Université du Québec
INRS-Institut Armand-Frappier

Identification of Growth-Dependent Genes in Brook Charr *(Salvelinus fontinalis)*

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Thèse présentée
pour l'obtention
du grade de Philosophiae doctor (Ph.D)
en Biologie

Jury d'évaluation

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ABSTRACT

Fish growth is important for fisheries management and aquaculture. The growth pattern of fish is a complex process which is influenced by environmental factors, hormones and genetic composition. Thyroid hormones (THs) play an important role in regulating fish growth. The identification of TH-dependant genes in fish could provide valuable information for predicting growth. Brook charr is an important species in aquaculture and therefore represents a good model to study fish growth. The objectives of this study were: (1) to identify TH-regulated genes in brook charr, (2) to identify growth responsive genes in brook charr and assess whether or not the expression of TH-dependent genes are altered in fast and slow growing fish using a genomics approach.

To identify TH-dependant genes, three experimental groups of immature charr were used: a euthyroid control group (n=10), a hyperthyroid group (n=10) in which brook charr were injected (i.p.) with 3, 5, 3'-triiodo-L-thyronine (T_3; 10 µg/kg body weight), and a hypothyroid group (n=10) in which fish were injected with sodium ipodate (50 mg/kg body weight) a TH inhibitor. Fish were injected on days 1 and 4 of the experiment and sampled on day 7. Serum T_3 levels were measured by radioimmunoassay. Results indicate that serum T_3 concentrations decreased from 2.20 ng/ml in controls to 0.85 ng/ml in the ipodate treated group. T_3 levels in the hyperthyroid group were 263.4 ng/ml. THs induced hepatic gene expression as measured by differential display PCR. The results show that over 100 transcripts were differentially regulated by THs. The expression of 18 of these genes was further confirmed by reverse dot blot. These were then cloned and

sequenced. Sequence homology comparisons revealed seven known genes: trypomyosin (3 isoforms), leukocyte-derived chemotaxin, mitochondrial NADH dehydrogenase subunit 5, basic transcription factor 3 and crystalline. Eleven genes had no significant homology to known gene and were therefore considered novel. Tissue distribution of the transcripts, as determined by Northern blot analysis, varied for each gene. While all genes were expressed in liver, clearly the functions of these genes were not restricted to hepatic function as most of these were expressed in multiple tissues.

Since THs are known to be important regulators of growth, we wanted to determine whether or not the expression of TH-dependent genes differed between animals exhibiting different growth rate. To characterize growth-dependent gene expression profiles, cDNA arrays (GRASP project, University of Victoria) containing 16,000 genes were used. Two families of immature brook charr that were known to exhibit different growth characteristics were compared. These fish had been raised under similar rearing conditions from egg incubation to the time of sampling (April, 2003). One family had fast growing fish (n=8; average weight 54.73 ± 12.14 g) while the second had slow growing fish (n=8; average weight 8.43 ± 1.59 g). Total RNA isolated from livers of fast and slow growing fish were used to prepare cDNA probes. The cDNA probes were labeled and hybridized to the cDNA microarrays. Microarrays were scanned and analyzed using GeneSpring software according to MIAME standards. A total of 94 genes were found to be growth-dependent exhibiting at least a 2-fold change in expression. There were 69 genes that were up-regulated and 25 genes were down-regulated in fast growing fish. Out of the 94 differentially expressed genes, 39 of these are known genes that have been implicated in regulating carbohydrate, fatty acid and protein metabolism,

as well as mitochondrial function and signaling pathways. Twenty of these have been shown in the literature to be TH-dependent. The remaining 55 genes have unknown functions. Only two of the genes (basic transcription factors and leukocyte-derived chemotaxin) identified in the first experiment were present on the microarrays. However, the microarray results did not indicate different expression for these two between our fast and slow growing fish. These results indicate that TH-dependent genes may be associated with growth.

In conclusion, we have identified 18 TH-dependent genes in brook charr. This has allowed us to show that THs are implicated in a wide variety of physiological processes as indicated by their function and tissue expression pattern. The genomic analysis of hepatic gene expression in fast and slow growing fish revealed that 20 genes that are known to be TH-dependent and regulated by growth. This supports previous reports that THs may be involved in the regulation of growth.

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ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Daniel Cyr, who guided me through my Ph.D studies with enthusiasm, dedication, insight and systematic training. I will always be indebted to him for introducing me to molecular biology research.

I would like to thank my co-supervisor Dr. Céline Audet for her encouragements, insight, suggestions and her guiding during my studied.

I would like to thank the committee members: Dr. Sandi McGeachy, Dr. Yvan Lambert and Dr. Michel Charbonneau for their valuable comments and advice.

I thank all of my colleagues: Jayaprakash, A., Michelle, P., Stephane, P., Isabelle, P., Noël, R., Benjamin, De M., Johanna, B., Jean-Marc, N., Valérie, P., Benjamin, A., Patrick, G., and my other friends who have given me help and support during my research period.

I am appreciative of Julie D. and Mary G., for their technical assistance and help in the laboratory.

I am grateful to the financial support from High Education Ministry of China and INRS during my PhD studies.

Finally, I would like to thank my family, for their understanding, perseverance and their continued support in the pursuit of my academic goals.

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LIST OF ABBREVIATIONS

Ah-R	Aryl hydrocarbon receptor
ApoA-I	Apolipoprotein A-I
11-KT	11-ketotestosterone
bp	Base pairs
BTF3	Basic transcription factor 3
BW	Body weight
ChM1	Chondromodulin 1
cDNA	Complementary deoxyribonucleic acid
CF	Condition factor
Ci	Curie
cpm	Counts per minute
DD-PCR	Differential Display Polymerase Chain Reaction
DI	Type I deiodinase
DII	Type II deiodinase
DIII	Type III deiodinase
DEPC	Diethyl-pyrocarbonate
DNA	Deoxyribonucleic acid
DHP	Dihydroprogesterone
E-cadherin	Epithelial cadherin
EDTA	Ethylenediaminetetraacetic acid, disodium salt
EtBr	Ethidium bromide

E_2	17ß-estradiol
EST	Expressed Sequence Tags
FSH	Follicle-stimulating hormone
FATP	Fatty acid transport protein
GH	Growth hormone
GnRHs	Gonadotropin-releasing hormone
GTHI	Gonadotropin I
GTH II	Gonadotropin II
GHRH	Growth hormone releasing hormone
GAPDH	Glyceraldehyde-3-phosphate-dehydrogenase
HDL	High-density lipoprotein
H-FABP	H-Fatty acid-binding protein
hr	Hour
Hox gene	Homeobox gene
IGF-I	Insulin-like factor-I
kb	Kilobase
µg	Microgram
µl	Microliter
LB medium	Luria-Bertani medium
LECT 1	Leukocyte-derived chemotaxin 1
LH	Luteinizing hormone
MT	Methyl-testosterone
mRNA	Messenger ribonucleic acid

msx genes	Muscle segment homeobox genes
mt-DNA	Mitochondrial DNA
mt ND 5	Mitochondrial NADH dehydrogenase subunit 5
MMLV	Moloney murine leukemia virus reverse transcriptase
ORF	Open reading frame finder
Otx gene	Orthodenticle-related homeobox gene
PCR	Polymerase chain reaction
PTU	Propythiouracil
PFK	Phosphofructokinase
PMT	Photomultiplier tube
PRL	Prolactin
RIA	Radioimmunoassay
RT	Reverse transcription
RT-PCR	Reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
rT_3	3, 3', 5'-Triiodothyronine
sec	Second
SCP2	Stercarol carrier protein 2
SRIF	Somatostatin
SGR	Specific growth rate
svp-40	Seven up -40
SPP2	Secreted phosphoprotein 2
T	Testosterone

T_3	3, 5, 3'-Triiodo-L-thyronine
T_2	3, 3'-Diiodothyronine
T_4	L-Thyroxine
Taq	DNA polymerase
TG	Thyroglobulin
TH	Thyroid hormone
THs	Thyroid hormones
TR	Thyroid hormone receptor
TRE	Thyroid hormone response element
TSC22	Transforming growth factor-β1-stimulated clone 22
TSH	Thyroid stimulating hormone
TM	Tropomyosin
X-gal	5-Bromo-4-chloro-3-indolyl-b-D-galactopyranoside

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INTRODUCTION

Fish Growth is an Important Element for Fisheries Management

Fisheries are an important resource for food, employment and revenue in many countries and communities. However, commercial catches have consistently declined from a maximum record in 1995 (FAO, 2004). Approximately 52% of major fish stocks or species have been fully exploited and production has reached or is close to reaching its maximum sustainable limits. Another 17% of stocks are overexploited and do not have any potential for future increase and with the likelihood that stocks will further decline. Among the 7% of stocks that have been depleted significantly, fish production has decreased and there is no potential for further increase (FAO, 2004). In order to protect the resource and to recover stocks from depletion and overexploitation, we must develop a controlled fisheries system response (FAO, 2002). Future development of fisheries will depend on improvements in proper and efficient management. The successful management of the fisheries resource needs to be based on the integration of biological characteristics, such as growth rate.

Importance of Fish Growth in Aquaculture

As the world's demand for fisheries products has been rapidly increasing, natural fish resources have been fully exploited while production has remained relatively stable (FAO, 2004). As a consequence, future demands for fish production must be met through the development of aquaculture. At present, both inland and marine aquaculture play an important role in meeting this increasing demand. The average annual growth rate in

aquaculture was 5.3% in the 1990's. It is believed that the potential for aquaculture exists in many areas and for many species (FAO, 2004). The application of genetics to improve fish traits including enhanced growth rate may also be an important element in the success of aquaculture. This will help aquaculture to maximise fish growth and minimise production costs.

Fish Growth as an Important Indicator of Physiological and Environmental Conditions

Fish growth can affect survival rates. Fast growing fish get priority access to food, territory (Riechert, 1998) and are less vulnerable to predation (Diana, 2004a; Werner et al., 1983). Growth also influences reproductive output (Roff, 1992), since larger females produce more eggs. Under different environmental conditions, differences in energy budget can result in dramatic growth variations (Diana, 2004a,b). Results of Feist et al. (2005) indicated that exposure to environmental contaminants may be affecting both growth and reproductive physiology of white sturgeon (*Acipenser transmontanus*) in the Columbia River. Such growth differences may be useful in determining adaptations of fish and provide interesting models for monitoring environmental conditions.

Fish Growth Regulation by Hormones

Fish growth is controlled by the endocrine system where hormone levels alter the expression of genes that regulate growth (Weatherley and Gill, 1987a; Yang et al., 2007). Since the 1970's, several hormones such as growth hormone (GH), thyroid hormones (THs) and anabolic steroids have been shown to influence fish growth and have been investigated as growth promoters, either for resource enhancement or fish

culture (Matty, 1985). It has been reported that many factors influence the efficacy of hormones on fish growth. Such factors include hormone dose, route of administration, water temperature, food quantity, feeding time, species and size of fish (Higgs et al., 1992; Higgs et al., 1982; Lam, 1982; Cyr et al., 1998; Tanangonan et al., 1989). However, inappropriate treatment can induce growth retardation, opercula abnormalities and lordosis (Higgs et al., 1992; Kang and Devlin, 2003). As a result, it is difficult to use hormones to increase fish growth for both aquaculture and stock enhancement. Furthermore, food safety concerns where hormones could be passed on to consumers represent an important consideration (Matty, 1985).

Enhance Fish Growth Using Traditional Genetic Methods

Genetic research has become increasingly important to ensure the long-term sustainable development of aquaculture. Growth rate is one of the major traits that requires improvement. Traditional methods have been achieved by domestication, selection, interspecific and interstrain crossbreeding, polyploidy and synthesis of monosex populations. However, while domestication of species without directed selection could improve the growth rate, this requires several generations and long-term investment (Dunham and Devlin, 1999). Selection is a powerful tool for improving fish growth. However, when the focus of selection is on increasing growth rate, other traits are often inadvertently selected against, which can affect selection responses, such as decreased ability to tolerate low concentrations of dissolved oxygen as reported for channel catfish (*Ictalurus punctatus*; Dunham and Devlin, 1999). Intraspecific crossbreeding and interspecific hybridization do not always result in improved growth

performance, such as in chum salmon (*Oncorhynchus keta*) crossbreeds (Dunham and Smitherman, 1983; Dunham and Devlin, 1999). Likewise polyploidy does not increase growth rate in some species when compared to diploids (Parsons et al., 1986). However, monosex, sex reversal and selected breeding can improve growth and may be induced by hormones (Donaldson and Hunter, 1982; Hunter et al, 1983).

Applied Modern Genetic Technology in Aquaculture

To complement traditional breeding programs, modern genetic technologies have been applied to aquaculture species (Houdebine and Chourrout, 1991). It has been reported that enhanced fish growth can be attained using GH genes. For several species, the insertion of the GH transgene can result in a dramatic increase in weight. For example, in the GH-transgenic coho salmon (*Oncorhynchus kisutch*) the growth rate was increased by 11-fold after one year (Devlin et al., 1994, Wu et al., 1994). However, GH-transgenesis in other species, such as rainbow trout (*Oncorhynchus mykiss*) did not improve growth rate (Penman et al., 1991). This may be due to the lack of available piscine gene sequences. Transgenic fish in the mid-1980's used existing mammalian GH gene sequences for transgenesis (Dunham and Devlin, 1999). The expression of gene constructs coding for mammalian hormones in fish may result in the expression of an inactive hormone for a given species (Bétancourt et al., 1993). This stresses the need to develop an 'all fish' promoter to target fish species. Furthermore, GH transgenesis can not only result in increased growth rate but also in morphological abnormalities and viability (Devlin et al., 2001). These indicate that the overexpression of the GH gene affects both growth pathways and normal morphology. Morphological abnormalities in

transgenic fish associated with increased growth suggest that physiological controls are not completely successful (Kang and Devlin, 2003) and that growth regulatory mechanisms need to be elucidated. Recently, molecular methods have provided the possibility of identifying genes that control economically important traits. This may offer new progress in the genetic improvement of fish growth.

Transcriptional Analysis

Biological function is determined by the level of expression of certain genes and therefore alterations of biological functions often result from changes in gene expression. In many cases, mRNA levels are the best surrogates available for protein abundance and determinants of function. Analysis of gene expression at the mRNA level could allow the identification of genes implicated in regulatory mechanisms. Differential display-polymerase chain reaction (DD-PCR) and genomics are both valuable approaches, which allow a much broader understanding of changes at the transcriptional level.

DD-PCR is used to determine expressed genes without previous genetic information (Liang and Pardee, 1992). It can be used to analyse the expression of a large number of mRNAs from a very small amount of sample. DNA microarray analyses is another powerful tool for studying the functional regulation of genes since it allows the simultaneous measurement and comparison of thousands of expressed genes (Meltzer, 2001). The 16K salmonid microarray (GRASP) contains cDNAs representing approximately 16,000 genes (http://web.uvic.ca/cbr/grasp/). This array is likely to become an important resource for genetic and physiological studies on salmonids. It offers the possibility of identifying large numbers of genes related to growth and

accelerates investment in genetic characters of fish in fisheries and aquaculture industries (Von Schalburg et al., 2005).

Brook Charr in Freshwater Aquaculture

Salmonids are an excellent animal model to study fundamental genetic mechanisms of growth (Thorgaard et al., 2002). Brook charr (*Salvelinus fontinalis*) is a member of the family Salmonidae, which has been selected for freshwater aquaculture in Quebec for both its biological characteristics and economical feasibility (http://www.aquaculture.ca). However, because of variable growth rate, the economic success of using brook charr in aquaculture has been limited (Boghen, 1995). This may depend upon the genetic composition of fish and environmental culture conditions. The limited information on the genetic background and physiological mechanisms of growth in farmed brook charr may seriously affect future studies on the improvement of economic traits for this species.

THs play an important role in regulating fish growth (Fagerlund et al., 1980; Higgs et al., 1992; Lam, 1982; Leatherland, 1994). In teleosts, T_3 regulates the expression of the GH gene (Moav and McKeown, 1992). The identification of TH-dependent genes expression pattern in fish could provide valuable information on the genes implicated in fish growth.

Objectives and Hypothesis of This Study

The objectives of this study were: (1) to identify expression pattern of TH-dependent gene in brook charr, (2) to identify growth responsive genes in brook charr and

detect the alteration of TH-dependent genes in fast and slow growing fish using a genomics approach. These results will provide a basic biological understanding of fish growth and will help to identify the mechanisms by which THs regulate fish growth. Finally, they will provide tools for the aquaculture industry to select fast growing fish using gene expression profiles.

My research hypothesis is that, THs regulate somatic growth by altering the pattern of gene expression and that these genes can be identified by altering the thyroid status of fish. Fast and slow growing fish will have different gene expression signatures, including changes in TH-dependent genes.

To test the first hypothesis, we first designed an experiment in which THs were manipulated in fish using intraperitoneal injections of T_3 and sodium ipodate. Fish were then sampled and hepatic genes that were either over-expressed in the T_3 gain and or down-regulated in the sodium ipodate group were identified as TH-dependent (Chapter 2). To address our second objective and to test if fish exhibiting different growth pattern also have different gene expression profiles, we compared the expression pattern of fish sampled from two families surveyed in a selection program that were characterized as fast and slow-growing. This allowed us to identify genes that were differentially expressed in the two families and, using data banks identify potential TH-dependent genes that could be altered by fish growth (Chapter 3).

CHAPTER 1. LITERATURE REVIEW

As indicated in the Introduction, there is international recognition that fisheries resources are being harvested to their limits and that their abundance may even decline in the future due to overfishing (FAO, 2004). Aquaculture is the major avenue for producing fish to meet the increasing worldwide demand. Genetic improvement of fish growth rate will allow fish to reched to the market size fast. It represents a profitable avenue for the aquaculture industry and enhancement of wild stocks.

Fish growth is a highly complex process that is determined by variable contributions from environmental (Diana, 2004a), nutritional (Sumpter, 1992), endocrine (Weatherley and Gill, 1987a,b), and genetic factors (Diana, 2004a). The study of genes involved in growth will help in selecting fast growing fish to meet the needs of the aquaculture industry and will be beneficial for managing wild fish stocks.

1.1 Fish Growth and Genetics

Studies on many fish species have clearly demonstrated that growth performance can vary between different populations (Sylven and Elvingson, 1992; Van der Meeren et al., 1994; Svåsand et al., 1996; Conover et al., 1997). In many fish species such as silversides, halibut, turbot, with a large north–south distribution, northern populations have been found to have higher growth potential as compared to southern populations (Conover, 1992; Imsland et al., 2000a,b; 2001a). Similar differences in growth performance between northern and southern regions have also been reported for salmon

(Torrisen, 1991; Torrisen et al., 1993), striped bass (Conover et al., 1997) and Arctic charr (Torrisen and Shearer, 1992; Imsland and Jonassen, 2001).

Genetic variation in commercial fish species has been studied for more than three decades. However, the significance of genetic variation is still unknown and very few studies have tried to link the functional relationship between such genetic variation and physiological parameters (Imsland and Jónsdóttir, 2002).

1.1.1 Genetic Control of Fish Growth

Genetic components have a role in determining the pattern of growth. Recent research on turbot and halibut where growth of different genotypes was investigated under variable environmental conditions have shown that approximately 30% of growth variations are caused by genetic factors (Imsland et al., 2000a,b; 2001a,c; Jonassen et al., 2000b). Several studies have shown that body size is a highly heritable trait in salmonids (Gall and Huang, 1988; Sylven and Elvingson, 1992; Elvingson and Johansson, 1993; Horstgenschwark, 1993; Pante et al., 2002). Genome-scale analyses in fish are in their infancy (Cossins and Crawford, 2005) and almost nothing is known on the genetic control of growth in brook charr.

1.1.2 Current State of Genetic Resources for Fish

Improved availability of genomic resources and tools will be critical for future advances in fish functional genomics. At the present time there are more high-quality annotated gene databases for humans (Schuler et al., 1996) and mice (Cossins and Crawford, 2005). However, much more work has to be done for genome-wide sequence

data annotation in fish. Thousands of EST (Expressed Sequence Tags) sequences are available for carp, catfish, salmon, trout, killifish, stickleback, and tilapia. In total over 1.5 million teleost EST sequences are now available in publicly accessible databases and the list is rapidly increasing. Four fish genome sequence projects are underway; genome sequences that will soon be available include those of zebrafish (*Danio rerio*; http://zgc.nci.nih.gov/Info/Summary), medaka (*Oryzias latipes*; http://dolphin.lab.nig.ac.jp/medaka/) and two pufferfish species (*Takifugu rubripes*; formerly designated as *Fugu rubripes*; http://fugu.biology.qmul.ac.uk and *Tetraodon nigroviridis*; http://www.genoscope.cns.fr/externe/tetranew). Genome sequences will be available for several key species in the near future, including the rainbow trout, various species of salmon, killifish (*Austrofundulus limnaeus*), sheepshead minnow (*Cyprinodon variegatus*), three-spined stickleback (*Gasterosteus aculeatus*), the European flounder (*Platichthys flesus*) and the common carp (*Cyprinus carpio*; Katagiri et al., 2001; Clark et al., 2003; Cossins and Crawford, 2005). Several salmonid genomic research projects have been carried out such as the one on Atlantic salmon (*Salmo salar;* GRASP, http:// grasp.mbb.sfu.ca), Salmon Genome Project (SGP; www.salmongenome.nocgi-bin/sgp.cgi) and Aquaculture Genome projects (NAGRP; http://www.animalgenome.org/aquaculture/salmonids). Research on salmon and trout would greatly benefit from the availability of complete salmonid genome sequences.

DNA sequence databases can be used as a point of reference where gene sequences from fish species that lack a sequenced genome can be searched. One of the most powerful techniques to identify novel genes is to search sequence alignments. With the production of new fish genome sequences, such comparative studies are likely to play

an important role in guiding the identification of functional elements, and in deciphering subtle sequence variations that may lead to phenotypic changes (Ahituv et al., 2004; Boffelli et al., 2004). Access to these sequences will also open up new comparative approaches to gene function across vertebrates classes (Boffelli et al., 2004). The collection of ESTs is essential for microarray–based and transcription level studies. Efforts directed at specific questions and specific tissues, using subtractive techniques, where appropriate, to enrich libraries for genes that show different expression patterns between physiological states, experimental conditions, and between different populations or species are needed. Such databases can be exploited to design studies on fish physiology including the endocrine regulation of growth. All of these studies will help us in understanding the evolution of genome in fish and provide an understanding of gene function.

1.1.3 Large Gene Families in Teleosts

There are more than 25,000 species of fish, and more than 120 families, which have been identified. When compared to only 4,000 mammalian species, the diversity of the fish genome is tremendous (Nelson, 1994). As well, fish inhabit an enormous range of aquatic ecosystems, from Tibetan streams to the abyss of the oceans. The significance of this is more understandable since water temperatures range from –2 to 45°C and there is a wide variation in salinity between freshwater and seawater. Fish species also have extraordinarily diverse body forms, lifestyles, and physiologies, which help them adapt to the most divergent ecological conditions (Nelson, 1994). Fish size also varies tremendously. For example, the whale shark (*Rhincodon typus*) can weigh as much as

10,000 kg while the dwarf goby *(Trimmatom nanus)* weighs only 0.1g. Divergence of fish species has occurred over 500 million years of evolution; it is therefore not surprising that there is such a large diversity in the genetic makeup of these species.

The larger number of genes and their interactions may help fish to adapt and respond to widely varying and adverse conditions (Wittbrodt et al., 1998). The size of fish genomes vary between 0.32 and 133 billion base pairs (Cossins and Crawford, 2005). The more complex genomic architecture of fish has allowed them to adapt quickly in response to changing environmental challenges and evolution. Therefore, fish genomes are more varied and show frequent genomic changes in comparison with other vertebrates.

Databases on characterised fish genes suggest that fish contain more genes than mammals. For example, it has been reported that there are four clusters of homeobox (Hox) genes in mammals. However, in zebrafish, there appears to be at least two additional clusters giving a total of at least six (Garcia-Fernandez and Holland, 1994; Prince et al., 1998). It has been proposed that in early teleost evolution the entire genome was duplicated, resulting in a sudden increase in gene number (Amores et al., 1998; Wittbrodt et al., 1998). Duplication of genes and the subsequent diversification of function might also have been important in the evolution of increasingly complex genes to the challenge experienced in water by the fishes.

1.1.4 Divergent Function of Some Genes between Fish and Mammal

Studies have shown that, in several cases, although fish genes appear to have sequence similarity with mammals, they have quite different expression patterns and

functions as compared to human or mice (Wittbrodt et al., 1998; Ekker et al., 1997). For example, muscle segment homeobox genes (msx) have been isolated from mammals, birds, amphibians, and zebrafish. Sequence analysis and studies on the expression pattern of genes have shown that both structure and function of msx genes diverged during evolution in these organisms (Ekker et al., 1997). There are three members msx family in mammals, birds, amphibians (msx1, msx2, msx3) and these participate in the regulation of body patterning and cell differentiation during embryonic development. These genes have distinct expression patterns in the neural crest, facial mesenchyme of crest origin, and in distal mesoderm of developing limbs. However, the zebrafish genome contains at least five msx homeobox genes (msxA, msxB, msxC, msxD, and msxE; Ekker et al., 1997). The expressions of zebrafish msx genes are necessary for the development of embryonic dorsal neuroectoderm, visceral arches, fins, and sensory organs. A similar situation is found for other genes such orthodenticle-related homeobox genes (otx). In zebrafish, three otx genes (otx1, otx2 and otx3) have been reported. The zebrafish otx2 homeoprotein is highly conserved in comparison with the mouse otx2, whereas the zebrafish otx1 and otx3 homeoproteins have not been identified in higher vertebrates (Wittbrodt et al., 1998). Comparison of the expression patterns of otx genes in zebrafish, medaka and mouse strongly suggest that structure and function of the otx genes are different between fish and mouse (Wittbrodt et al., 1998). As the early expression patterns of otx genes appear at the midbrain in the zebrafish, those genes are consistently expressed in the regions of midbrain and forebrain in the mouse (Mercier et al., 1995).

As previously cited studies have shown, genes in mammals can serve somewhat different functions than their homologues in fish. This highlights that the degree of evolutionary relatedness of genes is not a reliable predictor of their evolutionary conservation and their similarity of function. Thus it may not be possible to apply the concept of homology in order to interpret structural similarity or to extrapolate a known function from another taxonomic group to fish.

1.1.5 Future Prospects

Identifying growth–dependent genes in fish remains challenging. Homologous comparisons have been predominantly used to identify such genes in fish using degenerate primers for PCR or by hybridizing mammalian cDNA probes with fish mRNA. While such approaches have had a certain degree of success to identify transcripts in fish, the function of these genes needs to be carefully established to ensure that they have similar function as mammalian counterparts. Moreover, it is not possible to identify genes which have no sequence similarity to known genes

Recently developed microarrays can detect a large number of functionally regulated genes which yields an overview of the pattern of gene expression in a given tissue. However, this method is dependent on proper annotation of genes spotted on the microarray. Interpretation is ultimately dependent on the accurate identification of genes, homology search methods, the sequence databases used and gene function (Meltzer, 2001). The limitation of this approach is that large-scale genome resources are restricted to just a few key fish species (for example zebrafish), and for most other fish species resources are limited. For example, studies by Ton et al. (2003), used zebrafish to study

effects of hypoxia on gene expression even though this species is not regarded as possessing well-developed hypoxia-resistance mechanisms. Results obtained in this species may have limited value for understanding hypoxia-induced gene expression in different fish species. Therefore there is an urgent need to characterise more genes to provide a better understanding of the diversity of genes and their functionality in different fish species.

1.2 General Information on the Regulation of Fish Growth

1.2.1 Pattern of Growth

In fish, growth is continuous so that under suitable conditions, most fish continue to grow throughout their entire life (Diana, 2004a). While specific growth rate is high in young fish it decreases in older animals (Weatherley and Gill, 1987a). In temperate environments, most fish show a seasonal growth pattern with increased growth in the spring and low growth in the winter (Boeuf and Le Bail, 1999). Fish growth is usually faster in warm water and decreases during migration or spawning (Diana, 2004a). The manipulation of environmental factors such as temperature, salinity and photoperiod is currently used to control fish growth in aquaculture (Jobling, 1994). A number of fish species have been reported to display a compensatory growth phenomenon (Weatherley and Gill, 1987a). Fish which have been starved, or have reduced food ration, will have an increased growth spurt over and above normal growth rates when food levels are restored. For fish, which display compensatory growth, using cyclic feed restriction and refeeding could be one of the ways to maximise growth for aquaculture. However, the mechanisms that mediate compensatory growth are not well understood (Saether and

Jobling, 1999; Qian et al., 2000). There are several hypotheses that attempt to explain the increased growth rate that normally ensues following a starvation period. One explanation is that the response to TH during and following starvation drives growth (Gaylord et al., 2001).

1.2.2 Environmental Regulation of Growth

Many environmental factors can affect fish growth (Diana, 2004a). The major factors can be classified into two categories: (1) determining factors which are directly involved in the regulation of growth such as temperature (Burel et al., 1996), salinity (Boeuf and Payan, 2001) and photoperiod (Taylor et al., 2005; Leiner et al., 2001); (2) limiting factors such as oxygen, ammonia and pH (Stierhoff et al., 2006; Diana, 2004a). The interactive effects of any of these factors may be as important as any single factor in regulating growth (Jobling, 1983).

1.2.2.1 Determining Factors

Temperature has been shown to be the most important factor for regulating growth (Ottersen and Loeng, 2000; Burel et al., 1996). Generally, fish have temperature optima for growth and survival (Diana, 2004a; Gadomski and Caddell, 1991). Water temperature impacts fish growth through its effect on metabolic processes (Diana, 2004a; Burel et al., 1996) and food assimilation (Krohn et al., 1997).

Changes in salinity affect osmotic and ionic regulation (Varsamos et al., 2005). When the energetic costs of osmoregulation are low, the saved energy is substantial enough to increase growth. Conversely, growth will decrease when too much energy is

used for osmoregulation. The energetic cost of maintaining homeostasis, food intake (Le Bail and Boeuf, 1997), food conversion (Imsland et al., 2001b) and hormonal regulation are key elements in explaining the influence of salinity on the growth capacity of marine fish and many salmon species (Varsamos et al., 2005).

In certain latitudes, the growth rate of many fish has been demonstrated to be positively related to day length (Boeuf and Le Bail, 1999; Jonassen et al., 2000a; Imsland and Jonassen, 2001). Long photoperiods increase growth rates (Taylor et al., 2005; Taylor et al., 2006) and food consumption as well as enhanced food conversion efficiency (Saether et al., 1996). The mechanisms involved in photostimulation on growth are still not clear.

1.2.2.2 Limiting Factors

Under oxygen-deficient conditions fish experience a transient or long-term loss of appetite, depending on the severity of hypoxia and its duration (Pichavant et al., 2000). Hypoxia can also be lethal to fish (Jobling, 1994). Growth and feeding efficiency decrease when the pH is either lower or higher than 7.0; the mortality of bluegill fish (*Lepomis macrochirus*) increased when pH level is 5.5 (Brogowski et al., 2005). Fish growth is inhibited by high levels of ammonia as the energy is diverted to protect them from ammonia toxicity. When ammonia accumulates in water, even at non-lethal concentrations, it can alter numerous processes, such as ion exchange, acid-base balance, amino-acid metabolism, oxygen delivery and alter enzyme activity (Person-Le Ruyet et al., 1997, 1998; Rasmussen and Korsgaard, 1998).

Recently, few studies have addressed changes in gene expression associated with different environmental factors that were experimented on fish. The results indicate that gene expression patterns can be altered by environmental conditions. Studies on gene expression responses to variations in salinity in a euryhaline fish such as the common sea bass (*Dicentrarchus labrax*) indicated that the expression of 586 genes are altered depending on whether or not they were acclimated to salt or freshwater (Boutet et al., 2006). When killifish (*Austrofundulus limnaeus*) were exposed to either constant (20, 26 and 37°C) or fluctuating daily temperatures (between 20°C and 37°C) there were major differences in the gene expression in the liver. The genes that control cell growth and proliferation appear to be susceptible to changes in water temperature (Podrabsky and Somero, 2004). Results of these studies indicate that alterations in different environmental factors can have profound effects on gene expression.

1.2.3 Effects of Nutrition

The quality and quantity of food are relevant to the growth performance of fish (Weatherley and Gill, 1987a). Fish growth only occurs when sufficient food is eaten to account for all other processes such as metabolism, maintenance and reproduction (Diana, 2004a,b). Salmonids use dietary proteins as a source of metabolic energy (Halver and Hardy, 2002). Although they possess all of the necessary digestive and metabolic enzymes involved in carbohydrates digestion and metabolism, carbohydrates have limited value as a source of dietary energy (Thorgaard et al., 2002).

The liver is central to many essential physiological processes. Many fish species accumulate energy in the liver during periods of high-energy intake (Busacker et al., 1990). During periods of insufficient food supply, the energy obtained from food is less than the energy spent for body maintenance, fish reduce their metabolic rate and growth is decreased. During periods of starvation, lipid stores and protein breakdown from the liver to provide energy for body maintenance (Diana, 2004a,b). The nutritional status, enzyme activity and gene expression in liver can thus be used as indicators and predictors of growth (Metón et al., 1999). It is therefore reasonable to study growth-dependent gene expression in the liver. The control processes of energy reserves and metabolic rate are complex and are not completely understood at present. A more complete understanding of metabolic pathways and specific genes involved in metabolic regulation will be beneficial in generating diets suited for fish consumption and genetic selection programs for the production of growth enhanced fish.

1.2.4 Hormones Regulation of Growth

It is well documented that hormones control growth rates in fish. Many studies using salmonids have shown the importance of three families of hormones in regulating somatic growth: GH, anabolic steroids, and THs (Donaldson et al., 1979; Sumpter, 1992; Higgs et al., 1982; Higgs et al., 1992). The administration of these hormones to coho salmon increased the rate of food conversion (Higgs et al., 1982), modified protein and lipid metabolism in rainbow trout (*Oncorhynchus mykiss*) and coho salmon (Foster et al., 1991; Sheridan, 1986). There is a strong correlation between specific growth rate and

plasma levels of GH, anabolic steroids or THs in salmonids (Higgs et al., 1982; Eales and Shostak, 1985; McCormick and Saunders, 1990).

1.2.4.1 Growth Hormone

Growth Hormone (GH) is secreted by the anterior pituitary in response to hypothalamic regulatory hormones: somatostatin (SRIF) and growth hormone releasing hormone (GHRH). The secretion of GH is stimulated by GHRH and inhibited by SRIF (Björnsson, 1997; Wong et al., 2006). In teleosts, as in other vertebrates, GH acts by binding to the GH receptor, a single transmembrane protein on cells of target tissues. The liver is the most important target tissue of GH (Björnsson et al., 2002). The effects of GH in stimulating growth are also mediated through the insulin-like growth factor I (IGF-I; Björnsson et al., 2002; Reinecke et al., 2005). Circulating GH or IGF-I levels exhibit a negative feedback effect on GH secretion by the pituitary (Wong et al., 2006; Björnsson, 1997; see Figure 1-1).

