, reproduction, as well as in modulating learning, memory, and cognition [55, 220]. Whether or not brain insulin has similar functions in fish remains to be determined. However, the most studied effect of brain insulin in

mammals related to the suppression of food intake, is also observed in several fish species (for review see [232]). In mammals, this effect is mediated via insulin binding to its receptors in brain cells, which leads to an alteration in the production of feeding modulators by targeted cells. In this context, similar to accumulation of the insulin receptors in mammalian brain cells, high level of expression of insulin receptor genes [63] and insulin receptors themselves [122] have been detected in fish brain tissues. Moreover, at least one of the mammalian insulin-sensitive feeding modulators in brain, neuropeptide Y, could also be downregulated by insulin in fish brain [228]. In agreement with the feeding regulation concept, we found that in the brain the insulin gene is predominantly transcribed in the hypothalamus which is known to be a feeding regulatory centre in fish as well as in mammals [228]. Moreover, using *in situ* hybridization, we detected that high concentrations of insulin mRNA are associated with neuron bodies in nucleus recessi lateralis (mediators of the feeding response [228]). However, we also found its presence at lower levels in other brain regions, and at a higher level in the pituitary gland. A similar distribution pattern was described earlier in mammalian and fish brains for the insulin-like mRNA using *in situ* RNA hybridization [272] and for insulin-like protein using immunodetection [14, 71].

The other potential role of insulin in fish brain could be related to its original function as a regulator of energy homeostasis, and in particular to glycogen production and accumulation by glial cells. It is well known that fish brain is high in glycogen, which can be utilized as an alternative brain fuel during food deprivation and toxic stress (for review see [232]).

Finally, the function of brain and pituitary insulin could be associated with growth and reproduction [20, 129]. To support this view, expression of the GH gene and secretion of GH were inhibited by insulin in mammals [129]. Furthermore, expression of insulin receptors has been described in the pituitary tissue [129, 255], and neuronal-specific genetic disruption of those lead to impaired secretion of the luteinizing hormone [20]. Interestingly, we found that tilapia insulin expression in pituitary gland is colocalized with the region of gonadotropin (luteinizing and follicle-stimulating hormone) producing cells, indicating possible autocrine function of insulin in this area.

With regards to the cell type, we found that the insulin mRNA hybridization signals were generally associated with brain areas enriched in neuron cell bodies, and insulin immunoreactivity was also detected in neuronal cells. We can therefore speculate that in fish, neurons can at least partially contribute to the brain insulin production. In agreement with our results, mammalian immunoreactive insulin and insulin mRNA in brain have been mainly detected in neuronal cells [35, 215, 217].

In this study we were able to detect mature immunoreactive insulin only in the pituitary gland but not in brain tissues by Western blotting. In addition, only small number of cells showed immunoreactive insulin in brain immunohistochemical staining. Similarly, insulin mRNA, but not immunoreactive insulin, has been detected in neonatal rabbit brain [35]. This is most likely because the low level of insulin expression in the brain is below the sensitivity of detection with our tilapia insulin antibody. Indeed, the concentration of the mature insulin in the pituitary is near the level of detection limits and the level of insulin mRNA determined by qRT-PCR is several fold higher in the pituitary than in brain tissues. In addition, it is possible that, as described for mammalian brain [35], the insulin found in fish brain is not stored within the cells, but rather immediately secreted. This could result in a diffused staining (as had been observed by us in optic tectum) or often leads to a high background [35].

In the present paper we have demonstrated that in tilapia insulin transcription occurs in intact adult brain and in the pituitary gland. This suggests the existence of an extrapancreatic origin of insulin production in teleost species. Further experiments should clarify whether or not insulin biosynthesis in the brain and the pituitary gland is common for all non-mammalian vertebrates and whether the main function of the brain insulin indeed involves modulation of feeding behavior or reproduction.

Chapter 5

Studies of the Genomic Polymorphism of the Insulin Locus in Nile Tilapia (Sex-Related Variations of the Insulin Alleles)*

5.1. Abstract

Genetic sex determination in fish is of utmost importance for both aquaculture applications, such as early sex identification in commercially important fish, and for evolutionary biology in understanding the early stages of sex chromosomal development. Using Southern hybridization, we found that the 5' insulin gene flanking region in an African cichlid fish, Nile tilapia (*Oreochromis niloticus*), contains sex-linked sequence variations. We cloned, sequenced, and analyzed the sex-different regions from both sexes. We also designed PCR to rapidly identify the presence of a male-specific insulin allele in the tilapia genome. Using fluorescence *in situ* hybridization (FISH), we also found that the insulin gene in tilapia is located on the sex chromosome. Applying RT-

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* Manuscript in preparation by Olga Hrytsenko, James R. Wright Jr., David J. Penman, and Bill Pohajdak

PCR and quantitative RT-PCR (qRT-PCR) we have determined that the insulin gene is expressed at low levels in the testis and ovaries of adult tilapia. Moreover, the levels of insulin expression were different in gonads of adult fish and in tilapia fry prior to sex differentiation in male-allele-containing and male-allele-absent genotypes. Based on our results, we propose that in tilapia insulin might have acquired sex-related functions that are mediated via different levels of insulin expression regulated by sex-different sequences upstream of the insulin gene.

5.2. Introduction

Early gender identification in commercially important fish remains an extremely challenging subject for the aquaculture industry. Unlike mammals, where the XX-XY sex determination mechanism seems to be basal in the evolution of the group, fish utilize a remarkable diversity of sex determination mechanisms that vary from gonochorism to simultaneous or subsequent hermaphroditism, and from environmental sex determination to polyfactorial or monofactorial genetic sex determination (for review see [37]). The situation is even more complex, since in species where sex is genetically determined, sex-specific genes as well as sex determination systems themselves may be different in two closely related species. For example, the sex-specific *DMY* gene found in the genomes of Japanese medaka *(Oryzias latipes)* and Hynann ricefish *(O. curvinotus)* is absent from the genome of two other medaka *(Oryzias)* species, Celebes medaka *(O. celebensis)* and *O. mekongensis* [106, 141]. In the genus tilapias *(Oreochromis)*, both Nile tilapia *(O. niloticus)* and Blue tilapia *(O. aureus)* have a primarily monofactorial sex determination systems [11, 133, 134]. However, this system is XX-XY male heterogametic in Nile tilapia *(O. niloticus)*, but ZW-ZZ female heterogametic in Blue tilapia *(O. aureus)*. It appears likely that many of the sex determination mechanisms found in fish will be of recent origin, e.g. the *O. latipes* sex chromosome is approximately 10 million years old [107], compared to the 300 million year old human Y chromosome [229]. In most teleost species proto-sex chromosomes contain only limited regions of sex-specific sequences and therefore are homomorphic in shape, making classic karyotypic sexing impossible. For instance in Japanese medaka *(O. latipes)*, the only fish species in which the sex-specific region is fully sequenced and characterized, it spans only 1% of the Y

chromosome length and contains a single functional gene, the master sex gene *DMY* [159, 214]. In several other teleost species, sex-distinct sequences have been partially isolated, but no sex-determining genes have yet been found (for review see [37]). Therefore, identification of sex-specific sequences in different teleost species is important both for evolutionary biology, to explore mechanisms of sex chromosomal development, and for the aquaculture industry, to find genetic sex markers for rapid sex determination.

The Nile tilapia (*O. niloticus*) is an African freshwater fish, which breeds easily, grows rapidly, and tolerates variations in water temperature and oxygen levels. It is one of the most important commercial species available for food production worldwide. A cytogenetic analysis of metaphase chromosomes demonstrated that the tilapia karyotype consists of 22 chromosome pairs with no morphologically distinct sex chromosomes [135]. However, it has been established that sex determination in this species is governed by monofactorial genotypic factors with male heterogamety (XY) and female homogamety (XX) [133]. Moreover, it has been found [22] that the terminal region of the longest bivalent (chromosome 1) shows delayed pairing in meiosis and that the putative X and Y have limited sequence differences [69, 70]. Recently, several sex-linked microsatellite and AFLP markers have been isolated and the latter assigned to the middle of the long arm of chromosome one [47, 115].

In the present study we report an unexpected finding that insulin, a key hormone in regulation of the blood glucose homeostasis, may have acquired a new sex-related function in tilapia. We show that allelic variance in the insulin gene 5' flanking sequence is linked to the phenotypic sex in tilapia. The physical location of the tilapia insulin gene on metaphase chromosomal spreads was determined using the FISH technique. Finally, the level of insulin expression was examined in the gonads of adult tilapia and in tilapia fry prior to sex differentiation.

5.3. Materials and Methods

5.3.1. Fish Maintenance and Tissue Isolation

The Nile tilapia (*Oreochromis niloticus*) broodstock originated from Lake Nasser (Egypt). Fish were purchased from North American Tilapia INC (Lindsay, ON), and

were reared in 40-gallon aquaria with circulating aerated fresh warm (approximately 27°C) water in the Biology Department at Dalhousie University. Tissues were extracted from tilapia after anesthetizing with 2- phenoxyethanol (Sigma).

5.3.2. Isolation of Genomic DNA

For the Southern hybridization experiments, chromosome walking and construction of the tilapia genomic library, genomic DNA was isolated from the blood or liver tissues of the adult male and female tilapia using the proteinase K (Bio Basic Inc.) / phenol-chloroform method as described by Maniatis et al. [136] with slight modifications.

Genomic DNA employed in PCR with X- and Y-specific primers was extracted from blood of adult male and female tilapia using GenomicPrep Blood DNA Isolation Kit (Amersham Pharmacia Biotech Inc.) or from whole tilapia 10 days post fertilization (dpf) fry using TRIzol reagent (Invitrogen).

5.3.3. Southern Blots and Hybridizations to Study Allelic Polymorphism of the Tilapia Insulin Gene

Southern blots and hybridizations were performed as described by us earlier [196], according to Maniatis et al. [136] with minor modifications.

A 3.5 kb sequence (3.5INS) containing the tilapia insulin gene, cloned in our laboratory and described previously [138, 196], was used as a template in PCR with the In 40-In 41, In 6-In60 and In10-In15 primer pairs. All primer sequences and obtained products are listed in Table 1 (in appendices). PCR products were labeled with [^{32}P] dCTP (Amersham/ Pharmacia) and were used as the hybridization probes.

5.3.4. Construction and Screening of the Tilapia Genomic Library

The 2911 bp Y allele sequence upstream of the 3.5INS was isolated by constructing and screening a tilapia genomic library.

The tilapia genomic library was prepared using a 100 μg aliquot of the male tilapia genomic DNA digested with Sau3A, λGEM-11 genomic cloning vector digested

with BamHI (Promega), and the Plus Packagene System (Promega), according to the Genomic Cloning Technical Manual (Promega).

Approximately one million phage plaques were screened with the [^{32}P] dCTP (Amersham/ Pharmacia) labeled In40-In41 probe using general blotting and hybridization methods [136]. DNA from positive clones were isolated using the ultracentrifugation method [136]. After subsequent restriction mapping, a 3328 bp HindIII /PstI fragment that represents the Y insulin allele and 417 bp overlapping with the 5' end of 3.5 INS was subcloned into pUC18 (Fermentas) and commercially sequenced.

5.3.5. Chromosome Walking

The 2304 bp sequence of the Xa allele was obtained by two consecutive chromosome walking PCR. First, we amplified In44-SspI fragment and then the X3-EcoRI fragment.

To obtain the In44-SspI fragment, approximately 5μg of tilapia genomic DNA was digested with HindIII (Roche) + SspI (Roche), 5μg of the pUC18 vector (Fermentas) was digested with HindIII +HincII (Roche), and then both were ligated. Aliquots of the "plasmid library" were used as a template for the nested PCR: the first round was performed with the In40 insulin-specific primer and M13 reverse primer, followed by the nested PCR with the In44 insulin-specific primer and M13 reverse primer. Both PCR mixtures were composed of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM of each dNTPs, 200 nM of each of the primers, and 10 U of our Taq polymerase, isolated as described by Pluthero [195]. PCR were performed in a UNOII Thermocycler (BiometraLtd.) using the following conditions: 3 min general denaturing (94^0C); 10 cycles of denaturing (94^0C) for 30 s, annealing (68^0C) for 30 s, and elongation (72^0C) for 1 min; 20 cycles of denaturing (94^0C) for 30 s, annealing (68^0C-58^0C) for 30 s, and elongation (72^0C) for 1 min; and the final 10 cycles of denaturing (94^0C) for 30 s, annealing (58^0C) for 30 s, and elongation (72^0C) for 1 min. The 913 bp product was resolved on a 1% agarose gel, purified using QIAquick gel extraction kit (Qiagen), cloned into pCRII (Invitrogen) vector, and commercially sequenced.

The X3-EcoRI fragment was obtained using PCR *in vitro* cloning kit (Takara Shuzo Co., Ltd.) with EcoRI cassette, according to the manufacture instructions. The X2 and X3

X-allele-specific primers were used in two rounds of nested PCR. Both PCR mixtures were composed of 75 mM Tris-HCL (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2.5 mM $MgCl_2$, 200 μM each of dNTPs, 200 nM of each of the primers and 10 U of our Taq polymerase. PCR were performed using step-down conditions similar to those used for amplification of the In44-SspI fragment. The 1530 bp product was cloned into pCRII vector (Invitrogen), and commercially sequenced.

5.3.6. Polymerase Chain Reactions (PCR) to Determine XX and XY Fish Genotype

To develop PCR specific for the amplification of the X and Y insulin alleles, approximately 200 ng aliquots of the genomic DNA previously analyzed in Southern hybridizations were used as the templates with the following pairs of primers: Y1-XY, Y2-XY, Y3-Y4, X-XY. To determine population correlations of the phenotypic (gonadal) sex with the presence of the Y insulin allele in the fish genome, 200 ng aliquots of the genomic DNA were added as a template in each of three PCR: with Y1-XY, Y2-XY Y-allele specific primers, and with In49-In16 insulin-gene-specific primers (positive control). Sequences of all primers are listed in Table 1 (in appendices). PCR mixtures were the same as in the amplification of the In44-SspI fragment. PCR were performed using the following conditions: 50 cycles of denaturing (94^0C) for 20 s, annealing (59.5^0C) for 30 s, and elongation (72^0C) for 1 min. PCR products were resolved on 6% polyacrylamide gels, visualized by staining with ethidium bromide and photographed. Amplifications of the correct products were confirmed by cloning and sequencing (as described above) of the selective PCR products.

To determine genotypes of the adult tilapia, 200 ng aliquots of the genomic DNA were added as a template in each of the two PCR: with Y1-XY primers and with X-XY primers. To determine genotypes of the 10 day post fertilization (dpf) tilapia fry approximately 1-10 ng aliquots of the genomic DNA were added as a template in each of the two PCR: with Y1-XY1 primers (Table 1 in appendices) and with X-XY primers. PCR mixtures were the same as in the amplifications of the X3 /EcoRI fragment, only our Taq polymerase was substituted with 5 U of the Taq DNA polymerase (recombinant) (Fermentas). Reactions were performed under the same conditions as described earlier in this section. PCR products were resolved on 1% agarose or 6% polyacrylamide gels and

visualized by staining with ethidium bromide. Due to the low concentrations of the Y1-XY1 products, the presence of the Y allele in the tilapia fry was confirmed by the subsequent blotting and hybridization with DIG-labeled Y1-XY probe. Labeling, hybridizations, and detections were performed using DIG High Prime Labeling and Detection Starter Kit II (Roche Applied Science, Laval, QC) according to the provided protocol.

