EXPRESSION OF THE MYC PROTO-ONCOGENE IN THE PITUITARY GLAND OF THE RAINBOW TROUT

by

Joseph P. Panno

B.Sc., Simon Fraser University, 1980

M.Sc., Simon Fraser University, 1984

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

in the Department
of
Biological Sciences

SIMON FRASER UNIVERSITY

December, 1996

APPROVAL

NAME: Joseph Panno

DEGREE; DOCTOR OF PHILOSOPHY

TITLE OF THESIS:

EXPRESSION OF THE MYC PROTO-ONCOGENE IN THE PITUITARY GLAND OF THE RAINBOW TROUT

EXAMINING COMMITTEE:

CHAIR: Dr. A. P. Farrell, Professor

Dr. B. McKeown, Professor, Senior Supervisor
Department of Biological Sciences, SFU

Dr. R. Devlin, Research Scientist
Department of Fisheries and Oceans

Dr. N. Haunerland, Associate Professor
Department of Biological Sciences, SFU

Dr. A.H. J. Burr, Associate Professor
Department of Biological Sciences, SFU
Public Examiner

Dr. G. Iwama, Professor
Department of Animal Sciences
University of British Columbia
External Examiner

Date Approved: Dec. 4, 1996

PARTIAL COPYRIGHT LICENSE

I hereby grant to Simon Fraser University the right to lend my thesis, project or extended essay (the title of which is shown below) to users of the Simon Fraser University Library, and to make partial or single copies only for such users or in response to a request from the library of any other university, or other educational institution, on its own behalf or for one of its users. I further agree that permission for multiple copying of this work for scholarly purposes may be granted by me or the Dean of Graduate Studies. It is understood that copying or publication of this work for financial gain shall not be allowed without my written permission.

Title of Thesis/Project/Extended Essay

Expression of the myc Proto-oncogene in the Pituitary gland of the Rainbow Trout

Author:_
(signature)

Joseph Panno
(name)

Dec. 9, 1996
(date)

Abstract

The objective of this study was to test the hypothesis that the *myc* proto-oncogene is involved in controlling the production of hormones in the pituitary gland (PG) of rainbow trout. *Myc* expression was studied initially using a previously cloned genomic fragment from rainbow trout (tmyc1). The PG was shown to express an unusually small, 1.6 kilobase (kb), c-*myc* related transcript that increases nearly two-fold as the fish approach sexual maturity. The identity of the 1.6 kb *myc* transcript was confirmed by constructing and screening a PG cDNA library. Two *c-myc* related clones were isolated and sequenced. One of these (tmyc2) was shown to be expressed as a 1.9-2.0 kb transcript in the PG, brain and heart but was not expressed in the liver. The second clone (tmyc3) is expressed in the PG (1.6 kb), but not in brain, muscle, liver or heart. Tmyc3 expression level in the PG is much greater than that of tmyc2. The tMyc3 protein shows extensive homology to tMyc1 (88%) whereas tMyc2 is more divergent (72%). Southern blot analysis indicates that tmyc2 and tmyc3 are distinct c-*myc* genes. *In situ* hybridization and northern blot analysis showed that tmyc3 is expressed primarily in somatolactotrophs of the intermediate lobe (IL) of the PG. Tmyc2 is expressed at similar levels in both the IL and the pars distalis (PD). *In vitro* stimulation experiments show that releasing factors, known to control cells in the PD, failed to stimulate tmyc2 or tmyc3. However, Dopamine and norepinephrine (two neurotransmitters known to control hormone release from the IL) increased tmyc3 expression 2-5 times. Two other neurotransmitters (GABA

and Serotonin) failed to stimulate tmyc3 expression. None of the neurotransmitters tested affected tmyc2 expression. These results suggest that tmyc3 is involved in regulating expression of the somatolactin gene and hypertrophy of somatolactotrophs.

Dedication

To the memory of my good friend Karun Nair.

Acknowledgments

I would like to thank my senior supervisor, Dr. Brian McKeown, for giving me the opportunity to do a Ph.D. in his lab and the late Dr. Karun Nair for paving the way. Thanks are also due to Drs. Jay Burr, Norbert Haunerland, Robert Devlin and George Iwama for their careful attention to the details of the thesis and the many helpful suggestions that greatly improved the final version. I would also like to thank my wife, Diana Dowsley, for providing valuable information on *in situ* hybridization, assistance with photography and for proofreading the thesis. Finally, I would like to thank my daughter Eleanor for putting all of this in its proper perspective.