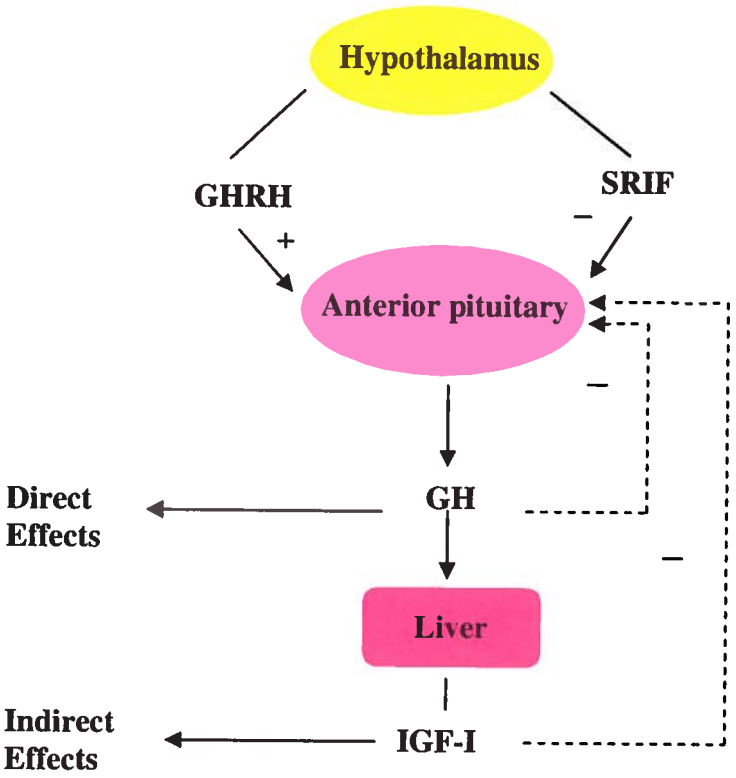


Figure 1-1. Hypothalamic and negative feedback regulatory mechanisms of pituitary GH secretion. GH action can be either direct or through an indirect pathway via IGF-1. GH and IGF-I levels exhibit negative feedback regulatory mechanisms to the anterior pituitary

The GH/IGF-I system is highly conserved during evolution from fish to mammals. IGF plays an important role in bone (Björnsson et al., 2002; Wood et al., 2005) and somatic growth (Reinecke et al., 2005; McCormick et al., 1992). Reducing IGF-I production causes a delayed response to GH treatment and leads to growth retardation in salmon (Duan, 1998). Skyrud et al. (1989) attempted to examine the *in vivo* effects of IGF-I in brook charr by using direct intraperitoneal injections (0.001-10.0 hIGF-I μg/g body wt). This led to an unexpected decrease in growth and an increase in

fish mortality, which likely resulted from the hypoglycemic effect of exogenous IGF-I. The effect of IGF-I on growth rate was also examined by McCormick and co-authors (1992) in juvenile coho salmon. Their results indicate that exogenous treatment with mammalian IGF-I can stimulate coho salmon growth under some conditions. High doses of IGF-I (greater than 0.13 μg/g.d) resulted in hypoglycemia and death while low doses of IGF-I (0.01 to 0.05 μg/g.d) did not alter growth rate.

GH in teleosts is known to regulate several important physiological processes. Many aspects of GH physiological effects have been studied intensively in samonids, cyprinids and sparids (Reinecke et al., 2005; Björnsson, 1997; Björnsson et al., 2002). GH regulates fish somatic growth by inducing a number of metabolic changes, such as the metabolism of proteins, lipids, carbohydrates, and regulation of ionic and osmotic balance (McLean and Donaldson, 1993; Björnsson, 1997). It also stimulates skeletal growth (Björnsson, 1997; Björnsson et al., 2002; Pérez-Sánchez, 2000), food intake (appetite) and improves food conversion in salmonids (Johnsson and Björnsson, 1994).

A large number of studies have demonstrated that exogenous GH (most often of mammalian origin), increases specific growth rate by increasing both fish weight and length (Down et al., 1988, 1989; Schulte et al., 1989; McLean et al., 1991; Johnsson and Björnsson, 1994; Björnsson, 1997). However, in salmonids, this is accompanied by a decrease in condition factor (the relationship between weight and length) indicating that fish become relatively leaner during GH treatment (Down et al., 1988, 1989; McLean et al., 1991). Studies on the effects of GH on growth also showed that excess administration of exogenous GH initially increases the growth above normal, but during long-term treatment growth becomes retarded (Johnsson and Björnsson, 1994). Maximal growth

rate may also induce increased mortality and occurrence of misregulation during development. The administration of GH can also affect several aspects of behaviour, including foraging, aggression, and predator avoidance in teleosts (Björnsson, 1997; Björnsson et al., 2002). Jönsson et al (1998) observed that rainbow trout treated for 4 days with GH increased swimming activity and aggression levels.

Many factors influence circulating GH concentrations such as temperature, food availability, ammonia and hypoxia. Increased water temperature tends to increase circulating GH levels (Boeuf and Le Bail, 1999; McCormick et al., 2000). Starved and stressed fish also exhibit high levels of GH (Sumpter et al., 1991; Pickering et al., 1991). GH levels were found to be significantly increased under high concentrations of ammonia (>20 mg/l; Boeuf et al., 1999), while GH synthesis was depressed under hypoxic conditions (< 3.5 - 5mg/l; Boeuf et al., 1999; Pichavant et al., 2000).

1.2.4.2 Steroid Hormones

Neurons from the hypothalamus secrete gonadotropin-releasing hormone (GnRH) into the hypophyseal portal system where it stimulates the release of pituitary gonadotropins (GTHs) by the anterior pituitary (Figure 1-2). There are two types of GTHs: GTH I and GTH II (Weltzien et al., 2004). Based on nucleic acid sequence analysis, amino acid sequence analysis and bioassays, fish GTH I and GTH II are homologous to tetrapod follicle–stimulating hormone (FSH) and luteinizing hormone (LH), respectively (Quérat, 1994). It appears that in fish, plasma GTH I levels are high during the early stages of gametogenesis and decrease in females during vitellogenesis and ovarian maturation. GTH II is undetectable during gametogenesis, and increases just

before spermiation or ovulation (Slater et al., 1994; Prat et al., 1996; Breton et al., 1998; Bon et al., 1999). Plasma levels of GTHs indicate that GTH I is involved in gametogenesis and steroidogenesis, whereas GTH II regulates the final stages of gametogenesis (Prat et al., 1996; Cyr and Eales, 1996). Both GTH I and GTH II are involved in gonadal development and act on the gonads, which stimulate the synthesis of steroids including testosterone (T), 11-ketotestosterone (11-KT), 17β-estradiol (E_2) and dihydroprogesterone (DHP; Kime, 1993). Sex steroids also have a negative feedback regulation at the hypothalamus or pituitary level and inhibit GTH I and GTH II secretion.

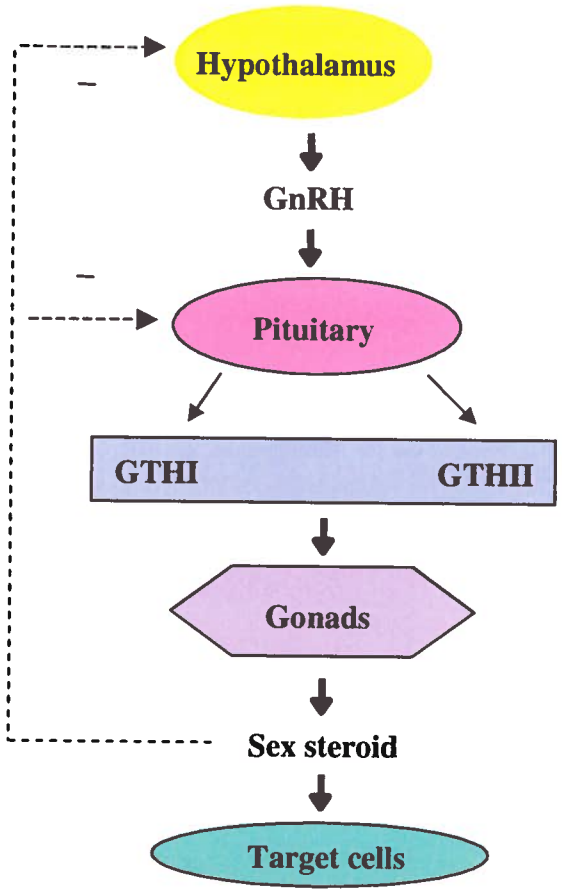


Figure 1-2. Endocrine regulation of gonadial steroidogenesis. Neurons from hypothalamus secrete GnRH. GnRH stimulates the anterior pituitary to release GTH I and GTH II. Both GTH I and GTH II are involved in regulating the gonads. While GTH I is secreted during early developmental stages of the gonads, GTH II is secreted in the final stages of gonadal development. GTHs stimulate steroidogenesis and the synthesis of T, 11-KT, E_2 and DHP, which can then act on the target tissues. Sex steroids also exert a negative feedback regulation at the hypothalamus or pituitary level.

A number of anabolic steroid hormones, both androgenic and estrogenic, increase growth and food conversion efficiency through their actions on the gonads and are involved in many processes affecting metabolism, reproduction and behaviour (Higgs et al., 1982). Studies have shown that when T and E_2 were individually incorporated in test diets (2.5 mg/kg dry diet) and fed to coho salmon for 14 weeks, it resulted in accelerated growth rate and increased feed conversion efficiency (Yu et al., 1979). Schrecka et al.

(1982) reported that administering T between 5 and 10 μg/g feed promoted growth without affecting male sexual development in chinook salmon. Several studies have indicated that the effects of anabolic steroids are markedly influenced by dose, water temperature, duration of treatment and quality of nutrition (Higgs et al., 1982). Sex steroids, in particular estradiol, play a role in regulating seasonal changes in circulating GH levels in goldfish; E_2 implants cause an increase in serum GH levels in females in a dose-dependent manner (Trudeau et al., 1992).

Steroids can be used to induce monosex or sterile fish (Donaldson and Hunter, 1982; Hunter et al., 1983). Such fish have been shown to grow faster because the energy required for reproductive activities and gonadal maturation is channelled to somatic growth. Maturing fish will allocate energy to liver and gonadal growth instead of somatic growth (Hansen et al., 2001)

1.3 Thyroid Hormones

1.3.1 Thyroid Hormones in Teleosts

T_4 and T_3 are small liposoluble molecules. T_4 is produced in thyroid follicles, which are typically located in the basibranchial region and made up of a single layer of epithelial cells surrounding a lumen (Figure 1-3). T_4 is formed within the thyroglobulin (TG) protein, which is secreted from the follicular cell into follicular colloid by a coupling reaction of two diiodotyrosyl residues (Eales et al., 1999). The production of T_4 is controlled by thyroid stimulating hormone (TSH) from the pituitary, which is itself controlled by the hypothalamus. TSH acts on the thyroid to promote the synthesis and release of the prohormone T_4. T_4 is probably the only secreted THs from the thyroid

follicles of teleosts (Eales et al., 1999). THs in fish can also be obtained directly from dietary sources (Eales et al., 1997).

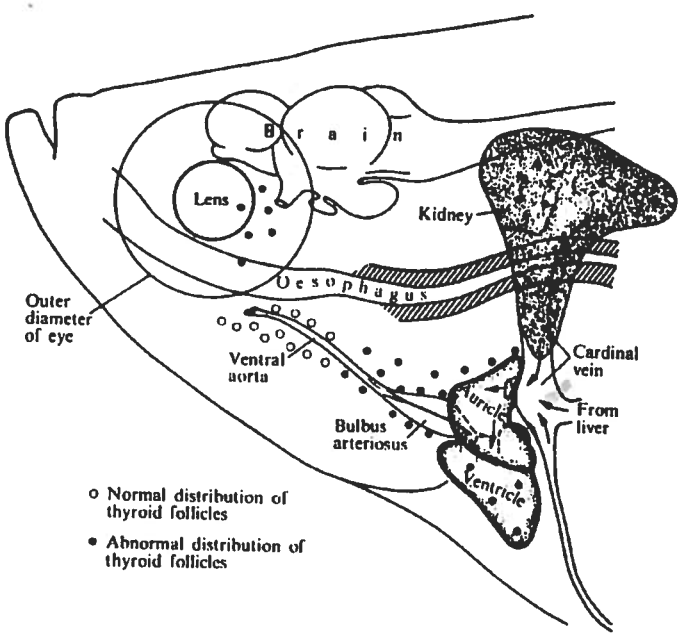


Figure 1-3. Diagram of the head region of a platyfish to show the distribution of normal and abnomal thyroid follicles. (Source: Matty, 1985)

Free plasma T_4 exerts a negative feedback regulation through the brain-pituitary-thyroid axis (Figure 1-4). Negative feedback of free T_4 at the level of the hypothalamus and pituitary regulates circulating free T_4 concentration. T_3 does not appear to have a negative feedback action on the hypothalamus-pituitary-thyroid axis in teleost (Eales et al., 1999). Approximately 99% of the circulating T_4 in plasma is reversibly bound to several plasma proteins (Cyr and Eales, 1992). Lipoproteins are the major binding proteins of T_4 in trout plasma (Babin, 1992; Cyr and Eales, 1992). Less than 1% of T_4 is in the free fraction in plasma.

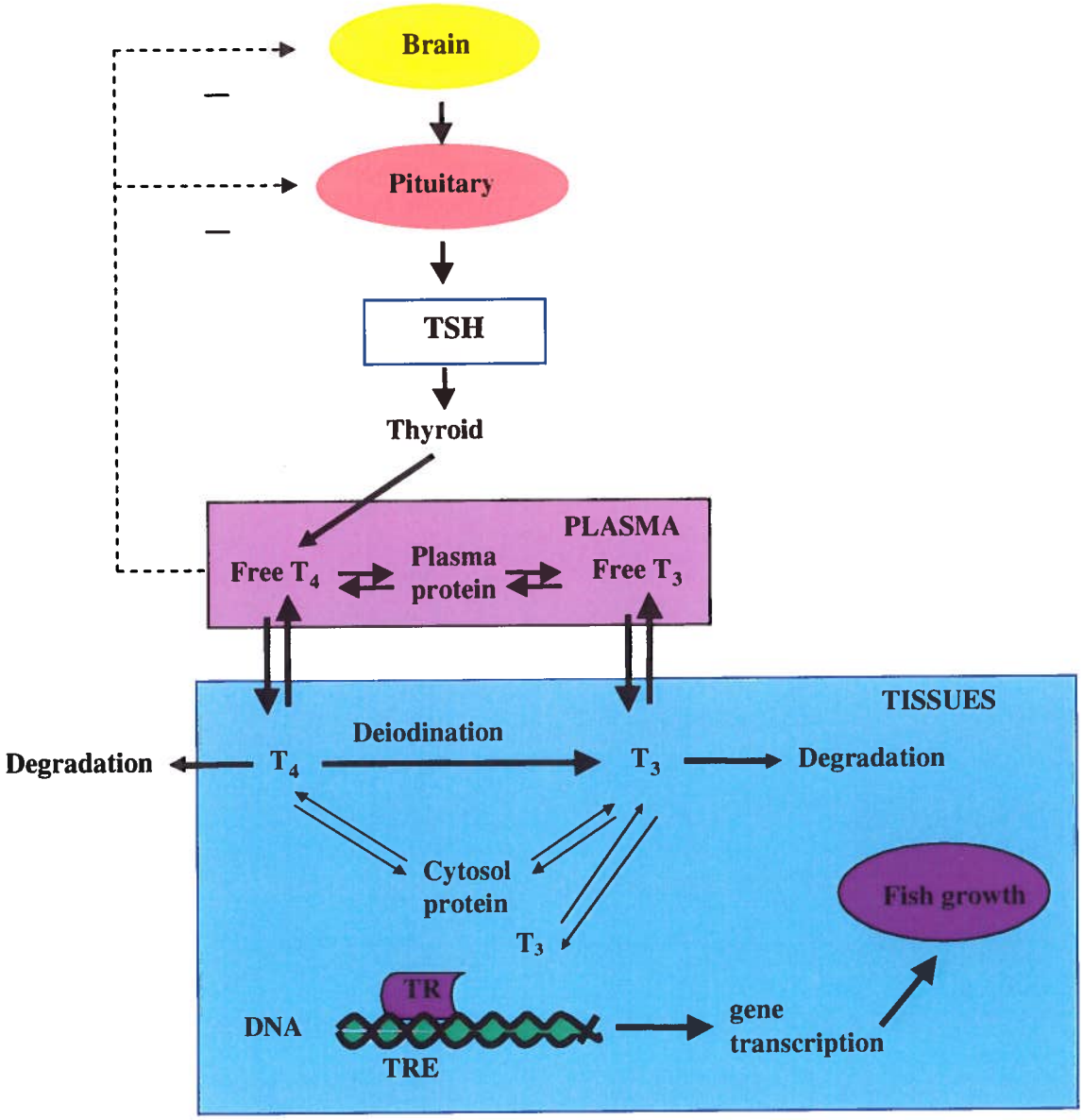


Figure 1-4. Schematic representation of thyroid hormone regulation in fish. T_4 is the only secreted THs from the thyroid follicles. The production of T_4 is controlled by TSH from the pituitary, which is controlled by the brain. TSH acts on the thyroid follicles to stimulate the synthesis and release of T_4. T_4 in blood is bound to several plasma proteins, but the small free fraction exchanges with tissues and can be converted to T_3 by deiodination. T_3 is the biologically active hormone. T_3 exchanges with blood or binds to thyroid hormone receptor (TR) which binds to the thyroid response elements on the DNA to initiate gene transcription to generate a biological response. T_4 exerts a negative feedback regulation at the brain or pituitary to inhibit TSH secretion. (Modified from Eales et al., 1999)

T_3 is the biologically active hormone (Eales et al., 1999). It is the free T_3 that enters target cells, is transported into the nucleus and binds to fish nuclear thyroid hormone receptors (TR) which then generates a biological response (Eales et al., 1999). T_3 is also rapidly and reversibly bound to plasma proteins in blood, but with a higher affinity than T_4, resulting in a proportion of free T_3 in plasma ten-fold lower than T_4. Approximately 1% of T_3 is free in plasma. The elevation of plasma T_3 does not depress plasma T_4 level in fish.

1.3.2 Thyroid Hormones Deiodination and Deiodinases

The prohormone T_4 is converted into the biologically active hormone T_3 (3, 5, 3'-triiodothyronine) mainly via the 5'-deiodination of the T_4 outer-ring (Eales et al., 1999; Eales and Brown, 1993; Moore VanPutte, et al., 2001; Figure 1-5). Alternatively, T_4 can be converted into the inactive metabolite rT_3 (3, 3', 5'-triiodothyronine) by the 5-deiodination of the T_4 inner-ring. T_3 and rT_3 can be further metabolised to 3, 3'-diiodothyronine (T_2); Deiodinations further down in the deiodination cascade have not been explored in fish (Eales et al., 1999). T_3 conversion to T_2 by the outer-ring deiodination pathway is negligible in trout liver, but is more active in brain (Frith and Eales, 1996). In fish, deiodination plays a more important role in the regulation of TH bioactivity than the central nervous system (hypothalamus-pituitary). Deiodination activities respond to numerous physiological, environmental and development stages (Eales et al., 1999).

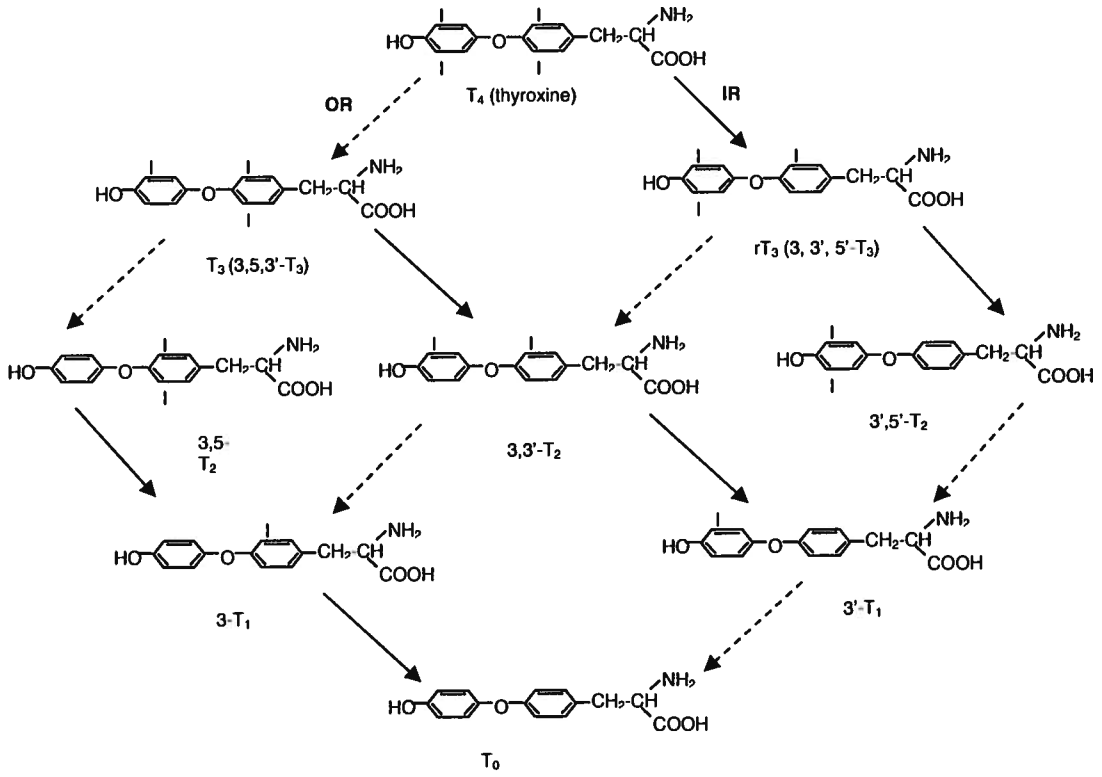


Figure 1-5. Structures of the thyroid hormones and their pathway of deiodinations. (-➤) represents the pathway of outer-ring deiodination (OR), while (⟶) represents the pathway of inner-ring deiodination (IR). T_4 is converted to the biologically active T_3 by deiodinases through outer-ring deiodination. T_4 can be converted to the inactive hormone rT_3 by inner-ring deiodination.

TH deiodination is accomplished by a family of enzymes termed deiodinases. There are three deiodinases: Type I, II and III. Deiodinases have been identified with distinct types and functions in teleosts (Eales and Brown, 1993; Mol et al., 1997, 1998). Type I (DI) possesses both outer-ring (5') and inner-ring (5) activity and converts T_4 to T_3. DI is important for maintaining circulation T_3 levels and is found primarily in the liver and kidney (Frith and Eales, 1996; Eales et al., 1999; Finnson and Eales, 1999; Van der Geyten et al., 2001). Type II (DII) also has outer-ring activity (5'D), and produces intracellular T_3 in certain tissues (Griffin and Ojeda, 2000). It has been reported to be

highly expressed in rainbow trout liver, gonads and at lower levels in the kidney (Finnson and Eales, 1999; Sambroni et al., 2001). Type III (DIII) has only inner-ring (5-deiodinase; IRD) activity and is involved in the inactivation of T_4 and T_3 by converting them to either rT_3 or T_2 (St. Germain and Galton, 1997). The primary function of DIII is to protect from overexposure to T_3. DIII is present at higher levels in the brain and lower ones in gills of rainbow trout, tilapia and Atlantic salmon (Eales et al., 1993; Sanders et al., 1999; Mol et al., 1998). The three deiodinases regulate the thyroidal status in fish by either forming or degrading T_3 (Eales et al., 1993).

1. 3. 3 Functions of Thyroid Hormones

Numerous diverse physiological responses to THs have been described in both salmonids and non-salmonids (Higgs et al., 1982; Gill et al., 1985; Eales, 1979). In general, THs are necessary for metabolism and have been reported in several fish species including Arctic charr, coho salmon, rainbow trout, Atlantic cod (*Gadus morhua)* and catfish (*Anabas testudineus*; Plisetskaya et al., 1983; Matty and Lone, 1985; Leatherland 1994; Varghese and Oommen, 1999). Their effects on lipid (Leatherland, 1994; Varghese and Oommen, 1999), carbohydrate and protein metabolism are particularly important in regulating the metabolic rate of coho salmon, brook charr and lake trout (*Salvelinus namaycush*; Matty and Lone, 1985; Leatherland, 1994). THs influence cartilage and bone growth in zebrafish (Brown 1997) and are also involved in development (Gavlik et al., 2002), growth (Gomez et al., 1997) and reproduction in salmonids (Cyr and Eales, 1996).

Early studies have demonstrated that the maintenance of adequate thyroidal status is a prerequisite for normal growth (Eales et al., 1999). Growth rate was significantly

correlated with daily T_3 concentrations in rainbow trout (Gomez et al., 1997). The positive correlation between growth and T_3 concentrations have been reported in other salmonids such as Arctic charr (Eales and Shostak, 1985) and Atlantic salmon (Boeuf and Gaignon, 1989; McCormick and Saunders, 1990). Exogenous TH administration has been reported to stimulate growth in several non-salmonid fish such as carp (Lam and Sharma, 1985), tilapia (*Saratherodon mossambicus*; Matty et al. 1982) and milk fish (*Chanos chanos;* Lam et al., 1985) as well as in salmonids species including coho salmon (Higgs et al., 1982; Shelbourn et al., 1992), chinook salmon (Higgs et al., 1983), Atlantic salmon (Lam, 1982) and rainbow trout (Higgs et al., 1992). For example treatment of coho salmon with T_3 (added to the feed at 4 mg/kg) yielded a 14% increase in body weight over controls after 12 weeks (Shelbourn et al., 1992). Matty et al. (1982) immersed tilapia fry in T_4 (10 μg T_4 /100 ml water) and found an increase in the final body weight of treated fish as compared to controls. After feeding immature Atlantic salmon with T_3-supplemented diets at a dose of 5 mg/kg for 45 days, their weight increased 7% over control (Lam, 1982). Fagerlund et al. (1984) observed that orally administered T_3 supplementation (1, 5 and 25 ppm) to juvenile steelhead for 42-days induced a significant increase in growth rate.

1.3.4 Factors Influencing Thyroid Hormones Levels in Fish

TH function in teleosts is influenced by temperature (Eales and Brown, 1993). Studies on coho salmon and male Atlantic cod (*Gadus morhua*) have demonstrated that the concentration of T_3 changes with season (Cyr et al., 1998; Comeau et al., 2000). T_3 levels were elevated with increasing water temperature in teleost such as in coho salmon

and male Atlantic cod (Larsen et al., 2001; Cyr et al., 1998). Temperature can increase the rate of T_4 to T_3 conversion in the liver of teleosts (Johnston and Eales, 1995). This may explain why temperature can alter circulating levels of THs in teleosts (Eales, 1995).

Salinity can also affect circulating TH levels. The endocrinology of osmoregulation has been intensively studied and underlines the importance of THs. Interactions between the thyroidal system and osmotic balance in teleost have been studied mainly in adult salmonids (Varsamos et al., 2005). Orozco et al. (2002) showed that a mild change in salinity resulted in significant and sustained changes in circulating levels of T_3 and T_4 as well as in renal DI and hepatic DII activities in rainbow trout. There is some evidence to indicate that THs have a role in controlling salinity tolerance in coho salmon as well as in other salmonids (Refsie, 1982; McCormick, 2001). Subash Peter et al. (2000) studied the involvement of THs in osmoregulation in tilapia and showed that both T_3 and T_4 play roles in osmoregulation by affecting the Na+/K+-ATPase activity, as well as plasma Na+, Cl– and K+ concentrations in both branchial and renal tissues.

Variations in growth, food consumption and food conversion efficiency with changing photoperiod suggest that photoperiod influences the secretion of growth promoting hormones, such as GH and THs (Leatherland, 1982; Björnsson et al., 1995). Several studies have investigated the relationship between THs levels and photoperiod in several fish species such as Atlantic cod (Comeau et al., 2000), red drum (*Sciaenops ocellatus*; Leiner et al., 2000; Leiner et al., 2001) and rainbow trout (Cook and Eales, 1987; Boujard and Leatherland, 1992). In general, these studies concluded that elevations in serum T_4 varied seasonally and that hormone levels were strongly correlated with

photoperiod. THs regulate growth through enhanced feeding efficiency (Higgs et al., 1992; Woo et al., 1991) and increased food consumption (Higgs et al., 1979; Woo et al., 1991). Studies have shown that photoperiod affects circulating levels of TH, although the relationship between these is not always consistent. A possible explanation for this may be that certain studies did not account for effects induced by temperature which may complicate the interpretation of data on circulating hormone levels (McCormick et al., 2000).

Fish growth can be manipulated in a positive or negative direction according to the dose of THs. Excessive doses of THs may induce growth retardation (Lam and Sharma, 1985), abnormalities in body morphology, such as enlarged skulls (Higgs et al., 1982), opercular abnormalities, lordosis and scoliosis in rainbow trout (Higgs et al., 1992; Kang and Devlin, 2003). Oral administration of T_3 is more effective than T_4. Thus, the dose of hormone and the method of administration (Higgs et al., 1982) as well as fish species and size of fish used in experiments may represent critical factors influencing experimental outcome.

Plasma T_3 levels have been reported to decrease during food deprivation in rainbow trout (Leatherland and Farbridge, 1992), tilapia (Toguyeni et al., 1996) and black seabream (Deng et al., 2004) and increase after refeeding (De Pedro et al., 2003; Raine et al., 2005). Higgs et al. (1992) observed that T_3 (8 or 12 ppm)-supplemented diets improved carbohydrate utilisation and feed efficiency in rainbow trout. These studies showed a relationship between nutritional status and THs levels. Reduced feeding or fasting has been associated with a reduction in plasma T_3 and reduced hepatic 5′-

monodeiodinase activity in several fish species (eg. rainbow trout (Finnson and Eales, 1999), tilapia (Van der Geyten et al., 1998), red drum (Leiner et al., 2000)).

1.3.5 Interactions between Thyroid Hormones with Other Hormones

1.3.5.1 Interactions with Growth Hormone

The interactions between THs and GH are complex. THs and GH are believed to play synergistic roles in the control of growth in teleosts (Valente et al., 2003; Donaldson et al., 1979; Miwa and Inui, 1985). At the pituitary level, thyrotropin releasing hormone was shown to stimulate not only TSH but also GH release in teleosts (Peng and Peter, 1997). At the molecular level, it has been observed that a THs response element (TRE) is present in the promoter region of the Atlantic salmon (Farchi-Pisanty et al., 1997) and zebrafish GH gene (Sternberg and Moav, 1999).

There are many reports which show that GH exerts effects on THs. GH increases circulating T_3 levels by stimulating the conversion of T_4 to T_3. This is the result of increased hepatic DII 5'-deiodinase activity in teleosts (MacLatchy and Eales, 1990; MacLatchy et al., 1992). However, there are few data available on the action of THs on GH. In the eel, THs not only reduced GH release but also significantly inhibited GH synthesis both *in vivo* and *in vitro* (Rousseau et al., 2002). In contrast, studies have reported that T_3 stimulated both the synthesis and release of GH in tilapia (Melamed et al., 1995) and increased the circulation level of GH in GH-transgenic coho salmon (Kang and Devlin, 2003). THs also stimulated GH release in cultured pituitary cell of rainbow trout in a dose dependent manner (Luo and McKeown, 1991). T_3 treatment *in vivo* increased the mRNA levels of GH in the rainbow trout (Moav and McKeown, 1992) and

in vitro in pituitary fragments of the carp (Farchi-Pisanty et al., 1995) but did not alter GH levels in either the pituitary or in plasma (Moav and McKeown, 1992). This suggests that while THs regulate the transcription of GH, the translation into proteins is regulated by other factors. These contradictory results may be due to species specific effects, stage of development or methods used to measure T_3 and GH levels.

1.3.5.2 Interactions between Thyroid Hormones and Sex Steroids

Studies have shown that TH and sex steroids can influence each other. An association between plasma T_3 levels and E_2 has been observed in several fish species including rainbow trout and Atlantic salmon (Leatherland, 1985; Cyr et al., 1988; Cyr and Eales, 1992; McCormick et al., 2005). Studies by Cyr et al. (1988) have shown that E_2 treatment (injected estradiol benzoate 0.5 mg/100 g) in immature rainbow trout significantly depressed plasma levels of T_3. This effect is the result of a decrease in hepatic 5'-monodeiodinase (5'D) activity, which is responsible for conversion of T_4 to T_3. E_2 treatment in rainbow trout also caused a significant decrease in free T_4 (Cyr and Eales, 1992) and T_4 kinetics suggesting a direct effect of estradiol on thyroid secretion (Leatherland, 1985; Cyr and Eales, 1996). Short-term treatment with Methyl testosterone (MT; injected ip 0.5 mg/100 g body wt) in Arctic charr significantly increased plasma T_3 level and hepatic T_4 5'-monodeiodinase activity (MacLatchy and Eales, 1988). In male common dentex *(Dentex dentex)*, T_3 was positively correlated with 11-KT (Pavlidis et al., 2000).

1.3.6 Thyroid Hormone Receptors

The biological effects of THs at the gene level are mediated by the TR. There are two major types of TRs (TRα, TRβ) that have been identified in several fish species (Power et al., 2001; Raine et al., 2005). Each type of TR has different isoforms (TRα1, TRα2, TRβ1, TRβ2; Kawakami et al., 2003) and these different isoforms have different roles and expression in different tissues and at different developmental stages (Yamano et al., 1994; Yamano and Inui, 1995; Yamano and Miwa, 1998). The expression pattern of each TR isoform has also been described in zebrafish (Essner et al., 1997). It is believed that the expression of different TR isoforms could allow for differential interactions with other proteins in regulating transcription. Differential interaction with other proteins, including other nuclear receptors (activators and repressors) may play a role in THs physiology of fish as it does in mammals.

1.3.7 The Mechanisms of Action of Thyroid Hormones

TRs are members of a large family of nuclear receptors, which acts as ligand-dependent transcription factors (Yen, 2001). TRs form monomers, homodimers or heterodimers with RXR, a receptor for retinoic acid, and these binds to the TH response element (TRE) on the DNA. The entire complex acts as a transcription factor to further initiate gene expression and thereby regulate specific protein synthesis. TRs modulate gene expression through silencing or activating transcription by recruitment of either co-repressor or co-activator complexes depending on the absence or presence of T_3 (Zhang and Lazar, 2000; Collingwood et al., 1999). TRs can enhance or inhibit gene expression depending on the nature of the TREs, the hormonal status and the cellular environment.

In mammals, in the absence of T_3, the RXR–TR heterodimer binds to co-repressors and represses transcription (Chen and Evans, 1995; Horlein et al., 1995). In the presence of T_3, the receptor undergoes a conformational change, which induces the release of the co-repressors and replacement of a corepressor complex by coactivators (McKenna et al., 1999). Marchand et al. (2004) reported that fish may have a similar regulation. This results in the positive or negative regulation of target-gene transcription and the subsequent regulation of protein synthesis. Thus, the nuclear actions of T_3 are sensitive to inhibitors of transcription and translation and have a latency of hours to days (Yen, 2001).

1.3.8 Thyroid Hormones Target Genes in Liver and Their Functions

The liver is a major target organ of THs. Several techniques have been used to identify THs target genes. For example some studies have used a microarray approach to identify THs target genes in mammals (Feng et al., 2000; Miller et al., 2001; Weitzel et al., 2001, 2003b; Clement et al., 2002; Wood et al., 2002; Flores-Morales et al., 2002; Stahlberg et al., 2005). Feng et al. (2000) used a quantitative fluorescent cDNA microarray which contained 2225 different mouse genes to study the THs target genes in liver and identified fifty-five THs dependent genes. Among them, forty-five were not previously known to be regulated by THs (Feng et al., 2000). Flores-Morales et al. (2002) found more than 200 genes that were regulated by T_3 in the mouse liver. Weitzel et al. (2003b) used fluorescent-labelled cDNA prepared from hepatic RNA of hypothyroid and T_3 treated hypothyroid rat, hybridised it to a DNA microarray containing 4608 different genes in order to characterise T_3-induced gene expression at different time points (6, 24,

48 hrs); sixty-two genes were found to be T_3 responsive. To our knowledge, there are no studies which have identified TH-dependent genes in brook charr.

Analyses of TH regulated genes have shown that THs affect multiple cellular pathways and function, including gluconeogenesis, lipogenesis, insulin signalling, adenylate cyclase signalling, cell proliferation, and apoptosis. THs have also been shown to regulate genes involved in cellular immunity, cell matrix, cell structure and mitochondrial function (Feng et al., 2000). T_3 has been shown to stimulate gluconeogenesis and glucose production in the liver (Arrondo et al., 1981; Feng et al., 2000). It has been found to increase glucose-6-phosphatase which is a key enzyme for converting glucose-6-phosphate to glucose.