5.3.7. RNA Isolation and Reverse Transcription (RT)

Total RNA was isolated from tilapia testis, ovaries, and 10 dpf whole fry using TRIzol reagent (Invitrogen) according to the recommended protocol. To remove contaminating DNA, aliquots of isolated total RNA were treated with 1U of DNAseI (Invitrogen) and then were reverse transcribed using a SuperScript III reverse transcriptase kit (Invitrogen) and oligo(dT) primer (Invitrogen).

5.3.8. PCR to Examine Expression of the Insulin, Prohormone Convertase 1/3 (PC1/3), Prohormone Convertase 2 (PC2) and Carboxypeptidase H (CPH) Genes in the Tilapia Gonads

To analyze expression of the insulin gene in the tilapia testis and ovaries, 2 μl of the gonadal cDNA and 2 μl of the diluted pancreatic islet cDNA (prepared by us earlier and used as a positive control) were added as templates in each of the two PCR - one with the insulin primers (In49 and In16) and another with the β-actin primers (actFOR1 and actREV1). To examine expression of the PC1/3, PC2, and CPH genes, the same cDNA samples were analyzed with the degenerative PC1/3, PC2, and CPH primers listed in Table 1 (in appendices). PCR conditions were similar to those used for the determination of genotypes of the tilapia adults and fry. PCR products were resolved on a 6% polyacrylamide or 1% agarose gels, visualized by staining with ethidium bromide, and photographed. Obtained PC2 and CPH products were extracted, purified, cloned, and sequenced as described above. Blastn (www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) analysis of the PC2 and CPH sequences revealed that those are 100% identical to the tilapia brain and pituitary PC2 and CPH partial transcripts (accession numbers EF081463, EF081464, EF081466, and EF081468) cloned by us earlier.

5.3.9. Quantitative PCR (qPCR) to Analyze the Levels of Insulin Expression in Testis, Ovaries and 10 dpf Whole Fry

Amplifications of transcripts were performed on a Rotor Gene 2000 real-time PCR machine (Corbett Research, Sydney, Australia) using a Platinum SYBRGreen qPCR SuperMix-UDG Kit (Invitrogen) in accord with the protocol provided. qPCR primers were the same as in the corresponding PCR. Quantitative amplifications of insulin and β-actin products and data analysis were performed as described by us [75]. Each cDNA sample was analyzed in three or four duplicated PCR representing three or four different template dilutions ranging from 1:2 to 1:10. In the preliminary experiments, a linear correlation between the inputted cDNA template and calculated transcript concentration were determined to occur within this range of dilutions. To compensate for any variance in the amount or quality of the input RNA, the presented levels of the insulin mRNA are shown relative to the levels of β-actin mRNA that was used as an internal control. All results are expressed as mean ±SE of three to four independent experiments. Data were considered significantly different at the level of $p<0.05$ using an unpaired Student's t test.

5.3.10. BAC Library Screening and Fluorescence In Situ Hybridization (FISH)

A BAC clone containing the insulin gene was isolated from the tilapia BAC library [95] using the In49-In16 probe described above. This was used as a probe in FISH performed on mitotic chromosome spreads. The probe labeling, hybridizations, and signal detections were performed as described previously [47].

5.3.11. Western Blot and Hybridization for Detection of the Immunoreactive Insulin in the Tilapia Testis and Ovaries

Adult tilapia testis and ovaries were homogenized in the lysis buffer (150 mM NaCl, 1% Triton X-100, 50mM Tris-HCl pH 7.5, 2 mM EDTA, 10% Glycerol) supplemented with protease inhibitors (1 tablet per 10 ml of buffer) (Roche) and then centrifuged. Supernatants were applied to Centricon centrifugal filter devices with ultracel YM-30 membrane (Milipore) and then filtrates were concentrated by acetone precipitation. Aliquots of the obtained protein samples (>30 kDa) were electrophoresed in a 4-15% SDS-polyacrylamide gel together with earlier prepared protein sample extracted from tilapia pancreatic islets. Next, samples were electroblotted onto PVDF transfer

membrane (Hybond-P, Amersham/ Pharmacia). Filters were hybridized for 12 h with rabbit anti-tilapia insulin 1^{o} antibodies developed by us [270], washed with PBS/0.1% Tween, and then incubated for 30 min with HRP-conjugated goat anti-rabbit 2^{o} antibody (1:10,000) (Santa Cruz Biotechnology). After hybridizations filters were processed for chemiluminescent detection using a Western Blotting Luminol Reagent kit (Santa Cruz Biotechnology).

5.4. Results

5.4.1. Analysis of the Genomic Polymorphism of the Tilapia Insulin Gene

The cloned tilapia insulin sequence (3.5 INS) contains the complete tilapia insulin gene (990 bp), as well as 1575 bp of 5' flanking and 994 bp of 3' flanking regions [138, 196]. In this study, the genomic polymorphism of the insulin locus was initially analyzed using the Southern hybridization technique. Genomic DNA was extracted from 16 randomly selected fish (8 phenotypic males and 8 phenotypic females), digested with restriction enzymes PstI, EcoRI, HindIII, and SspI, blotted, and hybridized with the insulin probes that represent different parts of the 3.5INS (Fig. 5.1 A). The multiple size signals were observed only in hybridizations with the probes that correspond to the sequences upstream of the restriction sites (e.g. probe A; and probe B in case of PstI, EcoRI, and SspI) (Fig. 5.1 B-E). These initial results suggest that some sequence variations exist in the region upstream of the 3.5INS locus, but not in the region downstream of it. As shown in Figure 5.1 C and D, using upstream probes we found at least three different insulin alleles in the tilapia genome. Two of them (named Xa and Xb) were detected in both the male and the female genomes. The third one (named Y) has never been detected in the female genome, but has been detected in the genome of six (from eight tested) male fish. Thus, results obtained with Southern hybridization suggest that the insulin gene may be sex linked in tilapia.

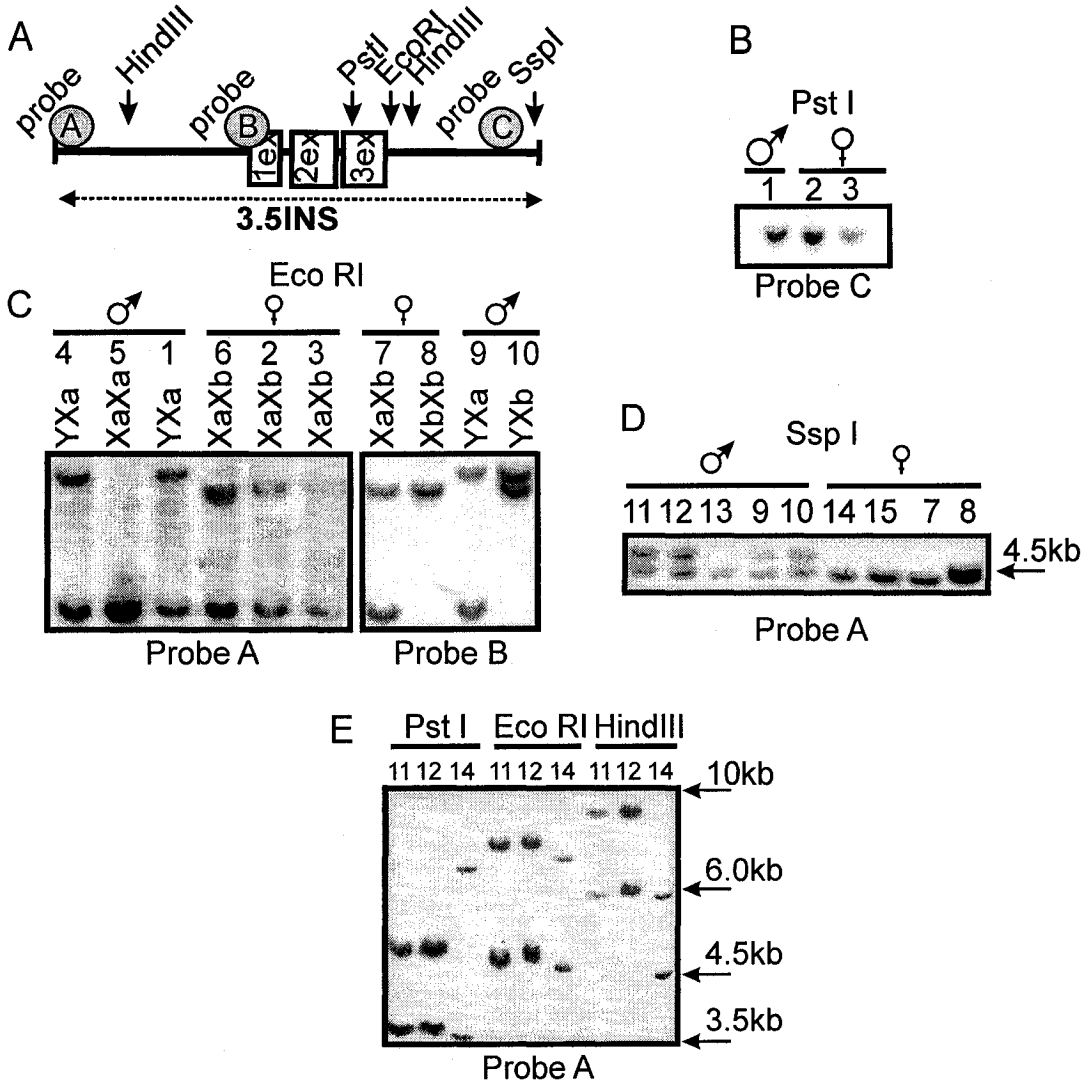

Figure 5.1. Allelic polymorphism of the tilapia insulin gene
(A) schematic representation of the 3.5 INS locus and location of the hybridization probes. *(B-E)* results of Southern hybridization of tilapia genomic DNA from 15 fish *(1-15)* digested with the indicated restriction enzymes and hybridized with the indicated probe; ♀-phenotypic (gonadal) female, ♂-phenotypic (gonadal) male; *(B)* a single hybridization signal was detected in hybridizations using downstream probes (representative results), indicating no sequence polymorphism in the 3'insulin flanking region. *(C* and *E)* three signals were found when genomic DNA was digested with EcoRI, HindIII, or Pst I, and hybridized with upstream probes, indicating sequence polymorphism in the 5'insulin flanking region. Three hybridization signals correspond to three different insulin alleles *(Xa, Xb, Y)*. *(C)* shows presence of fish with the different genotypes, and linkage of the Y insulin allele to the male sex (representative results). *(D)* two hybridization signals were detected when genomic DNA was digested with SspI, indicating no sequence differences between Xa and Xb insulin alleles within the SspI fragment. *(E)* summarizing results: genomic DNA from two YXa males *(11* and *12)* and one XaXb female *(14)* were digested with PstI, EcoRI, and HindIII and hybridized with probe A.

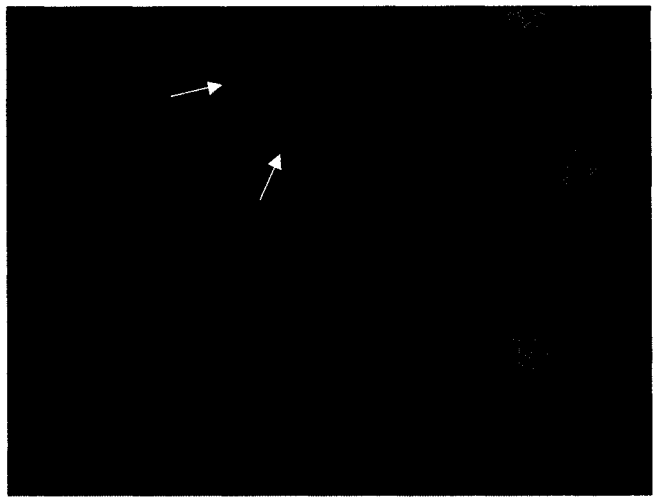

Figure 5.2. Chromosomal localization of the tilapia insulin gene
Results of the fluorescent in situ hybridization of the BAC clone containing the insulin gene against the tilapia mitotic chromosomes. Arrows indicate hybridization signals on the long arm of the chromosome one.

5.4.2. Chromosomal Localization of the Insulin Gene[*]

To further confirm the insulin-sex-linkage hypothesis, we mapped the tilapia insulin gene on mitotic chromosomal spreads using the FISH technique. A BAC clone containing the insulin gene was isolated from a tilapia BAC library and was used as a probe for hybridization. As shown in Figure 5.2, the fluorescent signal was detected on the long arm of chromosome one, indicating that the insulin gene is indeed located on the putative sex chromosome in the tilapia genome.

5.4.3. Cloning, Sequencing, and Analysis of the Insulin- Linked Sex-Different Regions

Based on the results obtained with the Southern hybridizations, we prepared restriction maps of the Xa, Xb and Y alleles (Fig. 5.3) and determined that the start of the sequence differences between the X and Y alleles should be within a 0.7-1.0 kb region upstream of the 5'end of the 3.5INS. Therefore, as a next step, we cloned, sequenced, and aligned 3 kb sequences from both Xa and Y alleles (Fig. 5.4 A, and appendices). Despite numerous attempts, we failed to obtain sequence of the Xb allele. However, according to

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[*] This experiment was carried out by Dr. Penman's laboratory

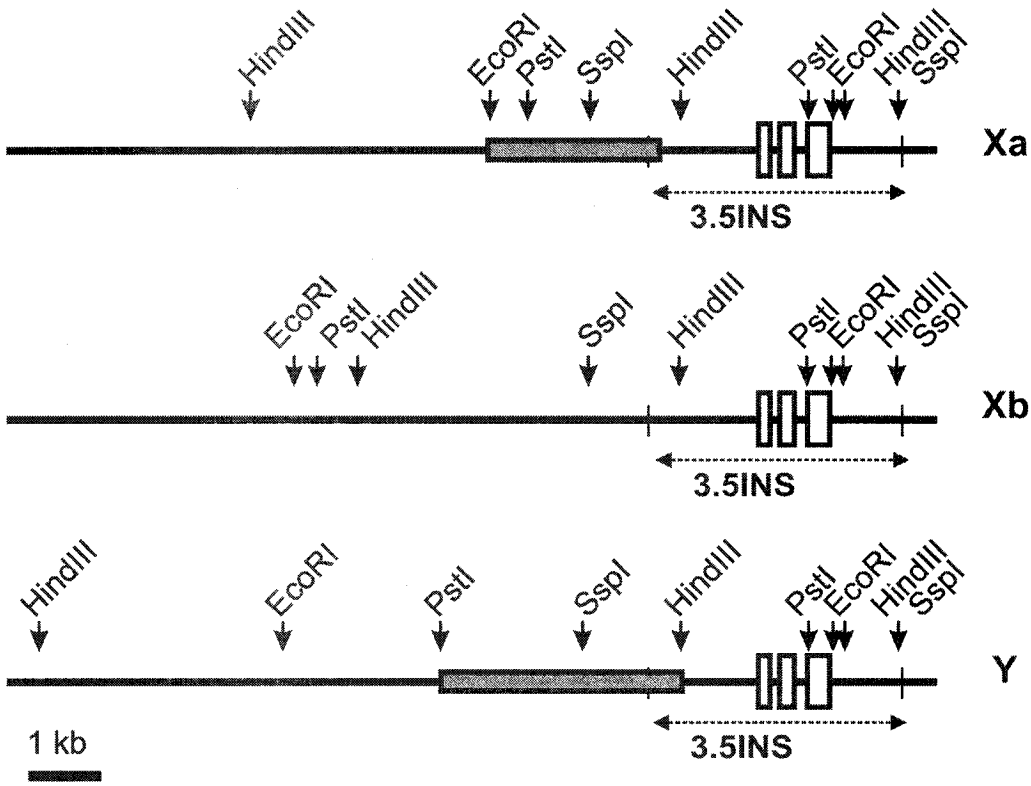

Figure 5.3. The restriction maps of the Xa, Xb and Y insulin alleles
Based on the results from Fig. 5.1 E and B we designed a restriction map of *Xa, Xb*, and *Y* insulin alleles; the grey rectangles represent cloned parts of the Xa and Y alleles.

the location of SspI sites (Fig. 5.1 C, and Fig. 5.3), there are no sequence differences between the two X alleles at least within 0. 8 kb region upstream of the 3.5INS.