Table of Contents

List of Tables

List of Figures

Chapter I
General Introduction

Oncogenes

The development of cancer is believed to be caused by a disturbance in a relatively small number of genes (proto-oncogenes) that are involved in maintaining or operating signal transduction and secretion pathways. Conversion of a proto-oncogene to an oncogene, capable of neoplastic transformation, occurs primarily through point mutations or inappropriate expression (1,2). Classification of the transformation is divided into three main groups; sarcomas (cancers of muscle, connective and neural tissue), carcinomas (epithelial cells) and leukemias (blood forming tissue).

Studies leading to the discovery of oncogenes began with Rous (3) who induced sarcomas in chickens by injecting them with millipore-filtered tumor extract. The transforming agent was eventually shown to be a retrovirus (4,5) that is now called Rous sarcoma virus (RSV). Infection of a cell by a retrovirus leads to the reverse transcription of the viral RNA genome into DNA, after which the DNA integrates into the host genome to produce the provirus. The integration step can alter the function of host genes and it can provide an opportunity for the virus to incorporate host genes into its own genome. Integration is also essential for the continual expression of viral genes (6).

RSV deletion mutants have been used to show that the transforming ability of this virus is due to a single gene, called *src* (from sarcoma), which is located at the 3' end of the RSV genome (4). Using the viral *src* (*v-src*) gene as a probe it was later discovered that this gene occurs, and is expressed, in a wide variety of invertebrate and vertebrate species including Drosophila (7), fish (8), birds (9) and mammals (10). It is now clear that *v-src* arose by transduction of genetic loci from the host genome and hence did not originate in RSV, nor is it common to all retroviruses (6).

Since the discovery of *src*, approximately 60 oncogenes have been isolated from retroviruses and tumor cell lines (11). Some of these genes are known to code for transcription factors (*myc, fos*), hormone receptors (*erbA*), growth factors (*sis, fms*), protein kinases (*src, kit*) and G-proteins (Table 1). Some of the oncogenes initiate cellular transformation much more frequently than others. *Myc*, and *ras*, for example, are associated with all of the major forms of cancer described above (12).

The ability of *myc* to induce neoplastic transformation is largely due to its involvement in signal transduction pathways. Two such pathways that have been studied extensively are those that generate cAMP and inositol trisphosphate (IP_3). The realization that proto-oncogenes code for, or maintain, the components of these signaling pathways has generated a considerable amount of interest in their relationship to basic cellular functions, particularly those involving growth, development and the hormonal control of these processes. The relationship between

Table 1

Examples of oncogenes that were isolated from retroviruses

ONCOGENE	VIRAL HOST	FUNCTION	TUMOR
erb-A	chicken	Thyroid hormone receptor	Leukemia
src	chicken	Tyrosine kinase (TK)	sarcoma
kit	cat	TK	sarcoma
ras	Rat	G-protein	sarcoma
sis	monkey	growth factor	sarcoma
myc	chicken	Transcription factor (TF)	carcinoma
fos	mouse	TF	osteosarcoma

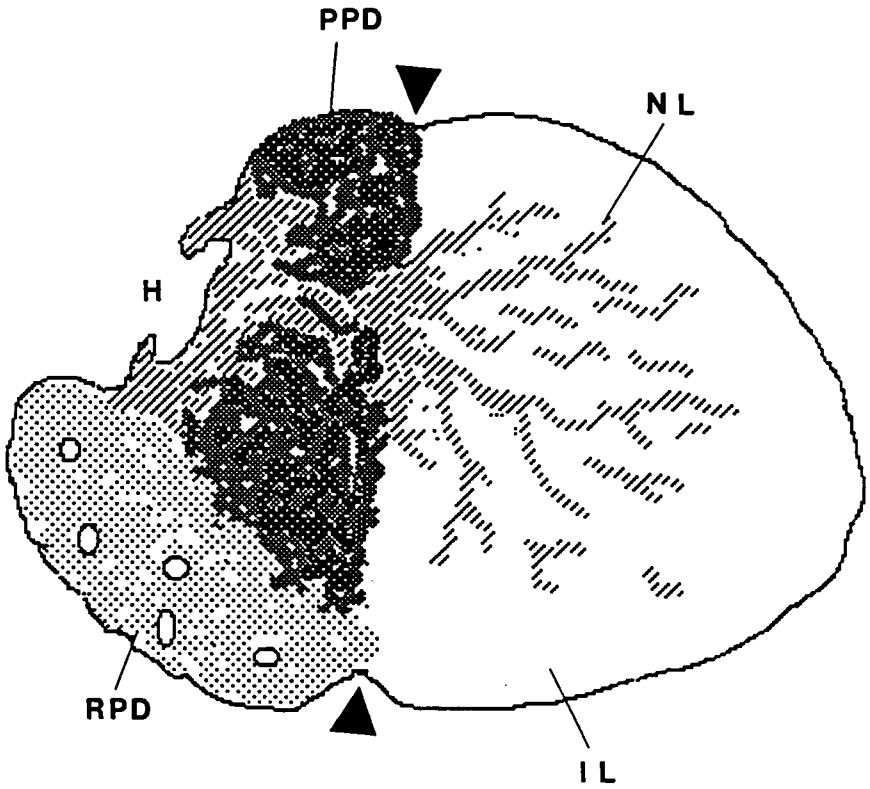
Oncogenes frequently initiate the formation of different types of tumors. *Myc*, for example, also induces sarcomas and myelocytomas (1,2,11).

proto-oncogenes, signal transduction pathways and the endocrine system is of special interest in view of the many physiological processes controlled by the pituitary gland and other endocrine tissue.