T_3 also regulates lipogenesis in liver. A number of T_3 target genes are involved in lipid metabolism such as appolipoprotein, L-FABP, fatty acid transport protein (FATP), and sterol carrier protein 2 (SCP2; Feng et al., 2000; Weitzel et al., 2003b; Flores-Morales et al., 2002). It has been reported that T_3 can down-regulate several genes in the rat including cytochrome P-450 4A (4A1, 4A2 and 4A3) and CYP 4A10. Consequently, fatty acid oxidation is reduced and lipogenesis increased (Webb et al., 1996; Feng et al., 2000). Furthermore, a number of lipogenic enzymes have been shown to be regulated by GH and THs in the liver of rat and mice (Oppenheimer et al., 1987). These include malic enzyme I, fatty acid binding protein 5, fatty acid syntheses, NADH dehydrogenase I subcomplex 5, hydroxysteroid sulfotransferase (Flores-Morales et al., 2001; Flores-Morales et al., 2002; Stahlberg et al., 2005). Other genes such as hepatic lipase and apoliporotein have also been identified as T_3 regulated genes (Sensel et al., 1990; Taylor et al., 1996; Yen, 2001). T_3 plays an important role in hepatocyte proliferation and cell

survival (Ohmura et al., 1997; Torres et al., 1999; Oren et al., 1999). All these studies show that THs play a role in regulating metabolism and this may be related with growth. T_3-dependent gene expression in liver occurs either via intermediate factors or the TR (Yen, 2001; Weitzel et al. 2003b; Flores-Morales et al., 2002). Most target genes are dependent on TRβ (Strait et al. 1990, Flores-Morales et al., 2002). T_3 can also regulate gene expression via intermediate factors. Some intermediate factors of T_3 have been identified including the nuclear respiratory factor 1, nuclear respiratory factor 2 (Scarpulla, 2002), peroxisomal proliferator-activated receptor and mitochondrial transcription factor A (Garstka et al., 1994; Weitzel et al., 2003a). There is currently no information on TR-dependent and TR-independent gene expression in fish.

1.3.9 Mitochondrial Actions of Thyroid Hormones

Mitochondria play a key role in metabolism as well as in the regulation of cellular differentiation. They have their own genome (mt-DNA; Casas et al., 2003) which encodes 13 enzymatic subunits of the respiratory chain, two rRNAs and 22 tRNAs. The majority of mitochondrial proteins such as carriers, enzymatic subunits, transcription factors, DNA and RNA polymerases, or proteins involved in RNA translation, are encoded by nuclear genes and are imported into mitochondria. T_3 is considered an important regulator of mitochondrial gene expression by both indirect and direct pathways (Pillar and Seitz, 1997; Feng et al., 2000; Wrutniak-Cabello et al., 2001; Weitzel et al., 2003a). THs have been shown to increase oxidative phosphorylation in rat liver by stimulating cytochrome-*c*-oxidase in mitochondria (Goglia et al., 1999; Thomas et al., 1987). T_3 has also been shown to directly regulate mitochondrial genes encoding

ATPase (Gouveia et al., 2002), several subunits of NADH dehydrogenase (Iglesias et al., 1995; Stevens et al., 1995) and subunits of cytocrome-*c*-oxidase (Wiesner et al., 1992).

1.3.10 Different Time Course Actions of Thyroid Hormones Target Genes

THs target genes can be classified into several groups according to differences in the time course in response to THs treatment: (i) The nongenomic actions of THs are detectable within minutes (Davis and Davis, 1996), have a short latency period and are unaffected by inhibitors of transcription and translation; (ii) Early response genomic effects of T_3 are typically detectable within a few hours after THs treatment (Pillar and Seitz, 1997) and their induction does not require protein synthesis; (iii) Late response genes induced by THs start approximately 24 h after treatment and require protein synthesis. For most late-response genes, there are indications of a direct mechanism involving the binding of T_3 to TR (Zilz et al., 1990; Norman et al., 1989; Petty, et al., 1990; Weitzel, et al., 2001) and these actions are sensitive to inhibitors of transcription and translation (Yen, 2001).

In summary, it is clear from the previously mentioned studies that THs are essential regulators of fish growth. The actions of THs are mediated through the regulation of gene expression. Many factors affect growth (temperature, photoperiod, salinity, and nutrition), via changes in circulating levels of THs and gene expression in fish. The hormones which regulate fish growth (GH, steroids) also interact with THs. The liver is a major target organ of THs action. However, only a small number of genes in the liver have been tested and a limited number of T_3-target genes have been identified (Feng et al., 2000; Flores-Morales et al., 2002; Stahlberg et al., 2005). A large-scaled profile of

THs transcriptional effects has not yet been undertaken in fish liver. Therefore there is limited information on the regulation of TH-dependent gene expression in fish liver, or for that matter, in any other TH-dependent organ in fish.

CHAPTER 2. MATERIAL AND METHODS

SECTION I: INDENTIFICATION OF TH-DEPENDENT GENE EXPRESSION

2.1 Thyroid Hormone Induced Gene Expression

To identify TH-dependent genes, immature brook charr were treated with either T_3 or the TH inhibitor, sodium ipodate (Na-ipodate), for 7 days (Cyr and Eales, 1986). Thyroid status was assessed by measuring circulating levels of T_3 using a solid phase radioimmunoassay (RIA). A differential display polymerase chain reaction (DD-PCR) approach was used to detect differentially expressed genes. Differentially expressed bands were cloned and sequenced. To rule out false positives from DD-PCR, reverse dot blots were used. Northern blot analyses were also used to identify the tissue distribution and expression patterns of TH-dependent genes.

2.1.1 Fish Treatment and Sampling Procedure

Thirty immature charr were divided into three groups (n=10 per group). The average body weight of each group was 48.3 ± 9.4; 49.1 ± 8.5 and 47.7 ± 6.1 g. Fish were kept in fibreglass tanks with continuous water flow for one-week at the Station aquicole de Pointe-au-Père (Institut des sciences de la mer de Rimouski). Fish were maintained under the water temperature of 8°C and photoperiod of 12.5L: 11.5 D. Fish were fed commercial food pellets each day (1% of their body weight), until 24 hrs before sampling.

Fish were divided into three experimental groups: a saline-injected control; a T_3-injected hyperthyroid group (10 µg/kg L-triiodothyronine free acid; Sigma Chemicals); and a Na-ipodate injected hypothyroid group (50 mg/kg; ORAgrafin, Squibb Canada Inc., Montreal). All compounds were given by intra-peritoneal injections on days 1 and 4 of the 7 day experimental period. Fish were tagged on the dorsal fin at the time of injection in order to follow individual fish.

At day 7 of the experiment, fish were anaesthetised with 0.18% 3-aminobenzoic acid ethyl ester (MS-222, Sigma, Chemicals). Individual fork length and weight of the fish were measured at the beginning and at the end of experiment. Blood was collected from the caudal vein using a heparinized syringe (Fisher Scientific, Unionville, ON). The serum was obtained by centrifugation and stored at -80°C. Fish were subsequently killed and the liver was quickly removed and frozen on dry ice. Blood and tissues were subsequently transferred on dry ice to INRS-Institut Armand Frappier and were stored at –86°C until RNA extraction.

2.1.2 Specific Growth Rate and Condition Factor

Specific Growth Rate (SGR) of the fish was calculated using the formula: 100 [(logW2 - logW1)/ t]; where W1 and W2 are initial and final body weights (g), and t is the time interval between the initial and final body weight measurement (days). Condition Factor (CF) was calculated using the formula: 100 x BW/BL^3, where BW is the body weight (g) and BL the fork length (cm). The data were analysed by one-way analysis of variance (ANOVA). Statistical significance was set at $p < 0.05$.

2.1.3 Radioimmunoassay

Serum T_3 concentrations were measured by RIA using 5 ml Quick-Sep columns (Isoclean Inc., Ohio) containing 0.3 g (dry weight) of Sephadex G-25 (GE Healthcare, Baie d'Urfe, QC, Canada; Cyr and Eales, 1986). Briefly, a 50 µl solution of [^{125}I]-T_3 (8000-10,000 cpm /50 µl; NEN-DuPont, Montreal, QC) was added to Sephadex columns. A 50 µl aliquot of serum was then added to the columns, gently mixed and subsequently allowed to drain onto the column. The free iodide fraction present in the tracer was removed by eluting 2 ml of phosphate buffer (100 mM Na_2HPO_4, 3 mM $Na_2H_2PO_4$, pH 7.4). The serum and tracer were incubated overnight at room temperature after the addition of 1 ml of T_3 antibody (Sigma Chemicals, Mississauga, ON). At the end of the incubation, 2 ml of phosphate buffer was added to elute the hormone bound fraction. The eluent containing antibody bound hormone was collected and the [^{125}I]-T_3 content was determined using a Canberra Packard gamma counter (Cobra 2, Model 5002). Non-specific binding was determined using columns that received neither serum nor antiserum. T_3 standards were assayed at the same time with serial T_3 concentrations (0.15, 0.31, 0.62, 1.25, 2.5, 5.0, 10, 20 ng/ml). Samples were analysed in duplicate for each assay. T_3 intra- and inter-assay coefficients of variation were 8% and 10%, respectively.

2.1.4 RNA Preparation

Total RNA was isolated from livers using the guanidium isothiocynate method (Sambrook and Russell, 2001). Liver samples (100-200 mg) were pulverised to a fine powder in liquid nitrogen with a mortar and pestle. They were then homogenised in a 1

ml solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate buffer, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol. Proteins and DNA were removed by phenol-chloroform extraction. The RNA was precipitated in isopropanol and the precipitation was collected by centrifugation. The resulting RNA pellet was washed in 70% ethanol. Finally, the RNA was dissolved in sterile DEPC (diethyl-pyrocarbonate)-treated water. Total RNA concentrations were determined using a spectrophotometer at a wavelength of 260 nm. The integrity of the RNA was determined by electrophoresis on a 1.5% denatured agarose formaldehyde gel stained with ethidium bromide.

2.1.5 Differential Display Polymerase Chain Reaction

To identify differentially expressed genes between the three experimental groups, DD-PCR was done using the RNA Image Kit (GenHunter, Nashville, TN, USA; Figure 2-1). RNA used for DD-PCR was further purified using the Message Clean kit (GenHunter, Nashville, TN, USA) to eliminate genomic DNA. Total RNA was treated with RNAase-free DNase (Message Clean kit, GenHunter) for 30 min at 37°C. DNase was removed by phenol/chloroform extraction, and the purified RNA was recovered by precipitation in ethanol (100%). The RNA was dissolved in DEPC-treated water and stored at -86°C. The integrity of the RNA was once again analysed by electrophoresis on a 1.5% agarose formaldehyde gel. A 0.2 μg aliquot of total RNA from each sample was used as a template for reverse transcription (RT). Three anchored oligo-dT primers (Table 2-1) H-T_{11}G, H-T_{11}A and H-T_{11}C were used as RT primers. The final RT reaction contained the following components: 5X RT buffer, 250 μM dNTP, one of the three oligo-dT primers, RNA template and Moloney murine leukemia virus reverse

transcriptase (MMLV) enzyme. The reaction was carried out at 65°C for 5 min, 37°C for 60 min, followed by 75°C for 5 min (RNAimage, GenHunter). RT and PCR reaction for each sample were performed in duplicate to control for random differences in cDNA synthesis (Liang and Pardee, 2003).

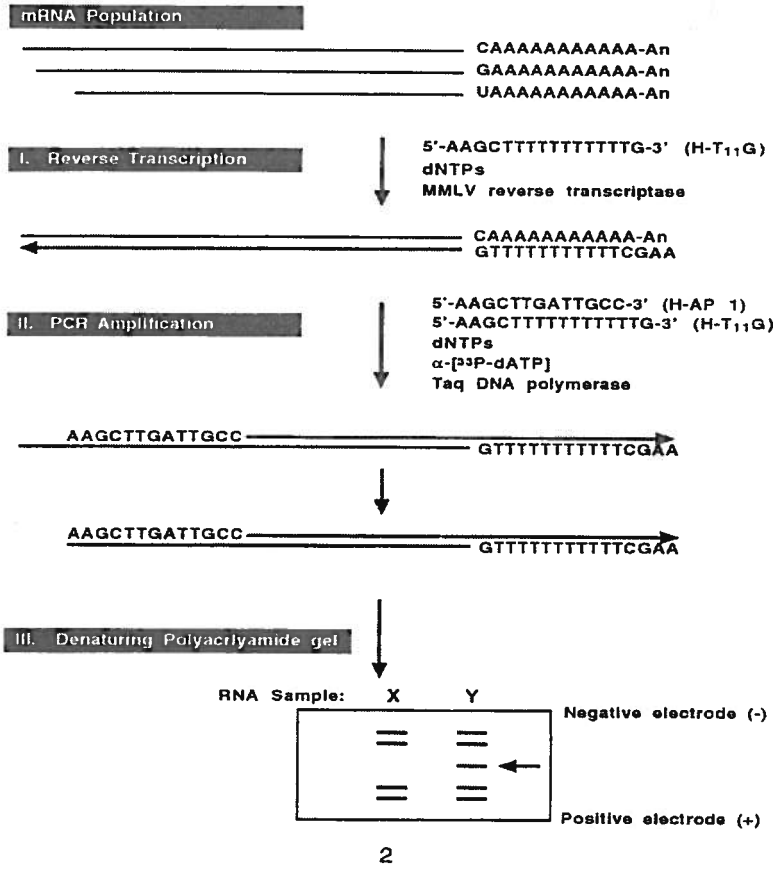


Figure 2-1. Schematic representation of the principle of DD-PCR (Adapted from http://genhunter.com/products). Total RNA was isolated from treated and untreated samples (X, Y). I. Reverse transcription was done to produce cDNAs. II. cDNAs were reamplified by PCR with arbitrary and anchored primer pairs. Labeled PCR products were seperated by electrophoresis on a denaturing polyacrylamide gel and exposed to an X-ray film. Differentially expressed genes between sample X and Y were identified.

Table 2-1. Anchored primer sequence for reverse transcription used in DD-PCR

Primer ID	RT primer sequences
H-T_{11}G	5'–AAGCTTTTTTTTTTG-3'
H-T_{11}A	5'-AAGCTTTTTTTTTTA-3'
H-T_{11}C	5'-AAGCTTTTTTTTTTC-3'

PCR amplifications were subsequently done using combinations of each original anchored oligo-dT primers from the RT with either one of the two arbitrary primers (H-AP_1 or H-AP_2; RNA image kit, GenHunter) giving a total of six different amplifications (Table 2-2). The amplifications were done in the presence of ^{33}P-dATP (GE Healthcare) in order to label the amplicons. The cycling parameters for PCR amplifications were: 94°C for 30 sec, 40°C for 2 min, 72°C for 30 sec for 40 cycles, followed by 72°C for 5 min. Radiolabelled PCR products were then separated on a 6% denaturing polyacrylamide sequencing gel for 3.5 hrs at 60 watts. After electrophoresis, the gel was blotted on a piece of 3 mm Whatman paper and covered with a plastic wrap, dried in a gel drier (Bio-Rad Laboratories, Inc., Toronto, ON) at 80°C for 1 hr under vacuum (Bio-Rad Laboratories, Inc.). The gel was then exposed to an X-ray film (Kodak, BioMax, U.S.A)

for 2 days. After developing the film, the differentially displayed bands were identified and excised from the gel.

Table 2-2. Primer pairs sequences used for the PCR reaction in DD-PCR.

Primer Combination	Primer Sequence
$H\text{-}AP_1$ $H\text{-}T_{11}G$	5'-AAGCTTGATTGCC-3' 5'–AAGCTTTTTTTTTTG-3'
$H\text{-}AP_1$ $H\text{-}T_{11}A$	5'-AAGCTTGATTGCC-3' 5'-AAGCTTTTTTTTTTA-3'
$H\text{-}AP_1$ $H\text{-}T_{11}C$	5'-AAGCTTGATTGCC-3' 5'-AAGCTTTTTTTTTTC-3'
$H\text{-}AP_2$ $H\text{-}T_{11}G$	5'-AAGCTTCGACTGT-3' 5'–AAGCTTTTTTTTTTG-3'
$H\text{-}AP_2$ $H\text{-}T_{11}A$	5'-AAGCTTCGACTGT-3' 5'-AAGCTTTTTTTTTTA-3'
$H\text{-}AP_2$ $H\text{-}T_{11}C$	5'-AAGCTTCGACTGT-3' 5'-AAGCTTTTTTTTTTC-3'

2.1.6 Elution and Reamplification of DD-PCR Products

The excised portions of gel containing the bands (attached to filter paper) were placed in ddH_2O (100 µl) for 10 min to hydrate the polyacrylamide gel. The cDNA was then diffused out of the gel by boiling the gel slice for 15 min in a capped microfuge tube. The microfuge tube was then spun for 2 min to pellet the gel and paper debris. The

supernatant was transferred to a new microfuge tube and the cDNA was precipitated by the addition of 100% ethanol (450 µl) in the presence of 0.3 M sodium acetate (10 µl), and 10 mg/ml glycogen (5 µl). The precipitate was pelleted by centrifugation and washed once with ice-cold 85% ethanol (200 µl) and re-dissolved in sterile H_2O (10 µl). A 4 µl aliquot of the eluted cDNA was reamplified by PCR with the same combination of primers and PCR conditions detailed in the DD-PCR section with the exception that at last cycle of the PCR, the extension step, was done at 72°C for 10 min to ensure that all PCR products were full length and 3'-adenylated for further cloning.

To verify whether or not the size of reamplified PCR products was consistent with their size on the DNA sequencing gel and to eliminate multiple bands, the products were separated by electrophoresis on a 1.5% agarose gel. The cDNA bands were cut and purified with the QIAEXII gel extraction kit (Qiagen, Chatsworth, CA). Briefly, gel slices were placed in microfuge tubes and weighed. A 300 µl aliquot of Buffer QX1 and 10 µl of QIAEX II were added to each sample (100 mg) and mixed. The cDNA was then solubilized by incubating the mixture at 50°C for 10 min and pelleted by centrifugation. After the pellet was washed with 500 µl of Buffer QX1 and subsequently with 500 µl of Buffer PE, the cDNA was eluted from the gel with 20 µl of 10 mM Tris-Cl (pH 8.5) and then stored at -20°C until subcloning.

2.1.7 Cloning and Sequencing of Differential Display Products

DD-PCR products were cloned using the TOPO TA Vector Cloning Kit (Invitrogen, San Diego, CA). The purified cDNAs were ligated into the PCR 2.1-TOPO vector (Cloning Kit, Invitrogen; Fig. 2-2). A 2 µl aliquot of this cloning reaction was

added into a vial of One Shot Chemically competent *E. coli* (Invitrogen) and gently mixed. After a 30 min incubation on ice, the cells were heat-shocked at 42°C for 30 sec without shaking, and then cooled on ice. Pre-warmed SOC medium (250 µl; Invitrogen) was transferred to the mixture. The cultures were then incubated at 37°C for 1 hr in a horizontal shaker (200 rpm). The cultures (50 µl and 25 µl) for each transformation were spread on prewarmed LB/AMP plates which contained 40 µl of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (40 mg/ml; X-gal; International Biotechnologies, Inc., New Haven, Conn.) and Ampicillin (50 µg/ml). The LB/AMP plates were then incubated overnight at 37°C.

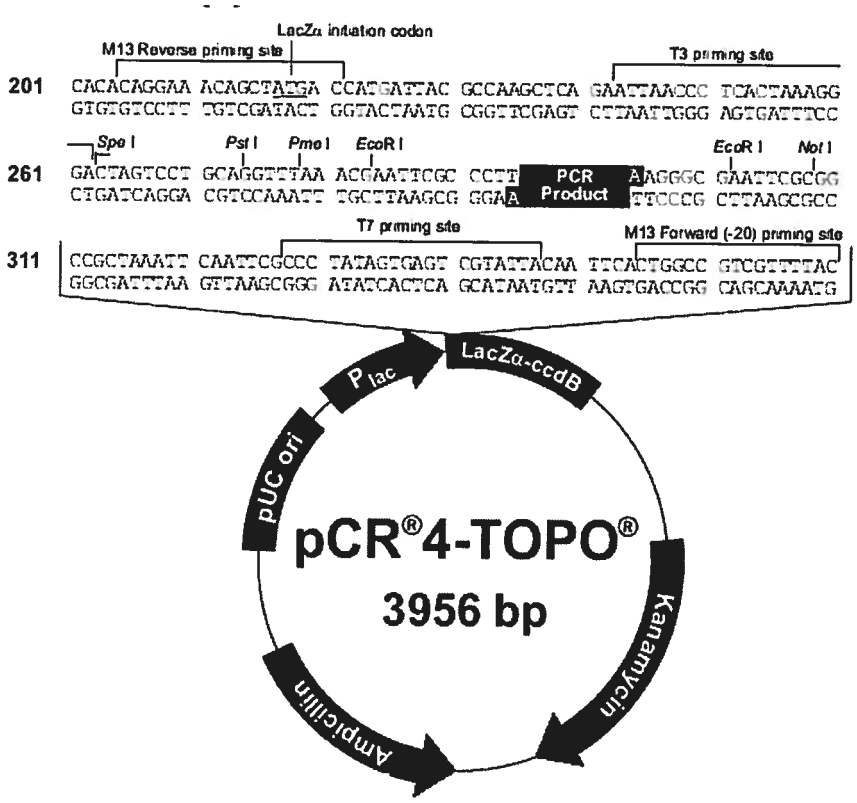


Figure 2-2. A schematic map depicting the features of pCRII-TOPO vector. Shown here are the genes coding for Ampicillin, kanamycilin, pUC ori, Piac and LacZα, and the digestion sites of several restriction enzymes (Adapted from the protocol of the TOPO TA Vector Cloning Kit, Invitrogen).

Single white colonies were picked from plates and inoculated into 2 ml LB medium, which contained ampicillin (50 μg/ml). The culture was left at 37°C to grow overnight. Plasmids containing the cDNA inserts were then extracted from the bacteria using the Plasmid Miniprep method (Sambrook and Russell, 2001) as follows: 1.5 ml of culture was centrifuged at 10,000x g for 15 min and the supernatant discarded. The cell pellet was resuspended in 150 μl of solution I (25 mM Tris-Cl, pH 8.0, 10 mM EDTA, pH 8.0 and 50 mM Glucose). Solution II (300 μl; 0.2 N NaOH and 1% SDS) was then added and the mixture was left at room temperature for 5 min before adding 225 μl of solution III (3 M CH_3COOK, pH 8.0). The mixture was then incubated on ice for 10 min, centrifuged at maximum speed for another 10 min and the plasmid DNA precipitated from the resulting supernatant by the addition of 1 volume isopropanol. Precipitated DNA was washed with 70% ethanol, dried and dissolved in 20 μl of 10 mM Tris-Cl, pH 8.0. RNA was degraded by the addition of 20μg/ml RNase A (Sigma Designs, Inc. CA). The extracted plasmid (10 μl) was digested with the appropriate restriction enzyme (*Eco*RI, *BamhI* or *EcoRV*; GE Healthcare). The digested products were then electrophoresed on a 1.5% agarose gel to determine the size of the cDNA.

Once the correct clones were identified, glycerol stocks were prepared for long-term storage. For this step, 850 μl of a single colony culture was mixed with 150 μl of sterile glycerol and stored at -86°C.

2.1.8 Computer Analysis for Sequence Homology

The cloned cDNAs were sequenced at the Sheldon Biotechnology Centre (McGill University). The cDNA sequence was determined using the dideoxy chain termination

method (Sanger et al. 1977). Sequencing was performed using a 377 Applied Biosystems Automatic Sequencer (Perkin Elmer-Applied Biosystems). The resulting sequences were analysed for similarity by comparing them with known genes or ESTs using the BLAST nr or EST database (NCBI: National Centre for Biotechnology Information; http://www.ncbi.nlm.nih.gov/BLAST/). The sequences were also analyzed using the ORF (open reading frame) finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The predicted ORFs were translated into putative proteins and were analysed by BLAST (NCBI). Finally, the genes were classified as matching known genes, matching unknown ESTs, or as being novel genes having no significant homology to any known sequences.

2.1.9 Reverse Dot Blot Analysis

Reverse dot blot was used to confirm differentially expressed genes that were detected by DD-PCR. Genescreen Plus membranes (Du Pont, NEN Research Products, USA) were immersed in 0.4 M Tris solution (pH 7.5) for 5 min, then placed into a Bio-Dot microfiltration apparatus (Bio-Rad) and clamped for cDNA dot blotting. The cDNAs obtained by DD-PCR, were cloned and released from the plasmids by restriction enzyme digest. The cDNAs were then separated on a 1.5% agarose gel and isolated with the QIAEXII kit (Qiagen). The purified cDNA were denatured in a solution of 0.25 N NaOH, 0.5 M NaCl for 10 min at room temperature. The total volume of denatured cDNA was adjusted to 410 µl with a solution of 0.1X SSC, 0.125 N NaOH. A 200 µl aliquot containing 250 ng DNA from each sample was loaded onto the nylon membrane in duplicate. The solution was allowed to remain on the membrane for 30 min, and a light vacuum was applied to the micro-filtration apparatus until the loading buffer was drawn

into the well. The membrane was then removed from the manifold and neutralised by rinsing in a solution of 0.5 M NaCl, 0.5 M Tris-Cl (pH 7.5). The cDNA was cross-linked onto membrane in a vacuum oven at 80°C for 1 hr. GAPDH and β-actin genes were also blotted onto the membrane to determine the hybridisation efficiency and to correct for sample variation.

2.1.9.1 cDNA Probes Synthesis

cDNA probes were synthesised using the ReversePrime^{TM} cDNA labelling kit (GenHunter). Hepatic RNA template (20 µg) was extracted from the (i) control (ii) T_3- and (iii) Na-Ipodate-treated fish. The reaction mixture consisted of 5X RT buffer (8.0 µl), 250 µM dNTP-dCTP (6.0 µl), 2 µM primer oligo dT_{20} (1 µl), radioactive ^{32}P-dCTP (5 µl; 3,000 Ci/mmol, GE Healthcare) and MMLV reverse transcriptase (4 µl; 100U/µl). The reaction was carried out at 65°C for 5 min, 37°C for 60 min, followed by 75°C for 5 min. The unincorporated ^{32}P-dCTP were removed from the labelled probes using NICK Columns (GE Healthcare). Each labelled probe was counted in a scintillation counter to adjust the amount of labelled probed used for the hybridisation

2.1.9.2 Hybridisation

Three membranes (one for each experimental group) containing the dot blots, were soaked in 2X SSC buffer for 1 min and placed in one of three different hybridisation bottles containing 10 ml of prehybridization buffer (1 mM EDTA, 6X SSC, 0.5% SDS, 5X Denhart's solution and 100 µg/ml denatured salmon sperm DNA). Prehybridization was done at 65°C for 2-4 hrs, followed by the addition of the denatured cDNA probes

(10×10^6cpm) from each of three experimental groups (control, T_3 and Na-Ipodate group). Hybridisation was carried out overnight (16-18 hrs).

Following the hybridisation, the membranes were washed in 2X SSC for 5 min at room temperature. This was followed by 3 washes in a solution containing 2X SSC, 1% SDS at 65°C for 30 min. After each wash, the background radioactivity of the membrane was monitored with a Geiger counter. The membranes were exposed to a phosphorous screen for 4-7 days. The screen then scanned in a phosphoImager (Molecular Dynamics, Sunnyvale, CA). The signal intensity of each spot on the membrane was quantified using the ImageQuant software (Molecular Dynamics).

2.1.10 Northern Blot Analysis

Northern blot analysis was used to determine the tissue distribution of differentially expressed genes. Liver, muscle, kidney, intestine, ovary, testis, brain, heart and spleen were sampled from adult charr at the early phase of gametogenesis (June 2003; n=10) at ISMER, UQAR. Tissues were frozen on dry ice and subsequently transferred on dry ice to INRS-Institut Armand-Frappier. Total RNA was extracted from all samples (9 tissues x 10 fish). To reduce individual variations, RNA was pooled (n=2) for each tissue. Equal amounts of two samples (100 mg) of each tissue were pulverised to a fine powder in liquid nitrogen with a mortar and pestle. RNA extraction was done using the guanidium isothiocynate protocol (Sambrook and Russell, 2001).

2.1.10.1 RNA Blotted on the Membrane

Total RNA (20 μg) from liver, muscle, kidney, intestine, ovary, testis, brain, heart and spleen were loaded on 1.5% agarose formaldehyde gels in triplicate and separated by electrophoresis. The fractionated RNA was transferred onto Genescreen Plus membranes (Du Pont, NEN Research Products, USA). The membranes were then washed in 0.5 M Tris–HCl (pH 7.5) and the RNA was cross-linked onto membrane in a vacuum oven at 80°C for 1 hr.

2.1.10.2 cDNA Probes Synthesis

To generate radiolabelled cDNA probes, a Ready-To-Go DNA Labelling kit (GE Healthcare) was used according to the manufacturer's instructions. Briefly, 25-50 ng differentially expressed cDNA's obtained from DD-PCR reactions, were dissolved in TE buffer (45 μl) and denatured by heating for 2-3 min at 95-100°C. The denatured cDNA was then added to the tube containing the reaction mixbead. The solution was mixed and incubated at 37°C for 30 min. The reaction was done in the presence of 50 μCi ^{32}P-dCTP (3000 Ci/mmol, GE Healthcare). After cDNA probe synthesis, the unincorporated ^{32}P-dCTP was removed using NICK columns (GE Healthcare). The amount of labelled probe was determined with a scintillation counter.

2.1.10.3 Hybridisation

The membranes containing the fractionated RNA were prehybridized in buffer (1% SDS, 10% Dextran sulfate solution, 1M NaCl, DEPC H2O, 50% formamide, and 100 mg denatured salmon sperm DNA) for 2-4 hrs at 42°C. The labelled probes ($10x10^6$

cpm) were denatured and added to the prehybridisation buffer. Hybridisations were carried out overnight. The membranes were then washed twice in wash solution I (2X SSC, 1% SDS) for 30 min, and then for 15 min in wash solution II (0.1X SSC, 1% SDS) at 42°C. The membranes were exposed to a phosphorous screen in a PhosphoImager cassette for 4-7 days and screen were then scanned with a PhosphoImager SF (Molecular Dynamics, Sunnyvale, CA). The signal intensities were quantified using the ImageQuant software (Molecular Dynamics). Membranes were then stripped of the probe by boiling in buffer (1% SDS and 0.1X SSC) for 20 min to eliminate the residual radioactivity. After checking with a Geiger counter, the membranes were exposed to phosphorous screens in PhosphoImager cassettes for 4 days. They were again scanned and checked for probe elimination. The stripped membranes were subsequently hybridised with an end-labelled 18S rRNA probe to standardise for RNA loading.

2.1.11 Statistical Analysis

Signal intensities obtained by reverse dot blot were normalized to GAPDH and β-actin. Northern blot results were normalized to the 18S rRNA signal. Means were compared by one-way ANOVA. P values < 0.05 were considered significant.

SECTION II: GENES EXPRESSION PATTERNS IN FAST AND SLOW GROWING FISH

2.2.1 Aquaculture and Fish Sampling

The Laval strain of charr used in this project originates from an anadromous population of brook charr from the Laval River (Quebec). A genetic selection program at the Station Aquicole de Pointe-au-Père in which charr were followed over three generations provided the biological material used for the present study. Twenty families from a F_3 generation produced in captivity were surveyed from hatch to 22 months of age (Andréane Bastien, Ph.D. student at ISMER). All fish were raised under similar conditions and the average length and weight of the fish were compared at different periods of development (Bastien et al., non-published data). Based on weight, fish (at 15 months age) from two families exhibiting different growth characteristics were sampled to investigate differences in gene expression between fast and slow growing fish (Figure 2-3). The two families that we used were Family 11 and Family 13. Family 11 represents one of the families exhibiting an elevated average weight, while Family 13 had the lowest average weight among the 20 families surveyed (Figure 2-3).

At the time of sampling (April 2003), fish had been maintained in 500 L tanks of fresh water, under natural photoperiod and temperature conditions. Fish were fed commercial food pellets (1% of their body weight per day). Eight immature fish (at 15 monthes age) from each of the two families were randomly selected from the tanks. Fish were anaesthetised with 0.18% 3-aminobenzoic acid ethyl ester (MS-222, Sigma, USA), weighed and measured. The liver was excised and immediately frozen on dry ice and

stored at -80°C. Samples were then transferred on dry ice to INRS-Institut Armand-Frappier where they were stored at -86°C until they were used for microarray and semi-quantitative PCR experiments.

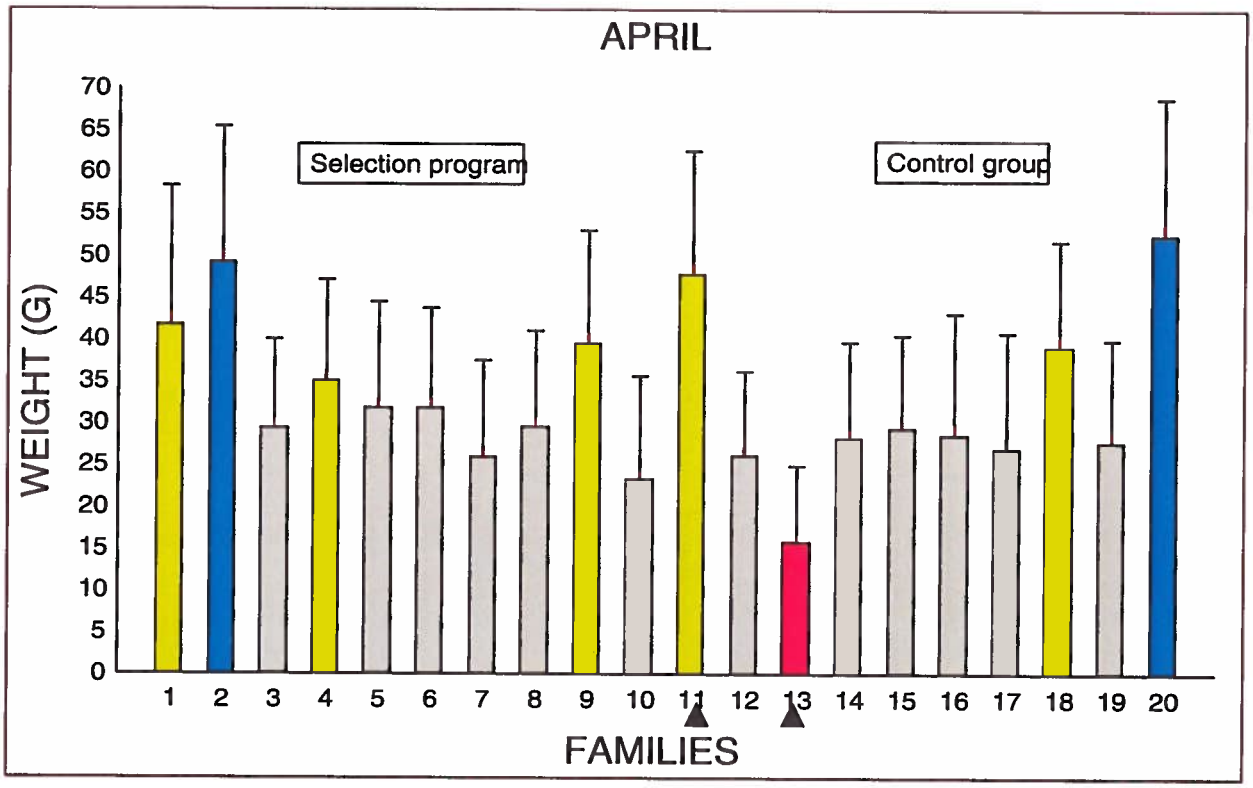


Figure 2-3. Average fish weight of 20 families of brook charr (at 15 months age) raised at the Station aquicole de Pointe-au-Père (Bastien et al. unpublished data). n=100 per family; different colors represent statistically different average weight based on Tukey mean comparisons test ($\alpha = 0.05$). Colors indicate homogeneous groups. Ten families (1-10) were from selection program. The selection was based on growth and absent of precocious sexual maturation. Ten families (11-20) were from control group (random cross).