Next, we analyzed the sequences of the male and female alleles using BLAST and RepeatMasker algorithms. Searches for repetitive elements with RepeatMasker identified only several small simple repetitive sequences. Analysis of the same sequences using BLAST N and BLAST X revealed no similarities to any known coding region. However, the sex-different sequence of the male allele starts with a polyA repeat and contains several regions of high similarity to the genomic intergenic sequences from Nile tilapia (*O. niloticus)* and from Burton`s Mouthbrooder *(Astatotilapia burtoni)* genomes. These include two partially overlapping long sequences: (1) 1-1389 bp region is 89 % identical to the sequence from Burton`s Mouthbrooder *(A. burtoni)* clone BAC 26M7 (accession

number DQ386648), and (2) 1026-1918 bp region is 92% identical to the Nile tilapia (*O. niloticus)* KLR genomic locus (accession number AY495714). Hence, the Y-specific insulin-linked sequence is present more than once in the genome of Nile tilapia and it is also found in the genome of another African cichlid fish. This suggests that the male-specific sequence most likely consists of unknown repetitive elements.

5.4.4. Development of Y-X Specific PCR to Analyze Correlations of Gonadal Sex with the Presence of the Y Allele in the Tilapia Genome

Based on the sequence alignment, we designed five primers: Y1, Y2, Y3, Y4, X, and XY, where Y1, Y2 and Y3 are forward primers and Y4 is the reverse primer that are specific to the sequence of the Y allele. XY is the reverse primer common for the X and Y alleles. X is the forward primer specific to the sequence of the X allele (Fig. 5.4 A). The PCR reactions with Y1/XY, Y2/XY, X/XY, and Y3/Y4 combinations of primers were performed on the genomic DNA previously used for the Southern hybridization. The PCR products specific for the male DNA (Y allele) were amplified with two sets of primers (Y1/XY, Y2/XY), but not with Y3/Y4 primers (Fig. 5.4 B-D). This further supports the idea that the Y-specific sequence is present more than once in the tilapia genome, but that its linkage to the insulin gene is male-specific. As expected, the X/XY PCR product was detected in all samples tested (Fig. 5.4 E). However, since in Southern hybridizations we have never observed fish with the YY genotype in our population, we cannot unequivocally confirm that the X/XY product is strictly X-allele specific and not due to the presence of the amplifying region somewhere else in the tilapia genome. In all reactions, amplifications of the correct products were further confirmed by cloning and sequencing.

To examine consistency of the phenotypic (gonadal) sex with the presence of the Y-insulin allele, we isolated DNA from the blood of 47 randomly selected tilapia (32 gonadal males and 18 gonadal females). Three sets of PCR with the Y specific (Y1/XY, Y2/XY) and insulin gene specific (positive control) primers were performed on DNA aliquots from each sample. Together (with 16 previously examined fish) we tested 63 fish in total: 38 phenotypic (gonadal) males and 25 phenotypic (gonadal) females. From 38 phenotypic males, the Y-specific product was detected in 23 (60.5%). The Y specific

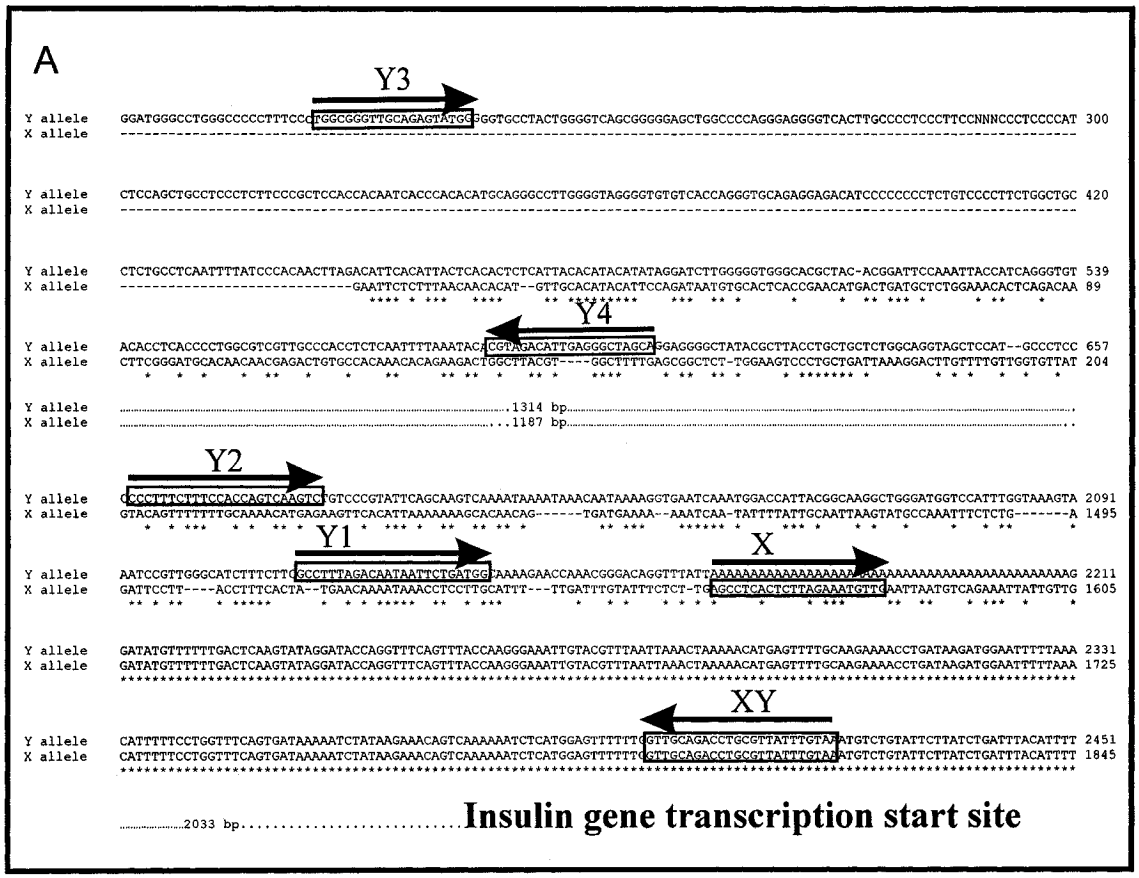

Figure 5.4. A sequence comparison of the Y and X insulin alleles and development of the Y- and X-specific PCR

(A) Alignment of the insulin gene 5' flanking sequences from Y and X alleles. Sequences that correspond to the designed primers are framed and shown with arrows. *(B-E)* results of the PCR using indicated pairs of primers. *(♀ and ♂)* fish phenotypic (gonadal) sex; *(XX and XY)*- fish genotype determined in Southern hybridizations; *(-)* negative control, *(+)* positive control (cloned part of the X or Y alleles used as a template), *(M)*-molecular weight marker.

PCR product was not amplified in 18 out of 25 tested phenotypic females (72%). Thus, overall, the consistency of identifying phenotypic sex along with genetic sex using the Y specific PCR was 65% (41 in 63 fish examined: χ^2[1d.f.] = 5.73, $P < 0.05$).

We also looked for the presence of the Y insulin allele in the tilapia population originating from Lake Manzala that was previously used to isolate sex-linked microsatellite and AFLP markers [47, 115]. Genomic DNA was extracted from ten XX females and ten YY males and used in PCR with Y1/XY and Y2/XY primers. We failed to detect any products corresponding in size to those amplified using genomic DNA from original tilapia population (results not shown).

5.4.5. Differential Expression of the Tilapia Insulin Gene in the Adult Gonads and During Ontogeny

In mammals, many genes within the sex-determining region are expressed preferentially in the gonads, and the corresponding proteins are involved in sex-differentiation / determination or carry other sex-specific functions [229]. Therefore, the location of the insulin gene on the sex chromosome near the sex-different sequence may indicate that insulin itself has acquired some sex-associated functions in tilapia. To test this, we examined expression of the insulin gene in testis of XX and XY adult males, in ovaries of XX and XY adult females, and in the whole tilapia fry prior to sex determination (10 dpf). Fish in each of two groups (fry group and adult tilapia group) were full siblings from the same brood. The XX or XY genetic sex of the adult fish was determined by PCR with Y1/XY and X/XY primers. Due to the very low DNA concentrations of the 10 dpf fry samples, the XX-XY fish genotype was determined by amplification of the short product in PCR with Y1/XY1 primers followed by Southern hybridization with Y1/XY probe. As shown in Figure 5.5 A, using the RT-PCR technique we detected insulin transcripts in the ovaries and in the testis of both XX and XY adult fish. This is surprising, since in mammalian adults expression of the insulin gene is restricted to the pancreatic β-cells. Next, we applied qRT-PCR to compare the levels of insulin mRNA in tilapia gonads. As shown in Figure 5.5 B, insulin expression was significantly higher in the testis of the sex-reversed males (XX) relative to that in the testis of normal males (XY). In the ovaries of normal females (XX), the level of insulin

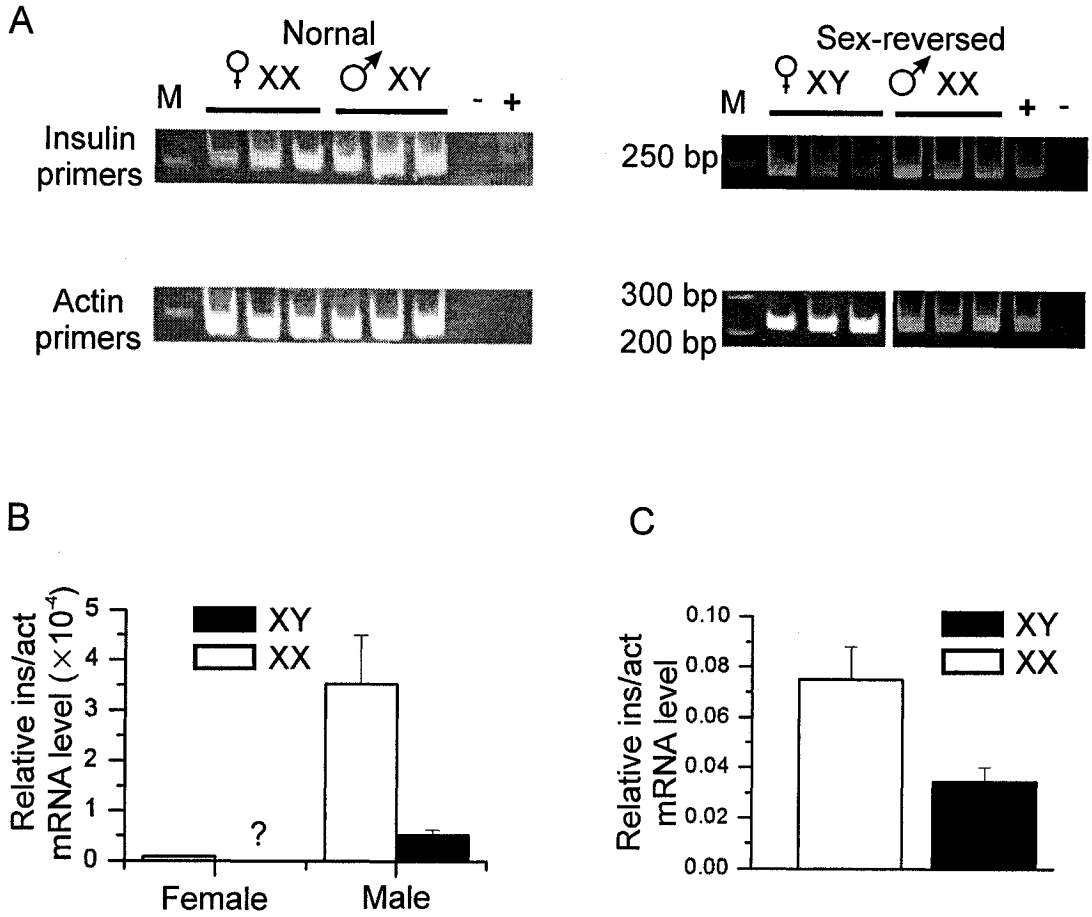

Figure 5.5. Expression of the insulin gene in tilapia gonads and tilapia 10 dpf fry
Total RNA was isolated from tilapia testis, ovaries, and tilapia 10 dpf fry and was used in RT-PCRs and in qRT-PCR. The *(XX)* and *(XY)* fish genotypes were determined by Y-specific PCR using the simultaneously extracted genomic DNA, as described in Materials and Methods. Male *(♂)* or female *(♀)* phenotypic sex was determined by gonadal examination. *(A)* results of the RT-PCR with the insulin-specific *(top panel)* and the actin-specific *(bottom panel)* primers, using total RNA isolated from ovaries and testis of three normal fish *(♂XY* and *♀XX)* and three sex-reversal fish *(♂XX* and *♀XY)*, *(-)* negative control, *(+)* positive control (insulin or actin products amplified using diluted pancreatic islet cDNA); *(M)*-molecular weight marker. *(B)* the relative levels of insulin mRNA in gonads of adult fish determined by qRT-PCR (n=3,4). *(C)* the relative levels of insulin mRNA in tilapia 10 dpf fry determined by qRT-PCR (n=4).

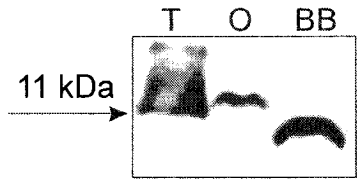

Figure 5.6. Identification of the immunoreactive insulin in the tilapia gonads
Results of the immunoblotting analysis of the protein lysates from the tilapia testis *(T)*, ovaries *(O)*, and Brockmann bodies *(BB)* hybridized with tilapia insulin antibodies.

expression was significantly lower than that in the testis of normal (XY) males. In most of the XY ovary samples (n=4), the level of insulin mRNA was below the transcriptional level necessary for reproducible quantification, and therefore significantly lower than that in the XX ovaries. However, in one sample the level of insulin expression was 6-fold higher than that in samples from normal ovaries. To verify these data, we repeated all RNA extractions from XY ovaries and obtained very similar qRT-PCR results. Currently, we do not know the reason for such fluctuations.

The levels of insulin mRNA in XX and XY fry at 10 dpf were approximately 2.2-fold lower in XY fish than in XX fish (Fig. 5.5 C).

Thus, the lower level of insulin transcription usually correlates with the presence of the Y allele in the tilapia genome, suggesting a potential negative influence of the Y-specific sequences on the transcriptional activation of the insulin gene.

The dimorphic levels of insulin expression during sex differentiation and in reproductive organs provide evidence that insulin in tilapia could have some sex related functions in addition to the well-known regulation of the glucose homeostasis.

5.4.6. Insulin Synthesis in the Tilapia Testis and Ovaries

At 10 dpf, tilapia fry have developed pancreatic islets and hence presence of mature insulin at that stage of development is expected [157]. Therefore, to further analyze potential involvement of insulin in the sex-related pathways we examined whether or not insulin mRNA is translated and processed in the tilapia testis and ovaries. A Western blot of the proteins extracted from the gonads and hybridized with tilapia insulin antibodies revealed the presence of a single signal in both tissues (Fig. 5.6).