The pituitary gland

The pituitary gland of rainbow trout consists of three, functionally distinct regions: the anterior lobe (pars distalis), a large intermediate lobe and a neural lobe that forms the core of the intermediate lobe (13, 14, Fig. 1). The pars distalis is further divided into the rostral pars distalis, consisting primarily of lactotrophs (prolactin-producing cells), and the proximal pars distalis consisting of several cell types; the gonadotrophs (gonadotropin hormone, GTH), thyrotrophs (thyroid stimulating hormone, TSH), corticotrophs (adreno-corticotropic hormone, ACTH) and somatotrophs (growth hormone, GH). The intermediate lobe consists of two cell types. One of these, the melanotrophs, produces melanocyte stimulating hormone (MSH) and the other, the somatolactotrophs, produces somatolactin (SL). The neural lobe consists entirely of nerve fibers that originate from cell bodies located in the hypothalamus. These fibers form a stalk, called the infundibulum, that connects the gland to the brain. Nerve fibers also pass through the anterior lobe and are responsible for delivering to the gland neuropeptides and neurotransmitters that control the release of all of the hormones referred to above. The neural-intermediate lobe is unusually large in teleosts accounting for more than two thirds of the gland, compared to less than 10% in higher vertebrates (13).

Figure 1. A sketch of a mid-saggital section of a pituitary gland from the rainbow trout. The arrows mark the approximate border between the distal lobe (Pars distalis) and the intermediate lobe. The gland is attached to the hypothalamus (H) through the infundibulum (two projections at upper left). The distal lobe is further divided into the rostral pars distalis (RPD, stippled) that consists primarily of prolactin cells and the proximal pars distalis (PPD, medium to dark shading) that consists of somatotrophs (cells that produce growth hormone, GH), thyrotrophs (thyroid stimulating hormone, TSH), corticotrophs (adrenal corticotropic hormone, ACTH) and gonadotrophs (gonadotropic hormone, GTH). The intermediate lobe (IL, clear) consists of melanotrophs (melanocyte stimulating hormone, MSH) and somatolactotrophs (somatolactin, SL). Both lobes are penetrated extensively by nerve fibers that originate from the hypothalamus (NL, cross-hatch). Drawn from a tissue section produced in this thesis and from other sources (13,14,64).


PPD
NL
H
RPD
IL

All of the hormones produced by the gland are under dual control. The release of GH, for example, is stimulated by GH-releasing factor (GRF) and inhibited by somatostatin. GRF will also stimulate the expression of the GH gene whereas somatostatin has the opposite effect (15). This simple scheme is complicated by the fact that other substances can also influence the release of GH; these include stimulation by the neurotransmitters dopamine (16) and inhibition by interleukin 2 (17). Dopamine is also involved in controlling the release of MSH from melanotrophs and prolactin from lactotrophs, although in this case the effect is inhibitory (18,19,20). Other neurotransmitters, such as norepinephrine (NE, 21), γ-aminobenzoic acid (GABA, 22) and serotonin (ST, 23) are also known to influence the production and release of MSH. Which of the positive or negative regulators dominate depends on complex interactions between the hypothalamus and the prevailing hormonal environment of the organism. Fluctuations in this environment can occur as the result of diurnal cycles (24), exercise (25), sexual maturation (26) and stress (27).

Signaling pathways in the pituitary gland

Hormone release from the distal lobe of the trout pituitary gland is controlled primarily by the cAMP or IP_3 pathways. The production and release of GH and ACTH occurs through the cAMP pathway (15) although the release of ACTH from the cell can also involve IP_3 (28). The release of the gonadotropins (GTH) and TSH occurs through the IP_3 pathway (29,30) and prolactin synthesis and release appears to be controlled using both

the cAMP and IP_3 pathways (19, 20). The control of MSH release from the intermediate lobe is complex, involving several neurotransmitters, as described above, and both the cAMP and IP_3 pathways (18). At present it is not clear which pathway controls the release of somatolactin.