2.2.2 RNA Preparation and Experimental Design

RNA extractions were done using the Agilent Total RNA Isolation Mini Kit (Agilent Technologies, Palo Alto, CA, USA). In order to minimise inter-animal variability, RNA was pooled (n=2). Each pool consisted of a balanced contribution of

RNA from two different fish of the same group selected randomly. Four RNA pools were prepared from each group. Frozen livers (100 mg) were homogenised in a glass tissue grinder with 2 ml of lysis solution (Agilent Total RNA Isolation Mini Kit, Agilent) for 30 sec. The homogenate was centrifuged through a mini prefiltration column for 3 min at 16,000x g. This step ensured complete homogenization of the tissue and removed all cellular contaminants. An equal volume of 70% ethanol was added to the filtrate, and mixed until the solution appeared homogenous. The ethanol/lysis mixture was added to the mini isolation column (Agilent Total RNA Isolation Mini Kit) and centrifuged for 30 sec at 16,000×g. The flow-through was discarded and the RNA-loaded column was placed in the collection tube. The column was washed twice with 500 µl of the provided wash solution and then centrifuged for 30 sec at 16,000×g. After ensuring that the mini isolation column was dry, the RNA was eluted into a 1.5 ml RNase-free collection tube using elution buffer.

RNA quality and quantity were analyzed using Lab-on-a-Chip technology with RNA 6000 Nano LabChip kit (Agilent). The system automatically calculates the ratio of ribosomal RNA in total RNA samples and shows the percentage of ribosomal impurities in mRNA samples. The highest quality RNA was then used for microarray analysis and semi-quantitative RT-PCR experiments. To minimise technical variability, all targets were synthesised in one round and each hybridisation experiment was conducted as simultaneously as possible. Each hybridisation experiment included dye swaps to compensate for cyanine dye effects.

2.2.3 cDNA Probes Synthesis and Purification

cDNA probes were synthesised and labelled using the Agilent Fluorescent Direct Labelling Kit (Agilent) according to the manufacturer's instructions. Briefly, 10 µg of total RNA samples from either fast or slow growing fish was reverse transcribed and their corresponding cDNAs were independently labelled by either Cyanine (Cy3)-dCTP or (Cy5)-dCTP (1.0 mM; Perkin Elmer). Finally, 1.0 µl RNase I was added to each reaction and incubated at room temperature for 30 min to degrade contaminating RNA. Cy3 and Cy5 -cDNA reactions were combined for each microarray hybridization and purified using the QIAquick PCR purification kit protocol (Qiagen). This step was done to remove unincorporated dye from the labelling mix and to reduce background on the microarray. The purified probes were then used for hybridization. The experiment was repeated and at each time point, dye-swap hybridizations were done (The cDNA sample from fast growing fish labelled with Cy5 and the cDNA sample from slow growing fish labelled with Cy3 were combined and hybridized on the first microarray. Conversely, the cDNA sample from fast growing fish labelled with Cy3 and the cDNA sample from slow growing fish labelled with Cy5 were combined and hybridized on the second microarray (Figure 2-4).

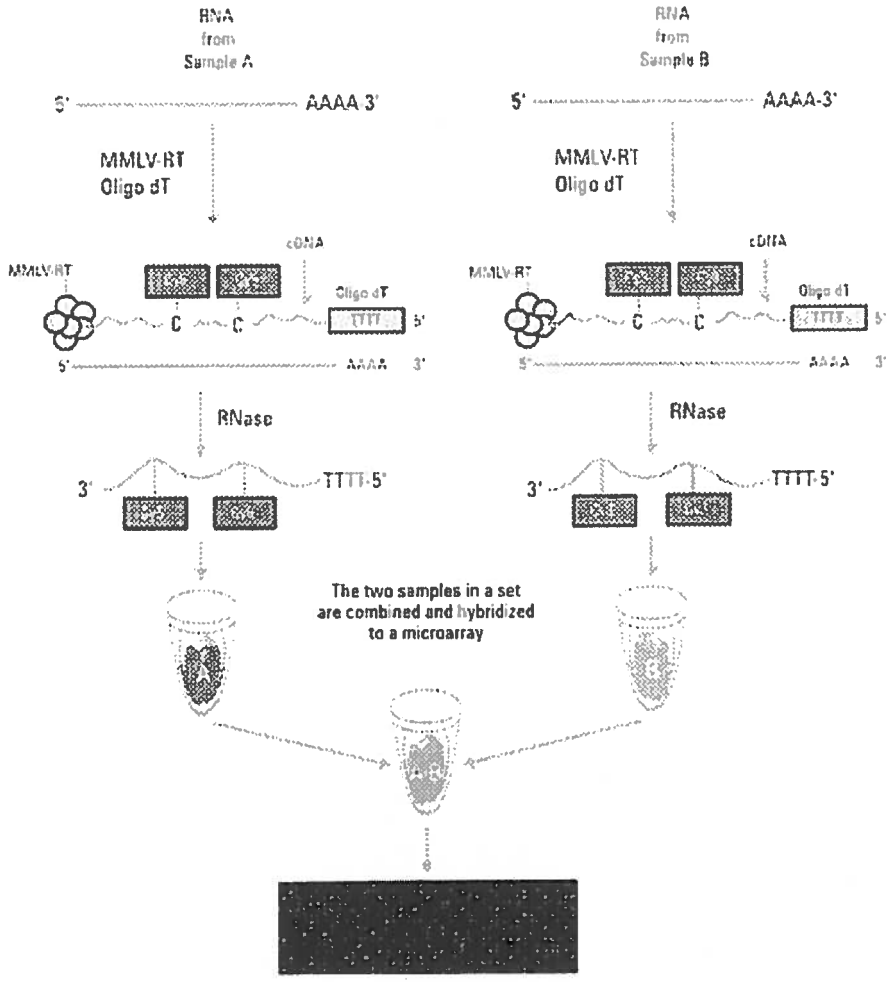


Figure 2-4. The procedure of RNA labelling and hybridizing to a microarray. Adapted from protocol of Agilent Fluorescent Direct Labelling Kit (Agilent). Cy3 was labelled with one of the RNA samples (control) and Cy5 was labelled with another RNA sample (treated). The two labelled samples were combined and hybridised to the cDNA microarray.

2.2.4 cDNA Microarray

The GRASP (University of Victoria) cDNA microarray used for this study contained 16,006 cDNA's which included 13,421 Atlantic salmon and 2,576 rainbow trout cDNA's and ESTs. These cDNA's have been selected from a wide variety of tissues at different developmental stages. The microarray was purchased from the University of Victoria Centre for Biomedical Research (http://web.uvic.ca/cbr/grasp). The binding of

cDNAs on the microarray to transcrips from other fish species has been reported (Von Schalburg et al., 2005).

2.2.5 Microarray Preparation and Hybridisation

To prepare the microarrays for hybridisation, the arrays were washed twice for 5 min in 0.1% SDS and 5 times for 1 min in MilliQ H_2O. The arrays were then immersed for 3 min in 95°C MilliQ H_2O, and dried by centrifugation at 500x g for 5 min.

After the labelled cDNA probe (80 µl) was denatured, it was added to the hybridisation solution which contained Arabidopsis control genes (25 µl) and 2x hybridisation buffer (105 µl; Agilent). The hybridisation mixture was applied directly to the gasket slide. The active side of the microarray was placed on top of the gasket slide to form a sandwiched slide pair. This was then placed on a microarray hybridisation chamber (Agilent microarray hybridisation chamber kit G2534A) and sealed. Hybridisations were done for 17 hrs at 60°C in a hybridisation oven. The hybridization gasket was removed from the array slide at 60°C in 1X SSC, 0.2% SDS solution. The hybridised arrays were washed once in a solution of 2X SSC, 0.1% SDS at 60°C. This was followed by two washes for 5 min in a solution of 2X SSC, 0.1% SDS; 2 washes for 5 min in 1X SSC and 2 washes for 5 min in 0.1X SSC at room temperature. Microarrays were then dried by centrifugation as described above.

2.2.6 Microarray Analyses

After washing, the microarrays were immediately scanned with a ScanArray Express (Perkin-Elmer) array reader at a resolution of 10 µm, and photomultiplier tube

(PMT) of 75 (Figure 2-5). After locating each spot on the slide, a digital record of the green and red fluorescence signal at each point on the array was determined by the image analysis software. The median and mean signal values were recorded for Cy3 and Cy5 separately. Normalisation ratios of Cy3/Cy5 were calculated for individual target genes. A scatter plot graph was generated to display the results of gene expression from each microarray slide. These graphs were used to evaluate the success of the experiment. In our study, image analysis was performed using the ImaGene 5.5 software (BioDiscovery, El Segundo, CA). Automatic and manual flagging were applied to localise poor quality spots (< 1.6 times above background). These spots were excluded from subsequent analysis.

Further analyses of the microarray data were performed using GeneSpring 7.0 (Agilent) software. Lowess normalisation per spot and per chip was carried out to normalise the data between arrays. The large quantities of expression data generated were sorted by filtering on flag, filtering on experiment level and advanced filtering. Finally, the fold change threshold was set at two folds or greater difference in Cy5/Cy3 ratio (<0.5 or > 2.0) for each microarray used in our study.

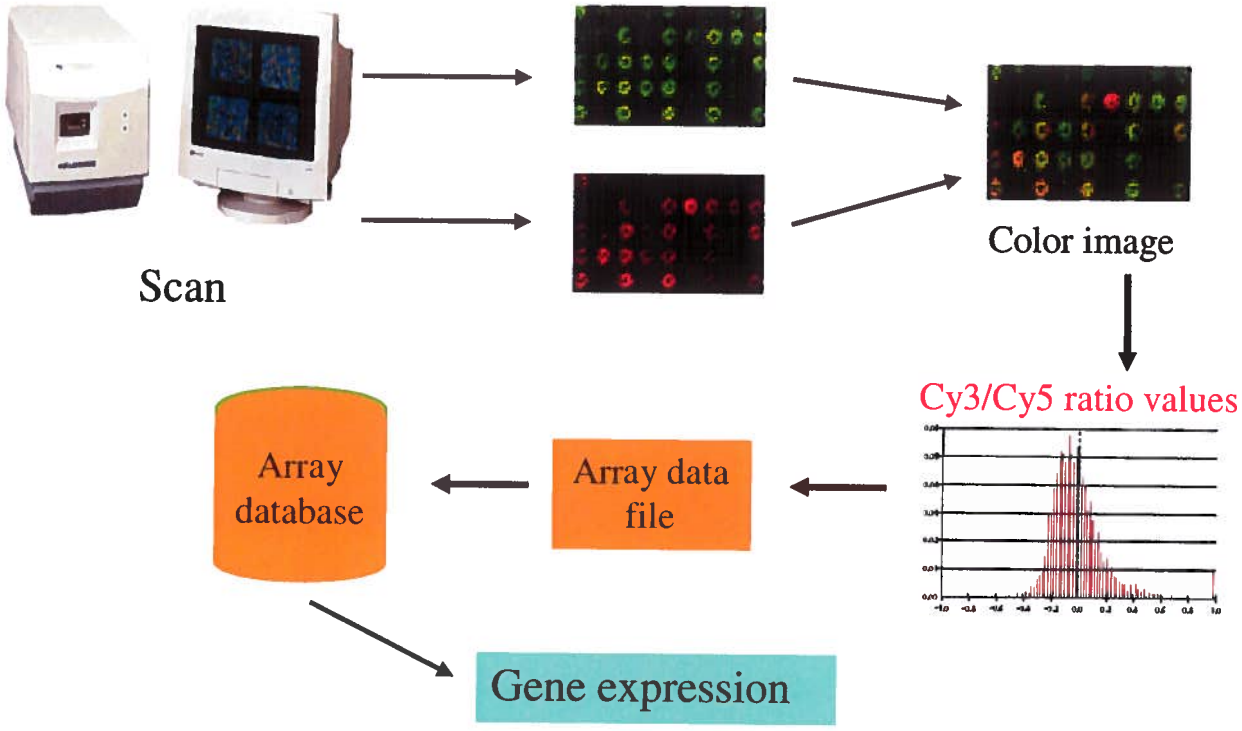


Figure 2-5. The process of gene expression analysis. Microarray technology makes it possible to simultaneously study the expression of thousands of genes in a single experiment. After hybridization, Cy3 and Cy5 labelled probes are scanned using different lasers and a composite colour image is generated. Cy3/Cy5 ratio values are generated and gene expression is sorted from the array database.

2.2.7 Semi-quantitative RT-PCR

Primer Selection and Tm Determination

Primer pairs were designed for each gene using the Primer 3 software (http://www-genome.wi.mit.edu). The sequences of the primers used for PCR amplification have a major effect on the specificity and sensitivity of the PCR reaction. We designed the PCR amplification primers, according to laboratory set guidelines. Primers were typically 18-25 nucleotides in length, melting temperatures for primers were in the 57-65°C range. The GC content of the primers was between 45 and 60% and the length of the predicted amplification products were between 200 and 500 bp. Primer

concentration is another important parameter for the semi-quantitative PCR. Lower concentrations of primer may result in amplifications that would not be quantitative, whereas higher concentrations may leave a large amount of unused primers which could give rise to non-specific amplification products. In our study, the primer concentration used was 0.5 μM. All designed primer pair sequences were analyzed with Blast (NCBI) to determine the specificity of the primers. The primers were finalized, when both primer sequences showed homology to the same gene. The sequences of the forward and reverse primers, annealing temperature (Tm), products size for this study are listed in Table 2-3. The primers were synthesised by Integrated DNA Technologies (Coralville, IA 52241 USA).

Table 2-3. Semi-quantitative PCR reamplified genes, size, Tm and primers Genes identified by microarray were confirmed by semi-quantitative PCR.

Gene name	Clone ID	PCR size (bp)	Tm (°C)	Primer sequences
Apolipoprotein	CB493958 [P]	542	60	5'-3' TATGGCACAGTCACTGACCAACCT 3'-5' TGCTTCAGAAGAGTGAGGAGCTGA
ATP syntheses lipid-binding protein	CB493213 [P]	574	55	5'-3' ATGGGAACATAAAAAGGCAGGATT 3'-5' GCTAAGTTTGTCACCTCACCTGCT
Cadherin 1, epithelial	CB517027 [P]	586	58	5'-3' ACTCTTCAGAAGCAAGCAAGAAAA 3'-5' TCTAACTGGACTGCCAGAATGAAC
TSC-22-like [Homo sapiens]	CB509522 [P]	379	55	5'-3' GTTTTCTGCAGTTGGACAGAATCA 3'-5' CCCCCTACAGGGTAGAGATGGTAT

2.2.7.1 RNA Extraction

The method used for RNA extraction and semi-quantitative RT-PCR has been described above (2.2.2 RNA preparation and experimental design).

2.2.7.2 RT-PCR

Total RNA was isolated from the livers of fast and slow growing fish and treated with DNase. cDNAs were synthesised from 0.5 μg of total RNA template by RT, as described above. The PCR primer pairs used for each gene are described in Table 2-3. PCR was carried out in a total reaction of 40 μl, containing 0.5 μM of 3' and 5' primers for the gene, 0.5 mM dNTP, 0.5 mM $MgCl_2$, and 2.5 U Taq (DNA polymerase). The PCR reaction was started with 5 min incubation at 94°C, followed by 30 sec at 94°C, 1 minute of annealing at the temperature indicated for the specific gene (Table 2-3), 1 min at 72°C and finally 7 min at 72°C. Linear amplification range for each gene was first determined by performing PCR reactions at 15, 20, 25, 30, 35, and 40 cycles.

2.2.7.3 Quantitative Analysis

The PCR products were electrophorised on 1.5% agarose gels. The intensities of the bands were evaluated with the Multi-Analyst software (Bio-Rad Laboratories, Inc. CA, USA), and plotted to establish the linear phase of amplification. PCR reactions were then done for the two groups within the linear phase of the amplification. RT-PCR values are presented as a ratio of the specified gene normalized to β-actin.

CHAPTER 3. RESULTS

SECTION I: IDENTIFICATION OF TH-DEPENDENT GENE EXPRESSION

3.1.1. Serum T_3 Concentrations

In order to identify TH-dependent genes, we treated fish with either T_3 or Na-ipodate, an anti-thyroid drug. After 7 days of treatment, serum T_3 levels were measured by RIA (Figure 3-1). T_3 concentrations were significantly higher in the T_3-treated group (269.4 ± 20 ng/ml) as compared to controls (2.24 ± 0.2 ng/ml). In the Na-ipodate-treated group, T_3 levels were significantly lower (0.85 ± 0.05 ng/ml). These results confirm that we have euthyroid (control), hyperthyroid (T_3-treated) and hypothyroid (Na-ipodate group) fish.

3.1.2. Effects of Thyroid Hormone State on Fish Growth

To evaluate the growth of immature brook charr after treatment with T_3 or Na-ipodate, individual body weight and fork length were measured in the three groups before and after treatment. SGR and CF were also calculated (Table 3-1). There were no significant differences in mean length and weight between the three experimental groups neither at the beginning nor at the end of the treatments. There are no significant differences between any experimental groups after 7 days treatment.

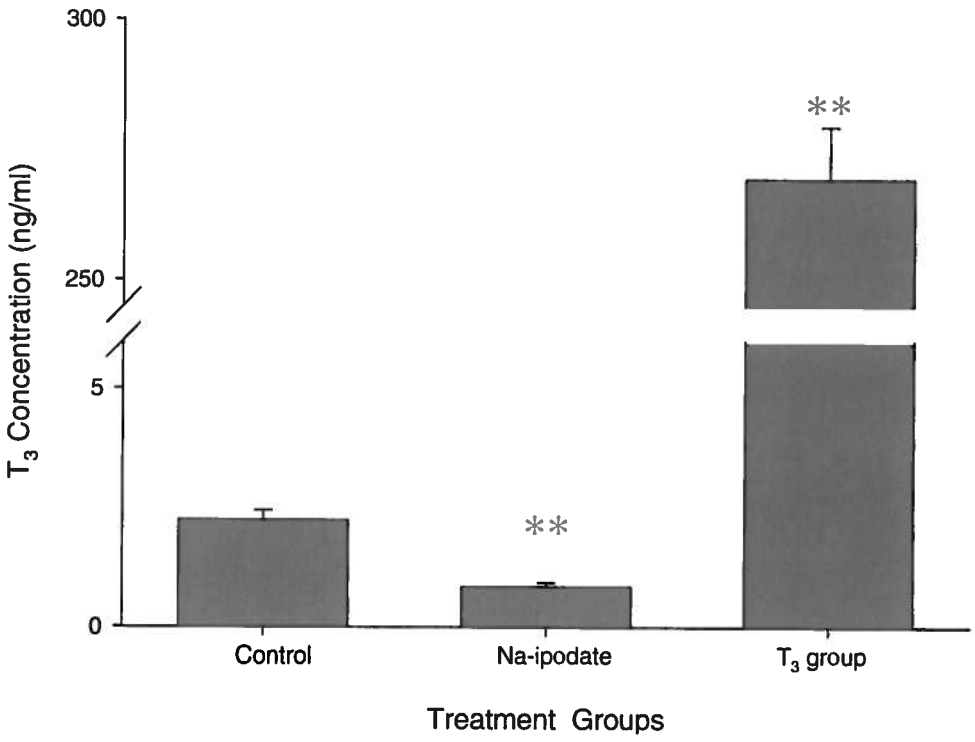


Figure 3-1. Serum T_3 concentration in fish treated to stimulate or to inhibit thyroid function. ** indicate significant difference from control, $p < 0.01$.

The CF was used to monitor the overall condition of the fish. There were no significant differences in CF between the three experimental groups at beginning and at the end of treatment. There were also no significant differences in CF between each of the experimental group.

Table 3-1. Effects of THs on fish weight, length, condition factor and specific growth rate

	Day 1			Day 7			
Group	Weight (g)	Length (cm)	CF	Weight (g)	Length (cm)	CF	SGR (%BW day^{-1})
Control (n=9)	47.7±6.1	17.7±0.6	0.9±0.04	47.7±7.4	17.5±0.6	0.9±0.07	0.1±0.22
T_3-Treated (n=10)	48.3±9.4	17.8±1.3	0.8±0.05	49.9±9.7	17.6±1.2	0.9±0.05	0.2±0.17
Na-ipodate Treated (n=9)	49.1±8.5	17.8±0.8	0.9±0.05	50.6±8.3	17.8±0.8	0.9±0.05	0.1±0.18

*(One control fish lost 5 g in weight over the 7 days; one fish was moribund in Na-ipodate group, these fish were excluded when calculated SGR)

3.1.3 Identification of Thyroid Hormone Dependent Genes by Differential Display PCR

DD-PCR was used to identify hepatic TH-dependent genes. The transcripts from the two treatments (T_3-treated group, Na-ipodate-treated group) and control group were compared. Differentially amplified genes were separated by polyacrylamide gel electrophoresis. A representative gel indicating differentially expressed genes is shown in Figure 3-2. The expression of most genes was not significantly altered by either treatment. However some genes were over-expressed in the T_3-treated group, while

others were down-regulated. Likewise genes in the Na-ipodate-treated group were also down- or up-regulated suggesting that these were THs responsive.

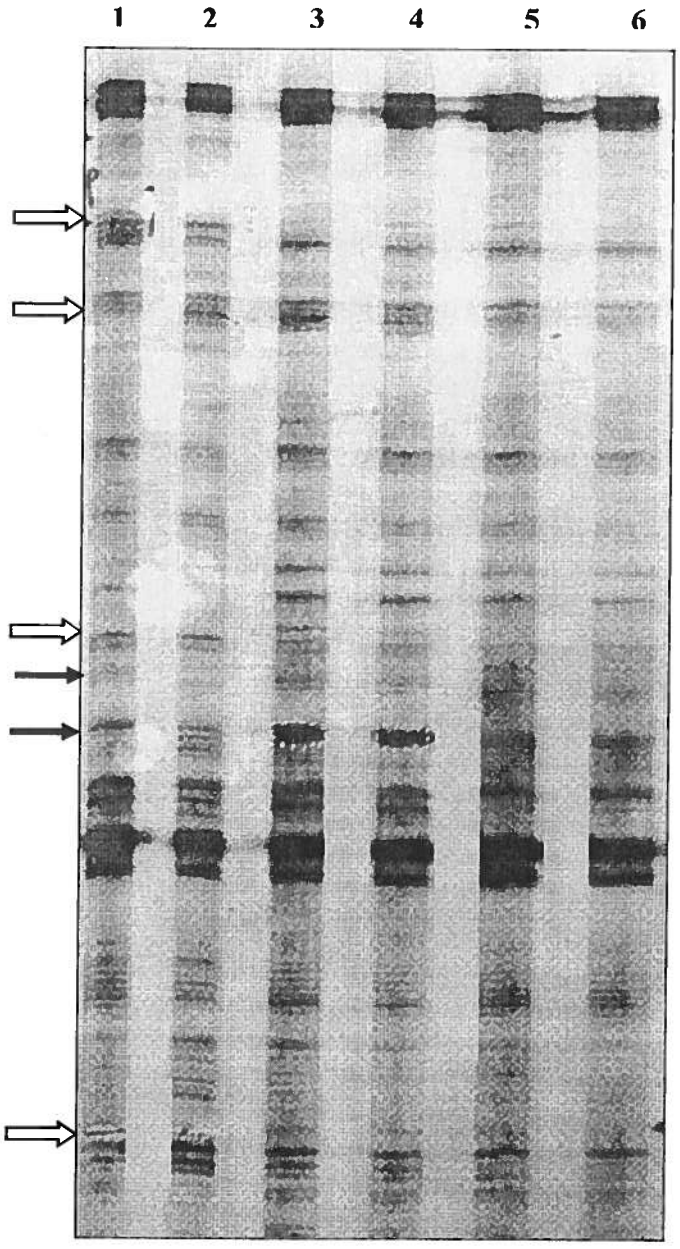


Figure 3-2. Representative gel obtained by DD-PCR derived from brook charr liver. The separated products on this gel were amplified with the H-T_{11}-G and H-AP_1 primers. Lanes 1 and 2 are cDNA from T_3-treated fish; lanes 3 and 4 from Na-ipodate-treated fish; lanes 5 and 6 are from control fish. Open arrows indicate up-regulated genes while black arrows indicate TH-repressed genes.

A total of 135 differentially expressed cDNA bands were identified and extracted from the gels. Of these 135 genes, 96 (71%) were successfully reamplified by RT-PCR with same primer pair combinations (see Table 2-2). The products obtained by PCR were highly reproducible. PCR products were purified on 1.5% agarose gels (Figure 3-3) and their size ranged from 120-600 bp. The number of differentially expressed cDNA, and primers used to generate these are listed in Table 3-2.

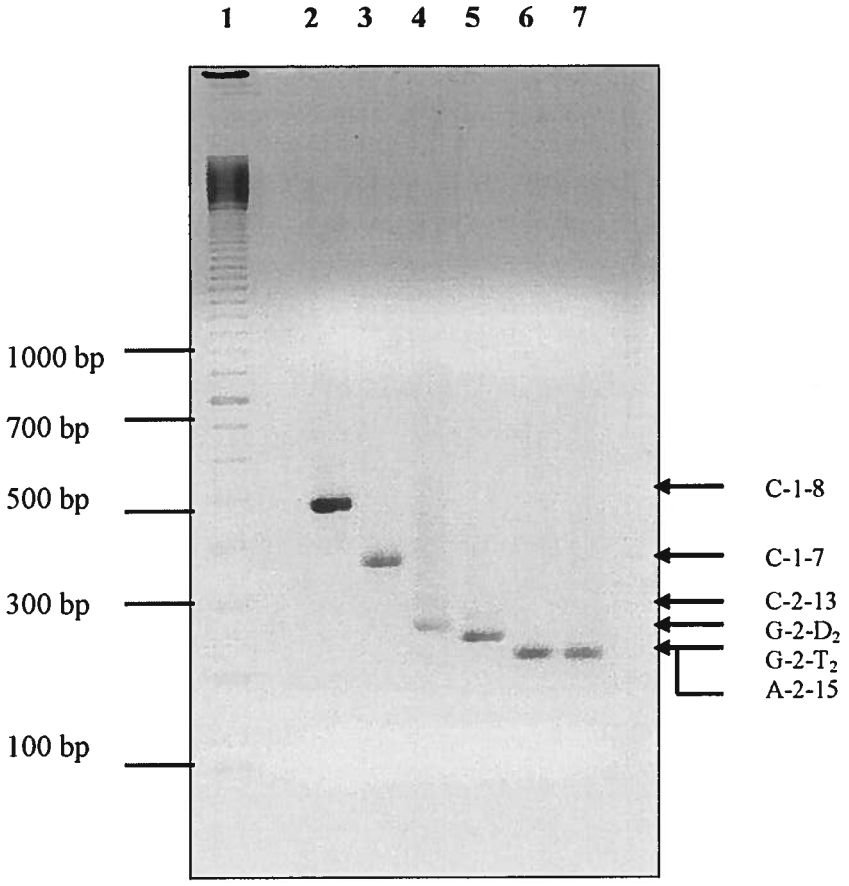


Figure 3-3. Representative gel showing cDNA bands reamplified by PCR and separated on a 1.5% agarose gel. Lane 1 is the DNA ladder. Lanes 2-7 are amplicons obtained from differentially expressed cDNAs with the same primer pair. The gene code is indicated on the right of the gel and corresponds to the genes listed in Table 3-3.

Table 3-2. List of differentially expressed bands extracted from the polyacrylamide gels. The 6 different primer pair combinations utilized for DD-PCR, the number of differentially expressed cDNA's obtained, the number of bands that have been reamplified and the range in amplified size of the cDNA's are listed.

Primer	No. of Bands extracted	No. of bands reamplified	Size range (bp)
$H\text{-}T_{11}\text{-}G$ $H\text{-}AP_1$	14	9	120-600
$H\text{-}T_{11}\text{-}G$ $H\text{-}AP_2$	20	19	120-400
$H\text{-}T_{11}\text{-}C$ $H\text{-}AP_1$	23	20	150-600
$H\text{-}T_{11}\text{-}C$ $H\text{-}AP_2$	25	25	120-600
$H\text{-}T_{11}\text{-}A$ $H\text{-}AP_1$	30	19	120-600
$H\text{-}T_{11}\text{-}A$ $H\text{-}AP_2$	23	4	130-590
Total	135	96	

3.1.4 Subcloning the Differentially Expressed cDNA

Of the 96 cDNA's that were reamplified, 20 cDNA's were randomly selected for cloning. Plasmid cDNA was extracted from three transfected positive colonies for each clone. The artificial products, which were the colonies with an inappropriate size, could be detected from this step and were removed from subsequent analyses. Inserts of the correct size were identified by electrophoresis on a 1.5% agarose gel (Figure 3-4). Three colonies with appropriate size inserts were sequenced for each gene. Two genes could not be cloned and therefore 18 cDNA clones were sequenced.

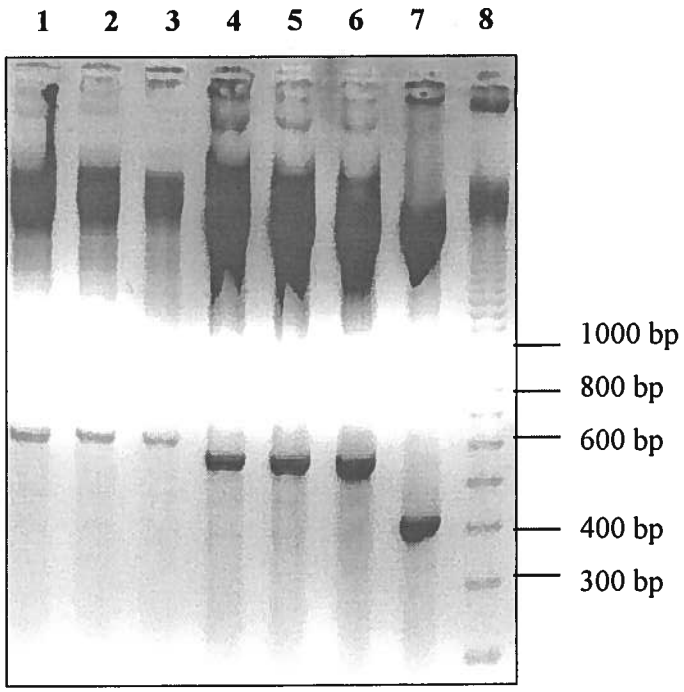


Figure 3-4. Representative gel of restricted plasma cDNA clones separated on 1.5% agarose gel which was used to verify the cDNA clone. Lanes 1 to 3 and lanes 4 to 6 represent three cDNA clones of C-2-2 and G-1-1, respectively. Lane 8 is the DNA ladder.

3.1.5 cDNA Sequence Analysis

The nucleotide sequences of the 18 cDNA clones are shown in Table 3-3. The cDNAs ranged in size from 249 to 568 bp. All cDNA sequences were flanked by the sequences of the arbitrary and anchored primers. The nucleotide sequences of the cDNA were connected to amino acid sequences using the ORF Finder. The sequences of certain cDNAs such as C-1-3, C-1-8 and C-2-9 were predicted to contain untranslated regions (Table 3-4).

Table 3-3. List of nucleotide sequences of cloned cDNAs. Sequences and size of 18 cDNAs obtained by DD- PCR are listed.