However, the detected insulin-like signal was larger (approximately 13 kDa) than the pancreatic insulin (6 kDa), suggesting that the gonadal insulin may not be fully processed. Furthermore, using RT-PCR we were able to detect only PC2 and CPH mRNA, but not PC1 transcripts in total RNA extracted from testis and ovaries (data not shown).

5.5. Discussion

Insulin together with insulin-like growth factor I (IGF-I) and insulin-like growth factor II (IGF-II) belong to a superfamily of structurally similar proteins that evolved from a common ancestor very early in the evolution of chordates [24]. Mammalian IGF-II, insulin, and tyrosine hydroxylase (TH) genes are organized as a 5'-TH-insulin-IGF-II-3' genomic locus, which is assigned to autosome 11p15 in humans, whereas IGF-I is linked to the phenylalanine hydroxylase (PAH) gene on the structurally paralogous part of autosome 12q22. This provides evidence that the insulin and proto-IGF genes likely arose via a tandem duplication of the common insulin/IGF hybrid gene, followed by partial chromosomal duplication and translocation. Subsequently, two protoPAH/TH–insulin-protoIGF loci diverged into TH-insulin-IGF-II locus and PAH–insulin-IGF-I locus and the insulin gene between PAH and IGF-I genes was silenced [183]. As a result of whole genome duplication, the genome of teleost fish contains two nonallelic insulin genes [85]. However, neither of them is located within the TH-IGF-II or PHA-IGF-I locus (see [29, 176, 204] and the chromosomal maps Zebrafish *(Danio rerio)*, Fugu fish *(Takifugu rubripes)* (www.ensembl.org), and Spotted green pufferfish *(Tetraodon nigroviridis)* (www.genoscope.cns.fr)), suggesting that in fish, the insulin genes have been translocated to other sites in the genome. In this study we found that in Nile tilapia the insulin 1 gene is located on the long arm of the putative sex chromosomes. The suggestion of a recent insulin gene translocation is further supported by the presence of a 137 bp tandem repeat sequence upstream (-444 bp to –307 bp) and downstream (405 bp to 542 bp) of the tilapia insulin gene that could have resulted from insulin gene insertion.

Our results indicate that the 2.3 kb sequence upstream of the tilapia insulin gene differs between male and female genomes and is produced by integration of a potential repetitive element in the Y allele. The finding of a repetitive element on the sex

chromosome is not surprising since their accumulation is postulated to be one of the critical steps in early sex-chromosomal differentiation [214]. It is a typical feature of almost all known sex determining regions, including those on chromosome one in tilapia [64, 69, 139]. In agreement with our findings, it has been proposed that sequence differences between tilapia proto-Y and -X chromosomes are probably mostly due to the number, distribution, and nature of the repetitive elements [64, 69].

In this study we have found that the insulin gene is expressed in both testis and ovaries, indicating a potential role of insulin in these tissues in tilapia. Insulin is the best known as a main hormone in regulation of the blood glucose homeostasis. To our knowledge, neither the gonosomal location of the insulin gene, nor its direct expression in gonads has ever been reported. However, sex-associated functions of insulin have been described in different animal species. In invertebrates, insulin orthologs (insulin-like peptides) regulate reproduction, sexual behavior, and potentially male sexual differentiation, which suggests an evolutionary early involvement of insulin in the sex controlling pathways [16, 137, 267]. In mammals during embryogenesis, the insulin signaling cascade is required for male sex differentiation [140, 163]. Furthermore, insulin also stimulates steroidogenesis (either directly or by potentiating effects of gonadotropins), oocyte growth and maturation by acting via its own or IGF-I receptors in mammalian [38, 197, 198] and also in fish [124, 233] ovaries. In the brain, insulin signaling pathways regulate secretion of the luteinizing hormone (LH) [20]. Recently, we co-localized insulin-producing cells with gonadotropin-producing cells (LH and follicle-stimulating hormone, FSH) in the tilapia pituitary [75] gland and also in preliminary *in situ* RNA hybridization experiments in ovaries. Coexpression of insulin and gonadotropins may indicate that in adult tilapia insulin influences gonadotropins production or steroidogenesis in an autocrine or paracrine fashion. The importance of local insulin synthesis is also supported by the fact that above serum levels of insulin were required for stimulating steroidogenesis in fish [233] and in mammals [51, 146, 155].

Unlike that in the tilapia pituitary, the immunoreactive insulin-like product in tilapia gonads had a higher molecular weight compare to the pancreatic mature insulin. Furthermore, we have found gonadal expression of PC2 and CPH genes, but no PC1 gene

expression, suggesting that the gonadal insulin precursor may not be fully processed to the mature insulin. It has been shown that while proinsulin exhibits a weaker metabolic potency than mature insulin, it is a strong stimulator of other effects, such as growth, differentiation, and cell survival [73]. Alternatively, we can speculate that insulin mRNA and/ or proinsulin may be stored in the tilapia oocytes or spermatozoa and delivered to zygotes where mature insulin is synthesized and functions during the early stages of development. Accordingly, the presence of maternal insulin mRNA has been described in zebrafish embryos at 1 hpf [178], in *Xenopus* mature oocytes (but not in its unfertilized eggs) [227] and the maternal insulin has been found in unfertilized chicken and *Xenopus* eggs [34, 227].

Thus, results presented here and by others indicate that in addition to the regulation of energy homeostasis, insulin is involved in the reproduction and sex differentiation pathways in a variety of animal species from invertebrates to mammals. We can speculate that the translocation of the insulin gene to the sex chromosome in tilapia further enhanced acquisition of sex-related functions by insulin in this species. In support, we have found insulin gene expression in tilapia testis and ovaries, and more importantly, the level of insulin mRNA was different in XX and XY gonads and in XX and XY fry prior to sex differentiation. The foregoing data interpretation seems consistent with Schartl's recent hypothesis of the evolution of the sex-determining genes [214]. Accordingly, gene duplication and translocation to the protogonosomes are the milestone events that relax selective pressure and thus allow accumulation of sex-determining functions for the gonosomal gene copy, while autosomal duplicate continues its original function. In this context, at least two insulin genes have been found in the genomes of teleost species [85], including tilapia (unpublished data). While the gonosomal tilapia insulin 1 gene is highly expressed in the tilapia pancreatic islets, its transcription is not up-regulated by physiological concentration of nutrients and is not as tightly restricted to β-cells as it is described for the mammalian insulin genes (unpublished results) [75]. Lost functions of the insulin 1 gene could at least partially be compensated by the duplicated homolog (insulin 2 gene), the transcription of which can also be detected in tilapia (unpublished data) and in *Danio rerio* pancreatic islets [178]. At the same time, translocation of the tilapia insulin 1 gene to the sex chromosome has

promoted a process of accumulation of sex-different sequences at the insulin locus. These different sequences probably control different levels of insulin gene transcription by enrolment of modifying secondary chromosomal structure or by evolution of transcription factor binding sites. In agreement with accumulation of transcriptional *cis*-elements, we determined that in contrast to the short (approximately 400 bp) mammalian insulin promoters/enhancers, the tilapia insulin regulatory elements are spread over a significantly longer region (unpublished data). On the other hand, consistent with the presence of heterochromatin or methylation, the cloned sex different sequence of the Y allele is 54% GC-rich compared to the 36% GC content of the cloned sex-different part of the X allele. Nevertheless, the lower level of insulin expression generally correlates with the presence of the insulin Y allele in the tilapia genome, suggesting a negative effect of the Y-specific sequences on the insulin gene transcriptional activation. However, in contrast to the hypothesis of male-specific insulin inhibition, we determined that the relative insulin/actin mRNA ratio was 5.4-fold higher in testis than it is in ovaries. This discrepancy may be explained by unequal abundance of the actin mRNA between the two tilapia tissues, similar to that detected between mouse ovaries and testis [5]. Therefore, the insulin mRNA level must be re-examined in the future experiments using alternative reference transcripts.

The physiological importance of the sexually dimorphic differences in insulin concentrations remains to be determined. We can speculate that insulin effects on sex-related pathways might be dose-dependent. For example, insulin induces expression of P450 aromatase gene and stimulates P450 aromatase enzyme activity in humans [51, 146, 193]. The ovarian P450 aromatase is a key enzyme in tilapia sex differentiation, functioning slightly downstream from the main sex-determination event [33, 111]. While ovarian P450 aromatase gene expression was observed in Nile tilapia embryos of both sexes from 3-4 dpf, the female specific gene up-regulation was found from 11 dpf [112], and was under control of environmental (temperature) and unidentified genetic sex-linked factors [33]. Since we found female specific up-regulation of the insulin expression at approximately the same time (10 dpf), it is tempting to speculate that one of the genetic factors that can induce P450 aromatase transcription in females might be insulin.

Finally, we developed Y-specific PCR and found that the Y insulin allele is present in the genomes of approximately 60% of males and absent from the genome of 72% females. Furthermore, when Y-specific PCR were tested on genomic DNA extracted from a different (Lake Manzala) population of Nile tilapia we failed to obtain any Y-like product. The relatively low rate of correlation of predicted genetic sex with phenotypic sex in the donor tilapia population and complete absence of Y1/XY or Y2/XY PCR products in a different Nile tilapia population are not entirely unexpected. Similar phenomena have been described earlier and most likely reflect an early/primitive stage of sex chromosome evolution. For example, population variations in sex-linked sequences were described in platyfish *(Xiphophorus maculatus)*, where Y-linkage of the repetitive element was found in one population of fish and was not detected in fish from a different river system [160]. Inconsistencies between phenotypic sex and expected genetic sex within a single population were also reported for tilapia previously, when microsatellite markers in linkage group 1 correlated with phenotypic sex in only two of three closely related Lake Manzala Nile tilapia families [115]. This is most likely since tilapia sex determination depends on more than one factor: both autosomal sequences and environmental factors have a powerful impact on sex determination in this species [133]. In fact, deviations from the standard rearing temperature (27°C), such as found in our tilapia facilities, during embryonic or larval development can cause significant alterations in phenotypic sex ratio towards production of sex-reversed males or sex-reversed females [1, 248]. Indeed, in the main Nile tilapia population studied here, sex ratios were far from the expected 50:50 and varied dramatically amongst different families.

In the present study we demonstrated that one of the Nile tilapia insulin genes is located on the putative sex chromosomes, 2.3 kb downstream from the sex-different region. We isolated and cloned insulin-linked sex-different regions from both sexes. Based on the sequence differences, we developed PCR for amplification of the male-specific allele. Because the levels of insulin expression were different in XX and XY tilapia fry prior to sex determination and in XX and XY tilapia gonads, we speculate that insulin itself could be involved in reproduction and/or sex determination pathways in tilapia. Future studies should attempt to elucidate the exact physiological role of the dimorphic insulin expression in the gonads of adult fish and in tilapia fry.

Chapter 6

Cloning and Characterization of the Second Tilapia Insulin Gene*

6.1. Introduction

In is generally accepted that the entire genome of ray-fined fish underwent a round of duplication during the early stages of evolution [6, 244, 245]. This event resulted in the presence of many paralogous genes in the genome of the teleost species. One of the retained functional duplicates is the insulin gene that is found in two non-allelic copies in the genome of several fish including fugu fish (*Fugu rubripes*) and zebrafish (*Danio rerio*) [85].

The physiological role of the second teleost insulin remains mostly undetermined. Recently, insulin 2 gene expression was detected in the zebrafish embryo, suggesting a potential functional significance during early development [178]. To our knowledge, there are no data available concerning insulin 2 gene expression in adult fish. Here, we

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* This chapter contains recent results and preliminary data

report the cloning, sequencing, and preliminary characterization of the insulin 2 (NTins2) transcript isolated from adult Nile tilapia *(Oreochromis niloticus)*.

6.2. Materials and Methods

6.2.1. Fish Maintenance and Tissue Isolation

Tilapia (*Oreochromis niloticus*) were produced in the Biology Department at Dalhousie University. Tissues were extracted as described by us earlier [75].

6.2.2. Isolation of the Genomic DNA, Southern Blot and Hybridization

Genomic DNA isolation, Southern blot, and hybridizations were performed as described in the previous chapter (sections 5.3.2 and 5.3.3).

6.2.3. Cloning of the Partial Sequence of the NTins2 Gene

A 898 bp product that represents the partial sequence of the NTins2 gene was amplified using tilapia genomic DNA as a template in a nested PCR: the first round was performed with In2-S1and In2-AS1 primers, followed by the second round with In2-S2 and In2-AS2 primers. Sequences of all primers are listed in Table 1 (in appendices). Both PCR mixtures were composed of 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2.5 mM $MgCl_2$, 200 μM each of dNTPs, 200 nM of each of the primers and 1 U of the Taq DNA polymerase (recombinant) (Fermentas). PCR were performed in a UNOII Thermocycler (BiometraLtd.) using the following conditions: 50 cycles of denaturing (94^0C) for 20 s, annealing (56^0C) for 30 s, and elongation (72^0C) for 1 min. PCR products was resolved on a 1% agarose gel, extracted and purified using QIAquick gel extraction kit (Qiagen), cloned into the pCRII (Invitrogen), vector and commercially sequenced.

6.2.4. RNA Isolation and Reverse Transcription

Total RNA was isolated from different tissues using TRIzol reagent (Invitrogen) according to the recommended protocol. To remove contaminating DNA, aliquots of isolated total RNA were treated with 1U of DNAseI (Invitrogen) and then were reverse

transcribed using a SuperScript III reverse transcriptase kit (Invitrogen) and oligo(dT) primer (Invitrogen).

6.2.5. Rapid Amplification of cDNA Ends (RACE)

To obtain 5' and 3' ends of the NTins2 mRNA, 3' and 5' RACE were performed using pancreatic islets total RNA and a GeneRacer Kit (Invitrogen) according to the provided protocol. For the 5'RACE, In2-AS5 and In2-AS6 NTins2-specific primers were used in the first and in the second rounds of PCR respectively. For the 3'RACE, In2-S2 and In2-S3 NTins2-specific primers were used in the first and in the second rounds of PCR respectively. The 527 bp 5' RACE product and 341 bp 3'RACE product were resolved on a 1% agarose gel, extracted and purified using a QIAquick gel extraction kit, cloned into the pCRII vector and commercially sequenced.

6.2.6. Cloning of Full Length Pancreatic Islet NTins2 Transcript

A 713 bp product that represents almost the complete sequence of the NTins2 transcript was amplified using pancreatic islet cDNA and In2-S4 / In2-AS4 NTins2-specific primers. PCR conditions were the same as for the amplification of the NTins2 gene product, except that primer annealing was performed at 58°C.

6.2.7. Polymerise Chain Reaction (PCR) to Study Tissue-Specific Expression of the Insulin Genes

In preliminary experiments, 2 µl of the cDNA from each tissue were used in the PCR with In2-S3 and In2-AS3 NTins2-specific primers. In the final experiment, 2 µl of the same cDNA samples were added in each of three PCR — with primers specific (1) to the tilapia insulin 1 (NTins1) transcript (In49 / In16); (2) to the NTins2 transcript (In2-S3/In2-AS4); and (3) to the tilapia the β-actin transcript (actFOR1/actREV1). Sequences of all primers are listed in Table 1 (in appendices). All PCR were performed in the same conditions as described above, except that elongation was 30 s. PCR products were resolved on 6% polyacrylamide gels, visualized by staining with ethidium bromide and photographed.