The cAMP pathway consists of a receptor, a trimeric G-protein, which binds and hydrolyzes GTP, and adenylate cyclase (31). Binding of a ligand to the receptor activates the G-protein which in turn stimulates adenylate cyclase to catalyze the formation of cAMP from ATP. Cyclic AMP, as a second messenger, activates protein kinase A (PKA), a serine/threonine-specific kinase that phosphorylates proteins located in the cytoplasm and in the nucleus. Some of the cytoplasmic substrates for PKA are themselves protein kinases that are involved in the metabolism of glycogen. Nuclear substrates includes a Myc protein (32, 33).

The IP_3 pathway consists of a receptor, a G-protein and a phosphodiesterase (PDE). Binding of a ligand to the receptor leads to the activation of PDE in a manner similar to the activation of adenylate cyclase in the cAMP pathway (34). PDE catalyzes the breakdown of inositol phospholipid to IP_3 and diacylglycerol (DAG). IP_3 initiates the release of calcium from internal stores and DAG activates protein kinase C (PKC). PKC, and its isoforms, are believed to have at least 45 substrates located in the cytoplasm and nucleus (35,36). One of the nuclear substrates for PKC is known to be a Myc protein (37).

The extent to which the cAMP and IP_3 pathways are used in the pituitary gland and the fact that both of these pathways are known to activate the Myc protein suggests that this proto-oncogene may be involved in regulating the production of pituitary hormones. The role of Myc may be to activate gene transcription for a particular hormone or to maintain and regulate gene transcription for components of the signal transduction pathway. It may also be essential for maintaining the extensive cell growth that occurs in some of the pituitary cells as the trout approaches sexual maturity (38, 39).

The myc *gene family*

The Myc family of proto-oncogenes codes for nuclear proteins that are believed to function as transcription and replication factors (1,2). Over-expression of these genes is associated with the development of carcinomas (40), sarcomas (41), and leukemias (42). This family consists of at least four members: c-(cellular) *myc* (43), N-*myc* (44), L-*myc* (45) and B-*myc* (46).

The *myc* oncogene was originally isolated from the genome of an avian leukemia retrovirus and is referred to as v-(viral) *myc* (1). Subsequent studies with v-*myc* have confirmed that the retrovirus acquired the *myc* gene from the avian host (43,47). C-*myc* is similar to v-*myc* except that it contains both introns and exons, whereas v-*myc* consists entirely of exon sequence. Human *c-myc* was used to isolate N-*myc* from a neuroblastoma, L-*myc* from a lung carcinoma and B-myc from a rat brain

cDNA library. Although L-myc was originally isolated from lung tissue it, like N-myc, is expressed primarily in the brain and kidney. B-myc shares this expression pattern to a limited extent, in that it is expressed at its highest level in the brain, but it is also expressed in several other tissues. Human c-, N- and L-*myc* genes are located on chromosomes 8, 2 and 1, respectively (1). The human locus for B-myc has not been determined but it is located on chromosome 11 in the rat (46).

Activation of the *myc* locus (converting it from a proto-oncogene to an oncogene) can occur through a translocation, gene amplification or proviral insertion within the host genome. Each of these events leads to abnormally high levels of *myc* transcription. This occurs, in the case of translocation and proviral insertion, because the *myc* coding region is brought under the control of an active promoter. For example, translocations between c-*myc* and immunoglobulin loci (which are actively transcribed) has been observed in lymphoid tumors where *myc* expression is increased 10-fold (2). Gene amplification can increase the level of the *myc* transcript to an even greater extent than translocation. In some tumors the level can be 20-70 times higher than it is in the normal cell (1).

All *myc* family members are encoded in a 3 exon structure with large portions of the first and third exons constituting 5' and 3' untranslated regions. In the case of B-*myc* the mature mRNA is derived entirely from exon 2 (1). Table 2 summarizes the range of transcripts that have been observed for the various *myc* family members. c-*myc* is expressed

with transcripts of 1.9 kilobases (kb) to 2.4 kb. L-*myc* produces the widest variety of transcripts, ranging from 2.0 kb up to 3.8 kb. B-*myc*, consisting primarily of exon 2 sequence, produces the smallest transcript of 1.3 kb. The variety of c-*myc* and L-*myc* transcripts is due to alternative processing of RNA precursors and to the existence of two or more promoters. c-*myc* is known to have three promoters, two of which are located at the 5' end of exon 1 and are responsible for the production of the 2.0 and 2.4 kb transcripts (1). The multiple transcripts of L-*myc* are produced primarily through alternative RNA processing or variations in the location of the poly-adenylation signal (48).