Name	Size (bp)	Cloned cDNA Sequence
C-2-9	336	AAGCTTCGACTGTGGCAGAGAGAGCCATAGGAGCAATCATTGGAGCAGCTGTGGCAGACGCAGCAGCCC AGCCCATGCATTGGATTTACAACCCAGAGCGACTGAAGGAGGTTCTGTCAGATCTGGAACCGTGTCCAG AGTTCCGCCCTCAGTCAGCTAACCCGTTCTACCGCAGAGAGACCGGAGAGCAGACGTGCTACGGAGACC AGGCCTACGTACTGCTAGAGTCACTCAGTCAATGTGGAGGTAGCATACTGACACCCAATAGGACGAACA TCCAATGGCCTAATGTCTTGCTAAATAAAGAACATTTCACGTCGAAAAAAAAAAAGCTT
C-2-11	314	AAGCTTCGACTGTTACATGCACTGACATCACTCTTAATACGATTTCATAGGAATCAGGAATGTGGTTAG CGTCAATGTGCAGGCCTATATAGGCTACATGTAGCCTATGTCGCCTGAATTTCTATTTCTAGAGGAGTT TTAAACATAGCATAAGTGTAAATTATTATTATTATTATTAGTAGTAGTAGTAGTAGTAGTATTTGTAGT ATGTTGTCCTAATGTTATTGAAACGAAACATTACTCATCAGGTGTGTCTGGGCTATCTTGAATCCAATA TAAATTGCACCTATTTTTATGGAAAAAAAAAAAAGCTT
C-2-13	264	AAGCTTCGACTGTAGGTAAGGGTTAAGTGACTAGGCAACAGGATAGATAATAAACAATAGCAGCAATAT TAGACTGTAATGTAAATGCTGTAAATGCACTCTATGTAGTTCTGTCCATGAGCTGTTCTTGTCTATTAA TGTTCTATATTATATCATGTTTTACTGTTTTGTGTGGACCTCAGGAAGAGTAGCTTCTGCTTTTGTAAC AGCTAATGGGGATCCTAATAAAATACCAAAATACCACCTGGAAAAAAAAAAAAGCTT

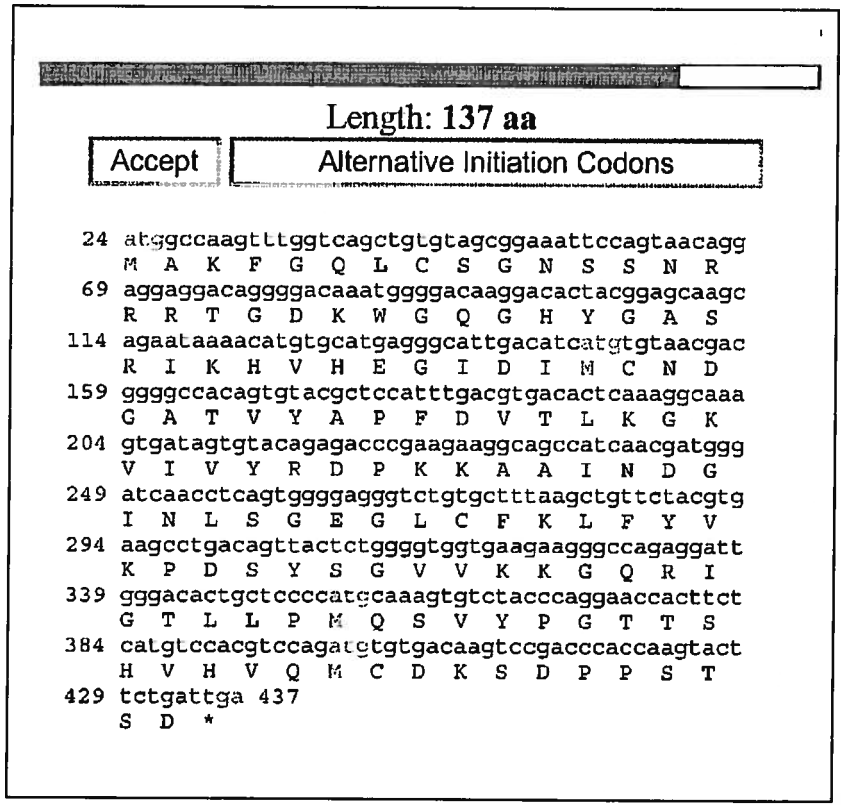
Name	Size (bp)	Cloned cDNA Sequence
G2T1	357	AAGCTTCGACTGTAGCACCAAGGGGTGTGGTCTACTCTAAAATGTGAGTCCCTCCATAATGTCACCTTG GGCCCTATTGACCCTGGTCAAAAGGAGTGCACTATAAAGGGAACGAGGTGCCATGTAGACATCTCTAGC AACGTGCTCGTGTGCCCCCCCCCAGCCTGTTTGCGGTGTCCCACTCAGCCAACCAATGTGCTGACCTCA TCCTGTTTATGCTAATCAATGTTTCTAACGTAATGACGGAGAGCTTGATGTGACCAATAATCACTGGCC TGACGGCCTCGCTGTTCAGCCTGAAACTGATCGGTCAATTTAACTGAAATAAAATGATCCCAAATCCAA AAAAAAAAAGC
G2D2	249	TAAGCTTCGACTGTGTAGATGAAATCGTTTTTCTGTGTTACTACTGGATGACATTAAATCCTGTGAATT AAAATGAATTAAATATTTAAACAAAGAAGCACTGTAATTGAATCAAATGACTCATGCAGGAGTTAGGCA CTGGTGTAGCTGGGATATATTTAGAAATGGAAATACATCTGACTTCATTGTACCTGACGTTTTAAAATC CTGAATAAATTCATTTCAGGCCTGGCAAAAAAAAAAAGCTT
G-1-1	568	AAGCTTGATTGCCACAATCGAGGAGAAGAACAATGGCAACTGTAAACAGATTCTGCAGATCATTGCACT CCAAAATCAAGTGACAAGATTACAGAGGCAAGAGTTAGAGTTCAATCAGTCATCTGAGGCCAAGATTGC TGAGCTAGAGAATCAGCTACAGGAGACGAGAGACCAGCTGAATGAAAAGAACGGCGAGCTGAAAGAAAC TGACAGTAGAATTCCACAACTCATTGCAGAGATCATGGGACTTCGTAACAAACTGACAGAGTTAGAAAG AACATTATCAGAGCTCAAACGGACAAGTGCTGACAAGATGGAAGATCTACAGAGTCTACTGAAGCAGAA GAGCAAGCAGCTTGAAGAAATCACTACACAACTGAGGGCTGTAGATGCCAAAAATGCAGAACAGATTCT GAAGATTATTGAACTCCAGAAAGAAATGAAAGCGATACAAACGGAAGCTTCAAAAGCCAAAGATAAAAA GTGTATCAGAAATCTCTGAGCTACAGAAACAACTTAAGGCCAAAGAGAAGCTGAATGCAGACTAGACTC CAAAAAAAAAAAGCTT
G-1-9	376	TAAGCTTGATTGCCCCCATAGAGGAAGAGGATGATGATGTTCCAGATCTGGTGGAGAATTTCGATGAGG CTTCCAAGGATGAGGCAAACTAAAGAAGAAAAAAAAACAGACCTTCAGGAGTCTCAACAGGACTGGGTT GGATAGGGGCACTAGGAGTTATTTTTTGTTTGTATTTTTATTTCTGAAATGCTATTTTGTGCACTGTGG TGTCTGTGTCCAGTTTGAGTCCGTGAGAGATTGTACGGGTTGTCTGAGGTTCGTGCTCTTCATATTCTC TTCAGCTGTTTCTTCCATGTTACTGAACCTGATTTCTTTGCAGTCTGGCTGAAACCATATCTGGAATAA ACATTTGCTATTGTCAAAAAAAAAAAGCTT
C-1-3	485	AAGCTTGATTGCCAGAGTGTCAGATGGCCAAGTTTGGTCAGCTGTGTAGCGGAAATTCCAGTAACAGGA GGAGGACAGGGGACAAATGGGGACAAGGACACTACGGAGCAAGCAGAATAAAACATGTGCATGAGGGCA TTGACATCATGTGTAACGACGGGGCCACAGTGTACGCTCCATTTGACGTGACACTCAAAGGCAAAGTGA TAGTGTACAGAGACCCGAAGAAGGCAGCCATCAACGATGGGATCAACCTCAGTGGGGAGGGTCTGTGCT TTAAGCTGTTCTACGTGAAGCCTGACAGTTACTCTGGGGTGGTGAAGAAGGGCCAGAGGATTGGGACAC TGCTCCCCATGCAAAGTGTCTACCCAGGAACCACTTCTCATGTCCACGTCCAGATGTGTGACAAGTCCG ACCCACCAAGTACTTCTGATTGATTGGTTAAATAAATAATCAAAATTATCACAATGAAAAAAAAAAAGC TT

Name	Size (bp)	Cloned cDNA Sequence
C-1-4	447	AAGCTTGATTGCCCAATCGAGGAGAACCCAATGGCAACTGTAAACAGATTCTGCAGATCATTGCACTCC AAAATCAAGTGACAAGATTACAGAGGCAAGAGTTAGAGTTCAATCAGTCATCTGAGGCCAAGATTGCTG AGCTAGAGAATCAGCTACAGGAGACGAGAGACCAGCTGAATGAAAAGAACGGCGAGCTGAAAGAAACTG ACAGTAGAATTCCACAACTCAATGCTTAGATCATGGGACTTCGTAACAAACTGACAGAGTTAGAAAGAA CATTATCAGAGCTCAAACGGACAAGTGCTGACAAGATGGAAGATCTACAGAGTCTACTGAAGCAGAAGA GCAAGCAGCTTGAAGAAATCACTACACAACTGAGGGCTGTAGATGCCAAAAATGCAGAACAGATTCTGA AGATTATTGAACTCCAGAAAAAAAAAAAGCTT
C-1-5	450	AAGCTTGATTGCCACAATCGAGGAGAAGAACAATGGCAACTGTAAACAGATTCTGCAGATCATTGCACT CCAAAATCAAGTGACAAGATTACAGAGGCAAGAGTTAGAGTTCAATCAGTCATCTGAGGCCAAGATTGC TGAGCTAGAGAATCAGCTACAGGAGACGAGAGACCAGCTGAATGAAAAGAACGGCGAGCTGAAAGAAAC TGACAGTAGAATTCCACAACTCATTGCAGAGATCATGGGACTTCGTAACAAACTGACAGAGTTAGAAAG AACATTATCAGAGCTCAAACGGACAAGTGCTGACAAGATGGAAGATCTACAGAGTCTACTGAAGCACGA AGAGCAAGCAGCTTGAAGAAATCACTACACAACTGAGGGCTGTAGATGCCAAAAATGCGAACAGATTCT GAAGATTATTGAACTCCAGAAAAACAAAAAAGCTT
C-1-6	399	AAGCTTGATTGCCGCCAACTTGCTCTTTGAAATGATGTGTGTCCTTCCAAAGACAAAATATTATGACTA ATTATGTGATGAGGATATGCACATACTTAATCGAAAGAATAGGACTTTTACTGAACGGATTCAATATGT AATCAAAGTTAATCACCGGTGTGACCATATCACACCAAACAAGGACTGGTAAAGCAGAAAAATAGAATG TCTCTAACATATGAACATCAAGCATTTATTACACACAGCAAGAGGATATGCACTGTAGCAGAACCTTTG AGACATCTGCAAATCAGATACATTTATTTAGTCAACATTGTACCAGGGTTATTAGTATAGTGATTTTGT AACATGTTTTATAATCAAATAAAACAACAGTATTGATGAAAAAAAAAAAGCTT
C-1-7	368	AAGCTTGATTGCCACTGAAAAACAGACTGACACAAGGACCAGAATCCTGTTTGGAGGATCTGAAACAAT GTAAGAAGGAAGAAGATTGGGGAAATACTGAGGGGTGTTTATGTTGATGTGCTCCATTATGTATTAATG AAGGATTGTGAGGGGAGAGGACATCAACTGCCTTTTTAACAGCATCTCAGTCAGTGTTACGGTCTCCCT TAAAGCTGCTTCTGTACTCACACACCCTTTGACATAACTCATTCGCCATGAGTTCAGATGTTCATTTCT AATGATGACATGCATTTTGTTTTAGATATCTTTCTATTTTTAAACAAATTGTTATATACCTAATAAATA CTTGTGTGAAAAAAAAAAAGCTT
C-1-8	478	AAGCTTGATTGCCCTCTACGTAACCTGATCTATTCTAGAATTTGCATCATGGTATATGCATGCCGACCC CAATATGAACCGATTCTTTAAATACCTTCTCCTATTTCTAATTGCTATGATTATCTTAGTAACCGCCAA CAACATATTCCAGCTATTTATTGGCTGAGAAGGAGTTGGCATTATATCGTTTCTCCTTATTGGTTGATG ACACGCCCGAGCTGACGCTAATACAGCTGCCATACAAGCTGTGATTTATAACCGAGTCGGAGACATTGG ACTTATCCTAAGTATAGCATGATTCGCAACAAACCTTAACTCCTGAGAGATTCAACAAATATTTGCCTC CTCAAAAGAGCTTGACCTTACACTTCCCCTCATAGGCCTCATTCTAGCCGCCACTGGTAAATCAGCGCA ATTTGGACTTCATCCGTGACTTCCTTCCGCGATAGAAGGTCCTACGAAAAACAACAAAAGCTT

Name	Size (bp)	Cloned cDNA Sequence
C-1-9	319	AAGCTTGATTGCCCTAATTTTATGTCGTAATGATGTTTTGTGTTCTCTAAAGACGAGGCATCACTCCTC ACGGGTTGTCATTCAGTCAGTTGAACAAGGCTGACCACTGAAGCTAAGAGGACGGGGAGCACAGAGAAT AGCAGGGCTCTGTGTGTGGGTGGTTGTATAATATGGGAAGGGTGTGTGTGTGAATGATTTGGTCAAATA CAGTCAAAAGAAACTTGTCTTTCCCCTCATGTCTCCCTCCCCATTTTCTCATCGGTCTCTTAAATAAAC AAACCTTGATTGGCCCATAACATTTAGAAAAAAAAAAAGCTT
C-2-2	576	TAAGCTTTTTTTTTTTCAAAATAGGTATATTTATAAAGTTCAATATATTTGCAAATTACCAACATCTAA TTATTAAACTATAACTTATTAAATGTTTTTGGGGGGGAATTAAGGGAAGGGAAACGGGGATACCTAGTC AGTTCCACAACTGAATGCATTCAACTGAAATGTGTCTTCCGCATTTAACCCAACCCCTCTAAATCAGAG CGTTGCGGAGTGGGGCTGCCTTAACCGACATCCACGTCATCGGGGAGCAGTTCTTGTTGGGGCTTAAAT TTAAGCACACAATACAAGGGAAGCCTTTTTCTACCCAGTCCTGGTAGCCTAGCAGTCTTTCCTAGAGTA CTGTATCCTAACCCTAGTACACCTGATTTAAAGGAATGGCGCTTGCTGATTAGTTCACATTTGGCAGGT GTGCTAAATACACTATAGAGAATGGCTAGAGGGCCCCAAGGACTGGTTTCAGACCGTAGGACGAAGTAT AGCGTCAAAATACAATAAACACTAGAGAAAATAAAACTATTCTCCAAGGTAGATGCCCTTCAAATGCAC TTTCATAACTCCTTTTTTAACAA
C-2-4	541	AAGCTTCGACTGTAAACTAAAAGCACAGCTATCAATCTTATAAACAAATGTTAGTGTTCAAAAACCATA TTAATAGATTTATTTGTATAAATAATATTTTGTTACATGTAACTTCCAAATGCTAAAGTAAAAATGCTC TTATTGAGGTCATTTTCTTAGTAGGTGGTCAGAAGTCAGCTACTGCAGAGTCGATAAAGTGGCAGGAAA CCATAGTTGAGCCATGCAGCGTCTGAGCAGGGGAACAACTTCCTGCCCCTGTCACCATTAACACGCCCA CAATTGATACTGTTAGGGGGAGGAGAAAGACTCAAAGCACAGAGACTAGAAGAGGAAATGACTGTTTAT ACACCCTCTTGTAGAAACCAGCCATCGTAGTGGAAGAGTCACATGACTTCATAGAAAATGTGGCTTTGC ATTAGAACCCCCTGCCCTCTAGACATGTTTGTGTTGACTCAAAAGTGTCCATAATGTATCAAGTTTCTA AGTTGATACAACGAGAGAATAAAAGAACTGCTGTGCATAGTGAAAAAAAAAAAAGCTT
C-2-7	475	AAGCTTCGACTGTACCAACTGCACCTTGGTGAACCAGTGAGCATGCATACAGGATTATCCACTAAGGGA TGGATGAGGGGTTACAGTGCCCCAAGATGGACGGCCTCAGCTCAGAGGAAACTGTTAGAGAATAATGGA AACTGAAGTTCAGAGATGGGGTCAGAGCAACTTGAGTTAACTACAAGGCTGATTGACTAAAACGTCTGT GTCTGTGGAGAGCTGGAGCACTGTGAACCCAGAACTAAATTATCTTGGGCTATTTTCACAGCCTTTCAT TGTCATCTGTGTCTGACCTAACATCCCTGTGCCTACTAAGTACATTTTTTTGGAATAAAATAATAGGTC CTGTGCTTCCAGCAACCAAAGGCTAAGACTGCTATCAGATAGTCCCCGAAGCCTACAACAGAAATTTCC CTGCTTTCCTATAACCACCTACCTGGGGTTGCCACTCATGTATTTGAAAAAAAAAAAAGCTT
C-2-8	476	AAGCTTCGACTGTACCAACTGCACCTTGGTGAACCAGTGAGCATGCATACAGGATTATCCACTAAGGGA TGGATGAGGGGTTACAGGCCCCAAGATGGACGGCCTCAGCTCAGAGGAAACTGTTAGAGAATAATGGAA ACTGAAGTTCAGAGATGGGGTCAGAGCAACTTGAGTTAACTACAAGGCTGATTGACTAAAACGTCTGTG TCTGTGGAGAGCTGGAGCACTGTGAACCCAGAACTAAATTATCTTGGGCTATTTTCACAGCCTTTCATT GTCATCTGTGTCTGACCTAACATCCCTGTGCCTACTAAGTACATTTCTTTGGAATAAAATAATAGGTCC TGTGCTTCCAGCAACCAAAGGCTAAGACTGCTATCAGATAGTCCCCGAAGCCTACAACAGAAATTTCCC TGCTTTCCTATAACCACCTACCTGGGGTTGCCACTCATGTATTTCGAAAAAAAAAAAAGCTT

Table 3-4. Deduced amino acid sequences of the three cDNAs obtained using the ORF Finder software. (A) The deduced 137 aa. amino acid sequence of C-1-3 and 484 bp nucleotide sequence including the predicted tga code. (B) The deduced 155 aa. amino acid sequence of the C-1-8 and 475 bp nucleotide sequence. (C) The deduced 87 aa amino acid sequence and 337 bp nucleotides sequence. All of three sequences were predicted to contain an untranslated region.

A



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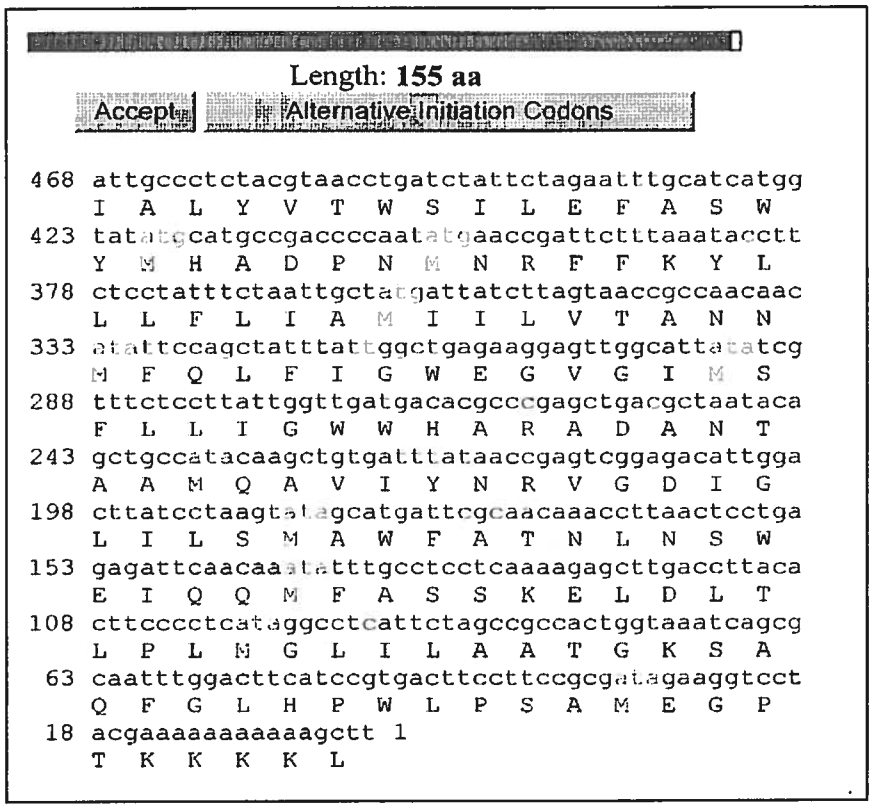
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Y M H A D P N M N R F F K Y L
378 ctcctatttctaattgctatgattatcttagtaaccgccaacaac
L L F L I A M I I L V T A N N
333 atattccagctatttattggctgagaaggagttggcattatatcg
M F Q L F I G W E G V G I M S
288 tttctccttattggttgatgacacgcccgagctgacgctaataca
F L L I G W W H A R A D A N T
243 gctgccatacaagctgtgatttataaccgagtcggagacattgga
A A M Q A V I Y N R V G D I G
198 cttatcctaagtatagcatgattcgcaacaaaccttaactcctga
L I L S M A W F A T N L N S W
153 gagattcaacaaatatttgcctcctcaaaagagcttgaccttaca
E I Q Q M F A S S K E L D L T
108 cttcccctcataggcctcattctagccgccactggtaaatcagcg
L P L M G L I L A A T G K S A
63 caatttggacttcatccgtgacttccttccgcgatagaaggtcct
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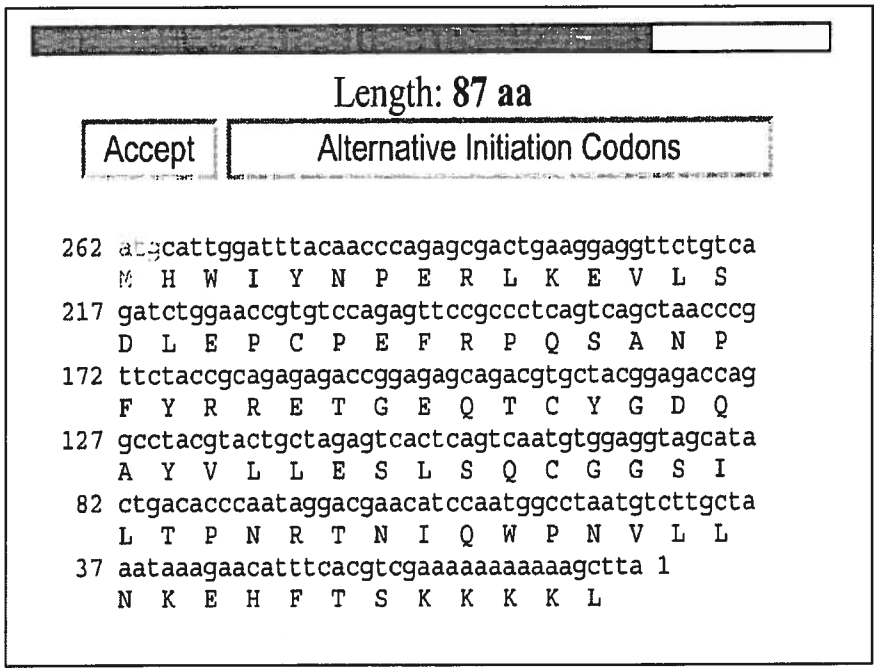
18 acgaaaaaaaaaaagctt 1
T K K K K L

C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Alternative Initiation Codons
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D L E P C P E F R P Q S A N P
172 ttctaccgcagagagaccggagagcagacgtgctacggagaccag
F Y R R E T G E Q T C Y G D Q
127 gcctacgtactgctagagtcactcagtcaatgtggaggtagcata
A Y V L L E S L S Q C G G S I
82 ctgacacccaataggacgaacatccaatggcctaatgtcttgcta
L T P N R T N I Q W P N V L L
37 aataaagaacatttcacgtcgaaaaaaaaaaagctta 1
N K E H F T S K K K K L

To identify the genes, the 18 cDNA sequences were subjected to BLAST homology search (NCBI); or in some cases the amino acid sequences were deduced and compared to known proteins using the BLASTP algorithm (NCBI). The 18 cDNAs revealed that 6 cDNAs (38.9%) had significant homology with known genes (E-values $<10^{-5}$; Table 3-5A, B).

The C-1-8 cDNA showed highly significant (99%) homology to mitochondrial NADH dehydrogenase subunit-5 gene (mt ND 5) of brook charr (GenBank accession number gi 5257509; E-value 0; Table 3-5A). The deduced amino acid sequence of the C-1-8 using BLASTP algorithm gave identical results as the nucleotide sequence (100% homology to the same gene; GenBank accession number AAD41382.1; Table 3-5B). The C-1-3 nucleotide and amino acid sequence were 89% and 90% homologous respectively with the Leukocyte-derived chemotaxin gene of rainbow trout (GenBank accession number gi11055325, E-value e-131; Table 3-5A; GenBank accession number AAG28030.1; Table 3-5B). The G-1-9 cDNA had 91% homology with the human basic transcription factor 3 (BTF3; GenBank accession number gi 30583078, E-value 6e-17; Table 3-5 A).

The C-2-9 nucleotide sequence had no significant homologies with any known gene. However the deduced amino acid sequence of C-2-9 was 58% homologous to the jellyfish (*Tripedalia cystophora*) crystalline protein J1 (gi 729210 with E-value 6e-7; Table 3-5B).

Table 3-5A. Identification of T_3 regulated hepatic genes using BLAST homology comparisons of differently expressed genes. The table indicates the ID, the size of the sequence, its regulation by T_3, E-value and gene homology.

Clone name	Sequence Length (bp)	T_3 regulation	E value	Maximum homology to known genes
C-1-8	475	up	0	99% homologous to brook charr NADH dehydrogenase subunit 5; gi 5257509
C-1-3	484	down	e-131	89% homologous to rainbow trout Leukocyte-derived chemotaxin; gi11055325
G-1-1	568	up	e-153	93% homologous to Atlantic salmon tropomyosin; gi15844724
C-1-4	471	up	e-141	97% homologous to Atlantic salmon tropomyosin; gi15844724
C-1-5	445	up	e-151	98% homologous to Atlantic salmon tropomyosin; gi12621679
G-1-9	375	up	6e-17	91% homologous to Human basic transcription factor 3; gi 30583078

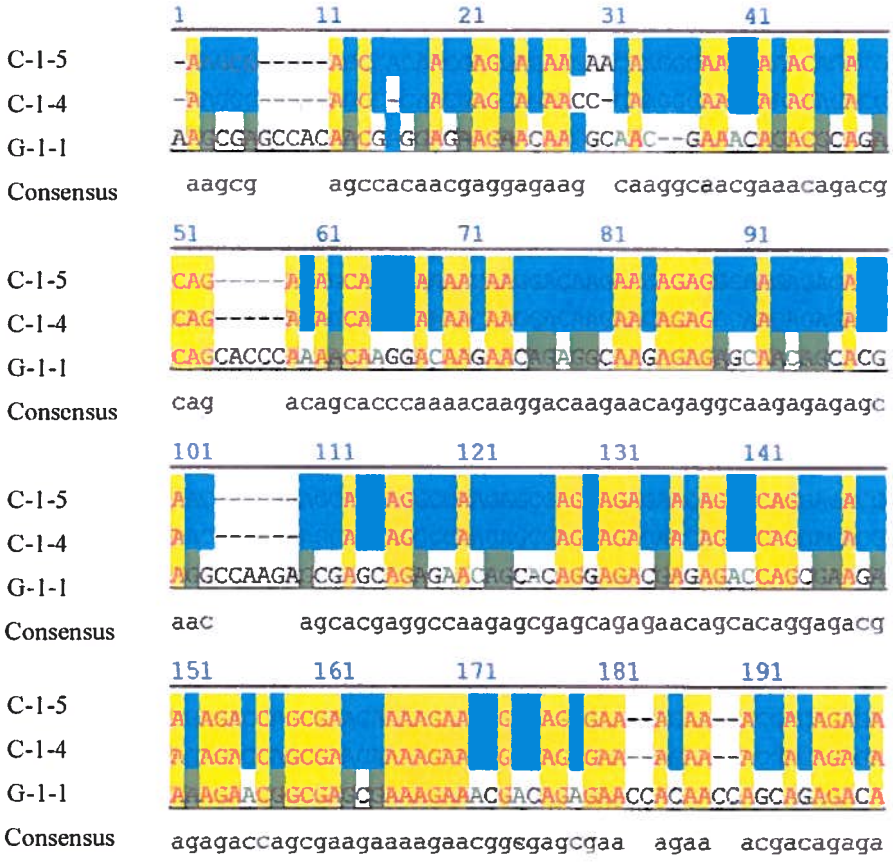
Table 3-5B. Identification of T_3 regulated hepatic genes using BLAST-P homology comparisons of differently expressed genes. The table indicates the clone name, the size of the sequence, No. of amino acids, it's regulation by T_3, E-value and known protein homology.

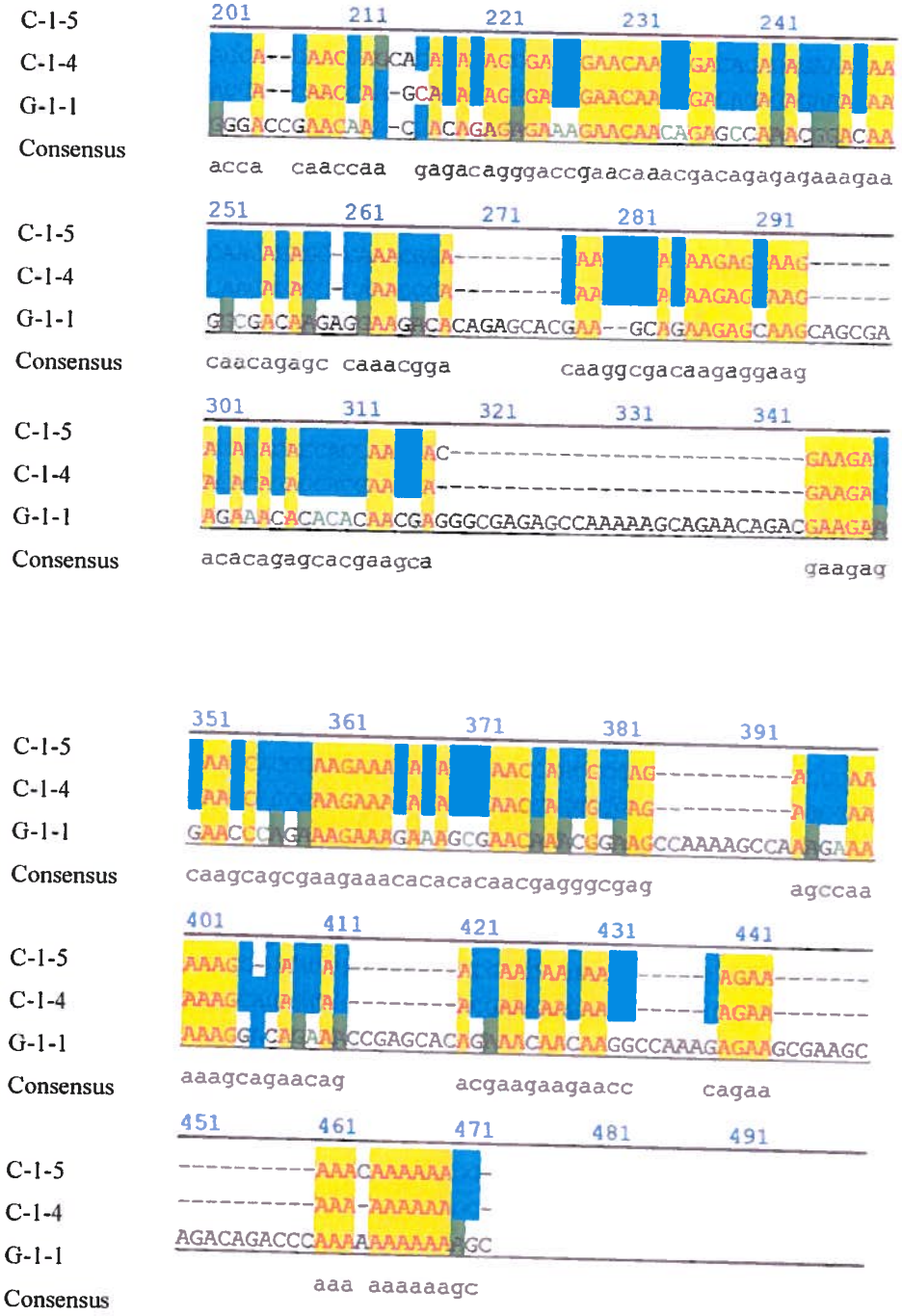
Clone name	Sequence length (bp)	No. of Amino Acids (aa)	T_3 regulation	E value	Maximum homology to known protein
C-1-3	484	137	down	5e-64	90 % homologous to rainbow trout Leukocyte-derived chemotaxin; AAG28030.1
C-1-8	475	156	up	4e-84	100% homologous to brook charr NADH dehydrogenase subunit 5; AAD41382.1
C-2-9	337	87	up	2e-05	58 % homologous to jellyfish crystalline protein J1; gi 729210

There were 3 cDNAs (G-1-1, C-1-4 and C-1-5) which had significant homology to Atlantic salmon tropomyosin (TM). While G-1-1 and C-1-4 were homologous to TM (Genbank Accession no.gi15844724), C-1-5 was homologous to a TM with a different Genbank Accession no. (gi 12621679). These genes were between 93 and 98% homologous. In order to understand the correlation between the three sequences of TM, the Jellyfish Software Program (www.btgenes.com) was used to compare the 3 cDNA sequences (Table 3-6). G-1-1, C-1-4 and C-1-5 had 38.4% homology with each other, while C-1-4 and C-1-5 were 97% homologous. This suggests that the cDNA's are likely to represent different regions of the same gene or may be different isoforms with similar

sequences. Further tests were done by Northern blot analysis to establish the size of transcripts and the tissue distribution of the genes.

Table 3-6. Sequence Alignment for cDNA C-1-4, C-1-5 and G-1-1. The blue color indicates identical sequences between two genes while the yellow color indicates identical sequences between the three cDNAs.




C-1-5
C-1-4
G-1-1
Consensus
201
211
221
231
241
acca caaccaa gagacagggaccgaacaaacgacagagagaaagaa
251
261
271
281
291
caacagagc caaacgga caaggcgacaagaggaag
301
311
321
331
341
acacagagcacgaagca gaagag
351
361
371
381
391
caagcagcgaagaaacacacacaacgagggcgag agccaa
401
411
421
431
441
aaagcagaacag acgaagaagaacc cagaa
451
461
471
481
491
aaa aaaaaagc

Seven cDNAs (38.9%) had significant homology to ESTs. These genes have unknown biological function and are listed in Table 3-7. Six of the seven cDNAs (C-1-6, C-1-7, C-1-9, C-2-4, C-2-2 and C-2-13) had 91-99% homology with different corresponding Atlantic salmon ESTs (GenBank accession number gi 24344594, gi 24385340, gi 24353059, gi 24342922, gi 24344518 and gi 29319330 respectively). One of the cDNA sequences, G-2-T_1, was 90% identical to a rainbow trout cDNA clone (GenBank accession number gi 24683984).

Table 3-7. Identification of T_3 regulated hepatic genes using BLAST(EST) homology comparisons of differently expressed genes. The table indicates the ID, the size of the sequence, its regulation by T_3, E-value and cDNA clone homology.

Clone name	Sequence (bp)	T_3 regulation	E value	Maximum homology to cDNA clone
C-1-6	399	up	e-148	95% homologous to Atlantic salmon cDNA clone; gi 24344594
C-1-7	369	up	e-165	97% homologous to Atlantic salmon cDNA clone; gi 24385340
C-1-9	319	down	e-129	95% homologous to Atlantic salmon cDNA clone; gi 24353059
C-2-4	514	up	2e-78	91% homologous to Atlantic salmon cDNA clone; gi 24342922
C-2-2	575	down	0	91% homologous to Atlantic salmon cDNA clone; gi 24344518
C-2-13	263	down	2e-41	92% homologous to Atlantic salmon cDNA clone; gi129319330
G-2-T1	357	up	3e-50	90% homologous to rainbow trout cDNA clone; gi 24683984

Four of the cDNA's (22.2%) had no significant similarity with previously identified genes or EST. These cDNAs (C-2-7, C-2-8, C-2-11 and G-2-D_2) may represent novel genes. Two of those genes were shown to be over-expressed in T_3-treated fish (C-2-7, C-2-8), while the other two were down-regulated by T_3 (C-2-11 and G-2-D_2).

3.1.6 Confirmation of Differentially Expressed cDNA Genes

To confirm that differentially expressed genes obtained by DD-PCR were not false positives, reverse dot blots were done. A total of 18 cDNAs were blotted onto nylon membranes in duplicate. The labelled cDNA probes were synthesised by reverse transcription using RNA from Control, T_3-treated or Na-ipodate-treated fish. Three hybridizations were done and analyzed. Representative hybridizations are shown in Figure 3-5. After normalising the signal intensities to the housekeeping genes (GAPDH and β-actin), all 18 cDNA showed a statistically significant differential expression relative to the fish thyroid status ($p<0.05$; Figure 3-6). Twelve genes (66.7%) were up regulated by T_3, including 6 known genes C-1-8 (mt ND 5); G-1-1, C-1-4, C-1-5 (TM), G-1-9 (BFT3); C-2-9 (crystalline protein J1), ESTs C-1-6, C-1-7, C-2-4, G-2-T1 and unknown genes C-2-7, C-2-8. The other 6 genes (33.3%) were down regulated by T_3. These included known gene C-1-3 (Leukocyte-derived chemotaxin), ESTs C-1-9, C-2-2 and C-2-13. Out of the 6 cDNA that were found to be down-regulated, two of them are unknown or novel genes (C-2-11 and G-2-D_2). These results confirmed our results obtained by DD-PCR. Functional classification of the 18 TH-dependent genes is shown in Table 3-8. 39.9% are known genes and have functions related with mitochondrial energy metabolism, signal transaction, immunity and others. 60.1% are unknown genes.