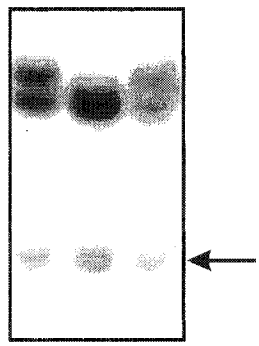

Figure 6.1. Non-allelic polymorphism of the tilapia insulin gene
Results of the Southern hybridization of the tilapia genomic DNA digested with SspI and hybridized with the insulin probe. Arrow indicates weak hybridization signal that represents a potential second insulin gene.

6.2.8. Quantitative PCR (qPCR) to Analyze the Levels of Insulin Expression in Different Tissues

Quantitative amplifications of NTins1, NTins2, and β-actin products and data analysis were performed as described by us earlier [75]. qPCR primers were the same as for the corresponding PCR. Data were considered significantly different at the level of $p<0.05$ using an unpaired Student's t test.

6.3. Results and Discussion

6.3.1. Non-Allelic Polymorphism of the Tilapia Insulin Locus

Recently, we employed Southern analysis to examine genomic polymorphism of the NTins1 gene. In several experiments, when genomic DNA was hybridized with the probe that represents the partial sequences of the first and second exons and the first intron of the NTins1 gene (probe B Fig. 5.1 A), three hybridization signals were detected in the single sample of the genomic DNA (Fig. 6.1). As described earlier (section 5.4.1.), two of them represent allelic polymorphisms of the NTins1 gene (X and Y alleles). The third signal was weak and was usually detected only after longer exposures, suggesting that the NTins1 probe may hybridize to some sequence similar, but not identical to part of the NTins1 gene encoding the signal peptide and B-chain. One of the possible candidate

sequences could be the second non-allelic insulin gene that may exist in the tilapia genome.

Recently Dr. Dooley (Dalhousie University) and Dr. Conlon (United Arab Emirates University) identified two insulin-like peptides in the tilapia Brockmann body extracts using reverse-phase high-performance liquid chromatography (personal communications). One had MW of 5716 Da, which corresponds to the MW of the known tilapia insulin. The other one was 5744 Da and could be either the second non-allelic insulin or a populational allelic variant of insulin 1.

6.3.2. Cloning and Sequencing of the Pancreatic Islet NTins2 cDNA

Based on the alignment of the available sequences for the fish insulin 2 genes we designed degenerative primers: In2-S1, In2-AS1, In2-S2, In2-AS2, and used them together with the tilapia genomic DNA in two consecutive rounds of PCR. The obtained 898 bp clone contained partial sequence of the NTins2 gene that included: 100 bp of the second exon, 146 bp of the third exon, and 682 bp of the second intron. The exon sequences of this product were used to design NTins2 gene specific primers In2-S3, In2-AS3 and to perform preliminary tissue expression analysis. This experiment revealed the presence of NTins2 mRNA in the total RNA extracted from the pancreatic islets. Therefore, pancreatic islet RNA was used to obtain 5' and 3' ends of the NTins2 cDNA employing RACE techniques. Finally, to confirm the entire sequence of the NTins2 transcript, we performed PCR using pancreatic islet cDNA and In2-S4/In2-AS4 primers that correspond to the 5' and 3' terminal sequences of the NTins2 RACE products. The obtained complete NTins2 transcript was 750 bp long and contained 333 bp of the main open reading frame, as well as 295 bp of the 5' and 122 bp of the 3' untranslated regions (Fig. 6.2). Interestingly, an additional 12 bp reading frame was detected in the 5'end of the NTins2 cDNA (113-124 bp). The deduced amino acid sequence of the main reading frame encoded for a protein of 111 aa with MW of 12.6 kDa. Analysis of the primary protein organization revealed that precursor molecule consists of the four typical insulin regions, including a signal peptide (23 aa), B chain (32 aa), C chain (31 aa), and A chain (21 aa). Signal peptide cleavage site was predicted using SignalP software (http://www.cbs.dtu.dk/services/SignalP/). The prohormone convertase processing sites

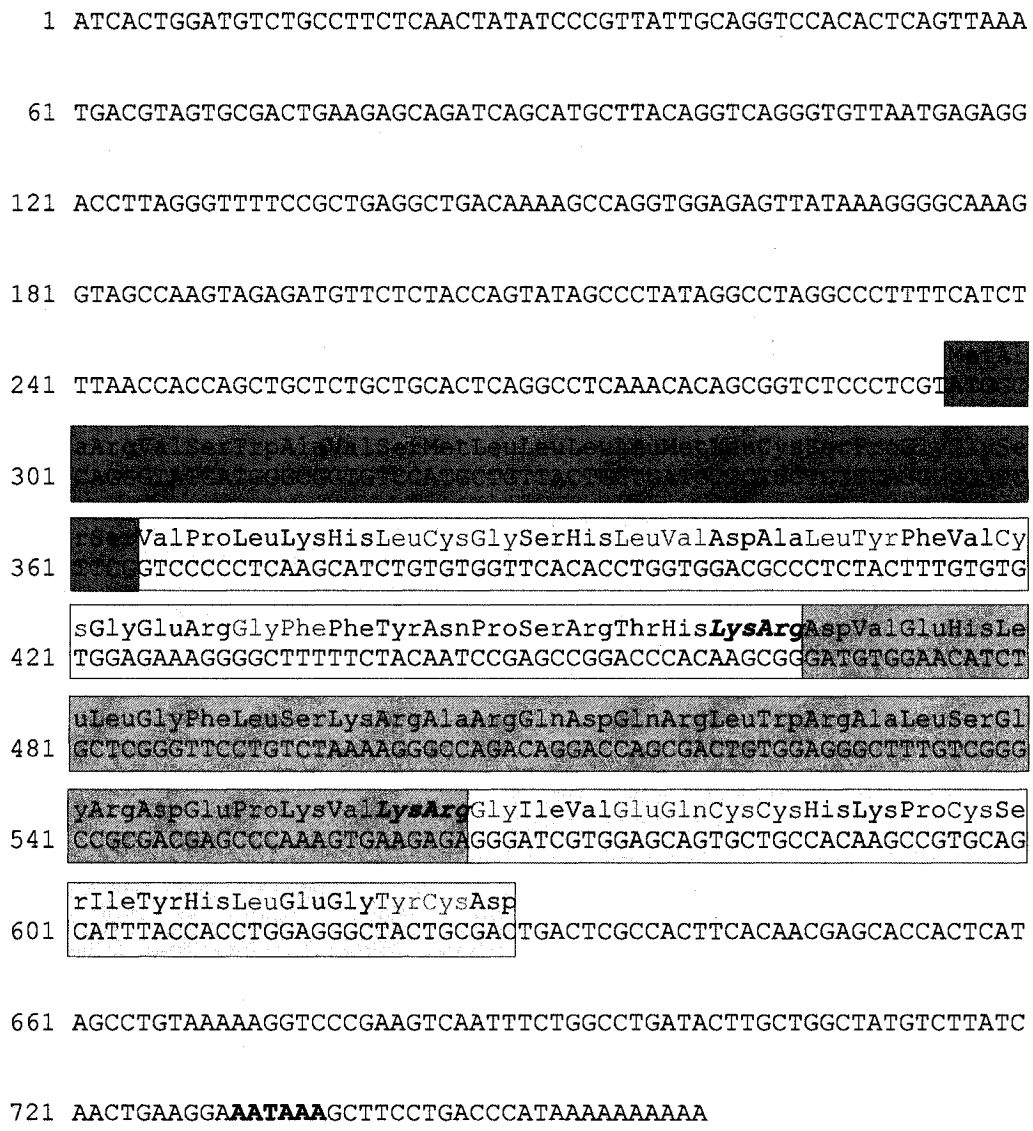

Figure 6.2. Nucleotide and deduced amino acid sequence of the pancreatic islet NTins2 cDNA