Structure and function of the Myc protein

The Myc protein belongs to the helix-loop-helix (HLH) and leucine zipper (LZ) families of transcription factors that form an active complex through dimerization (Fig. 2). Myc forms a heterodimer with a smaller HLH protein called Max (49, 50). Dimerization is mediated by the carboxyl end of the protein which contains the HLH and LZ motifs. Each member of the dimer has a DNA binding site (the basic region, BR) on the amino side of the HLH motif that binds specifically to the DNA sequence GTGCAC (51). This sequence is an enhancer, often referred to as the Myc binding site (MBS). The Binding of Myc to the enhancer makes it possible for two trans-activation domains, at the amino terminus of the protein, to interact with transcription factors IIH or IID (55). Once activated by Myc,

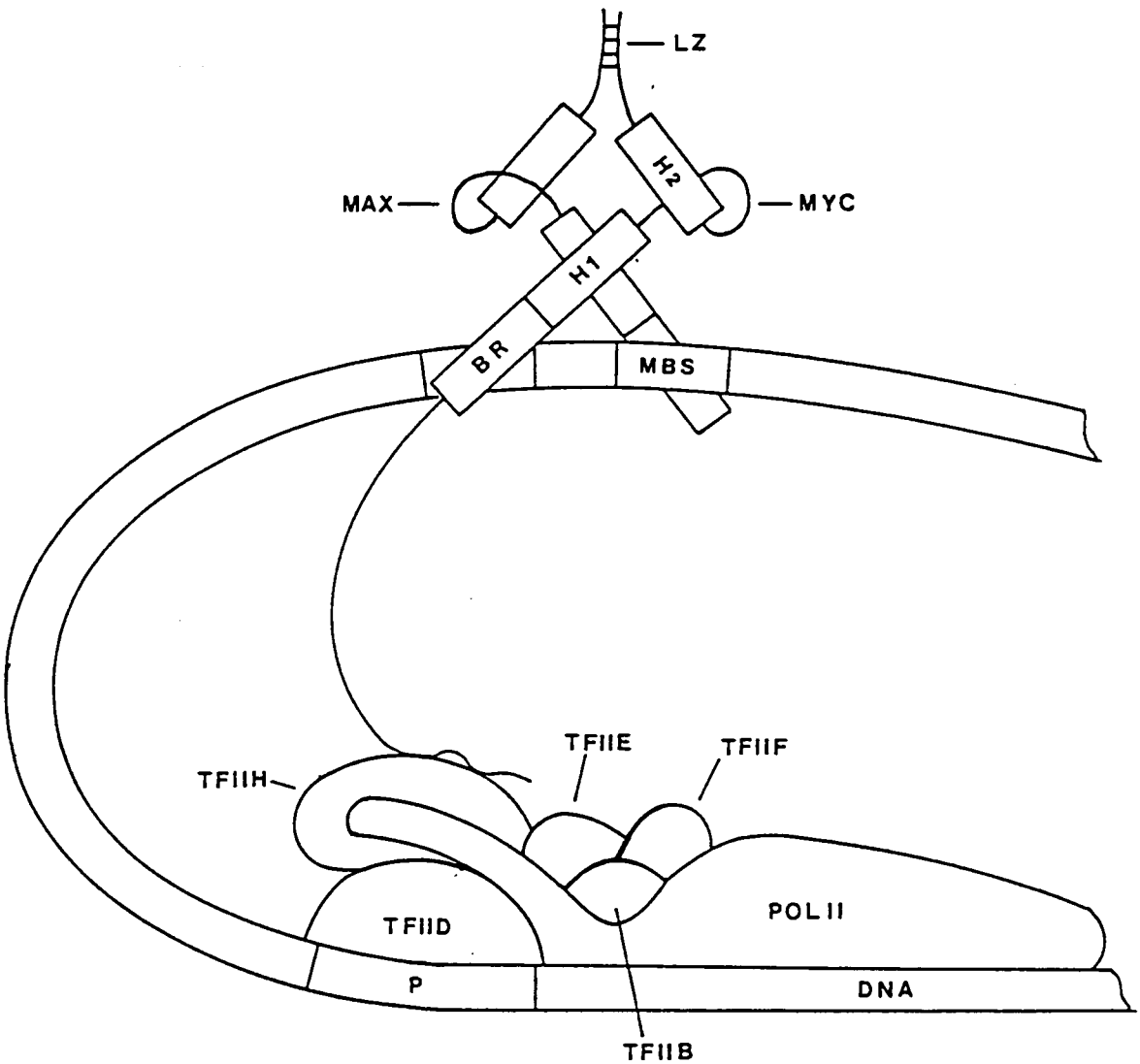
Table 2

Messenger RNA transcripts of *myc* family members

Oncogene	Transcript size (kb)	CDS
c-*myc*	1.9	2, 3
	2.0	2, 3
	2.4	2, 3
N-*myc*	3.0	2, 3
L-*myc*	2.0	2
	2.2	2
	3.5	2, 3
	3.8	2, 3
B-*myc*	1.3	2

The data shown was compiled from several sources (1, 46,48,77). The third column gives the exon composition of the coding sequence (CDS) in the mature mRNA.