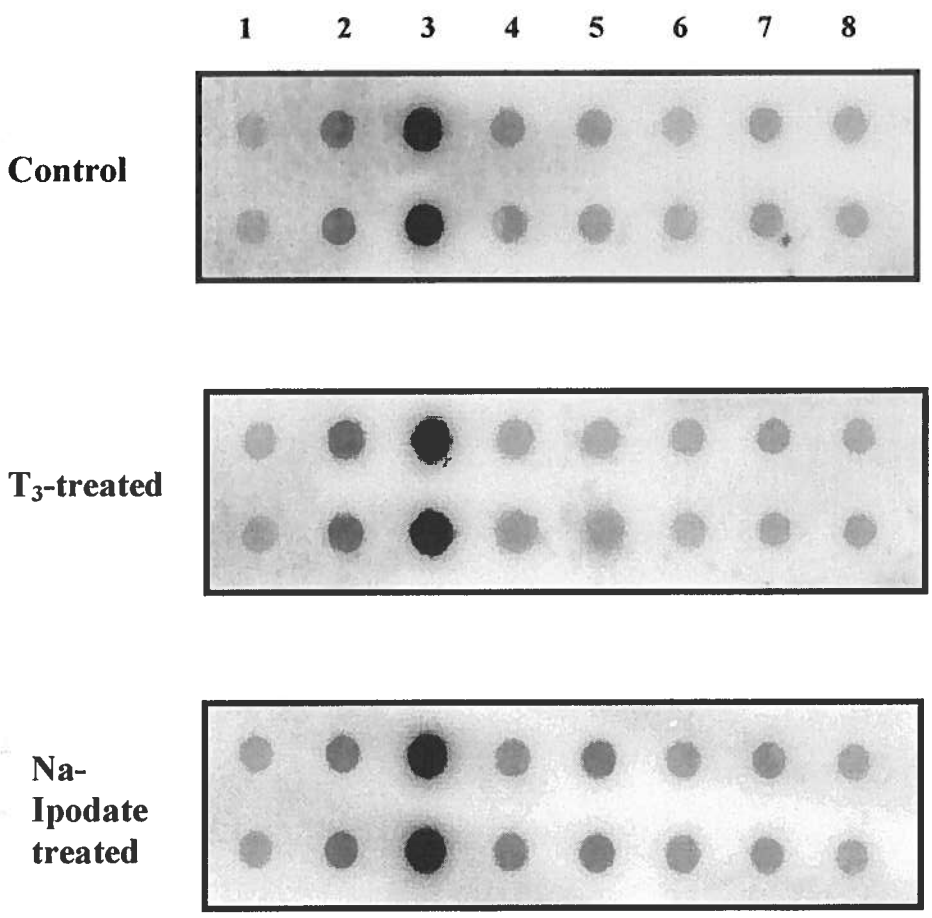
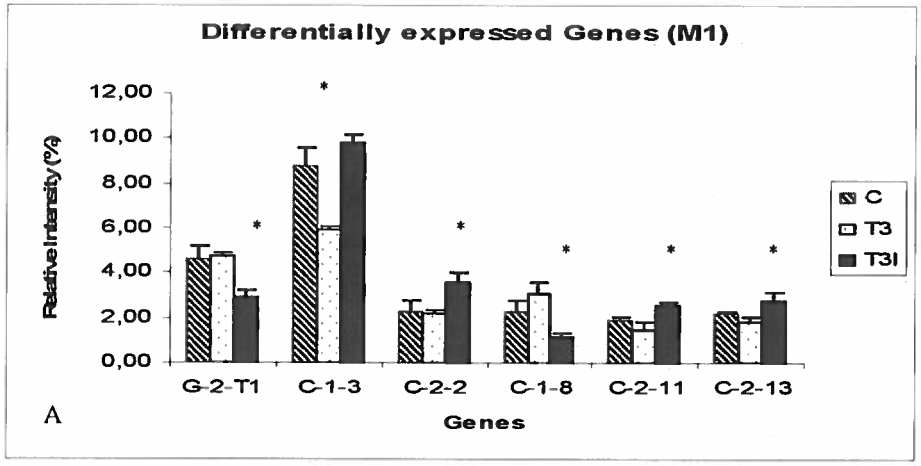
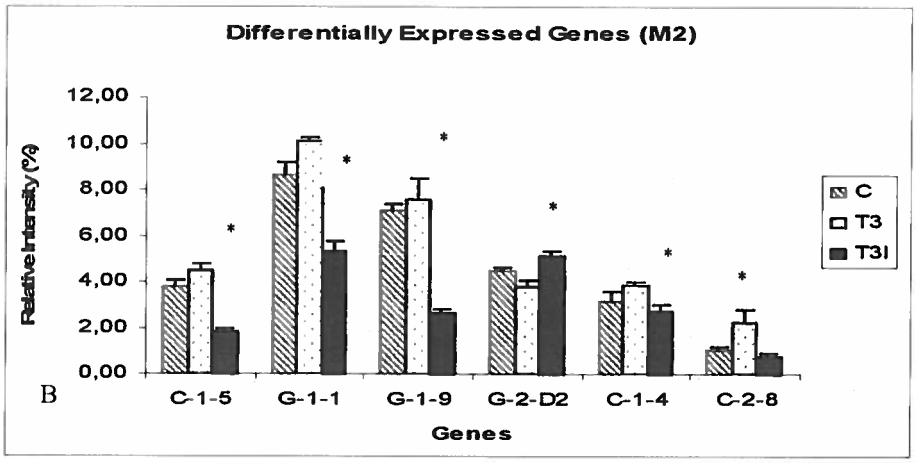


Figure 3-5. Representative reverse dot blot of cDNAs generated for hepatic RNA from control, T_3-treated and Na-Ipodate treated fish. Lane 1, GAPDH; Lane 2, G-2-T_1; Lane 3, C-1-3; Lane 4, C-1-4; Lane 5, C-2-2; Lane 6, C-2-11; Lane 7, C-2-13; Lane 8, β-actin (duplicate on the membrane). GAPDH and β-actin were used as house-keeping genes. Three identical membranes were hybridized separately with labelled cDNAs prepared from each of the three experimental groups.





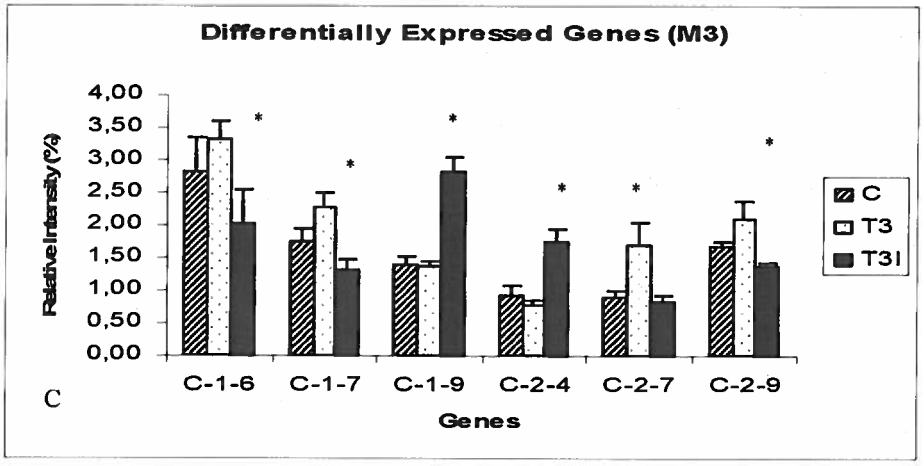


Figure 3-6. The expression of the 18 cDNAs was confirmed by reverse dot blot. Mean fold–change in intensity following normalization is depicted in each graph. M_1 (A), M_2 (B) and M_3 (C) represent the three different membranes. * Indicates a significant difference from control ($p<0.05$).

Table 3-8. Summarized functional classification of the 18 TH-dependent genes.

Biological Function	Number of genes		Gene Name
	Up	Down	
Mitochondrial Energy Metabolism	1	0	mt ND 5
Signal Transduction	1	0	BTF 3
Cellular Immunity	0	1	Leukocyte-derived chemotaxin
Others	4	0	TM (3); Crystalline pro J1.
Unknown	6	5	
Total	12	6	

3.1.7 Northern Blot Analysis

To assess the tissue distribution of TH-dependent genes, Northern blot analyses were done. All 18 differentially expressed cDNAs were tested in 9 adult brook charr tissues: brain, heart, intestine, spleen, liver, ovary, testis, muscle and kidney. Representative Northern blots that were used to assess the tissue distribution and transcript size are shown in Figures 3-7 A and B.

All cDNAs were expressed in liver even though, the tissue distribution of each of these varied widely (Table 3-9). Some genes such as G-1-9 (BTF 3), C-1-8 (mt ND 5) and C-2-8 (unknown gene) were expressed in all the tissues. Others such as G-1-1, C-1-4, C-1-5 (TM), C-1-3 (Leukocyte-derived chemotaxin), G-2-D_2 (unknown gene) and C-2-13 (cDNA clone) were detectable only in the liver.

Some genes such as C-2-11 (unknown gene) were expressed in liver, brain, heart, muscle, testes and ovaries but not in intestine, spleen and kidney. The tissue distribution of each gene is summarised in Table 3-9.

The size of the transcripts for each of the 18 cDNAs varied from 1.35 kb to 9.0 kb (Table 3-10). For some genes, the transcripts had different sizes in different tissues. The size of the transcripts in each tissue is also indicated in Table 3-10. Northern blot analysis revealed that tropomyosin had three isoforms corresponding to 4.4 kb, 7.5 kb and 9.0 kb

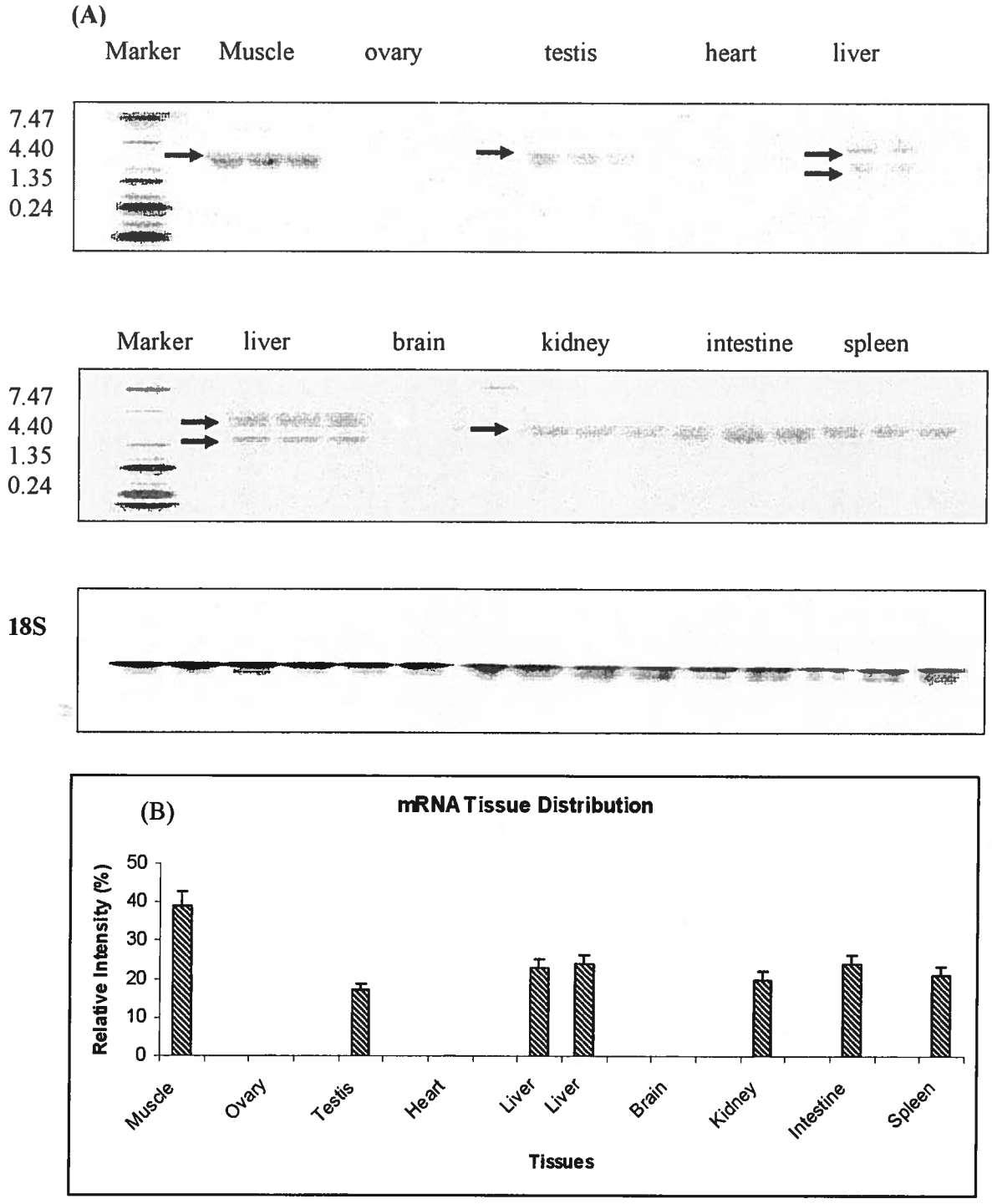


Figure 3-7. (A) Northern blot analyses of C-2-4 cDNA in various adults brook charr tissues. The arrows indicate positive signal. Equal amounts of total RNA were loaded in each lane. The marker shows the mRNA size. (B) Normalization was done using 18 S rRNA and the relative intensity was calculated.

Table 3-9. Distribution pattern and relative expression level of TH-dependent genes in adult brook charr. Charr were sampled in July, 2003. Expression levels were normalised to the 18 S rRNA. One asterisks (*) indicates a relative level of 0-25%; ** indicates an expression level of 26-50%; *** indicates an expression level of 51-75% relative to 18S.

cDNA ID	Genes Name	Brain	Heart	Intestine	Kidney	Liver	Muscle	Ovary	Testis	Spleen
G-1-1	Tropomyosin					** *** **				
C-1-4	Tropomyosin					** *** **				
C-1-5	Tropomyosin					** *** **				
C-1-8	mt NADH 5	*	*	*	***	**	***	*	*	*
C-1-3	chemotaxin					***				
G-1-9	BTF 3	**	**	***	***	***	***	***	***	***
C-2-9	Crystalline J1				*	*		*	**	**
C-1-6	cDNA clone	*	*			*		*		
C-1-7	cDNA clone					*			*	*
C-1-9	cDNA clone	**		*	**	*		*	**	*
C-2-2	cDNA clone	*				*				
C-2-4	cDNA clone			*	*	*	**		*	*
C-2-13	cDNA clone					**				
G-2-T_1	cDNA clone	*	*		*	*		*		
G-2-D_2	Unknown					*				
C-2-7	Unknown				*	*			*	*
C-2-8	Unknown	*	*	*	*	*	*	*	*	*
C-2-11	Unknown	*	*			*	*	*	*	

Table 3-10. Size of differentially expressed mRNA in brook charr. Northern blot analysis was done to identify the size of the mRNA transcripts for each of the TH-dependent genes. Multiple weights indicate the presence of multiple bands.

Name of cDNA	Name Of Genes	Brain	Heart	Intestine	Kidney	Liver	Muscle	Ovary	Testis	Spleen
G-1-1	Tropomyosin					4.4 kb 7.5 kb 9.0 kb				
C-1-4	Tropomyosin					4.4 kb 7.5 kb 9.0 kb				
C-1-5	Tropomyosin					4.4 kb 7.5 kb 9.0 kb				
C-1-8	mt NADH 5	7.0kb	7.0kb	7.0kb	7.0kb	7.0kb	7.0kb	7.0kb	7.0kb	7.0kb
C-1-3	chemotaxin					1.7 kb				
G-1-9	BTF 3	2.4 kb	2.4 kb	2.4 kb	2.4 kb	2.4 kb	2.4 kb	2.4 kb	2.4 kb	2.4 kb
C-2-9	Crystalline J1				2.4kb	2.4kb		2.4kb	2.4kb	2.4kb
C-1-6	cDNA clone	2.4 kb	2.4 kb			2.4 kb		2.4 kb		
C-1-7	cDNA clone					4.4kb			4.4kb	4.4kb
C-1-9	cDNA clone	7.4kb		7.4kb	7.4kb	7.4kb		7.4kb	7.4kb	7.4kb
C-2-2	cDNA clone	5.0kb				5.0kb				
C-2-4	cDNA clone			5.0kb	5.0kb	4.0 kb 6.0kb	5.0kb		5.0kb	5.0kb
C-2-13	cDNA clone					1.35kb				
G-2-T	cDNA clone	7.47kb	7.47kb		7.47kb	7.47kb		7.47kb		
G-2-D	Unknown					5.0kb				
C-2-7	Unknown				4.4kb	4.4kb			4.4kb 7.0kb	4.4kb
C-2-8	Unknown	5.5kb	5.5kb	5.5kb	5.5kb	5.5kb	3.0kb 5.5kb	3.0kb	3.0kb	5.5kb
C-2-11	Unknown	7.47kb	7.47kb			7.47kb	7.47kb	7.47kb	7.47kb	

SECTION II: GENE EXPRESSION PATTERNS IN FAST AND SLOW GROWING FISH

3.2.1 Fish Growth Rate

To identify and characterize growth-dependent genes in brook charr, a 16 K microarray (GRASP) was used. All the fish used in this experiment were raised in identical condition to eliminate environmental effects on growth. Immature fish (n=8) from two families were selected based on their growth rate. The average weight in fast growing fish was 54.73 ± 12.14 g and slow growing fish weighed 8.43 ± 1.59 g. The differences in average weight between families were significantly ($p<0.01$).

3.2.2 cDNA Microarray Analysis, and Hepatic Genes Expression Profile Analysis

Microarrays (GRASP) were obtained from the University of Victoria and contained 16006 salmonid genes. Genes were derived from multiple tissues of Atlantic salmon (13421 cDNA) and rainbow trout (2576 cDNA; von Schalburg et al., 2005). Pools of RNA from fast or slow growing fish liver (4 pools of 2 fish) were used to generate cDNA probes and those were hybridised to microarrays. The genes were considered expressed when they were detected in at least three of four samples. There were 12910 (80.7%) genes present on the microarray that were expressed in brook charr liver. A representative microarray is shown in Figure 3-8. As expected, some genes were up-regulated in fast growing fish while other genes were down-regulated.

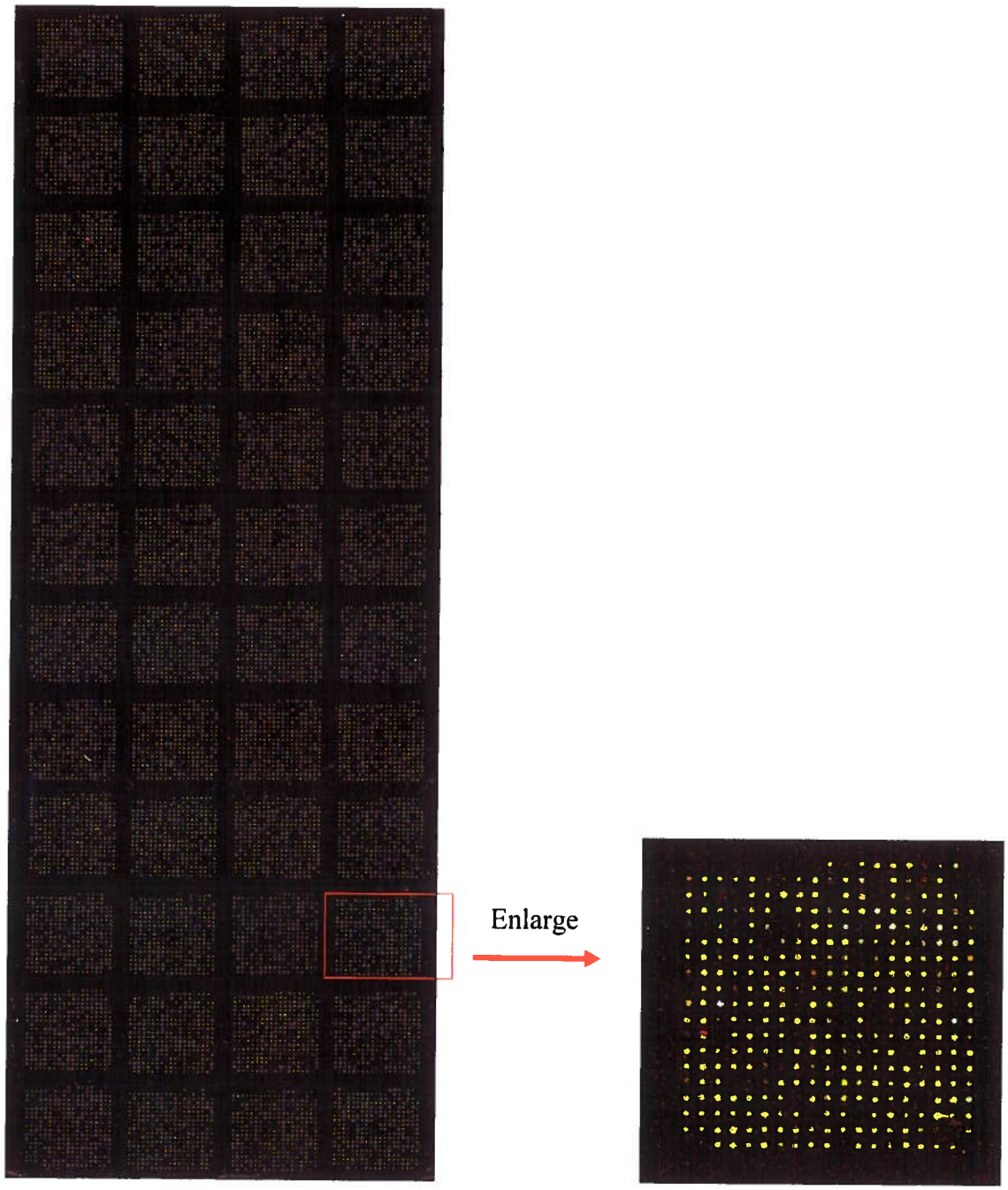


Figure 3-8. Representative microarray (contain 4x12 of sub-block). RNA from slow growing fish was labelled with Cy3 (green); RNA from fast growing fish was labelled with Cy5 (red). mRNAs that were more abundant in slow growing fish are shown in green, while the mRNA that is more abundant in fast growing fish are shown in red. Yellow spots represent genes whose expression was similar between the two samples. Enlarged sub-block was inserted to show details; each sub-block contains 19 rows and 19 columns.

3.2.3 Gene Expression Patterns

To understand the gene expression pattern related with growth and to identify growth dependent genes, global gene expression in slow and fast growing fish was examined using the QuantArray software (Packard Biosystem). The normalized ratio of Cy5 to Cy3 was calculated for individual target genes. The scatter plot represents the log ratio of Cy5 and Cy3 (Figure 3-9) which was used to confirm the results from the microarray image. As expected, the majority of genes did not change between the two groups with a Cy5/Cy3 ratio of approximately 1.0.

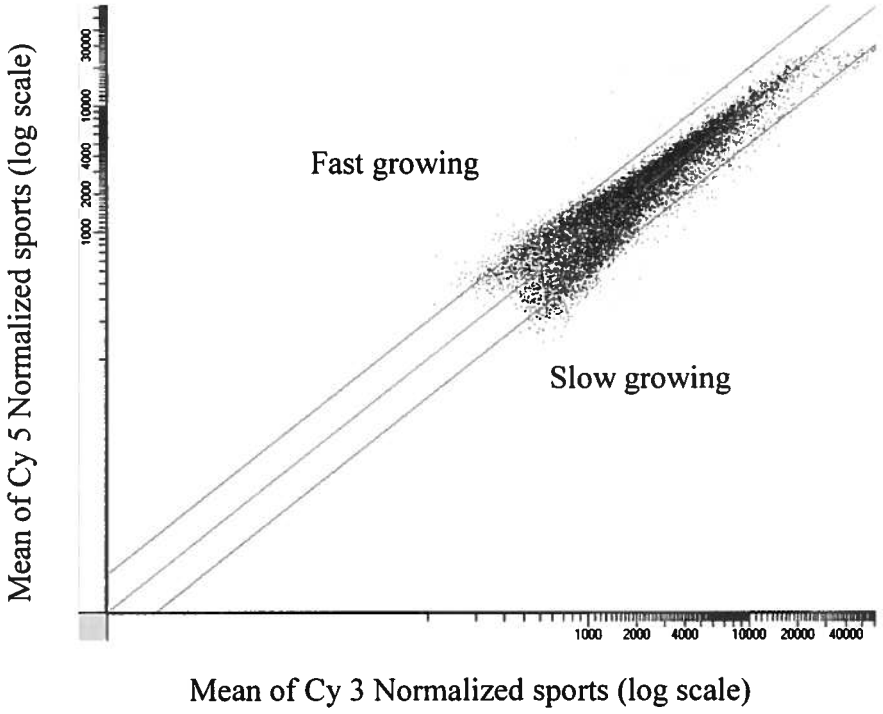


Figure 3-9. Scatter plot. Scatter plot of the mean log ratio between Cy5 and Cy3 representing a comparison of the expression ratio between fast and slow growing fish. Each spot represents one gene. The two lines indicate a two-fold difference. Genes above this line are two fold up-regulated; the genes below this line are two fold down regulated.

3.2.4 Analysis of Gene Expression Patterns

The data from 4 separate microarrays were analyzed using GeneSpring (Ver.7; Agilent). There were 94 genes which were differentially expressed between slow and fast growing fish. Twenty-five genes were down-regulated with a fold change ranging from 0.338 to 0.497 (Figure 3-10; Table 3-11). These included ten known genes (40%) such as prostaglandin D syntheses (3), fast myotomal muscle actin, collagen alpha 2(I) chain precursor, helix-destabilizing protein, cytochrome P450 IA1, cytochrome P450 3A45, prolactin precursor (PRL), ribosomal protein L11. The fifteen others (60% of the down-regulated genes) were unknowns. Sixty-nine genes were up-regulated in fast growing fish (Figure 3-10; Table 3-12). The fold change of up-regulated genes ranged from 2.028 to 4.237. Among these, 31 were known genes (45%) and they are listed in Table 3-12. There were 38 unknown genes, which represent 55% of the up-regulated genes. Overall, more genes were up-regulated than down-regulated in fast growing fish. Each of these genes represents a potential molecular actor related to fish growth.

Among the growth-related genes, the higher fold change genes were unknowns (CA038946, 4.237 fold; CB496650, 3.731 fold) and ATP syntheses lipid-binding protein (CB493213, 3.62 fold; Table 3-12). Both TSC-22 (3.584 fold) and cadherin 1 (3.4 fold; Table 3-12) also had large increases. These genes are implicated in cell growth and differentiation (Choi, et al, 2005, Babb and Marrs, 2004).

The results were reproducible as different probes for the same gene spotted independently at different positions on the microarray, gave similar results. For example, three probes (CA043176, CB510500 and CB511307) corresponding to gene prostaglandin D syntheses were all down-regulated in fast-growing fish, with fold

changes of 0.379, 0.379 and 0.311 respectively. Another gene, H-FABP, also had three probes (CA043176, CB510500 and CB511307) on the microarray, and all of them were up-regulated in the fast growing fish with fold changes of 2.151, 2.179 and 2.326, respectively.

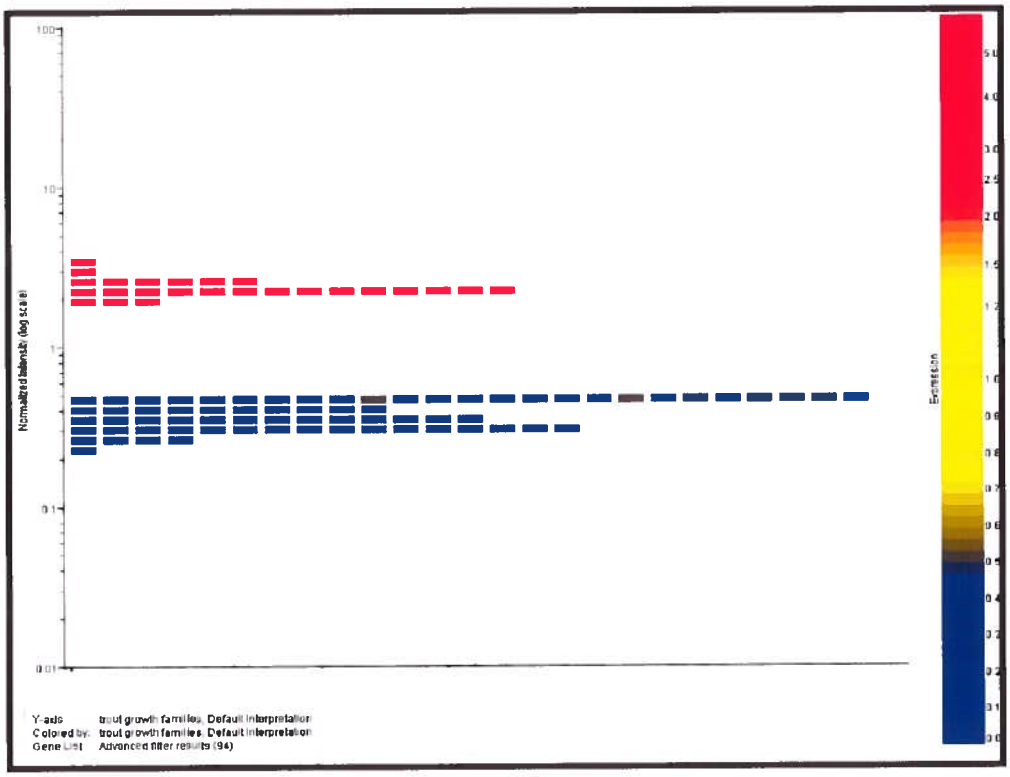


Figure 3-10. Differentially expressed genes between fast and slow growing fish. Each band represents one gene. There were 94 differentially regulated genes.

Table 3-11. Down-regulated genes in fast growing fish. The genes ID and fold change of 25 genes identified by microarray analyses are indicated.

Gene ID	Fold Changes	Genes Name
1. CB489828	0.338	cDNA clone SK1-0960
2. CA043176	0.379	prostaglandine D syntheses
3. CB510500	0.379	prostaglandine D syntheses
4. CA038313	0.380	UNKNOWN
5. CA063490	0.381	cDNA clone SK1-0315
6. CB511307	0.411	prostaglandin D syntheses
7. CB496977	0.418	fast myotomal muscle actin
8. CA059189	0.431	UNKNOWN
9. CB507670	0.433	Collagen alpha 2(I) chain precursor
10. CA049935	0.439	UNKNOWN
11. CK990274	0.439	UNKNOWN
12. CA038363	0.458	UNKNOWN
13. CA059065	0.458	UNKNOWN
14. CA042038	0.462	Helix-destabilizing protein
15. CA052005	0.462	UNKNOWN
16. CB497960	0.462	cytochrome P450IA1
17. CA038065	0.467	UNKNOWN
18. CA041423	0.469	UNKNOWN
19. CB517306	0.480	cDNA clone SK1-0674
20. A039642	0.480	UNKNOWN
21. CB499154	0.485	cDNA clone k16F03
22. CA052038	0.486	cytochrome P450 3A45
23. CB490586	0.493	cDNA clone
24. CA048029	0.497	Prolactin precursor (PRL)
25. CB511063	0.497	ribosomal protein L11

Table 3-12. Up-regulated genes in fast growing fish. The gene ID and fold change of 69 genes identified by microarray analyses are indicated.

Gene ID	Fold Changes	Genes Name
1. CA051408	2.028	UNKNOWN
2. CB497318	2.041	UNKNOWN
3. CB493696	2.049	UNKNOWN
4. CA060526	2.070	cystatin precursor
5. CN442545	2.079	cytochrome-c oxidase mitochondrion
6. CB497341	2.088	precerebellin-like protein
7. CA052927	2.092	UNKNOWN
8. CB505738	2.114	beta-globin
9. CB491130	2.114	ubiquinone-binding protein QP-C;
10. CA051739	2.119	UNKNOWN
11. CB510681	2.146	UNKNOWN
12. CB509749	2.146	Pentraxin
13. CB493958	2.151	28kDa-1e apolipoprotein
14. CA769854	2.151	fatty acid binding protein H-FABP
15. CB498195	2.165	UNKNOWN
16. CA062843	2.169	cDNA clone s09F03
17. CB489347	2.179	fatty acid binding protein H-FABP
18. CA051872	2.227	Coregonus albula DNA
19. CA052047	2.232	RIKEN cDNA 2410001P07
20. CB510409	2.252	UNKNOWN
21. CB512374	2.262	RIKEN cDNA 4931406C07
22. CB517150	2.262	hydroxysteroid (17-beta) dehydrogenase
23. CA041073	2.268	UNKNOWN
24. CA038612	2.283	Serotransferrin II precursor
25. CB496849	2.299	hypothetical protein
26. CB496931	2.326	fatty acid binding protein H-FABP
27. CB510628	2.38	Secreted phosphoprotein 2,

Gene ID	Fold Changes	Genes Name
28. CB507958	2.519	UNKNOWN
29. CA044945	2.525	UNKNOWN
30. CB516895	2.551	UNKNOWN
31. CB511983	2.558	cDNA clone k13H11
32. CA038900	2.611	UNKNOWN
33. CA063639	2.639	Putative
34. CA062479	2.653	UNKNOWN
35. CA039685	2.667	O-methyltransferase
36. CB495080	2.703	UNKNOWN
37. CB493533	2.732	O-sialoglycoprotein endopeptidase
38. CA045868	2.801	cDNA clone k05G09
39. CB492462	2.825	UNKNOWN
40. CB490833	2.833	Phosphofructokinase
41. CB493296	2.950	UNKNOWN
42. CA056074	2.976	p60
43. CB511162	2.985	beta-1,2-N-acetylglucosaminyltransferase
44. CB498131	2.994	UNKNOWN
45. CA055890	3.021	UNKNOWN
46. CB499980	3.021	UNKNOWN
47. CB515417	3.040	cDNA clone s02F10
48. CK990702	3.067	gastrulation specific protein
49. CB501602	3.077	UNKNOWN
50. CA052278	3.096	RNA 3'-terminal phosphate cyclase
51. CB517743	3.106	aryl hydrocarbon receptor 2 delta
52. CB516689	3.135	UNKNOWN
53. CB515004	3.175	seven-up related 40
54. CA046017	3.175	UNKNOWN
55. CA043849	3.185	cDNA clone SS1-0917

Gene ID	Fold Changes	Genes Name
56. CB493494	3.236	My016 protein
57. CA048685	3.367	cDNA clone k20B07
58. CB518078	3.390	UNKNOWN
59. CB516447	3.413	Glycosylase
60. CA053335	3.413	UNKNOWN
61. CB507658	3.425	UNKNOWN
62. CB517027	3.436	cadherin 1, epithelial
63. CB498113	3.460	transcription elongation factor B
64. CB515712	3.460	cDNA clone s19G10
65. CB509522	3.584	TSC-22
66. CB497064	3.610	UNKNOWN
67. CB493213	3.623	ATP synthesis lipid-binding protein
68. CB496650	3.731	UNKNOWN
69. CA038946	4.237	hypothetical protein

3.2.5 Classification of Genes by Molecular Function

Genes were classified based on their molecular function. Clusters of genes which were found to be differentially expressed in fast growing fish, and which have been implicated in various functions, are listed in Table 3-13. Eight genes (8.5%) are related to cellular immunity; among those, 6 (6.4 %) were up-regulated and 2 (2.1%) were down-regulated. Nine genes (9.6%) are involved in carbohydrate and fatty acid metabolism; three (3.2%) are implicated in mitochondrial energy metabolism and the other three (3.2%) are related to signal transduction. The genes whose functions are involved in carbohydrate and fatty acid metabolism; mitochondrial energy metabolism and signal

transduction were found to be up-regulated in fast growing fish while no genes were found to be down-regulated. Of the three protein metabolism genes (3.2%), 2 genes (2.1%) were up-regulated, and 1 gene (1.1 %) was down-regulated. Fourteen additional genes (14.9%) have other functions (Figure 3-11). Therefore, growth regulated genes are related to a wide range of cellular functions, including cellular immunity, carbohydrate and fatty acid metabolism, mitochondrial energy metabolism, signal transduction, protein metabolism and others. The genes that are involved in carbohydrate, fatty acid metabolism, mitochondrial energy metabolism and signal transduction are positively associated with growth. Twenty genes were TH-dependent genes according to literature (Table 3-13).

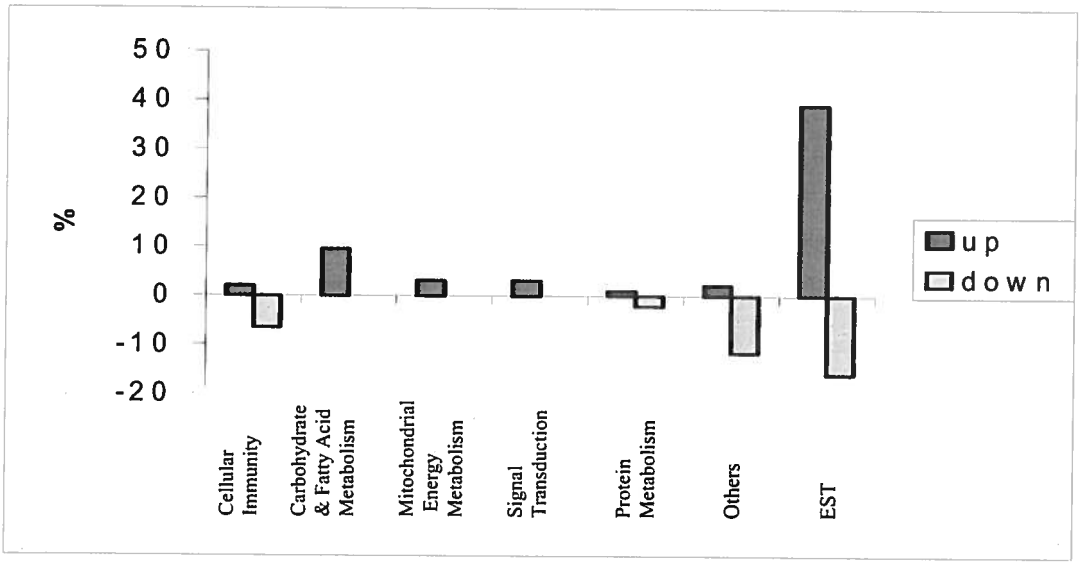


Figure 3-11. Differentially expressed genes classified based on their molecular function in fast growing fish.