Start codon and polyA signal are in bold. Predicted structural organization of the NTins2 precursor is shown in boxes: *(blue)*-signal peptide, *(yellow)*-B chain, *(pink)*-C peptide, and *(grey)*-A chain. Dibasic residues *(Lys Arg)* potentially recognized by prohormone convertases and carboxypeptidase H are in bold and italic. Conserved residues important for the peptide folding are in red and conserved residues essential for the receptor binding are in blue.

```
NTins1          MAALWLQAFSLLVLMMVSWPGSQAVGGPQHLCGSHLVDALYLVCGDRGFFYNPR--- -R D 56
FRins1          MAALWLQSVSLLLLMVVSSPGSQAMAPPQHLCGSHLVDALYLVCGDRGFFYNPK--- -R D 56
ZFins1          -MAVWLQAGALLVLLVVSS-VSTNPGTPQHLCGSHLVDALYLVCGPTGFFYNPK--- -R D 54
NTins2          -MARVSWAVSMLLLLMLCSPGGSSV-PLKHLCGSHLVDALYFVCGERGFFYNPSRTH KR D 58
FRins2          -MARL-WEVSALLLLVLSSPGVSPF-PAQHLCGSHLVDALYIVCGERGFFADPDRRH KR D 57
ZFins2          -MVLL-LQASVLILLLASLPGSQSS-PSQHLCGSSLVDALYLVCGPRGFFYTNRG-- RR D 55
                  .      : *:*:: .          :***** ******:***  ***        :* *

                                                      A1
NTins1          VDPLLGFLPPKAGGAVVQGGENEVTFKDQMEMMV KR GIVEECCHKPCTIFDLQNYCN 113
FRins1          VDSMMG------GAAGVDNEVAEYAFKDQMEMMV KR GIVEQCCLRPCNLLDLQNYCN 107
ZFins1          VEPLLGFLP---PKSAQETEVADFAFKDHAELIR KR GIVEQCCHKPCSIFELQNYCN 108
NTins2          VEHLLGFLS----KRARQDQRLWRALSGRDEPKV KR GIVEQCCHKPCSIYHLEGYCD 111
FRins2          VEDLLGFLS----NRARRQQRLWKVLSGHNEPKV KR GIVEQCCHKPCSIHHLQRYCD 110
ZFins2          LETLLALLS----NLAGYEAADADPLK-EKVMKM KR GIVEQCCHRPCTIYHLEDYCS 107
                :: ::.::                 :. .      ** ****:** :**.: .*: **.
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Figure 6.3. Alignments of the tilapia, zebrafish and fugu fish insulin precursor sequences

Insulin A and B chains are in bold. Residues important for the peptide folding are in red and residues essential for the receptor binding are in blue. Prohormone convertases and carboxypeptidase H processing sites are space separated. The second potential prohormone processing site of the fugu fish insulin 2 precursor is shown in italic.

were determined by identification of the consensus sequences recognized by these enzymes (RR or KR) [79, 235] and based on the sequence similarity with insulin precursors from other species (Fig 6.3). The location of the prohormone processing sites and the size of signal peptide, B-, C-, A-chains of the TNins2 were similar to those found in the other known insulin precursors.

The MW of the mature NTins2 is 6044 Da, and is different from the MW of the second insulin-like peptide (5744 Da) identified by Dr. Dooley and Dr. Conlon. The MW of the mature NTins2 could be 5748 Da, which is within the limits of error of mass spectrometer used (personal communications), if its B chain sequence contains an additional serine at the N terminus, but excludes arginine-threonine-histidine from the C terminus (*e.g.* it is NH_2-SVPLKHLCGSHLVDALYFVCGERGFFYNPS-C). However, in this case NTins2 prohormone processing sites would be different from the predicted consensus sequences, which seems unlikely. Therefore, the second insulin-like molecule found in the tilapia Brockmann body extracts is likely a polymorphic variant of the NTins1 and is not the second insulin.

As shown in Figures 6.2 and 6.3, the NTins2 sequence possesses the conserved amino acid residues important for the correct folding, formation of disulfide bonds and

receptor binding [24], suggesting that similarly to the zebrafish and fugu fish insulin 2 precursors [85], NTins2 precursor could be correctly processed to the bioreactive mature insulin.

According to the alignment of the tilapia, zebrafish, and fugu fish preproinsulin sequences (Fig.6.3), the NTins2 precursor shares 52% identity with the NTins1 precursor, 54% and 75% identity with zebrafish and fugu fish preproinsulin 2, respectively. The higher sequence similarity of the NTins2 precursor to the insulin 2 precursors from other fish species supports the hypothesis that the two non-allelic fish insulin genes aroused before radiation of the teleost fish [85].

The greater divergence amongst NTins2 precursor and other known insulin precursors was found within the signal and connective peptides, whereas mature NT ins2 (A- and B- chains) showed 72% identity with the NTins1 and zebrafish insulin 2, and 80% identity with the fugu fish insulin 2.

6.3.3. Tissue-Distribution Analysis of the NTins2 Transcripts

In adult mammals the expression of the insulin gene is almost exclusively restricted to the pancreatic islet β-cells. In this study we examined the tissue-specific expression of the tilapia NTins2 gene and compared it with the expression pattern of the NTins1 gene. Total RNA was isolated from different tilapia tissues including gall bladder, kidney, pancreatic islets, liver, brain, adipose tissue, muscles, small intestine, and heart and used in RT-PCR with the NTins2-specific (Ins2-S4/Ins2-AS3), NTins1-specific (In49/In16), and β-actin-specific (actFOR1/actREV1) (positive control) primers. As presented in Figure 6.4 A, NTins2 transcript was detected in all tissues except for the adipose tissue. An almost uniform distribution pattern of the NTins2 mRNA was in the contrast to the recently reported restriction of insulin 2 transcription to the head and pancreatic islets in zebrafish at 1 and 2 days post fertilization (dpf) [178]. In comparison, using the same cDNA samples, NTins1 mRNA was detected in kidney, liver, pancreatic islets, small intestine, adipose tissues, and brain, but not in gall bladder, heart and muscles (Fig. 6.4 A). The finding of the NTins1 transcript in kidney, liver, small intestine, and adipose tissues is surprising since previously NTins1 expression was detected only in the pancreatic islets and brain tissues [75]. The discrepancy in the RT-

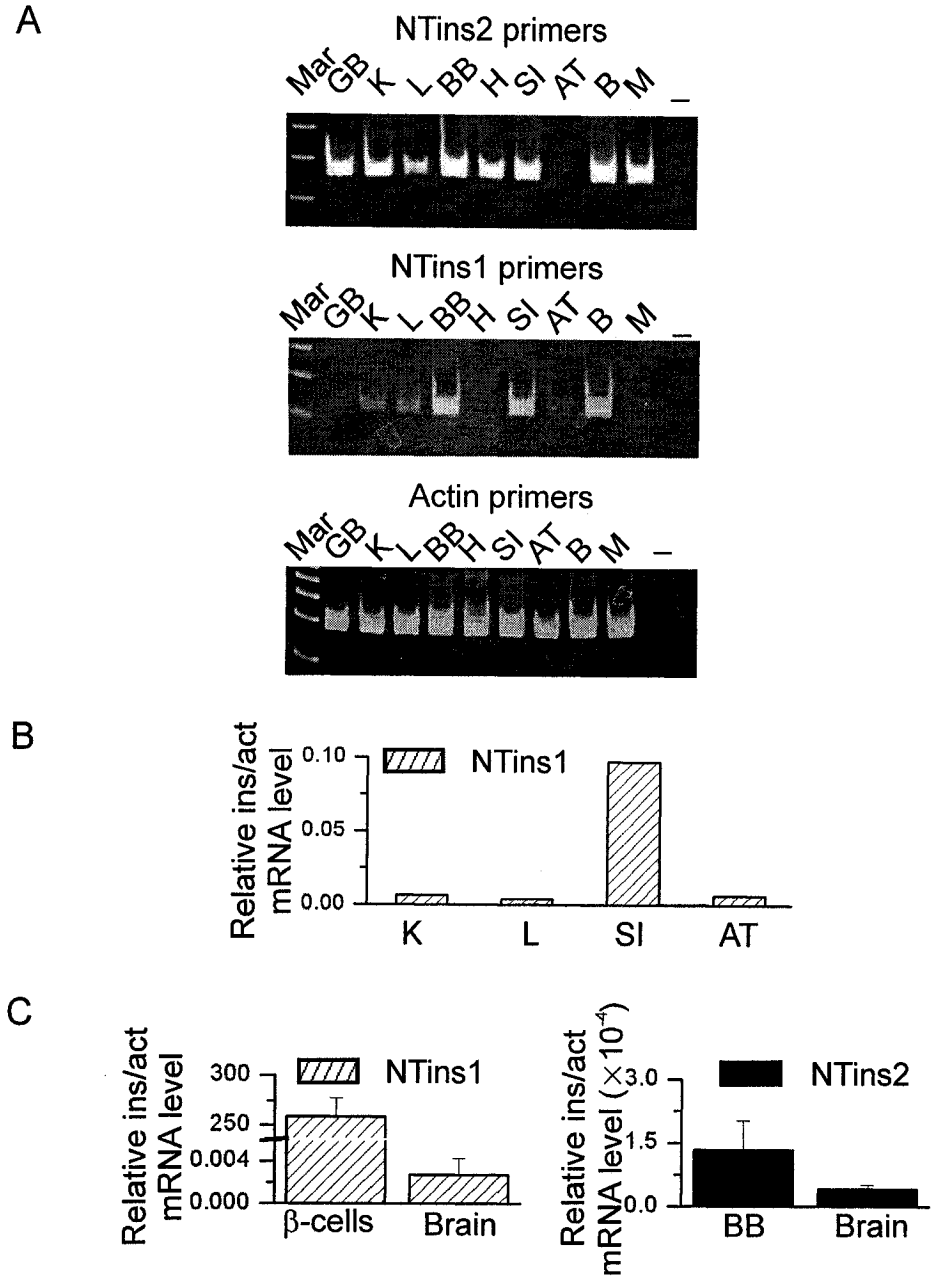

Figure 6.4. Tissue-specific expression of the NTins1 and NTins2 genes
Total RNA was isolated from several tilapia tissues: gall bladder *(GB)*, kidney *(K)*, liver *(L)*, Brockmann body *(BB)*, heart *(H)*, small intestine *(SI)*, adipose tissue *(AT)*, brain *(B)*, and muscles *(M)* and was used *(A)* in RT-PCR with indicated primers and *(B* and *C)* in qRT-PCR. In *(A) (-)* negative control, *(Mar)* molecular weight marker. In *(B* and *C) (stroked bars)* represent levels of NTins1 mRNA and *(black bars)* represent levels of NTins2 mRNA. Results of the qRT-PCR are presented as the insulin/actin mRNA ratios and in *(B)* are expressed relatively to the insulin/actin mRNA ratio of the brain sample (not shown), which is arbitrary set as 1. Results of *(C)* are expressed as means ±SE of at least three independent experiments.

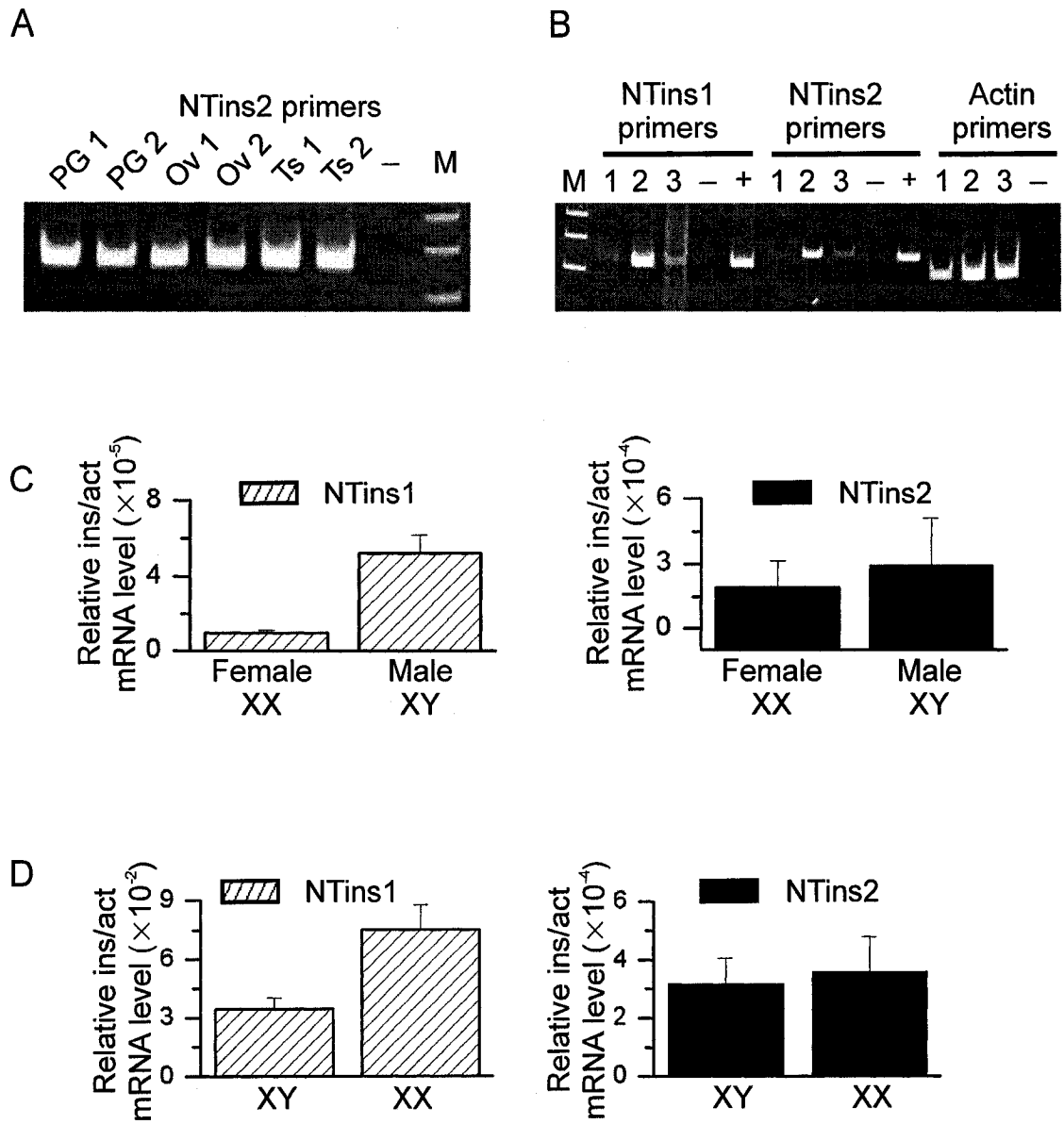

Figure 6.5. NTins1 and NTins2 expression in the tilapia gonads, pituitary gland and in whole 10 dpf tilapia fry

(A and B) total RNA was isolated from two pituitary gland *(PG1* and *PG2)*, two ovaries *(Ov1* and *Ov2)*, and two testis *(Ts1* and *Ts2)*; and three *(1,2,3)* 10 dpf tilapia fry and used in RT-PCR with the indicated primers. *(-)* negative control, *(+)* positive control (cloned NTins1 or NTins2 cDNA), *(M)* molecular weight marker. *(C* and *D)* the relative levels of NTins1 mRNA *(stroked bars)* and NTins2 mRNA *(black bars)* in tilapia gonads *(C)* and in the tilapia whole 10 dpf fry *(D)*, quantified by qRT-PCR and presented as insulin/actin mRNA ratios. Results of *C* and *D* are expressed as means ±SE of at least three independent experiments.

PCR results is most likely due to the very low level of NTins1 expression in kidney, liver, small intestine, and adipose tissues that were detected only with more sensitive SuperScript III reverse transcriptase enzymes used in this study, relatively to the older version of SuperScript II that was employed by us earlier. Indeed, preliminary results of qRT-PCR revealed that the levels of NTins1 mRNA in small intestine, kidney, adipose tissues, and liver were respectively 10-, 167-, 167- and 250-fold lower than that in brain (Fig. 6.4 B). The low observed NTins1/actin mRNA ratios indicate that insulin expression in these tissues is most likely restricted to a small number of cells. Similarly, occasional insulin-like immunoreactivity was detected in intestine cells of several teleost species including tilapia [4, 43, 156, 237] and insulin and insulin mRNA was found in adipose tissue [208]. Nevertheless, the negligible levels of the NT1ins mRNA in the extrapancreatic and extraneuronal tissues are questioning the physiological significance of such an expression.

Using qRT-PCR we preliminarily examined the levels of the NTins2 expression in gall bladder, kidney, pancreatic islets, liver, brain, muscles, small intestine, and heart. With the exception of brain and pancreatic islet tissues, all tissues contained the NTins2 mRNA at levels below required for reproducible quantification. As shown in Figure 6.4 C, similarly to the previously determined levels of NTins1 expression, the level of the NTins2 mRNA was higher in pancreatic islets as compared to that in the brain. However, in contrast to the almost 10^5-fold difference of the NTins1 mRNA levels between the brain and β-cells the difference in NTins2 mRNA levels in these tissues was only within 3-folds and not statistically significant. Moreover, the levels of NTins2 transcription in both brain and pancreatic islet tissues were substantially lower than the brain NTins1 mRNA level. Therefore, the preliminary results obtained with qRT-PCR suggest that although NTins2 mRNA species are present in multiple cell types, the level of NTins2 transcription is very low in all tissues tested including the main supplier of blood insulin, the pancreatic islets, which produce enormous amounts of NTins1. The extremely low level of NTins2 mRNA found in Brockmann bodies additionally indicates that the second insulin-like peptide (5744 Da) extracted by Dr. Dooley and Dr. Conlon is unlikely NTins2.

Recently, NTins1 expression was detected in tilapia ovaries, testis, and pituitary gland. Therefore, in this study we examined expression of the NTins2 gene in the same tissues using cDNA samples previously employed to analyze the NTins1 expression. As shown in Figure 6.5 A, insulin transcript was detected by RT-PCR in all samples tested. In the previous study we also determined that the level of NTins1 transcription (expressed as NTins1/actin mRNA ratio) is higher in testis than in ovaries, possibly due to the lower level of actin transcription there. By taking the last assumption to account for the difference, it is not surprising that we found a similar overexpression of the NTins2 gene in the testis as compared to that in ovaries.

Previously, the higher levels of NTins1 transcription were found in the tilapia 10 dpf fry with XX insulin allele genotypes, compared to those in the tilapia fry with XY insulin allele genotypes. In this study we preliminary examined expression of the NTins2 in the same fry cDNA samples, previously used to analyze NTins1 expression. As shown in Figure 6.5 C, similarly to the NTins1 transcript, NTins2 transcript was found in all samples tested. However, in contrast to the NTins1 mRNA expression, there were no significant differences in the NTins2 mRNA levels between fry with XX and XY insulin allele genotypes (Fig. 6.5 D).

In conclusion, our results revealed that in contrast to most mammalian genomes and similar to the genomes of other teleost fish, the tilapia genome contains two non-allelic insulin genes that both encode for a potentially bioactive proteins. In adult tilapia insulin 2 gene expression occurs in all tested tissues except for adipose tissue. This almost uniform pattern of NTins2 expression resembles the expression pattern for house keeping genes, and is in contrast to the tightly restricted β-cell-specific transcription of insulin gene in mammals. It is most likely that the NTins2 gene is transcribed at low levels or only in few cells in all extrapancreatic tissues tested and its transcription is only slightly up-regulated in the pancreatic islets. Future studies will focus on the characterization of the NTins2 promoter, nutrient-stimulated and developmental regulation of the insulin 2 biosynthesis and secretion, and should help to determine physiological role of insulin 2 in teleost species.

Chapter 7

Conclusions

This study focuses on characterization of the insulin genes in Nile tilapia *(Oreochromis niloticus)*. Using different molecular techniques, we cloned and sequenced the tilapia insulin 2 cDNA from pancreatic islets, examined tissue distribution of the insulin 1 and 2 mRNA in adult tilapia, analyzed the regulation of the insulin 1 biosynthesis and secretion, and investigated allelic variations and chromosomal localization of the insulin 1 gene in the tilapia genome.