Figure 2. A model of a transcription intiation complex being activated by a Myc-Max dimer. Assembly begins with the binding of transcrition factor TFIID to the promoter (P) followed by TFIIB. Once these factors are assembled TFIIF, TFIIE, TFIIH and RNA polymerase II (POL II) joins the complex. The Myc-Max dimer activates the complex when two transactivation domains, located near the amino terminus of Myc, makes contact with TFIIH or TFIID. This interaction leads to the phosphorylation of POL II which then proceeds to transcribe the gene. The Myc-Max dimer binds to an enhancer sequence referred to as the myc binding site (MBS). The interaction between Myc-Max and the MBS is mediated by a basic region (BR) located on each protein. Myc and Max dimerize though helix (H1)-loop-helix (H2) and leucine zipper (LZ) motifs located near the carboxyl end of each protein. Drawn from several sources (49,50,51,54).


LZ
MAX
MYC
H2
H1
BR
MBS
TFIIH
TFIIE
TFIIF
TFIID
POLII
P
DNA
TFIIB

TFIIH or TFIID phosphorylates RNA polymerase II to initiate transcription (53, 54). As indicated in Fig. 2, Max lacks the transactivation domains so that Myc-Max dimers are able to activate transcription whereas Max-Max dimers can function as Myc suppressers (55).

Although the model describes Myc-Max activation of transcription, the Myc protein is also known to be associated with the activation of replication, and may involve the binding of a Myc dimer (or heterodimer) to an origin of replication (2). The role of Myc as a replication factor has been implicated in the induction of cellular proliferation (1,2) and hypertrophy (56,57).

Project objectives

The *myc* gene and the protein that it codes for have been studied extensively since it was first isolated in 1983 (47). A great deal has been learned about the general expression profile of this gene, both in adult tissues and during embryonic development, and about the structure and function of the Myc protein.

However, very little is known about the activity of this gene family in the endocrine system and, in particular, in the pituitary gland. This is surprising in view of the many physiological processes that are controlled by this tissue. A single paper has appeared that describes the stimulation of *myc* expression by estrogen in the rat pituitary gland (58). However, this study was carried out using dot blot analysis without northern blot

validation. Consequently, it is not clear which family member was being studied, nor was there any attempt to localize the expression to a specific region or cell type. As a result, the authors were unable to draw any conclusions regarding the role that *myc* may have in controlling hormone production in this tissue.

The main objective of this thesis is to examine the possibility that the *myc* proto-oncogene is involved in controlling the production of hormones in the pituitary gland of rainbow trout. This organism was selected because of the extensive work that has been done to clarify the control of hormonal release from the pituitary gland of salmonids, both *in vivo* (24,25,38,59,60) and *in vitro* (61,62,63,64).

The experimental portion of the thesis is divided into four parts: A *myc* expression survey of trout tissues (Chapter II); cloning of *myc* genes from the pituitary gland (Chapter III); application of *in situ* hybridization to localize *myc* expression to a specific cell type (Chapter IV) and *In vitro* experiments to test various substances for their ability to either inhibit or stimulate *myc* expression (Chapter IV).

The experiments in parts 1 and 2 were designed to answer two questions: first; is *myc* expressed in the pituitary gland of rainbow trout? Second; if it is, which family members are present? The second question is extremely important in view of the known diversity of the *myc* family. As indicated above (Fig. 2), Myc function is understood for those family members that are translated from exons 2 and 3, but is unclear for those

members, such as the B-myc protein, that are translated from exon 2 (thus lacking the dimerization motifs).

Information from part 3 is essential for the design of experiments in part 4. This is particularly important for a tissue such as the gland, which consists of many cell types, each of which is controlled by multiple factors that originate in the hypothalamus, and in various other tissues of the body, including the immune system.

The *in vitro* experiments in part 4 were included for several reasons: First; the results of such experiments can be used to tentatively assign the *myc* gene to specific signaling pathways and can be used to correlate the expression of this gene with the production of a specific hormone. Second; activation of the myc locus (converting it from a proto-oncogene to an active oncogene) is known to be associated with an elevated expression level. Any substance that stimulates the expression of *myc* is a potential candidate for the *in vivo* transformation of normal cells to a cancerous phenotype. Conversely, any substance that inhibits *myc* expression may be useful as a tumor suppresser. Third; as indicated above, the Myc protein is known to function both as a transcription and replication factor. The later role is not restricted to cell division but can involve endopolyploidization, a process that can occur as part of cellular hypertrophy, where mitosis does not occur. Several of the cells in the pituitary gland are known to increase in size during sexual maturation (26,64, 65,91) and it is possible that *myc* may have a role in stimulating the growth of specific cells within the gland.