Table 3-13. Classification of genes based on molecular function. Asterisks (*) indicate genes which have been previously reported to be regulated by thyroid hormone.

Function	Gene ID	Genes Name	Fold Change
Cellular Immunity	CA043176(3)	prostaglandin D syntheses (O.mykiss) *	0.330
	CB507670	Collagen alpha 2(I) chain precursor	0.430
	CB497960	cytochrome P450IA1 mRNA, (S.gairdneri) *	0.462
	CA052038	cytochrome P450 3A45 (O. mykiss) *	0.486
	CB497341	precerebellin-like protein (O. mykiss)	2.088
	CB509749	pentraxin (Salmon salar)	2.146
Carbohydrate and Fatty Acid Metabolism	CB493958	28kDa-1e apolipoprotein (A. japonica) *	2.151
	CA769854(3)	H-FABP (O. mykiss) *	2.151
	CB511162	acetylglucosaminyltransferase II (R.norvegicus)	2.998
	CB517743	AhR2d mRNA, (S. salar)	3.106
	CB516447	glycosylase (G. gallus)	3.413
	CB517150	hydroxysteroid (17-beta) dehydrogenase 12 (Danio rerio)	2.262
	CB490833	phosphofructokinase(Homo sapiens) *	2.833
Mitochondrial Energy Metabolism	CB491130	ubiquinone-binding protein QP-C; complex III subunit VII (M.musculus) *	2.114
	CN442545	cytochrome-c oxidase chain III *	2.079
	CB493213	ATP synthesis lipid-binding protein*	3.623
Signal Transduction	CB510628	secreted phosphoprotein 2, mRNA*	2.381
	CB515004	seven-up related 40 (D. rerio) *	3.175
	CB498113	transcription elongation factor B polypeptide 1-like; (Homo sapiens) *	3.460

Function	Gene ID	Genes Name	Fold Changes
Protein Metabolism	CA042038	hnRNP core protein A1	0.459
	CB511063	ribosomal protein L11 (l. punctatus)	0.497
	CB493533	O-sialoglycoprotein endopeptidase (Homo) *	2.732
Others	CB505738	beta-globin (S. salar)	2.114
	CA060526	cystatin precursor (c. salmon)	2.070
	CB496977	fast myotomal muscle actin(S.salar)	0.411
	CA048029	PRL*	0.493
	CA038612	Sterotransferrins II (STF II)	2.283
	CA039685	O-methyltransferase (N.sp.) *	2.667
	CA056074	p60	2.976
	CK990702	gastrulation specific protein	3.067
	CA052278	RTC domain containing 1(Homo sapiens)	3.096
	CB493494	My016 protein [Homo sapiens]	3.236
	CB517027	cadherin 1, epithelial [Danio rerio]	3.436
	CB509522	TSC-22 *[Homo]	3.584

3.2.6 Validation of the Microarray Data

To validate the microarray data, the expression of 4 genes, which were regulated by growth, were selected to confirm the array by semi-quantitative RT-PCR (Table 3-14). The expression of the apolipoprotein gene (GenBank accession number CB493958 [P]) was up-regulated 2.15 times in fast growing fish using the microarray method and this was confirmed by semi-quantitative RT-PCR (5.62 ± 1.87). ATP synthesis lipid-BP (GenBank accession number CB493213 [P]) was found to be 3.62 times up-regulated in fast growing fish by microarray analysis and 3.90 ± 1.23 times by semi-quantitative RT-

PCR. Cadherin 1 (GenBank accession number CB517027 [P]) was found to be 3.44 times up-regulated by micro-array and 3.62 ± 0.89 times by semi-quantitative RT-PCR. TSC-22 (GenBank accession number CB509522 [P]) was 3.58 times up-regulation by microarray and 3.85 ± 0.71 times by RT-PCR. Thus results of semi-quantitative RT-PCR showed a similar trend as the microarray results, after normalized to the housekeeping gene β-actin.

Table 3-14. Semi-quantitative RT-PCR of differentially expressed genes obtained using the cDNA array. The genbank accession number of the gene, gene name, primer sequence, and fold changes in both RT-PCR and microarray are listed.

Clone ID	Gene name	primer sequences used for RT-PCR	Micro-array fold change	RT-PCR fold change
CB493958 [P]	Apolipo-protein	5'-3' TATGGCACAGTCACTGACCAACCT 3'-5'TGCTTCAGAAGAGTGAGGAGCTGA	2.15	5.62±1.87
CB493213 [P]	ATP synthesis lipid-BP	5'-3'ATGGGAACATAAAAAGGCAGGATT 3'-5'GCTAAGTTTGTCACCTCACCTGCT	3.62	3.90±1.23
CB517027 [P]	Cadherin 1, (epi.)	5'-3' ACTCTTCAGAAGCAAGCAAGAAAA 3'-5' TCTAACTGGACTGCCAGAATGAAC	3.44	3.62±0.89
CB509522 [P]	TSC-22	5'-3' GTTTTCTGCAGTTGGACAGAATCA 3'-5'CCCCCTACAGGGTAGAGATGGTAT	3.58	3.85±1.01

CHAPTER 4. DISCUSSION

Fish growth is influenced by many factors (Devlin et al., 2001; Diana, 2004a,b). THs have been shown to be important regulators of fish growth (Eales et al., 1999; Gomez et al., 1997). Since THs like other hormones, regulate the expression of specific genes, the identification of these genes will provide essential information on the physiological processes implicated in growth regulation (Hoopfer et al., 2002; Yang et al., 2007).

SECTION I: INDENTIFICATION OF TH-DEPENDENT GENE EXPRESSION

4.1.1 Fish Growth Rate and Gene Expressions Related with Thyroid Hormones

Exogenous T_3 administration has been reported to stimulate growth in several salmonid species. In coho salmon, body weight was increased by 14% compared to controls after 12 weeks of treatment with T_3 (Shelbourn et al., 1992). Likewise, immature Atlantic salmon treated for 45 days with T_3 showed a 7% increase in body weight over control (Lam, 1982). Conversely, hypothyroidism has been shown to cause growth retardation (Burel et al., 2000a,b; Leatherland, 1982; Reddy and Lam, 1992). Our results have shown that SGR was not significantly different between euthyroid, hypothyroid and hyperthyroid brook charr after 7 days of treatments. A possible explanation for this may be that the seven-day treatment period was too short to detect any effects of TH on growth. However, the significant changes in plasma hormonal levels may have been sufficient to elicit changes at the gene level.

4.1.2 Identification of TH-Dependent Genes in Brook Charr

THs regulate genes involved in growth such as the genes coding for basic transcription element binding protein, Fos-related antigen 2 and TR (Hoopfer et al., 2002; Yang et al., 2007). Down-regulation of hepatic TRβ expression has been shown to be associated with a decrease in growth rate while an increase in muscle TRα expression was accompanied with muscle accretion and myofiber hypertrophy (Yang et al., 2007). In the present study, 135 transcripts were differentially expressed between euthyroid, hypothyroid and hyperthyroid groups. From this pool, 18 genes were cloned and sequenced. Among the TH-regulated genes, that have been identified in our first experiment (Table 3-8), some known genes have been previously described as TH regulated in mammals. Our study has shown that those genes are also regulated by TH in fish.

4.1.2.1 Tropomyosin

TMs are ubiquitous proteins of 35 to 45 KDa associated with the actin filaments of myofibrils and stress fibers. In vertebrates, 4 known TM genes code for diverse isoforms of the transcript that are expressed in a tissue-specific manner and which are regulated by alternative splicing (Schachat et al., 1990). Although TMs are present primarily in muscle cells (Schachat et al., 1990; Schachat et al., 1985), they can also be expressed in non-muscle cells. There are over 40 TM isoforms that are derived from alternative splicing (Schevzov et al., 2005), and the TM isoform population is dramatically influenced by tissue activity and function (Prabhakar et al., 1999). Akutsu et al. (2002) first reported that several TM isoforms were present in the chicken liver and

several studies have shown that the expression of TM may be related to growth (Prasad et al., 1993; Akutsu et al., 2002). For instance, forced expression of Tm1, a TM isoform, suppresses neoplastic growth in mouse NIH 3T3 cells (Prasad et al., 1993). This suggests that TM may play a role in tissue development and growth. TM was also reported to be regulated by TH in bullfrog muscle (Dhanarajan et al., 1988), mice muscle (Prabhakar et al., 1999), and in human cardiac myofibrils (Machackova et al., 2005). It has been reported that TH regulates TM by modulating the α- and β-myosin heavy chain promoter through two thyroid regulatory elements present on the promoter of the genes (Prabhakar et al., 1999; Machackova, 2005).

Information on fish TM genes is very limited as compared to those from other vertebrates (Toramoto et al., 2004). A study of TM genes in pufferfish (*Fugu rubripes*) has shown that at least 6 TM genes are present in this species with respective sequences that are similar to each other (Toramoto et al., 2004). TM isoforms in skeletal muscle have also been reported in several fish species such as salmonids, herring and tuna (Heeley and Hong 1994; Huang and Ochiai 2005). Our data have indicated that there are three different sizes of the TM transcript (4.4; 7.5; 9.0 kb) in brook charr liver. Although the three TM genes (G-1-1, C-1-4, C-1-5) were only expressed in liver, the expression levels of the transcripts varied with the 7.5 kb transcripts being expressed at a higher level than the other two. This may provide new insights on the molecular mechanisms by which TH regulate TM in brook charr that there are at least 3 isoformes TM present in liver which share part of similar sequences each other.

4.1.2.2 Mitochondrial NADH Dehydrogenase Subunit 5

NADH dehydrogenase subunit 5 is one of the 7 mitochondrial DNA encoded subunits (mt ND1, mt ND2, mt ND3, mt ND4L, mt ND4, mt ND5, mt ND6) and is a member of the respiratory complex I (www.ncbi.nlm.nih.gov/OMIM). An up-regulation of mitochondrial gene expression could allow a rapid adaptation to increase respiratory and ATP requirements (Bai et al., 2000). In contrast, interferon-induced down-regulation of mitochondrial gene expression reduces cellular ATP levels in human Daudi lymphoblastoid cells. This reduction in cellular ATP levels appears to be responsible for inhibiting cellular growth (Lewis et al., 1996). Mitochondrial respiration is tightly regulated by the mt ND5 gene in human and mouse cells. It appears that decreasing mt ND5 level decreases the rate of respiration (Bai et al., 2000).

THs exert profound effects on energy metabolism and different mechanisms may underlie these effects. One of the mechanisms that has been suggested is through the regulation of mitochondrial gene expression and enzyme activity (Enriquez et al., 1999). Pillar and Seitz (1997) showed that many genes involved in mitochondrial energy metabolism were up-regulated by TH including enzymes associated with the citric acid cycle. Several mitochondrial genes have been reported to be regulated by THs. These include cytochrome c oxidase subunit in rat liver and skeletal muscle (Wiesner et al., 1992), cytochrome b, and subunits 1, 3, 5, 6 of the NADH dehydrogenase in mouse liver and brain (Iglesias et al., 1995; Stevens et al., 1995).

In the present study, mt ND 5 was up regulated by T_3 and down regulated by Na-ipodate. This suggests that THs may influence mitochondrial function and gene

expression in brook charr liver by regulating mt ND 5. Our study has also identified several known genes which have not been previously reported to be regulated by TH.

4.1.2.3 Crystalline Protein J1

Crystalline proteins are a diverse group of multifunctional proteins. Differences in expression of crystalline genes suggest that they have divergent physiological roles. For example, αB2-crystalline protein is not only expressed in the eye, but also in other tissues such as heart, brain, skeletal muscle, liver (Smith et al., 2006; Atomi et al., 1991; Bhat and Nagineni, 1989) and ovary (Beckemeyer and Shirk, 2004). These proteins are responsible for the optical properties of the lens but they may have non-refractive roles when crystalline genes are expressed in other tissues (Tomarev and Piatigorsky, 1996). Another member of this family of proteins, J1 crystalline protein was discovered in jellyfish (*Tripedalia cystophora*), but its function is unknown.

cDNA cloning experiments showed that the J1 crystalline subfamily is composed of three similar proteins (J1A, J1B and J1C; Piatigorsky et al., 1993). Each J1 crystalline gene is highly expressed in the lens of the eye. The structure of the gene reveals a potential retinoid X receptor-binding sequence present in the J1B-crystallin promoter. The regulation of J-crystalline genes may involve by the retinoid X receptor (Tomarev and Piatigorsky, 1996). The present study is the first to report that THs regulate this gene in brook charr liver. Since TRs form heterodimers with the retinoid X receptor and acts as a transcription factor to further initiate the TH-dependent gene expression (Yen, 2001), it is thus not surprised that this gene was to be regulated by THs. In charr, the tissue distribution of J1 crystalline protein suggests that it is not only present in the liver, but

also expressed in kidney, ovary, testis and spleen. However its role remains unclear as whether or not it plays the multifunctional role in these tissues.

4.1.2.4 Basic Transcription Factor 3

Four basic transcription factors (BTF1, BTF2, BTF3 and STF) have been found to be necessary for the initiation of transcription of eukaryotic genes from proximal promoter elements (Zheng et al., 1990). BTF3 can form a stable complex with RNA polymerase II for the initiation of transcription. The gene structure of human BTF3 has been identified (Klockars et al., 1997). This gene has homology with a bovine nascent-polypeptide-associated complex (NAC; Wiedman et al., 1994), and NAC has been reported to prevent apoptosis. The loss of this gene leads to inappropriate apoptosis in developing and differentiated cells in various tissues (www.ncbi.nlm.nih.gov/OMIM). We report for the first time that BTF3 is regulated by TH in brook charr. Northern blot analysis has shown that it is ubiquitously expressed in all the 9 tissues that were sampled (brain, heart, intestine, kidney, liver, ovary, testis, spleen and muscle). The level of expression of this gene is higher in the intestine, kidney, liver, ovary, testis, spleen and muscle than it is in the brain and heart. However, to determine its function will require further investigation.

4.1.2.5 Leukocyte-Derived Chemotaxin 1

Chemotaxin is an immune-relevant gene identified in the liver of rainbow trout (Bayne et al., 2001). Leukocyte-derived chemotaxin 1 (LECT 1) is identical to bovine chondromodulin 1 (ChM-I; Yanagihara et al., 2000). Northern blot analysis revealed that

the ChM-I mRNA transcript is approximately 1.7-kb and is expressed both in cartilage and other tissues (Yanagihara et al., 2000; Hiraki et al., 1999). In mouse, ChM-I was detected not only in cartilage but also in the heart (Yoshioka et al., 2006), thymus and eyes (Shukunami et al., 1999). Other studies suggest that ChM-I expression depends on the differentiated state of chondrocytes (Hering, 1999).

ChM-I mRNA levels were markedly increased in response to growth and differentiation of cultured chondrocytes (Shukunami and Hiraki, 1998). However, ChM-I also possesses inhibitory activity on the growth of vascular endothelial cells (Hiraki et al., 1997). Nakamichi et al. (2003) have shown that ChM-I$^{-/-}$ null mice have no overt abnormalities with the exception of a significant increase in bone mineral density with lower bone resorption in adults. This study revealed that ChM-I was not essential for normal cartilage formation and development. Indeed, ChM-I is more likely to be a bone-remodelling factor.

C-1-3 is 90% homologous to rainbow trout leukocyte-derived chemotaxin 1. It is only expressed in liver where the transcript is1.7 kb in size. The regulation of ChM-I in the charr liver by THs suggests a novel pathway in fish, not previously been reported. Since THs can influence cartilage and bone growth in zebrafish (Brown, 1997), it could be hypothesised that TH may be involved in bone development in fish through the regulation of ChM-I gene expression. In order to understand the function of ChM-I in fish, further investigations will be needed.

4.1.3 Identification of Novel Genes in Brook Charr

We have identified several unknown genes that are regulated by THs, which may suggest unidentified THs-regulated pathways. Seven genes were homologous to cDNA clones that presented in the EST database. Four additional genes had no homology to sequences available in the Genbank databases. These may be novel fish genes or represent specific brook charr genes which are sufficiently divergent from genes found in other organisms to preclude identification. DD-PCR can amplify sequences of genes containing the 3' untranslated region, which are often less conserved than the sequence of protein-coding region of a gene (Li, 1997).

4.1.4 TH-Regulated Genes and Novel Mechanisms of THs Actions

Northern blot analysis revealed that some unknown genes regulated by TH are present only in the ovary and not in testis (eg. C-1-6; G-2-T_2). On the other hand, genes like C-1-7 and C-2-4 are present in the testis but not in ovary. We have also observed that there are size differences of the C-2-4 mRNA in different organs. In intestine, kidney, testis, spleen and skeletal muscle, the major species of this transcript was 5 kb while in liver it exists as two different isoforms of 4 kb and 6 kb. The tissue specific distribution of C-2-4 raises the possibility that there is a mechanism for tissue-specific regulation. THs may regulate gene expression through different pathways in different tissues. The variation in transcript size among different tissues may be related to their different functions. Similarly, C-2-8 was also present in different organs. In brain, heart and intestine, kidney, liver, the mRNA size was 5.5 kb while in ovary and testis it was 3.0 kb.

Both of these isoforms were present in muscle. Getting information on these novel genes should provide potential gene models to study the mechanism of THs action in fish.

In summary, our study suggests that THs regulate different aspects of cellular metabolism and function, such as mitochondrial energy metabolism, gene transcription, immunity as well as bone development. These results are consistent with previous reports which suggest that THs mediate many metabolic effects in fish (Varghese and Oommen, 1999). They also suggest that certain molecular mechanisms of THs regulation may be conserved from fish to mammals.

SECTION II: GENES EXPRESSION PATTERNS IN FAST AND SLOW GROWING FISH

4.2.1 Growth-Related Genes and Expression Pattern

Subsequent to the identification of genes regulated by THs using DD-PCR, we used a 16 K cDNA microarray (GRASP) to identify genes that could be involved in growth regulation. The advantage of using cDNA microarray is that they enable simultaneous measurement and comparison of expression levels of thousands of genes (Schena et al., 1995, 1996).

A total of 12910 (80.7%) genes on the microarray were found to be expressed in brook charr liver. This proportion is higher than previous reports: 54.0% of adult Atlantic salmon and 63.3% of adult rainbow trout liver genes were bound on the 16 K microarray (von Schalburg et al., 2005). This may be due to the species and strain of fish used for this study.

The liver is central to many essential physiological processes (Busacker et al., 1990) and genes expression in the liver can be considered as good indicator and predictor of growth (Metón et al., 1999). We identified 94 genes that were up or down regulated in the liver of fast growing charr. The maximum changes in gene expression between fast and slow growing fish were around four-fold, data that were validated by semi-quantitative PCR. Growth regulated genes were classified according to their function: cellular immunity, carbohydrate and fatty acid metabolism, mitochondrial energy metabolism, signal transduction, protein metabolism and other functions. Among the 94 genes, 39 are known genes, and 55 (59%) are unknown. Among the 39 known genes, we found that 20 genes (51%) are THs-dependent genes. These results are in agreement with previous studies, which suggest that the regulation of fish growth by TH may occur through modification of specific genes (Eales, 1979; Donaldson et al., 1979; Higgs et al., 1982; Eales et al., 1999; Yang et al., 2007). Interestingly, we also found that genes related to mitochondrial energy metabolism and signal transduction were up regulated in T_3-treated fish as well as in the fast growing fish. In each functional subclass, we were able to identify genes that are regulated by TH. This is consistent with the notion that THs are involved in regulating growth.

4.2.2 Up-Regulated Genes in Fast Growing Fish

4.2.2.1 Genes Involved in Mitochondrial Energy Metabolism

The microarray analysis indicated changes in the expression of mitochondrial genes like cytochrome-C oxidase and ATP synthesis lipid-binding protein which are involved in energy metabolism. These genes are implicated in mitochondrial metabolic

pathways and are regulated by T_3 (Clement et al., 2002). This result is consistent with the idea that T_3 has a profound influence on mitochondrial biogenesis and metabolic balance. This may contribute to the marked effect of T_3 on cellular respiration (Clement et al., 2002) which in turn produces more ATP for cellular metabolism leading to growth. Interestingly, we also identified mitochondrial genes like mt ND 5 in the first section of this study. Together these results suggest that THs may regulate growth and that mitochondrial genes may play a role in this regulation.

4.2.2.2 Genes Involved in Carbohydrate and Fatty Acid Metabolism

Proteins and lipids are major sources of energy in fishes (Morais et al., 2006). Fish have low glucose tolerance and preferentially use body lipids instead of glycogen to derive energy even during starvation (Halver and Hardy, 2002). The rate of lipid and protein metabolism has been shown to be related to growth in several species (Morais et al., 2006). In this study, the expression of several lipid metabolic-related genes was altered in the liver of fast growing fish.

4.2.2.3 H-Fatty Acid-Binding Protein

We found that H-Fatty acid-binding protein (H-FABP) is up-regulated in fast growing fish. FABPs are abundant constituents of the cytoplasm that regulate lipid transport and metabolism. They play an important role in the uptake and transport of long-chain fatty acids, fuel utilisation and the interaction with other transport and enzyme systems (Stewart, 2000; Liu et al., 2003). Fourteen members of FABP family have been identified in mammals and named according to the initial site of isolation, e.g. brain (B-

FABP), heart (H-FABP), intestine (I-FABP), liver (L-FABP), and others (Stewart, 2000). In addition, FABP is known to be regulated by THs (Weitzel et al., 2003b; Stahlberg et al., 2005). Few studies have focused on the tissue-specific expression patterns of FABP in fishes (Stewart, 2000). Recently, Liu et al. (2003) reported that zebrafish H-FABP exhibited the greatest amino acid sequence identity to mammalian heart-type FABPs and that the H-FABP mRNA was localized in the ovary and liver, but not in the heart, muscle or brain as reported in mammals. The physiological function of this gene in fish may also differ from those observed in mammals as lipid storage and utilization are different between mammals and fish. The functions of H-FABP in fish need to be further studied.

4.2.2.4 Phosphofructokinase

Phosphofructokinase (PFK) was also found to be increased in the liver of fast growing fish. PFK converts fructose 6-phosphate and ATP to fructose 1,6-bisphosphate and ADP and is an important regulatory enzyme of glycolysis (Cowey et al., 1981; Panserat et al., 2001). Three types of PFK have been identified in mammals (muscle, liver, and brain type). The liver phosphofructokinase mRNA levels are regulated by nutritional and hormonal factors (Metón et al., 2000; Panserat et al., 2001; Seki et al., 2006). PFK gene expression was increased when previously starved mice were re-fed (Gehnrich et al., 1988). Studies on hepatic PFK in gilthead sea breams (*Sparus aurata*) demonstrated that the dietary composition regulates the expression of PFK suggesting that fish can adapt their metabolism by stimulating liver glycolysis to partially substitute proteins by carbohydrates in their diet (Metón et al., 2000). Therefore, the up-regulated

PFK in fast growing fish suggests that there is increased glycolytic activity in fast growing fish.

4.2.2.5 Apolipoprotein A-I

Apolipoproteins are important structural components of plasma lipoprotein and have been reported to participate in lipoprotein assembly, secretion and catabolism. Human apolipoprotein A-I (apoA-1) is primarily expressed in the intestine and liver (Ando et al., 2005). One of the major apolipoproteins, apo-28 k Da A-I is present in most fish high-density lipoprotein (HDL) and has homology with mammalian apoA-1, based on sequence similarity (Ando et al. 2005). However it remains to be determined whether apoA-1 has similar functions in fish and mammals. Interestingly there are studies which indicate that apoA-1 has THs binding properties (Benvenga 1997). Apolipoprotein 28 kDa is also a specific plasma component in fish.

4.2.2.6 Arylhydrocarbon Receptor

The arylhydrocarbon receptor (AhR) is a ligand-activated transcription factor. Several reports have suggested that the AhR may play an important role not only in the regulation of xenobiotic metabolism but also in the maintenance of homeostatic functions (Shimba et al., 2002). Evidence suggests that one of the possible physiological roles of the AhR is the regulation of cell proliferation. When A549 cells were treated with the AhR agonist, cell proliferation was stimulated. The clones that overexpress AhR grow faster than control cells, and their growth rate is proportional to the amount of AhR. So AhR, presumably in collaboration with the aryl hydrocarbon receptor nuclear

translocator, activates DNA synthesis and subsequent cell proliferation in A549 cells (Shimba et al., 2002). An increase in AhR indicates that there is an up-regulation of genes involved in proliferation and growth. How this could result in increased body mass has yet to be determined.

4.2.2.7 Cell Proliferation and Differentiation

There are several genes whose function is related to cell proliferation and differentiation such as epithelial cadherin (E-cadherin) and transforming growth factor-β1-stimulated clone 22 (TSC-22), that are up-regulated by more than 3-fold in the liver of fast growing fish.

E-cadherin was the first member of the cadherin family to be identified and is crucial for strong cell adhesion. It has been reported to be expressed in most vertebrate species. Cadherins are a gene superfamily of integral membrane glycoproteins mediating calcium-dependent cell adhesion via homophilic interactions. Thus, it controls the transfer of information between cells, controls cell growth, and cell motility (Gumbiner, 1996). Inhibiting E-cadherin protein expression reduces early zebrafish embryo survival (Babb and Marrs, 2004). E-cadherin gene knockout mice die at a very early stage, demonstrating its fundamental role in the formation of epithelial tissues (Larue et al., 1994). The expression pattern of E-cadherin in mouse, chick, and zebrafish indicate that it is implicated in many developmental processes, including early embryogenesis, epithelial tissue formation, and neurogenesis (Babb and Marrs, 2004). Recently, Dasgupta et al. (2005) studied the function of E-cadherin in liver ES cells and show that the expression of E-cadherin in ES cells can elicit a marked response to growth factor

stimulation and lead to the induction of later stages of hepatocyte maturation. Thus, E-cadherin could be used to harness the cross talk between the hepatotrophic and cadherin-based signalling pathways for controlled acceleration of ES hepatodifferentiation. E-cadherin mRNA levels were regulated by the thyroid inhibitor propythiouracil (PTU) in epididymidis. For example, treating rats with PTU, resulted in decreased E-cadherin mRNA levels in the cauda epididymidis, whereas levels were increased in caput-corpus epididymis (St-Pierre et al. 2003).

TSC-22 gene influences cellular differentiation and is expressed in almost all organs of mice and humans (Choi et al., 2005). Its expression is induced by many different factors including transforming growth factor-β (Shibanuma et al., 1992). The function of TSC-22 gene in fish and its mechanism of action are unknown. The increased E-cadherin and TSC-22 gene expression in fast growing fish suggest novel signalling pathways implicated in cell proliferation and differentiation.

4.2.2.8 Genes Involved in Cellular Immunity

Eight genes that were differently expressed between fast and slow growing fish are involved in cellular immunity. Among those, six are down regulated in fast growing fish. A study on immuno-stimulated fish has shown that chronic immune stimulation reduces muscle growth efficiency in rainbow trout. An increased energy demand for immunity could deplete energy stores and then decrease growth (Johansen et al., 2006). However, the two others were up regulated.

4.2.2.8.1 Pentraxins

Pentraxins are a family of pentameric serum proteins. There are two pentraxins that are present in mammals and are the products of homologous genes (Bayne et al., 2001). In fish, the presence of two distinct pentraxin genes has been questioned (Seery et al., 1993; Rubio et al., 1993; Jensen et al., 1997). The number of pentraxin genes present appears to be different among various fish species. One pentraxin has been reported in salmon, wolf-fish, cod and halibut (Lund and Olafsen, 1998). However, two pentraxins have been reported in dogfish and plaice (Bayne et al., 2001; Bayne and Gerwick, 2001). The difference in the number of pentraxins may be related to the different functions. Pentraxins have been implicated in innate immunity, inflammation and matrix deposition. They are important components of the immune response in mammals (Jensen et al., 1997). Although pentraxins have been isolated from a number of fish species (Cook et al., 2003), few studies provide evidence of their immunological role in fish (Cook et al., 2005). It has been found that pentraxins are a *negative* acute phase protein and they were shown to be down-regulated in the liver of rainbow trout after fish received an intraperitoneal injection of bacterium (*L. anguillarum;* Gerwick et al., 2007). Further studies need to be done to understand the function of these genes.

4.2.2.8.2 Precerebellin-Like Protein

Precerebellin-like protein in rainbow trout belongs to the C1q — globular domain family of proteins and is synthesised in the liver (Bayne et al., 2001). A phylogenetic analysis indicates a close relationship between trout precerebellin-like protein and the precerebellin protein sequences from rat and mouse (Bayne et al., 2001). The

physiological function of precerebellin has yet to be determined completely but it would appear that the precerebellin-like protein in trout is involved in the innate immune response (Kodama et al., 1989). This suggests that precerebellin-like protein in trout has a defensive immune function. Further study of this gene would be interesting and help in determining its function in humans, mice and fish.

4.2.2.9 Genes Involved in Signal Transduction

Three signal transduction genes were up-regulated in fast growing fish. They were also previously reported to be regulated by T_3 (Feng et al., 2000; Miller et al., 2001; Weitzel et al., 2001, 2003b; Clement et al., 2002; Wood et al., 2002; Flores-Morales et al., 2002; Stahlberg et al., 2005). The influence of T_3 on somatic growth may also occur through cellular trafficking and tissue remodelling through increased expression of genes involved in protein transport and maturation.

4.2.2.9.1 Secreted Phosphoprotein 2

Secreted Phosphoprotein 2 (SPP2) encodes a 24-kD secreted phosphoprotein that shares sequence homology with members of the cystatin family of thiol protease inhibitors and is expressed in bone, as cystatin. Bennett et al. (2004) identified protein sequences similar to SPP2 in several mammalian species and in chicken, salmon, and trout. These phosphoproteins are implicated in a wide variety of cellular processes, including carbon and energy metabolism, transport, stress and development.

4.2.2.9.2 Seven Up -40

The protein encoded by the zebrafish seven up -40 (svp-40) gene is a member of the steroid hormone receptor superfamily. This gene is expressed in specific regional and segmental domains within the developing brain. The function of svp-40 is not clear and it could be involved in neural patterning during early embryonic development (Fjose et al., 1995). Kanai et al. (2005) showed that svp-40 controls switching of transcription factors in *Drosophila neuroblasts*. Svp-40 has also been shown to be regulated by THs (Feng et al., 2000). The svp-40 gene is over expressed by more than 3-fold in fast growing fish liver. This is the first report suggesting that this gene could be related to growth

4.2.2.10 Transcription Factors

Transcription elongation factor B encodes an elongation protein which is a subunit of the transcription factor SIII complex. The SIII complex is composed of elongation factors A/A2, B and C (http://www.ncbi.nlm.nih.gov/OMIM). Elongations assist RNA polymerase II by suppressing transient pausing of the polymerase at many sites within the transcription units. Elongation A functions as the transcriptionally active component of the SIII complex. Elongation B and C are regulatory subunits (Aso et al., 1995). Transcription elongation factor B was found to be up regulated in fast growing fish. This suggests that increased fish growth resulted by up-regulation of nuclear factors involved in transcriptional control in the liver.

4.2.3 Down-Regulated Genes in Fast Growing Fish

4.2.3.1 Prolactin

Prolactin (PRL) is a glycoprotein hormone, which is secreted by the anterior pituitary. In higher vertebrates, the PRL gene is expressed not only in the pituitary but also in several PRL target tissues. It has been reported that PRL is synthesized in the mammary gland, brain and spinal cord (Nolin and Witorsch, 1976; Fields et al., 1993; Kurtz et al., 1993; Le Provost et al., 1994; Gabou et al., 1996; Emanuele et al., 1992; Wilson et al., 1992). In teleosts, PRL has been purified from five species (Leena et al., 2001). Gene expression has also been detected in several tissues of the sea bream (*Sparus aurata*), such as the intestine, liver, ovary, and testes (Santos et al., 1999). PRL plays an important role in freshwater osmoregulation by preventing both loss of ions and water uptake (Manzon, 2002). It also influences several important physiological processes, such as growth, reproduction, mucus production and metabolism (Manzon, 2002; Prunet et al., 1990). Research on the effects of PLR on lipid metabolism showed that PRL has an inhibitory effect on lipid metabolism in climbing perch (*Anabas testudineus*) through significant inhibition of malic enzyme, glucose-6-phosphate dehydrogenase and NADP-isocitrate dehydrogenase activities (Leena et al., 2001). In the present study, the PRL gene was down regulated in fast growing fish.

4.2.3.2 Cytochrome P-4501A1, P-450 4A3

The CYP genes encode enzymes of the cytochrome P-450 superfamily, which are expressed mainly in the liver and are active in mono-oxygenation and hydroxylation of various xenobiotics. Cytochrome P-450 1A1 and cytochrome P-450 3A45 are members

of this family. The total level of expression of CYP genes is up or down regulated by several circulating hormones (Yoshida et al., 1996). Both GH and THs (T_3 and T_4) down-regulate CYP forms in rats (Oinonen and Lindros, 1998). THs also suppress the expression of cytochrome P-450 4A3 mRNA (Feng et al., 2000). We observed that in fast growing fish, expression of cytochrome P-450 1A1 and cytochrome P-450 3A45 decreased which in turn, could reduce fatty acid oxidation and increase lipogenesis.

4.2.4 Identification of Novel Genes Related with Growth

The analysis and interpretation of microarray data depends on previous knowledge such as annotation of genes spotted on the microarray, the accurate identification of genes and the sequence databases used. The GRASP salmon microarrays contained 16006 salmon cDNAs. Among those cDNAs, more than 50% are EST which have unknown function (von Schalburg et al., 2005). Despite these potential limitations, we were able to demonstrate the utility of cDNA microarrays to identify novel target genes in fish and extend the applicability of this powerful technology. Characterisation of EST's will contribute to better annotations for the fish microarray. The information will contribute to a deeper understanding of biological diversity in many areas including genetics, physiology, growth, development, and evolution. Based on the data analysis, we found that 40 unknown genes were up-regulated and 15 were down-regulated with growth. The unknown genes correspond to novel putative target genes and novel pathways. The identification of these growth-related genes can also be added to fish databases for further study and could be used to study on other species. They could also be included in gene arrays specifically targeted to study growth.

Eighteen gene sequences have been identified as THs-regulated based on the DD-PCR study. Seven of them showed significant homology to genes previously described in other species. Eleven were unidentified or novel EST sequences. These ESTs should provide useful data for the development of DNA microarray analysis of fish gene expression, and the addition of further genetic research.

Unfortunately some genes which were found to be differentially expressed with the use of the DD-PCR technique were not included on the GRASP array, for example, the mt ND5, MT, J1 crystalline protein and other unknown genes which were found to be regulated by THs. While some genes which were detected as TH-dependent genes were blotted on the GRASP microarray, such as LECT1, BTF 3 but were not detected as differentially expressed in the microarray slides. This may be due to the lower sensitivity microarray analyses.

From this study, we identified that TH-dependent genes in brook charr and the function of some of these are related to growth. Among the 39 known growth-dependent genes which have been identified in brook charr, 20 genes (51%) are TH-dependent as demonstrated in previous publications (Day and Maurer, 1989; Feng et al., 2000; Clement et al., 2002; Flores-Morales et al., 2002; Weitzel et al., 2003b; Stahlberg et al., 2005; Table 3-13). Therefore, some TH–dependent genes in brook charr are also growth dependent. Regulation of gene expression is a key mechanism whereby hormones exert their regulation. Results from gene expression profile in fast versus slow growing fish support previous studies which indicate that THs are implicated in the regulation of fish growth.

In our first experiment, we detected 135 transcripts which were regulated by THs, but only 18 were cloned and sequenced. Most of these 18 genes are unknown genes and were not spotted on the 16 K microarray. However, 58.5% of growth-dependent genes obtained from the microarray data have unknown functions. This incomplete knowledge represents an important obstacle to understand the relationship between growth-dependent and TH-dependent genes. In the future, it should be interesting to clone and sequence the remaining 117 transcripts which were detected in the first experiment. This could allow the identification of more TH target genes and provide a better understanding of the regulation of growth by THs in fish.