The genomes of most mammalian species contain a single copy of the insulin gene that is located on one of the autosomal loci (11p15 in human). In contrast, the genomes of teleost fish, including the tilapia genome, contain two non-allelic insulin genes. The tilapia insulin 1 gene is located on the long arm of chromosome one (sex chromosome). The chromosomal location of the insulin 2 gene remains to be determined.

In this study, we have found that at least three different insulin 1 alleles exist in our tilapia population. Two of them are present in the genome of both male and female fish and the third one is mainly male-specific. The differences amongst insulin 1 alleles

comprise sequences located distally upstream of the insulin 1 gene (2.3 kb) and likely do not include the insulin gene itself and its proximal upstream and downstream regions. The allelic polymorphism of the tilapia insulin 2 gene remains to be elucidated.

It is generally accepted that in mammals insulin production is tightly restricted at the level of transcription initiation of the insulin gene to the β-cells of pancreatic islets. In this study, we have found that expression of both insulin genes in tilapia is not strictly β-cell-specific. Although in adult tilapia the insulin 1 gene is predominantly expressed in the β-cells of pancreatic islets, low levels of insulin 1 mRNA were also detected in the pituitary gland, brain and gonads as well as, in some cases, in small intestine, liver, kidney and adipose tissues. This indicates that the transcriptional control of the tilapia insulin 1 gene should be less tight than the control of insulin expression in mammals. The β-cell-specific transcriptional control of the insulin 2 gene is completely lost since the low levels of insulin 2 mRNA were detected in almost all tested tissues including gall bladder, kidney, pancreatic islets, liver, brain, muscles, small intestine, heart, pituitary gland and gonads.

The mammalian β-cells maintain a large insulin mRNA cytoplasmic pool that depends on highly activated transcription of the insulin gene. β-cell-specific insulin transcription is regulated via a complex and specific interaction of transcription factors with *cis*-elements located within a relatively short sequence upstream of the insulin gene (promoter and enhancer regions are approximately 400 bp). In this study, we have found that concentration of the insulin 1 mRNA in tilapia β-cells is several fold higher than that in mammalian β-cells. The enriched insulin 1 mRNA pool also relies on β-cell-specific activation of insulin 1 transcription. However, the tilapia insulin 1 promoter and enhancer regions are longer than those in mammals. The high level of the tilapia insulin 1 transcription in β-cells requires cooperative interactions between regulatory elements within the proximal (-1 to -396 bp) and the distal (-396 bp to -1575 bp) insulin 1 promoter regions. Moreover, it is likely that gender-related sequences located 2.3 kb upstream of the insulin 1 gene are also involved in the regulation of insulin 1 transcription and induce different levels of insulin expression in the gonads of adult fish and in tilapia fry prior to sex determination.

It is most likely that the β-cells of the islets of Langerhans produce all circulating insulin, emphasizing their critical role in the maintaining of glucose homeostasis. The dramatically higher level of insulin 1 expression compared to that of insulin 2 expression in tilapia pancreatic islets suggests that insulin 1 is a major component of the plasma insulin and therefore points toward its prevalent contribution to the regulation of energy homeostasis in tilapia. In mammals, β-cells of pancreatic islets "sense" elevation in the blood glucose concentrations and react immediately by secreting stored insulin. To compensate for the secreted insulin and to maintain a constant intracellular insulin level, β-cells also increase transcription and translation rates of insulin biosynthesis. Our results indicate that, similar to that in mammals, the tilapia β–cells secrete insulin in response to an elevated nutrient concentration and at the same time they manage to retain insulin stores at a constant level. Currently we do not know whether the measured insulin concentrations represent combined levels of both insulins (due to potential cross-reactivity of insulin 1 antibodies with the second insulin), or only the level of insulin 1. In our experiments, a constant level of the intracellular insulin was maintained without activation of tilapia insulin 1 gene transcription even over long-term stimulation. Only very high concentrations of nutrients can significantly potentiate transcription of the insulin 1 gene. Therefore, it is most likely that insulin biosynthesis in tilapia β–cells does not rely on transcriptional activation of the insulin 1 gene, but depends on a large insulin 1 mRNA storage pool. On the other hand, the effects of nutrients on insulin 2 biosynthesis and secretion are unknown. Therefore, the alternative explanation could be that intracellular insulin homeostasis in tilapia β-cells relies on the nutrient-stimulated induction of insulin 2 transcription and translation. However, the very low level of insulin 2 mRNA in tilapia pancreatic islets makes this mechanism unlikely.

In this study, we have also found that in addition to the regulation of energy homeostasis, tilapia insulin 1 could be involved in reproduction and/or sex differentiation. The insulin 1 gene is expressed at low levels in gonads and in the gonadotropin producing cells in the pituitary gland of adult fish. More importantly, it is located on the sex chromosome downstream from the gender-related sequences. These sequences likely induce different levels of insulin expression in tilapia gonads and in whole tilapia fry prior to sex differentiation.

In brain, insulin 1 gene expression was found in all regions with the highest level detected in the hypothalamus, suggesting that the neuron-associated insulin 1 production may also be important for the modulation of fish feeding behavior or some other brain functions.

The physiological role of insulin 2 remains mostly undetermined. Recently, insulin 2 gene expression was detected in the zebrafish embryo, suggesting a potential functional significance during early development [178].

Finally, considering applications of the tilapia pancreatic islets for the potential treatment of insulin-dependent diabetes, the results of this study suggest that "humanized" tilapia Brockmann bodies carrying human insulin under control of the tilapia insulin promoter/enhancer would secret human insulin in response to glucose stimulation in a dose-dependent manner. However, the production of the transgenic tilapia that synthesizes only "humanized" insulin could be more difficult than was initially expected since (1) there are at least two insulin genes actively transcribed in the β-cells of the tilapia Brockmann bodies and therefore both must be silenced; (2) the knockout of the insulin 1 gene may affect fish reproduction; and (3) the effect of the silencing of the insulin 2 gene is unknown. However, it potentially may affect fish development at early stages.

Future work will include studies of insulin 2 regulation and functions, insulin 1 involvement in reproduction and sex differentiation, and the effect of the nutrients (other than glucose and arginine) on insulin 1 and 2 biosynthesis and secretion in tilapia Brockmann bodies.

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Appendix 1.

PCR and qPCR Primers

Table 1. PCR and qPCR Primers

cDNA or DNA Product	Primer	Primer Sequence
Insulin 1 (3.5INS)	insFOR	5'ACAACCCCAGGAGAGATGTG3'
	insREV	5'AGGGTTTGTGACAGCATTCC3'
	In6	5'TGTAGAAGAAGCCTCTCTCCCC3'
	In10	5'AAACGGAGCAGATGGATCAG3'
	In15	5'GCCATTATTTGAATTGACAACATTGG3'
	In16	5'GGGACTCAGACCTGAATAGC3'
	In17	5'GCTCTTTTCCAGCATGGCAG3'
	In40	5'GCTTAATGGGTGTCAGAGGTGTG3'
	In41	5'GTTTGATTGTGGGGTGACAGCG3'
	In44	5'CGCTGTCACCCCACAATCAAAC3'
	In49	5'GAGATGTGGACCCTCTGCTTG3'
	In60	5'TCGTCTCGCCATCTCTTCA3'
X and Y insulin 1 alleles	X	5'AGCCTCACTCTTAGAAATGTTG3'

cDNA or DNA Product	Primer	Primer Sequence
	X2	5'CAACATTTCTAAGAGTGAGGCT3'
	X3	5'AGGTTTATTTTGTTGATAGTGAAAGG3'
	Y1	5'GCCTTTAGACAATAATTCTGATGG3'
	Y2	5'CCCTTTCTTTCCACCAGTCAAGTC3'
	Y3	5'TGGCGGGTTGCAGAGTATGG3'
	Y4	5'TGCTAGCCCTCAATGTCTACG3'
	XY	5'TTACAAATAACGCAGGTCTGCAAC3'
	XY1	5'TTCCCTTGGTAAACTGAAACCTGGT3'
Insulin 2	In2-S1	5'TCCMKCCCAGCAYCTGTG3'
	In2-AS1	5'YRCAGTATCYCTSCAGGTGGT3'
	In2-S2	5'GGYTCMMRCCTGGTGGA3'
	In2-AS2	5'GGCYTRTGGCAGCACTGCTCCAC3'
	In2-S3	5'GAGCCGGACCCACAAGCG3'
	In2-S4	5'CTGGATGTCTGCCTTCTCAACTATATCCC3'
	In2-AS4	5'AAGACATAGCCAGCAAGTATCAGGCC'3
	In2-AS5	5'GTCGCGGCCCGACAAAGCCCTCCA3'
	In2-AS6	5'TCCACAGTCGCTGGTCCTGTCTGGCCC'3
actin	actFOR	5'CTCTTCCAGCCTTCCTTCCT3'
	actREV	5'GTGTTGGCGTACAGGTCCTT3'
	actFOR1	5'GAGCGTGGCTACTCCTTCAC3'
	actREV1	5'AGGAAGGAAGGCTGGAAGAG3'
	act1	5'TGCCTGATGGACAGGTCATCAC3'
	act2	5'ACATGGTGGTACCTCCAGACAG3'
PC1	PC1FOR1	5'GYGGAGACTACACYGACCAGAG3'
	PC1REV1	5'KGCCAGGTCCACCAGGGC3'
	PC1FOR2	5'GCCCTGGTGGACCTGGC3'
	PC1REV2	5'GCTSGCCTCCACCTGCAC3'

cDNA or DNA Product	Primer	Primer Sequence
PC2	PC2FOR1	5'CGTCAGAAGCGAGGCTAC3'
	PC2REV1	5'RTCRATRCCATCATCCAT3'
	PC2FOR2	5'CATGACCTCDCCCATGGG3'
	PC2REV2	5'GGTCTATRTAAGGGGCACTTTGTGT3'
CPH	CPHFOR1	5'GAYGTGGTGGCMAACTACCC3'
	CPHREV1	5'TGCCVTCTTTGAAGCTGGAGTC3'
	CPHFOR2	5'CCAGCTTCAAAGABGGCATCAC3'
	CPHREV2	5'AGTTGCGGTTYTGSTCCCAGT3'

Appendix 2.

Alignment of the Insulin 1 Gene 5'Flanking Sequences from Xa and Y Alleles

Locations of the insulin 1 gene transcription start site *(insulinTSS)*, 5' end of the 3.5 kb insulin locus *(3.5INS)* and restriction endonuclease sites (***HindIII, PstI, SspI, EcoRI***) are shown.

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     PstI
Y    CTGCAGGAGGGGGAGATACAGGAGAGGTGGAGGAAGATCTCAGCCTGGGCGTCTATTGTC 60
Xa   ------------------------------------------------------------

Y    TTGTGTAGTCTGGAAGATGAGTGGATGATGGGGTGGGTGCAGTTTTCTCTGTGGTGGGGT 120
Xa   ------------------------------------------------------------

Y    CGGGTGGACTGTCCCGGGCTCTGTGGGGCTGGGCGGCGTTGCTGCACTGGGCCCCGGTCC 180
Xa   ------------------------------------------------------------

Y    GGATGGGCCTGGGCCCCCTTTCCCTGGCGGGTTGCAGAGTATGGGGGTGCCTACTGGGGT 240
Xa   ------------------------------------------------------------

Y    CAGCGGGGGAGCTGGCCCCAGGGAGGGGTCACTTGCCCCTCCCTTCCNNNCCCTCCCCAT 300
Xa   ------------------------------------------------------------

Y    CTCCAGCTGCCTCCCTCTTCCCGCTCCACCACAATCACCCACACATGCAGGGCCTTGGGG 360
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Xa  ------------------------------------------------------------

Y   TAGGGGTGTGTCACCAGGGTGCAGAGGAGACATCCCCCCCCCTCTGTCCCCTTCTGGCTGC 420
Xa  ------------------------------------------------------------

                                  EcoRI
Y   CTCTGCCTCAATTTTATCCCACAACTTAGACATTCACATTACTCACACTCTCATTACACA 480
Xa  ----------------------------GAATTCTCTTTAACAACACAT--GTTGCACA 29
                                  **** * ***   ****     ** ****

Y   TACATATAGGATCTTGGGGGTGGGCACGCTAC-ACGGATTCCAAATTACCATCAGGGTGT 539
Xa  TACATTCCAGATAATGTGCACTCACCGAACATGACTGATGCTCTGGAAACACTCAGACAA 89
    *****    ***  ** *      *     *  ** *** *      * **    *



Y   ACACCTCACCCCTGGCGTCGTTGCCCACCTCTCAATTTTAAATACACGTAGACATTGAGG 599
Xa  CTTCGGGATGCACAACAACGAGACTGTGCCACAAACACAGAAGACTGGCTTACGT----G 145
       *   *  *    *  **   *    *    **     ** **  *   ** *    *

Y   GCTAGCAGGAGGGGCTATACGCTTACCTGCTGCTCTGGCAGGTAGCTCCAT--GCCCTCC 657
Xa  GCTTTTGAGCGGCTCT-TGGAAGTCCCTGCTGATTAAAGGACTTGTTTTGTTGGTGTTAT 204
    ***     * **  ** *     * ******* *        * * *   *  *   *

Y   TGGGTTTTAAATGCACCTTAGAACACACATACATCAACACTACATAAGAGCGGGTGGAGG 717
Xa  AATACTGTTATTGTGTCTTTTCAC-TTCATGTGTCATACCTTGTTTGAAGAACATGGTAT 263
         * * * **   ***   **   ***   ***   **   *   **    ***

Y   GAGGTTTGGAGTCTTCCTACACCCCCGTTCTCTGCGGCCTGCTGGAGCGGGGGGGGCTAG 777
Xa  CAAAAATGGTAACTGCTTCAATTCATGCTTACATCACCCTATCACAG------------A 311
     *    ***   ** * *  *  *  * *  *  *  ***     **

Y   GAGGAGGAGTTGGCCGTCCGACTGGGGTCTGGAATGTGGGGCCTCCCTGCTGCTGCGGAG 837
Xa  ATGATGAAGATGATAATGAGACAGACACTTGGA-TATAA------CATACT--CACAGAA 362
      *  * ** **    *  *** *     **** * *        * * **    * **

Y   TCGGGGCAGTCTGCTTCTCCCCACCGCAGGAAAAAGGGTAACATCACCTGGGTCTGGGCG 897
Xa  CCCA------CTATTTTATGATGTCTTGGCTAAAGATGT--------CTGCAAGTGGCCA 408
     *        **  **        *   *  ***   **        ***    *** *