Chapter II

Expression of the myc proto-oncogene in the rainbow trout

Introduction

Studies in mammals (rat and mouse) have shown that c-*myc* and B-*myc* are expressed in a wide variety of cell types (46, 66), whereas L-*myc* and N-*myc* transcripts appear predominantly in post-mitotic tissues such as brain, kidney and heart (67, 68). In addition, some of these studies, and others, have shown a general decline in *myc* expression after embryonic development, and that the levels remain low during growth and maturation (66, 67, 68, 69).

Very little expression data are available for the pituitary gland as it was not included in any of the studies cited above. At present, it is not clear whether the *myc* proto-oncogene is expressed in the pituitary gland of any vertebrate species, although there is some indication that it is expressed in the gland of adult rats (58).

A partial *myc* genomic clone has been isolated from the rainbow trout (tmyc1) and was shown to have extensive homology to c-*myc* of higher vertebrates (70). Tmyc1 is expressed in the liver, but the expression in other tissues was not examined. The work described in this Chapter further characterizes the expression of c-*myc* in various trout tissues, including the pituitary gland, during early growth and sexual maturation.

Three *myc* transcripts are described, of which one is specific to the pituitary gland and another is expressed only in the heart. Some of the results in this chapter have been published (71).

Materials and Methods

Pituitary glands, brains, skeletal muscle, liver and heart were collected from 200 g (12 months old) and 1.0 kg (24 months old) rainbow trout. Ten individuals were sampled from each group. Pituitary glands were also collected from four additional groups of fish weighing 25 g (2 month old) to 600 g (18 months old). 10 to 40 glands were collected per group. All fish were reared in outdoor tanks, fed standard fish pellets at 1% body weight/day, five days a week and were allowed to acclimate to the tank for one week before tissue samples were collected. The water temperature ranged from 15° to 18°C.

Multiple tissue expression of myc

Total RNA was extracted from all tissues using the guanidinium-acid phenol protocol (64). The RNA (20 µg/lane) was fractionated on a 1.2% agarose MOPS-formaldehyde gel and transferred to Genescreen in 10X SSPE (1.50 M NaCl, 1.15 mM NaH_2PO_4 and 10 mM EDTA). The RNA was crosslinked to the filter by exposing it to 254 nM UV radiation for 3 minutes. Filters were pre-hybridized in a buffer consisting of 5X Denhart's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum

albumin, fraction V), 5X SSPE, 0.3% SDS and 30% formamide for 2-4h at 37°C. Hybridization was carried out in the same buffer and at the same temperature for 24 h (73, 74). The filters were probed with a 475 bp EcoRI-BamHI fragment tmyc1 (probe ***M1a***, 62, Glossary). This fragment contains nearly all of exon 2, excluding both the second intron and exon 3 of the original clone. The probe was labeled to high specific activity (10^9 dpm/μg) using an oligolabelling kit (75, Pharmacia, LKB). Filters were washed at medium stringency, which consisted of one 20 min wash at 50°C in 1X SSPE, containing 0.1% SDS. The filter was sealed wet in plastic bags and exposed to X-ray film for 3 days at -80°C with intensifying screens. Transcript levels were quantified with a scanning densitometer (Hoefer Scientific Instruments).

Expression of myc during sexual maturation

Total RNA was extracted from pituitary glands obtained from 25 g-600g trout. The RNA (10 μg/lane) was fractionated, blotted to Genescreen and probed for the expression of *myc*, using ***M1a***, as described above. The blot was exposed to X-ray film for 24 h and then stripped at 70°C, for 30 minutes, in a buffer consisting of TE (10 mM Tris, 10mM EDTA, pH 7.0), 1.0% SDS and 50% formamide. The blot was re-probed for the expression of GH. The GH probe (***GHa***) is a 200 bp Kpn I- Hind III fragment from the 5' end of a cDNA clone isolated from rainbow trout (76). The blot was exposed to X-ray film for 1h and the transcript levels of *myc* and GH were

quantified with a densitometer. *Myc* transcripts were standardized by dividing the total optical density (OD) of each band by the OD of the control (GH) band in the corresponding lane. All ratios were then divided by the value obtained for the smallest fish examined.

GH and myc expression ratio

Total RNA (10 μg) from the pituitary gland was fractionated, in duplicate, on a 2.5% agarose gel and transferred to Genescreen. The blot was cut down the middle, and one half was probed for the expression of GH and the other half was probed for *myc* expression. The two halves were aligned and exposed to the same piece of X-ray film for 4 h. For a control, both pieces were stripped, re-probed for the expression of GH and exposed to X-ray film for 1h. The film was scanned and standardized as described above.

Transcript sizes were estimated on all northern blots by constructing a standard curve based on the migration of known size markers (28S, 18S rRNA and GH mRNA). Distance migrated (mm) was plotted as a function of $\log_{10}$ molecular size, where molecular size is the number of nucleotides in the RNA.