CONCLUSION

Importance of Genomic Studies to Understand THs Biology and Growth in Fish

Brook charr is a major aquaculture species in Quebec and is heavily used in stock enhancement programs. Therefore, there are both important scientific and economic reasons for conducting genomic research on this species. The genetic regulation of fish growth for commercially important fish species has been studied. However, very few studies tried to link the functional relationship between genetic variation and physiological parameters. THs have been reported to regulate fish growth. A limited number of THs target genes have been identified. Moreover, most previous studies focused on rodent and human (Feng et al., 2000), and studies on fish are lacking.

We identified 135 transcripts regulated by THs. This represents a significant amount of new data that can be further explored. Among these genes, 18 were sequenced and identified. We have functionally classified the differentially expressed genes to explain the effects of T_3. The data also revealed new gene targets for the biologic action of T_3, extending beyond the classic metabolic effect of THs. We also identified 94 growth-dependent genes in brook charr liver. Among them, a number of transcripts were detected, which are regulated by THs. These growth-dependent genes were further classified based on their molecular functions and on the basis of expression patterns regulated by TH and growth rate in fish liver. All of those works could have the potential to improve our understanding of cellular mechanisms and transcriptional changes involved with certain physical characteristics in brook charr. We believe that it is important to improve this type of knowledge in aquaculture.

Significance of understanding the mechanism of THs biology and growth

Previous studies have shown that although fish and mammalian genes appear to have similar sequences, they have quite differential expression patterns and functions in fish when compared to humans or mice (Currie and Ingham, 1996; Zardoya et al, 1996). It may not be possible to apply the concept of homology to those genes in order to interpret structural similarity or to extrapolate a known function from one taxonomic group to fish. In the present study, we found that 7 genes and 11 ESTs were regulated by THs. Further 55 unknown genes and 39 known genes were related to growth in brook charr. Most of them are novel target genes of THs. This knowledge database can be exploited to design studies on fish endocrine and growth regulation. The 11 ESTs had been submitted to the EST bank. The value of the EST submissions resulting from this study is to increase the fish EST resources and to provide new insights on genetic and functional studies in other fish species. Those EST can be used to explore orthologous relationship with vertebrate species including humans and mice.

Teleosts, the most diversified group of all vertebrates, comprise over 25000 species classified in more than 120 families (Nelson, 1994). The complex genetic architecture has allowed fish to develop morphogenic variations and different phenotypes in order to adapt to different environmental conditions. Salmonids are known as 4N teleosts. It had been proposed that in early teleosts, the entire genome was duplicated and the increasing database on characterised fish genes also strongly suggests that fish contained more genes than higher vertebrates (Amores et al., 1998; Wittbrodt et al., 1998). Gene duplication may be responsible for some genes for having numerous subfunctions and perform a variety of tasks in different tissues and at different

developmental times. Our data revealed the gene expression patterns in response to T_3 and growth in brook charr liver. The functions and tissue distributions of some genes are different between fish and mammal. Some genes might have multiple functions in fish. All this work will contribute to a better understanding of gene functions in fish and will enhance our awareness of the genes and signalling pathways related either to THs or growth in fish species.

The selection and genetic improvement of fish stocks require new techniques that allow a more precise understanding of the cellular and physiological aspects that underlie traits of interest. We combined DD-PCR and microarray technologies to detecte both known and unknown genes, which are related with TH and growth. Our study illustrates that these techniques could be applied to many other important fields. For example, it could be used to understand regulation of other traits of interests as disease resistance or food conversion efficiency and could favourably impact cost of production.

In summary, the study of growth is important in aquaculture and fisheries management. The genomic information gained from brook charr by this study will be useful for improving the growth traits of cultured species. The novel TH-dependent genes (11) and their pathways of action deserve further investigation. The remaining 117 transcripts which were related with THs will need to be cloned and subsequenced. The 39 known genes which were related with growth and several TH-dependent genes could be used as potential growth predicting tools. The 55 unknown growth dependent genes also deserve further investigation.

SYNTHÈSE DE LA THÈSE

Problématique et objectifs

La pêche demeure une très importante source de nourriture, d'emplois et de revenus dans de nombreux pays et communautés. Toutefois, les pêches commerciales ont connu un fort déclin depuis le record de captures enregistré en 1995 (FAO, 2004) et les espèces visées sont actuellement soit pleinement exploitées, soit surexploitées (FAO, 2004). La gestion des ressources halieutiques se doit d'intégrer les caractéristiques biologiques des différentes espèces incluant leur taux de croissance.

L'aquaculture, marine et d'eau douce, joue un rôle prépondérant dans notre capacité à répondre aux besoins toujours croissants. L'utilisation de la génétique pour améliorer les traits de performance des poissons d'élevage est l'un des éléments qui nous permettront de maximiser la productivité tout en minimisant les coûts de production. Parmi ces caractéristiques, le taux de croissance est certainement un trait à considérer.

Les poissons à forte croissance acquièrent un accès prioritaire à la nourriture et aux meilleurs territoires (Riechert, 1998) et sont moins vulnérables à la prédation (Diana, 2004a, Werner et al., 1983). La croissance affecte également le succès reproducteur (Roff, 1992) et dans certains cas les variations de taux de croissance peuvent être utilisées dans des programmes de suivis environnementaux. Les caractéristiques génétiques et environnementales influencent la croissance des poissons, laquelle est régulée par le système endocrinien.

Depuis 1970, de nombreuses études ont montré l'effet de diverses hormones dont l'hormone de croissance, les hormones thyroïdiennes et les stéroïdes anabolisants sur la

croissance des poissons et plusieurs ont été étudiées en tant que facteurs de croissance dans des buts d'ensemencement ou de production aquicole (Matty, 1985). Toutefois, de nombreux facteurs influencent l'efficacité d'un traitement hormonal sur la croissance incluant la dose, la voie d'administration, la température de l'eau, la qualité de la nourriture, le mode d'alimentation, l'espèce et la taille des poissons (Higgs et al., 1982; 1992; Lam, 1982 ; Cyr et al., 1998 ; Tanangonan et al., 1989). Il est donc difficile de mettre en œuvre un traitement hormonal efficace permettant d'augmenter le taux de croissance. De plus, on sait encore peu de choses sur l'expression des gènes associés aux fonctions endocriniennes.

L'amélioration génétique est devenue de plus en plus importante pour assurer le développement durable de l'aquaculture et la croissance est un des principaux traits de performance visés. Les méthodes traditionnelles telles que la domestication, la sélection, les croisements inter spécifiques et inter souches, la polyploïdie et la création de populations mono sexuées ont été utilisées afin d'améliorer la performance des stocks d'élevage. Cependant, l'application pratique de ces techniques connaît des limites et les croisements intra- et inter spécifiques, l'hybridation et la polyploïdie ne conduisent pas toujours à une amélioration du taux de croissance (Dunham et Smitherman, 1983 ; Dunham et Devlin, 1999).

Les techniques génétiques récentes ont été appliquées à l'aquaculture afin d'améliorer le taux de croissance (Houdebine et Chourrout, 1991). Devlin et al. (1994) ont montré que l'insertion d'un transgène de l'hormone de croissance (GH) pouvait augmenter la croissance des poissons de façon dramatique. Ce résultat n'a pu cependant être répété chez d'autres espèces de poissons (Penman et al., 1991). Ceci pourrait résulter

du manque de séquences génétiques disponibles chez les poissons. Il apparaît donc important de développer un promoteur universel capable d'être reconnu par toutes les espèces de poissons.

Les techniques récemment développées en biologie moléculaire offrent la possibilité d'identifier les gènes qui participent à la régulation de traits à valeur économique importante. Leur utilisation combinée aux méthodes plus traditionnelles offre de nouvelles perspectives dans le domaine de l'amélioration génétique chez les poissons d'élevage. Les techniques de criblage différentiel par réaction en chaîne de la polymérase (ddPCR) et les biopuces à ADN sont deux approches utiles qui permettent une meilleure compréhension des changements génomiques au niveau transcriptionnel.

Au Québec, l'omble de fontaine est une des principales espèces produite en aquaculture dulcicole. L'acquisition de connaissances sur l'expression des gènes liés à la croissance chez cette espèce pourrait s'avérer utile dans l'amélioration de traits de performance à valeur économique.

Les hormones de la thyroïde jouent un rôle important dans la régulation de la croissance chez les poissons (Moav et McKeown, 1992). L'étude des patrons d'expression des gènes thyroïdo-dépendants pourrait fournir des informations très utiles pour la prédiction des performances de croissance chez les poissons.

Les objectifs de la présente étude sont : 1) d'identifier les patrons d'expression de gènes thyroïdo-dépendants chez l'omble de fontaine ; 2) d'identifier par une approche génomique des gènes qui répondent de façon différentielle chez des ombles de fontaine à faible et à forte croissance. Les résultats nous permettront de mieux comprendre la régulation de la croissance chez les poissons et aideront à identifier les mécanismes par

lesquels les hormones thyroïdiennes participent à cette régulation. Ils pourront également fournir à l'industrie des outils permettant de sélectionner des individus à forte croissance par l'utilisation des profils d'expression génique.

Résultats

Afin d'identifier les gènes thyroïdo-dépendants, nous avons traité des ombles de fontaine soit avec de la triiodo-thyronine (T_3) soit avec de l'ipodate de sodium (drogue anti-thyroïdienne). Après 7 jours de traitement, les concentrations en T_3 telles que mesurées par radioimmunoessai indiquaient des niveaux plus élevés chez les animaux traités à la T_3 (269.4 ± 20 ng/ml) que chez les contrôles (2.24 ± 0.2 ng/ml) et significativement plus faibles chez ceux traités à de l'ipodate de sodium (0.85 ± 0.05 ng/ml).

La technique de ddPCR a été utilisée pour identifier les gènes thyroïdo-dépendants au niveau hépatique. Au total, 135 transcrits différentiels ont été identifiés et 18 clones ont été séquencés. En comparant ces séquences par homologie avec les bases de données existantes, six séquences présentaient une forte homologie avec des gènes connus : la sous-unité 5 de la NADH déshydrogénase d'omble de fontaine, la chémotaxine dérivée des leucocytes et pré-identifiée chez la truite arc-en-ciel, une séquence présentant une forte homologie avec le facteur de transcription 3 chez l'humain, une séquence proche de la protéine « crystalline J1 » et 3 séquences présentant une homologie significative avec le gène de la tropomyosine du saumon atlantique. Dans ce dernier cas, ces trois séquences pourraient représenter soit différentes régions d'un même gène, soit différentes isoformes. Sept ADN complémentaires (ADNc) ont montré une

forte homologie avec des « expressed sequence tags » (EST), gènes de saumon atlantique (6) ou de truite arc-en-ciel (1) dont la fonction est inconnue. Finalement, aucune homologie avec des gènes connus ou des EST n'a pu être trouvée pour les 4 derniers, qui pourraient donc représenter de nouveaux gènes. Les analyses par buvardage Northern ont montré une distribution et une expression variables en fonction des tissus testés.

Afin de caractériser les profils d'expression de ces gènes, une biopuce à ADN de 16,000 gènes a été utilisée pour comparer les profils de poissons à croissance rapide avec ceux de poissons à croissance lente. Tous les animaux utilisés pour cette partie de l'étude ont été élevés de façon identique et des poissons de deux familles ont été échantillonnés, celles-ci ayant été sélectionnées sur la base des taux de croissance mesurés. Le poids moyen des poissons issus de la famille à croissance rapide était de 54.73 ± 12.14 g et celui des poissons issus de la famille à croissance lente de 8.43 ± 1.59 g. L'ARN hépatique provenant des deux poissons de chacun des groupes a été utilisé pour préparer une sonde d'ADNc (n = 2). Après marquage avec des sondes Cy3 et Cy5, l'ADNc fut utilisé pour une hybridation avec la biopuce à ADN de 16K. Les lames ont été étudiées à l'aide du logiciel QuantArray (Packard Biosystem) et les données analysées à l'aide de GeneSpring 7.0 (Agilent). Quatre analyses indépendantes par biopuce ont mis en évidence un total de 94 gènes exprimés de façon différentielle (69 régulés à la hausse et 25 régulés à la baisse). Ces résultats furent confirmés par Q-PCR. Des 94 gènes exprimés de façon différentielle, 8 sont impliqués dans l'immunité cellulaire, 9 dans le métabolisme des sucres et des acides gras, 3 dans le métabolisme protéique, 3 dans la transduction des signaux, les autres ayant des fonctions encore inconnues. De ces gènes, 20 sont thyroïdo-dépendants si on base sur les données de la littérature.

Discussion

La première portion de cette étude a permis d'identifier de nouveaux gènes dont l'expression est régulée par les hormones thyroïdiennes. Pour certains, leur lien avec les hormones thyroïdiennes avait déjà été montré chez d'autres espèces. Cependant, notre étude est la première à montrer que ces gènes, tel que la tropomyosine (TM), sont sous contrôle thyroïdien chez les poissons. Il est également intéressant de noter que certains des gènes identifiés dans cette étude n'avaient pas été auparavant identifiés comme étant thyroïdo-dépendants. Quatre de ces gènes sont déjà connus et sept possèdent une homologie avec des séquences ADNc déjà répertoriées dans la base de données EST.

La protéine J1 crystalline a déjà été décrite chez la méduse (*Tripedalia cystophora*) chez qui sa fonction est inconnue. La présente étude est la première à démontrer la régulation de la protéine J1 par les hormones thyroïdiennes chez l'omble de fontaine. Chez cette espèce, le gène de la protéine J1 est exprimé dans le foie, le rein, la rate, les ovaires et les testicules. Cette large distribution suggère que cette protéine pourrait être impliquée dans de nombreuses voies de régulation.

Plusieurs gènes mitochondriaux, telles que les sous unités 1, 3, 5, 6 de la NADH déshydrogénase dans le foie et le cerveau de souris sont sous contrôle thyroïdien (Iglesias et al., 1995 ; Stevens et al., 1995). Nous montrons pour la première fois que la sous unité 5 de la NADH est régulée par les hormones thyroïdiennes chez le poisson.

Le rôle du facteur de transcription 3 est d'initier la transcription des gènes chez les eucaryotes. Cette activité implique l'assemblage ordonné d'un complexe multi protéinique sur l'élément promoteur proximal (Zheng et al., 1990). Nous sommes les premiers à rapporter la régulation de ce gène par les hormones thyroïdiennes chez

l'omble de fontaine. Les analyses de type Northern montrent que son expression est largement distribuée dans les 9 tissus analysés : cerveau, cœur, intestin, rein, foie, ovaires, testicules, rate et muscle. Nous avons également montré que la chémotaxine dérivée de leucocytes est régulée à la baisse par les hormones thyroïdiennes dans le foie de l'omble de fontaine.

Quatre des gènes que nous avons clonés ne sont homologues avec aucune des séquences disponibles dans les banques de données. Ces gènes pourraient être des gènes non précédemment identifiés chez les poissons. Ces gènes hépatiques régulés par les hormones thyroïdiennes constituent un outil potentiel pour l'étude de la régulation transcriptionnelle. Le fait que ces gènes ne soient pas encore identifiés suggère que les hormones thyroïdiennes pourraient agir via des voies non encore connues. Les résultats présentés soulignent également l'utilité d'une approche basée sur la technique de ddPCR pour détecter des gènes répondant à une induction hormonale chez des espèces spécifiques.

Les hormones thyroïdiennes jouent un rôle prépondérant dans la régulation de la croissance chez les poissons et de ce fait ont fait l'objet de nombreuses études (Eales, 1979 ; Higgs et al., 1982 ; Lam, 1982 ; Leatherland and Farbridge, 1992). Les résultats présentés ici indiquent une action des hormones thyroïdiennes sur l'expression des gènes de la sous unité 5 de la déshydrogénase mitochondriale, de la tropomyosine, et de la chémotaxine dérivée de leucocytes en accord avec les résultats publiés précédemment. Une régulation à la hausse de l'expression des gènes mitochondriaux pourrait permettre de s'ajuster à un accroissement de la demande respiratoire et d'ATP (Bai et al., 2000). Lewis et al. (1996) ont montré qu'une régulation à la baisse de l'expression des gènes

mitochondriaux conduit à une détérioration du fonctionnement des mitochondries dans les cellules lymphoblastoïdes de Daudi humaines. L'inhibition de la croissance cellulaire pourrait être partiellement dûe à une réduction de la concentration cellulaire en ATP. Ainsi, l'inhibition de la fonction mitochondriale pourrait être au moins partiellement responsable de l'inhibition de la croissance. Une étude récente indique une régulation de la respiration mitochondriale par le gène ND5 (Bai et al., 2000). La démonstration que, chez les poissons, le gène ND5 est soumis à une régulation thyroïdienne pourrait ouvrir de nouvelles voies de recherche pour la compréhension de la fonction de ce gène en relation avec la croissance.

Le gène de la chémotaxine 1 dérivée des leucocytes (LECT 1) est identique à celui de la chondromoduline (ChM1) bovine (Yanagihara et al., 2000). L'analyse par buvardage de type Northern indique que la taille de son ARNm est d'environ 1,7 kb et qu'il est exprimé dans le cartilage ainsi que dans d'autres tissus (Yanagihara et al., 2000; Hiraki et al., 1999). Chez la souris, ce gène est également exprimé dans le thymus et les yeux (Shukunami et al., 1999). Les activités bifonctionnelles de ce gène ont été démontrées lors d'études in vitro. Les travaux de Nakamichi et al. (2003) avec des souris transgéniques ChM-I-/- démontrent que la ChM-I n'est pas essentielle à la formation et au développement normal du cartilage. En effet, la ChM-I est plus probablement impliquée dans le remodelage osseux. Il se peut que la ChM-I soit impliquée dans le développement osseux ou qu'il existe des différences dans la fonction de ce gène chez divers groupes d'animaux.

Chez l'omble de fontaine, certains gènes régulés par les hormones thyroïdiennes ont été dupliqués lors de l'évolution. Nos résultats démontrent la présence de trois

produits de transcription pour le gène de TM, soit de 4,4 kb, 7,5 kb et 9,0 kb, qui sont tous exprimés uniquement dans le foie. La présence d'isoformes de ce gène a déjà été démontrée dans le muscle squelettique d'autres espèces incluant des salmonidés, le hareng et le thon (Heeley et Hong 1994; Huang et Ochiai 2005). La duplication de gènes a peut-être ainsi permis la séparation de certaines fonctions. Dans la présente étude, l'expression étant limitée au foie, ceci suggère une régulation à la hausse dans cet organe via les hormones thyroïdiennes.

Nous avons observé une différence dans la taille de l'ARNm de C-2-4 selon le tissu étudié. Dans l'intestin, le rein, les testicules, la rate et le muscle squelettique, la principale forme d'ARNm exprimée était un fragment de 5 kb alors que dans le foie deux isoformes, respectivement de 4 et 6 kb, étaient présentes. Les différences tissulaires d'expression du C-2-4 suggèrent qu'il existe un mécanisme de régulation tissu-spécifique. Les hormones thyroïdiennes pourraient réguler l'expression de ce gène par différentes voies de signalisation selon le type de tissu visé. La présence de différentes isoformes suggère également la présence de différentes fonctions selon le tissu concerné.

Nos résultats indiquent la présence de certains gènes inconnus et régulés par les hormones thyroïdiennes dans l'ovaire, mais non dans le testicule (C-1-6 et G-2-T_2). À l'inverse, certains sont exprimés dans le testicule, mais non dans l'ovaire (C-1-7 et C-2-4). Ces gènes pourraient être impliqués dans une voie de signalisation inconnue dans laquelle les hormones thyroïdiennes agiraient sur d'autres hormones, dont les hormones stéroïdiennes, pour induire l'expression d'autres gènes. Ces interactions pourraient avoir des effets non seulement au niveau de la fonction reproductrice mais également au niveau du métabolisme, de l'apport énergétique et de la croissance somatique.

En résumé, notre étude suggère que les hormones thyroïdiennes régulent différents aspects du métabolisme cellulaire ainsi que différentes fonctions incluant le métabolisme énergétique mitochondrial, la transcription génique, la fonction immunitaire et le développement osseux (Tableau 3-8). Les résultats de cette étude concordent avec des études précédentes qui démontrent de nombreux effets des hormones thyroïdiennes sur le métabolisme des poissons (Varghese and Oommen, 1999). Ils suggèrent également que certains mécanismes de régulation moléculaire des hormones thyroïdiennes ont été conservés des poissons aux mammifères.

Expression génique chez des poissons exprimant différentes performances de croissance

Suite à l'identification par ddPCR de gènes régulés par les hormones thyroïdiennes, nous avons utilisé les biopuces à ADN pour identifier les gènes impliqués dans la régulation de la croissance. La biopuce utilisée contenant 16 mille gènes de saumon, n'avait jamais été utilisée pour étudier le profil d'expression de gènes impliqués spécifiquement dans la croissance. L'avantage de cet outil moléculaire est qu'il permet de mesurer et de comparer simultanément les taux d'expression de milliers de gènes (Schena et al., 1995; 1996). Dans notre étude, 94 gènes ont été identifiés comme étant régulés à la hausse ou à la baisse dans le foie d'omble de fontaine à forte croissance.

Parmi ces 94 gènes, 39 gènes étaient connus et 55 encore inconnus. Parmi les 39 déjà connus, 20 (51 %) avaient déjà été identifiés dans la littérature comme étant régulés par les hormones thyroïdiennes (Eales, 1979; Donaldson et al., 1979; Higgs et al., 1982; Eales et al., 1999; Yang et al., 2007). Il est intéressant de noter que, comme chez les

poissons traités à la T_3, nous avons observé chez les poissons à forte croissance une surexpression de gènes liés au métabolisme énergétique mitochondrial et à la transduction des signaux. Dans chacune des sous-classes fonctionnelles, nous avons pu identifier des gènes régulés par les hormones thyroïdiennes, ce qui confirme la notion de régulation de la croissance par les hormones thyroïdiennes.

Ainsi, nous avons détecté plusieurs gènes mitochondriaux régulés à la hausse chez les poissons à croissance rapide, dont la cytochrome-C oxidase et l'« ATP synthase lipid-binding protein » tous les deux étant impliqués dans les voies métaboliques mitochondriales et régulés par la T_3 (Clement et al., 2002). Ce résultat concorde avec le fait que la T_3 a une profonde influence sur la biogenèse mitochondriale et l'équilibre métabolique. Ces processus pourraient contribuer à l'effet de la T_3 sur la croissance.

Les protéines et les lipides sont les principales sources d'énergie chez les poissons (Morais et al., 2006). Chez les poissons à croissance rapide, la « H-Fatty Acid-Binding Protein » (FABP) est surexprimée. Les protéines de type FABP sont abondantes dans le cytoplasme où elles régulent le transport et le métabolisme des lipides. Elles lient et transportent les acides gras à longue chaîne et jouent un rôle important dans l'utilisation des sources d'énergie et dans les interactions avec d'autres systèmes de transport ou enzymatiques (Stewart, 2000; Liu et al., 2003). Ces protéines sont par ailleurs connues pour être régulées par les hormones thyroïdiennes.

La phosphofructokinase (PFK) est également surexprimée dans le foie des poissons à croissance rapide. Cette enzyme est importante dans la régulation de la glycolyse (Cowey et al., 1981; Panserat et al., 2001). Nos résultats suggèrent la présence d'une activité glycolytique accrue chez les poissons à croissance rapide.

Les apolipoprotéines sont des composés structurels importants des lipoprotéines plasmatiques et participent à l'assemblage, la sécrétion et au catabolisme des lipoprotéines. L'apolipoprotéine A-1 (apoA-1) humaine est principalement exprimée dans l'intestin et le foie (Ando et al., 2005). Chez les poissons, une des plus importantes apoliprotéines, l'apo-28 k Da A-I, est présente dans la plupart des lipoprotéines à forte densité (HDL) et sa séquence est similaire à celle de l'apo A-1 que l'on trouve chez les mammifères (Ando et al. 2005). Toutefois, il reste encore à déterminer si son rôle chez les poissons est similaire à celui observé chez les mammifères. Certaines études indiquent que l'apoA-1 possède des propriétés de liaison avec les hormones thyroïdiennes (Benvenga, 1997).

L'AhR est un facteur de transcription activé par des ligands. Plusieurs publications ont suggéré que l'AhR pouvait jouer un rôle important dans la régulation des xénobiotiques et des fonctions homéostasiques (Shimba et al., 2002). Par ailleurs, d'autres éléments suggèrent que cette molécule joue un rôle dans la régulation de la prolifération cellulaire. Quand des cellules A549 sont traitées avec des agonistes du AhR, la prolifération cellulaire est stimulée. Ainsi, la croissance est beaucoup plus rapide chez des clones qui surexpriment l'AhR que chez des cellules contrôles et leur taux de croissance est proportionnel à la quantité d'AhR. L'AhR, possiblement en collaboration avec l'Arnt, active la synthèse d'ADN et donc, la prolifération cellulaire des cellules A549 (Shimba et al., 2002). L'augmentation observée en AhR indique qu'il y a une surexpression des gènes impliqués dans la prolifération et la croissance, le mode d'action sur la masse corporelle demeurant cependant inexpliqué.

La cadhérine épithéliale (E-cadhérine) a été la première protéine à être identifiée dans la grande famille des cadhérines et joue un rôle important dans les processus d'adhésion cellulaire. La superfamille des cadhérines, des glycoprotéines membranaires, initie l'adhésion cellulaire calcium-dépendante via des interactions homophiliques. Ces glycoprotéines contribuent ainsi au transfert de l'information à travers les cellules, au contrôle de la croissance cellulaire et aux processus de motilité cellulaire (Gumbiner, 1996). L'étude des profils d'expression de la E-cadhérine chez la souris, le poulet et le poisson zèbre indique son implication dans plusieurs processus de développement incluant les débuts de l'embryogenèse, la formation du tissu épithélial et la neurogenèse (Babb et Marrs, 2004). Récemment, Dasgupta et al. (2005) ont étudié la fonction de la E-cadhérine dans les cellules ES du foie et ont montré que l'expression de la E-cadhérine dans ces cellules peut induire une stimulation des facteurs de croissance et l'atteinte des derniers stades de maturation hépatocytaire. Dans notre étude, le niveau d'expression du gène de la E-cadhérine est presque 3 fois plus élevé chez les poissons à croissance rapide. L'augmentation de l'expression de E-cad chez les poissons à croissance rapide pourrait indiquer de nouvelles voies de signalisation impliquées dans la prolifération et la différenciation cellulaire.

Les pentraxines font partie d'une famille de protéines sériques pentamériques. Les pentraxines sont impliquées dans l'immunité, l'inflammation et la mise en place de la matrice et sont d'importants composantes de la réponse immunitaire chez les mammifères (Jensen et al., 1997). Bien que les pentraxines aient été isolées chez différentes espèces de poissons (Cook et al., 2003), peu d'études ont pu fournir d'évidences d'une fonction immunologique chez ces derniers (Cook et al., 2005). Deux types de pentraxines codées

par des gènes homologues sont présents chez les mammifères. Chez les poissons, il n'est pas certain qu'il existe deux gènes distincts (Seery et al., 1993 ; Rubio et al., 1993 ; Jensen et al., 1997). En effet, les résultats diffèrent selon l'espèce étudiée. Seulement une pentraxine a été détectée chez le saumon, la morue, le flétan et le loup de mer (Lund et Olafsen, 1998). Cependant, deux pentraxines ont été découvertes chez la roussette et la plie (Bayne et al., 2001 ; Bayne et Gerwick, 2001). La différence dans le nombre de pentraxines pourrait indiquer des fonctions différentes chez les mammifères et les poissons. D'autres études seront nécessaires à la compréhension de la fonction de ces gènes.

Chez la truite arc-en-ciel, la protéine de type précérébelline appartient à la famille C1q — famille de protéines à domaine globulaire synthétisées dans le foie (Bayne et al., 2001). Une analyse phylogénétique indique une étroite relation des séquences entre la protéine de type précérébelline de la truite arc-en-ciel et la précérébelline du rat et de la souris. La fonction physiologique des précérébellines doit encore être déterminée mais il semblerait que la protéine de type précérébelline de la truite arc-en-ciel soit impliquée dans la réponse immunitaire (Kodama et al., 1989). Des études complémentaires sur ce gène seraient intéressantes pour en comprendre la fonction chez l'humain, la souris et les poissons.

Notre étude révèle que trois gènes impliqués dans la transduction étaient surexprimés chez les poissons à croissance rapide, gènes par ailleurs connus pour être régulés par la T_3 (Feng et al., 2000; Miller et al., 2001; Weitzel et al., 2001, 2003b; Clement et al., 2002; Wood et al., 2002; Flores-Morales et al., 2002; Stahlberg et al., 2005). L'influence de la T_3 sur la croissance des poissons pourrait se faire via les voies de

signalisation cellulaire et le remodelage des tissus consécutivement à l'expression de gènes impliqués dans le transport protéique et la maturation.

Le gène de la phospholipoprotéine 2 (SPP2) encode une protéine de 24-KD qui partage une homologie de séquence avec les membres de la famille des cystatines, des inhibiteurs des thiol-protéases et, comme celui de la cystatine, est exprimé dans les os. Ces phosphoprotéines sont associées à une large variété de processus cellulaires incluant le métabolisme énergétique et du carbone, le transport, le stress et le développement.

La protéine encodée par le gène « seven up-40 » (svp-40) du poisson zèbre appartient à un groupe distinct de la super famille des récepteurs stéroïdiens. Ce gène est exprimé dans des régions spécifiques et des domaines segmentaux du cerveau en développement. La fonction du svp-40 n'est pas claire ; il pourrait être impliqué dans la formation de la voie neurale durant les premiers stades de développement embryonnaire (Fjose et al., 1995). Kanai et al. (2005) ont montré que le svp-40 contrôle les facteurs de transcription dans les neuroblastes de drosophile. La régulation de ce gène par les hormones thyroïdiennes a déjà été démontrée (Feng et al., 2000) et il est surexprimé par un facteur supérieur à 3 dans le foie des poissons à forte croissance. Il s'agit de la première évidence suggérant un lien possible entre ce gène et la croissance.

Les hormones thyroïdiennes régulent à la hausse les facteurs de transcription tel que le « transcription elongation factor B » qui assiste l'ARN polymérase II en tant que facteur d'élongation auxiliaire. Ce gène est surexprimé chez les poissons à croissance rapide ce qui suggère que la stimulation de la croissance pourrait être consécutive à une régulation à la hausse des facteurs nucléaires impliqués dans le contrôle de la transcription au niveau du foie.

La prolactine (PRL) est une hormone glycoprotéique synthétisée dans l'hypophyse. Elle a été purifiée chez cinq espèces de téléostéens (Leena et al., 2001). Chez ces derniers, la PRL joue un rôle important dans l'osmorégulation des poissons d'eau douce en prévenant la perte d'ions et l'absorption d'eau (Manzon, 2002). Elle influence également plusieurs processus physiologiques importants tels que la croissance, la reproduction, le métabolisme et la production de mucus (Manzon, 2002; Prunet et al., 1990). Il semble que la prolactine ait également un effet inhibiteur sur le métabolisme des lipides chez la perche (*Anabas testudineus*; Leena et al. 2001). Dans notre étude, le gène de la PRL était réprimé chez les poissons à forte croissance.

Le cytochrome P-450 1A1 et le cytochrome P-450 4A3 appartiennent à la superfamille des P-450. Les gènes CYP encodent des enzymes de la superfamille des P-450 qui sont exprimés principalement dans le foie et impliqués dans les réactions de mono-oxygénation et d'hydroxylation de nombreux xénobiotiques. Les niveaux d'expression des gènes CYP sont induits ou réprimés par plusieurs hormones circulantes (Yoshida et al., 1996). Chez le rat, les gènes CYP sont réprimés par la GH et les hormones thyroïdiennes (T_3 et T_4) (Oinonen et Lindros, 1998). Les hormones thyroïdiennes répriment également l'expression de l'ARNm du cytochrome P-450 4A3 (Feng et al., 2000). Nous avons observé que chez les poissons à croissance rapide, le cytochrome P-450 1A1 et le cytochrome P-450 3A4 sont réprimés, ce qui pourrait provoquer une réduction de l'oxydation des acides gras et une augmentation de la lipogenèse.

Selon l'analyse d'expression différentielle des gènes régulés par la croissance, 40 gènes inconnus étaient régulés à la hausse et 15 gènes inconnus étaient régulés à la

baisse. Ces gènes présentent un intérêt particulier dans la recherche de nouvelles voies de régulation. Ces gènes inconnus reliés à des caractéristiques différentielles de croissance pourront être ajoutés aux bases de données existantes chez les poissons et servir lors d'études futures. Ils pourront aussi être inclus dans de futures puces à ADN conçues pour l'étude des processus liés à la régulation de la croissance.

Les nouvelles séquences EST et leur étude fonctionnelle contribuent à l'avancement de la recherche sur les poissons. La caractérisation des EST contribuera à l'amélioration de l'annotation des gènes des poissons sur cette puce à ADN. Dans cette étude, nous avons identifié 55 EST qui sont potentiellement impliqués dans la croissance. De plus, le changement d'expression de plusieurs gènes individuels a été confirmé. Ceci contribue à l'avancement des connaissances sur les voies de régulation de la croissance des poissons. Ces informations contribueront au développement d'outils qui serviront dans l'étude de la génétique et de la physiologie des espèces exploitées en aquaculture. Ces connaissances permettront également d'accélérer les investissements dans le développement de biopuces et dans les industries reliées à la pêche et à l'aquaculture.

L'omble de fontaine est une espèce importante dans l'industrie piscicole québécoise et il existe des raisons tant économiques que scientifiques de mener des recherches en génomique chez cette espèce. Les outils génétiques sont nécessaires pour améliorer les caractéristiques des stocks de géniteurs, entre autres au niveau de la croissance. Le manque de données représente un obstacle dans l'utilisation de la génétique pour faire avancer à la fois l'aquaculture des salmonidés ainsi que les activités de conservation.

Lors de nos travaux de ddPCR, nous avons identifié 18 séquences de gènes régulés par les hormones thyroïdiennes. Ces séquences contribueront à approfondir nos connaissances et élargir les bases de données sur les gènes de poissons. Lors de notre étude, nous avons utilisé la puce à ADN de 16 milliers de gènes GRASP pour identifier les gènes de poissons impliqués dans la croissance. Nos résultats concordent avec les études antérieures, à savoir que ce genre de microréseau à ADNc permet de prédire l'induction et la répression d'expression de gènes avec une grande fiabilité. En combinant les techniques de ddPCR et de biopuces, nous avons obtenu des résultats utiles non seulement pour étudier la croissance des poissons mais aussi dans la recherche de caractéristiques qui contribueront au développement économique des industries reliées à l'aquaculture et à la production alimentaire.

L'utilisation des biopuces à ADN dépend de connaissances déjà acquises telles que l'annotation des gènes sur la puce, l'identification exacte des gènes et des séquences utilisées dans les bases de données. La puce GRASP contient une forte proportion d'EST (58,5%) qui sont des gènes inconnus. Malheureusement, certains gènes qui ont été identifiés comme ayant une expression différentielle lors de la première expérience n'étaient pas inclus sur ce microréseau, comme la « mitochondrial NADH dehydrogenase subunit 5 », la tropomyosine, la crystalline, ainsi que certains gènes inconnus régulés par les hormones thyroïdiennes. Par ailleurs, certains gènes qui ont été identifiés comme modulés par les hormones thyroïdiennes étaient présents sur la puce GRASP, comme le LECT 1, le « basic transcription factor 3 » et le C-1-6. Cependant, lors de notre analyse par microréseau, ils n'ont pas montré d'expression différentielle en fonction des performances de croissance. Le ddPCR est une technique très sensible puisqu'elle

comporte une étape par PCR, alors que la technique des biopuces comporte une étape d'hybridation qui est moins sensible. Certains gènes pourraient alors être détectés par ddPCR mais non par biopuces.

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