Y   CAGTTTCCCCCTCCAGGGGCAAGGGTACCTAGACCCGGGGGCTWAGAGTACGCTTGGGGA 957
Xa  TAGACTGATGCGCTAGTGGTTGCTATGTACATATATAGAAGGAAAGATCAAACAACAGGA 468
     **  *    * * ** **      *    * *    *  *   ***  *  *    ***

Y   GTGTGATTGTGTGTACACCGTCTCTTTATGTCCGTCTCCACGTTGGGTGAGTGTTGAGTA 1017
Xa  GGGAA------TGTTAAGGGATTTCTTA----------------GGAT-----TTTAGGA 501
    * *        ***  *  *  *  ***               ** *     ** ** *
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Y   ATTGTATATGAGAGCATGAKGGGTGGGAATAGATGTTTGTATCTGTGTGTGCCTGTTTGT 1077
Xa  TTTTTATT-----ATGTTATGCTTAAAAGTACATTTCATTATCTAAATGTGTTTGCTCTT 556
     ** ***         * * *  *   * ** ** *   *****   ****  ** *  *

Y   CTGTGTCTTTATGTCAGGTTGGGNATCAAGANGCCACCTCTT---TGGGGACNTCTCAAG 1134
Xa  TCTCAT--TCAGCTTAACCTCTGCAACTCTGTGCAGACTCCTAAATGGGGAAGTTACA-- 612
         *  * *  * *   *  * * *     **   *** *   ******  *  **

                PstI
Y   GCCCTCCAAGGTTTGGAGGCCCATCTCCCCCCACCACTTCCCCTGCCGGTGGCAAGACGC 1194
Xa  ----CATGAAGCCTGCAGTTCTATTTTCTCTCTTCCTGTATGTGCTCTCTGAATAAAGAC 668
           * *  ** **  * ** * * * *  *   *       *  **   * *  *

                PstI
Y   CCTCAGNCATCGGTGNGTTGGTGGTTTTTTGTGTCCGGGGGTGGGCGCCCAGGTACCCAC 1254
Xa  TCTTCCCTTTTACTGCAGCGGTGCGAGTCTCCCTC---GACCGTGTACACGTAAACCTGT 725
     **      *   **    ****    * *   **   *   * *  * *    ***

Y   CGGCTCACTCCTTGGCGGCTGCTTATCGGGGCNTGGAGCCTGGGGCTCGCTCGGGCCACT 1314
Xa  CTGAGCCTTTTTATTTTAGTGCCTGTAGG---TAGAAATAACGAGTTATCTTTATTGATG 782
    * *  *  *  *       *** * * **     * *     * * *  **      *

Y   TCGGGNATGGGGTGCCCTNGGCCTCTCAGCCCGGGGCTCGGTCACTCAGGCACAGCTGGC 1374
Xa  TCAGTCTCC---TGTTCTGGTTTTCCCGACACTTTACTTACTTCCTCCATTCTCTTCAGC 839
    ** *        **  ** *   ** *  * *    **   *  ***           **

Y   TGCCGGNGGAGCTCANGGNCANGTCTCTGCAACCCCCCCNGGCTTATGNACCGCNGGTNG 1434
Xa  TTCTA------CTTTCCAGTAGATTCACATTAGGCACTAAAATCTCTCTTTTGCCTCCAT 893
    * *        **       *  *       *  * *       * *     **

Y   CTGAGTGACCCCTCATTNGGGACTNTCNTCAGCTCTTTGTGGNAAAGTGGCACNAAAANC 1494
Xa  CTT-----TTTCCAGTTCACAGCCATATTCTGTACTT-ACAGTATGATGGGACAATGGCA 947
    **         *   **     *  *  ** *  ***    * *   *** ** *

Y   CCCTNTGTTGGGTCTTCCTTGGGTCTCTTGTGTTCTGGGGCCTCTGGGATGTCTGGAGTT 1554
Xa  CCATCATGTGGTCAGTTATTGAGCAAAAAGCAAGCAAG-----CAAGAAAGAATAAAAGA 1002
    ** *    ***    *  *** *      *    *  *     *  * * *  *  *

Y   TTGATCTCCTCCATACCTGCTTCATGCCTTGGAGGACGGG-GCAGTGGCCCCCCACACCC 1613
Xa  GCAAACCATCTGACTTCCACATCCTGTGCTCCAAAATAAGCATAGTG------------- 1049
       * *      *   *  * ** **   *  *  *   *   ****

Y   TNTAGCAGATCATTACATGAAGGAACCTTTTAAAAACAAGCGCGTTCATGCTCACAGGTG 1673
Xa  -ATAGTA-ATAGTGATGTTAAAAAAGTT----ACATTTATTTCTTGTATATTTCCACAAC 1103
      *** * **  * *  * **  **  *    * *   *   * *  **  *  **

Y   CACACACGGGTGATCACACACACAAACTACACCCTTTTTGGCTCCTACCTCAAAGCACAC 1733
Xa  CCTGTATCCATAATCACATACTGTGTTTGCTACCATTGTATGTAGTGTATTATGTTA--- 1160
    *    *    * ****** **      * *  ** ** *   *  *   * *    *
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                                                  SspI
Y   TGTGCGCTGTCGATCCTACGTGCTGCACA-ATAATGTTTAATATTTAGTATTTACTGTCA 1792
Xa  TTTATTTTGT-ATTTTTTTGTGTTTTATTTGTATTTTTTTGAATTTTCCTTTTGCTATC- 1218
    * *    ***   *  *  *** *  *    ** * ***   ****    *** ** **

Y   TATTCCCATATATCATTGTGATGTTGTTTATTACTCTCGTTTTCTTCTGCTTGCTTTCTT 1852
Xa  TGTACC--TGAGCTGAGGTGACACACACATTTCCCCACAGTGGGATCAATAAAGTCTTAT 1276
    * * **  *        ****         ** * * *  *    **       * *  *
                     SspI                        SspI
Y   TTTTTCTTTCTCAACAGGTGATCCAGGTGATCGATA-TATGTATTTTTTGTCTGCTTATT 1911
Xa  CTTATCACTTTAAATATAATATTAAAAAAAACTGTGCTGCATGCAATATTTTTGGTAAAT 1336
     ** **  * * ** *    **  *    * *  *  *   *    * * * ** * * *

Y   CTGTTGGTTTTTGGTTTTTGCCCTTCTCCCCCGTCCCTCTTCTCAGCTGTTTTTCTTTCT 1971
Xa  TATATGTTTTTTGG-----GGTGCTGAATCCAAAGAATGATCTCCATTTCCCTCCATTAC 1391
        ** *******     *    *    **      *  ****   *    * * **

Y   CCCCTTTCTTTCCACCAGTCAAGTCTGTCCCGTATTCAGCAAGTCAAAATAAAATAAACA 2031
Xa  GTACAGTTTTTTTGCAAAACATGAGAAGTTCACATTAAAAAAAGCACAACAG------TG 1445
       *  * ***   * *  ** *       *  *** *  **  ** ** *
                SspI
Y   ATAAAAGGTGAATCAAATGGACCATTACGGCAAGGCTGGGATGGTCCATTTGGTAAAGTA 2091
Xa  ATGAAAA--AAATCAA-TATTTTATTGCAATTAAGTATGCCAAATTTCTCTG-------A 1495
    ** ***    ****** *     *** *    * *   *     *   * **       *

Y   AATCCGTTGGGCATCTTTCTTCGCCTTTAGACAATAATTCTGATGGCAAAAGAACCAAAC 2151
Xa  GATTCCTT----ACCTTTCACTA--TGAACAAAATAAACCTCCTTGCATTT---TTGATT 1546
     ** * **    * *****      *  * * *****  **  * ***         *

Y   GGGACAGGTTTATTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAG 2211
Xa  TGTATTTCTCT-TGAGCCTCACTCTTAGAAATGTTGAATTAATGTCAGAAATTATTGTTG 1605
     * *    * * * *     *     * ***     **  **    * ***  *     *

Y   GATATGTTTTTTGACTCAAGTATAGGATACCAGGTTTCAGTTTACCAAGGGAAATTGTAC 2271
Xa  GATATGTTTTTTGACTCAAGTATAGGATACCAGGTTTCAGTTTACCAAGGGAAATTGTAC 1665
    ************************************************************

Y   GTTTAATTAAACTAAAAACATGAGTTTTGCAAGAAAACCTGATAAGATGGAATTTTTAAA 2331
Xa  GTTTAATTAAACTAAAAACATGAGTTTTGCAAGAAAACCTGATAAGATGGAATTTTTAAA 1725
    ************************************************************

Y   CATTTTTCCTGGTTTCAGTGATAAAAATCTATAAGAAACAGTCAAAAAATCTCATGGAGT 2391
Xa  CATTTTTCCTGGTTTCAGTGATAAAAATCTATAAGAAACAGTCAAAAAATCTCATGGAGT 1785
    ************************************************************

Y   TTTTTGGTTGCAGACCTGCGTTATTTGTAAATGTCTGTATTCTTATCTGATTTACATTTT 2451
Xa  TTTTTGGTTGCAGACCTGCGTTATTTGTAAATGTCTGTATTCTTATCTGATTTACATTTT 1845
    ************************************************************
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Y   ACACATGACTCAATTGTTATTTTTGTTTAATCTATCACAATCTACTACAGAAAACATTAC 2511
Xa  ACACATGACTCAATTGTTATTTTTGTTTAATCTATCACAATCTACTACAGAGAACATTAC 1905
    ************************************************** ********

Y   AACAGAAAATAATGAAACAGCTCTACAATAATCATCCGACATCTACCAGTTAAAACTACA 2571
Xa  AACAGAAAATAATGAAACAGCTCTACAATAATCATCCGACATCTACCAGTTAAAACTACA 1965
    ************************************************************

Y   TTGGTAAATGTATCTGCACACTCATCTGTCAGTAACTGGAATACATGTAAATGATACTGT 2631
Xa  TTGGTAAATGTATCTGCACACTCATCTGTCAGTAACTGGAATACATGTAAATGATACTGT 2025
    ************************************************************

Y   TGTCCATAGTAGACCTGCCTCATTTGTTAAATATAGACATTGATTTTTTTATTGCTTTGT 2691
Xa  TGTCCATAGTAGAC-TGCCTCATTTGTTAAATATAGACATTGATTTTTTTATTGCTTTGT 2084
    ************** ********************************************

Y   TAGAAAATGTAAAACTGTGAGACAGAGAAACAGAGAGTGGGGAGTTTGTTCTCATTTCCA 2751
Xa  TAGAAAATGTAAAACTGTGAGACAGAGAAACAGAGAGTGGGGAGTTTGTTCTCATTTCCA 2144
    ************************************************************

Y   GAATATATTCAGTACTTTATCATAAATATAATGCATCTTCTTCACACCTATTTCTAGTAT 2811
Xa  GAATATATTCAGTACTTTATCATAAATATAATGCACCTTCTTCACACCTATTTCTAGTAT 2204
    ********************************** ************************

Y   CTCTAATGAATGCAACATATGAATTAAAACAAAATTCGAAAAGAAAATCAAGAGCAGAAA 2871
Xa  CTCTAATGAATGCAACATATGAATTAAAACAAAATTCGAAAAGAAAATCAAGAGCAGAAA 2264
    ************************************************************
                                                  5'end of 3.5INS
Y   CGATAAAAGTTGAGCTGTAGTATTCAGCATTTAATGGAGTGAATTTACATCGATTGTGCA 2931
Xa  CGATAAAAGTTGAGCTGTAGTATTCAGCATTTAATGGAGTGAATTTACATCGATTGTGCA 2324
    ************************************************************

Y   GCTTTGACAGAAAATGTTTTTATAGTTTGGTTGTGGGGTGACAGCGGCTCTGGTTTCTGG 2991
Xa  GTTTTGACAGAAAATGTTTTTATAGTTTGATTGTGGGGTGACAGCGGCTCTGGTTTCTGG 2384
    * ************************** ******************************

Y   TTCATTGGTGGAGCAGATGCAGATGAGAAAACACAAAGTTGTCTGAAACACGCGTCCTCC 3051
Xa  TTCATTGGTGGAGCAGATGCAGATGAGAAAACACAAAGTTGTCTGAAACACGCGTCCTCC 2444
    ************************************************************

Y   TTCGTCATGGACAGCTTGTCATGGTAACGCTCTTTCTCGGTCAGTTGAGCAGCTTCTTTA 3111
Xa  TTCGTCATGGACAGCTTGTCATGGTAACGCTCTTTCTCGGTCAGTTGAGCAGCTTCTTTA 2504
    ************************************************************

Y   AGAGACTAACAAGCTGGAACAAGAGCTCTGTCAGCACACCTCTGACACCCATTAAGCACT 3171
Xa  AGAGACTAACAAGCTGGAACAAGAGCTCTGTCAGCACACCTCTGACACCCATTAAGCACT 2564
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Y   CTTTGGATGGCAGATGGTTTGATGAGGCTCTGGGTTTTTGTGCAGTCGGGCTCTTTCTAC 3231
Xa  CTTTGGATGGCAGATGGTTTGATGAGGCTCTGGGTTTTTGTGCAGTCGGGCTCTTTCTAC 2624
    ************************************************************

Y   ACTGCGTGACTGAAAAATACAAACTTGAACTAGAGCTGAAGTCATCTTAGACCCATAACT 3291
Xa  ACTGCGTGACTGAAAAATACAAACTTGAACTAGAGCTGAAGTCATCTTAGACCCATAACT 2684
    ************************************************************
                                    HindIII
Y   ACAATAAACTATTTGTAACATCAGGACAGTCAAGCTTTTGTCTTTGTGTTTCATGCTGTC 3351
Xa  ACAATAAACTATTTGTAACATCAGGACAGTCAAGCTTTTGTCTTTGTGTTTCATGCTGTC 2744
    ************************************************************

Y   TGCGTAGTTCAGGGTTGTCAAACATCAGGCCCGGGGGCCGAGAAATTGGCCACCAAAGAC 3411
Xa  TGCGTAGTTCAGGGTTGTCAAACATCAGGCCCGGGGGCCGAGAAATTGGCCACCAAAGAC 2804
    ************************************************************


Y   TCTAAGCCACCTCATTGAATGACTTTGAACAATGTGATGAAGGGCATGAGTTTTAAACTT 3471
Xa  TCTAAGCCACCTCATTGAATGACTTTGAACAATGTGATGAAGGGCATGAGTTTTAAACTT 2864
    ************************************************************

Y   CATATTCATGAGTTTTACAGTTTTTCCAGCTGATAAAGAACTCGCCTGTAAGTAAGTAAT 3531
Xa  CATATTCATGAGTTTTACAGTTTTTCCAGCTGATAAAGAACTCGCCTGTAAGTAAGTAAT 2924
    ************************************************************

Y   AAAAAAAAAACCAATGTGCATATGAAATAGATCTTTTATACAATCTGTCCACATTAAAAA 3591
Xa  AAAAAAAAAACCAATGTGCATATGAAATAGATCTTTTATACAATCTGTCCACATTAAAAA 2984
    ************************************************************

Y   AAATAAATAAATAAATAAATCTGAAATTTTCTTTTTATTAACAAAAATTTCAGTTTTATA 3651
Xa  AAATAAATAAATAAATAAATCTGAAATTTTCTTTTTATTAACAAAAATTTCAGTTTTATA 3044
    ************************************************************

Y   ACTACAGGACATTTTAGCAGTTTTTTTTCTACTGAAATTGTGCTTTTTTCAGATCTTCTT 3711
Xa  ACTACAGGACATTTTAGCAGTTTTTTTTCTACTGAAATTGTGCTTTTTTCAGATCTTCTT 3104
    ************************************************************

Y   TTTCCTTTTTTCTGATCTTCTGAGCTCTGTCAGGAATTACATGTGTAATTAAACTTCTTT 3771
Xa  TTTCCTTTTTTCTGATCTTCTGAGCTCTGTCAGGAATTACATGTGTAATTAAACTTCTTT 3164
    ************************************************************

Y   ACAGTTACACGACTGAGTTTGAAATACTTTGAAATACTTTGAAATCTTCTGACATGTTTC 3831
Xa  ACAGTTACACGACTGAGTTTGAAATACTTTGAAATACTTTGAAATCTTCTGACATGTTTC 3224
    ************************************************************

Y   GCTTCACTCTGAGCTCTGCTGCACATCCTGATTTCTTTTTACAAACGTTCAGTCACACAT 3891
Xa  GCTTCACTCTGAGCTCTGCTGCACATCCTGATTTCTTTTTACAAACGTTCAGTCACACAT 3284
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Y   TTCATCACAAATATCAGCTCTTTGACGAAAAGACAGCTTGGACTTATTTTCATGTCTGTT 3951
Xa  TTCATCACAAATATCAGCTCTTTGACGAAAAGACAGCTTGGACTTATTTTCATGTCTGTT 3344
    ************************************************************

Y   AACGTCAGGCGTGATGGAGGATAGGAGATGCTGCATTATGTGAACACATCTTGTAAAAAA 4011
Xa  AACGTCAGGCGTGATGGAGGATAGGAGATGCTGCATTATGTGAACACATCTTGTAAAAAA 3404
    ************************************************************

Y   GCTGAATAAAAATGATTTCTACGACTGTTATCTGCTTTAAACTAATGAGCTGAGCAGATG 4071
Xa  GCTGAATAAAAATGATTTCTACGACTGTTATCTGCTTTAAACTAATGAGCTGAGCAGATG 3464
    ************************************************************

Y   GAGCAGAAGGTTAATAGCTGATCAGATCATGTCGGCTCATTAGCTTCAGTTTGTTTTACT 4131
Xa  GAGCAGAAGGTTAATAGCTGATCAGATCATGTCGGCTCATTAGCTTCAGTTTGTTTTACT 3524
    ************************************************************

Y   AAGTGCTGTAACCAGTCAATCAGAAACACACTGGCACTTAATATGTGCTGATGGCAGCGC 4191
Xa  AAGTGCTGTAACCAGTCAATCAGAAACACACTGGCACTTAATATGTGCTGATGGCAGCGC 3584
    ************************************************************

Y   ATCTGTTTGTCCACACACACACACACACACACACACACACACACAGATTCGTCTCGCCAT 4251
Xa  ATCTGTTTGTCCACACACACACACACACACACACACACACACAGATTCGTCTCGCCAT 3644
    ************************************************************

Y   CTCTTCACAGGGCTGTTCATTGACTAACGTTCAATTTCTGAAAGTTAAACCAAATCTTTC 4311
Xa  CTCTTCACAGGGCTGTTCATTGACTAACGTTCAATTTCTGAAAGTTAAACCAAATCTTTC 3704
    ************************************************************

Y   ACCTCAGGTTTAATAAATCATATTAAGGGTATTTTTGCAGAGTCCCCATAATCCGTAATC 4371
Xa  ACCTCAGGTTTAATAAATCATATTAAGGGTATTTTTGCAGAGTCCCCATAATCCGTAATC 3764
    ************************************************************

Y   GCACACAAGTCCCCACAATGTAGGTGAAATAGGTTCCACGGAAACACGTGGAACAGGGGG 4431
Xa  GCACACAAGTCCCCACAATGTAGGTGAAATAGGTTCCACGGAAACACGTGGAACAGGGGG 3824
    ************************************************************

                                                  insulinTSS
Y   TGTGTCAGGTGGTGCTGGTGGAGTATAAATGGAGAGAAGGCTCTTGGTTCTGC 4484
Xa  TGTGTCAGGTGGTGCTGGTGGAGTATAAATGGAGAGAAGGCTCTTGGTTCTGC 3877
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