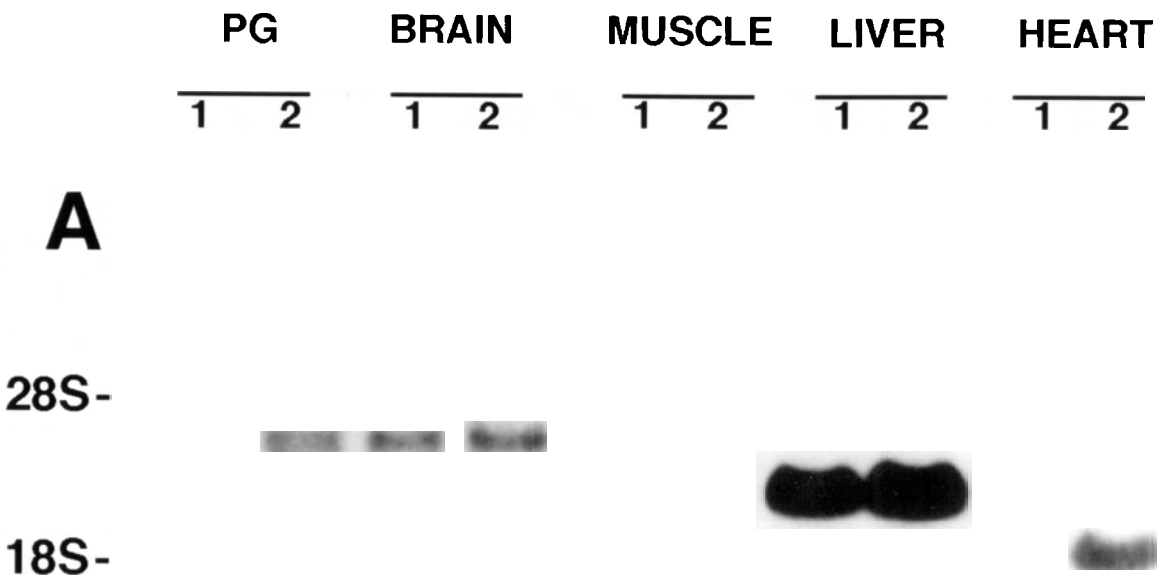
Results

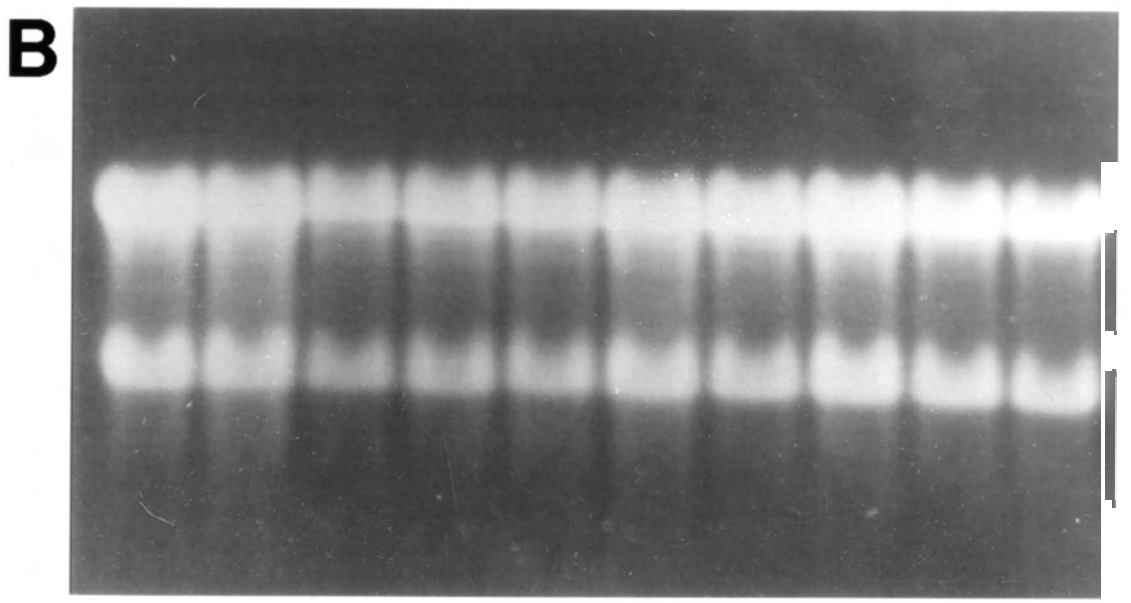
Fig. 3A shows the results obtained after probing an RNA blot for the expression of *myc* in the pituitary gland (PG), brain, skeletal muscle, liver

and heart from 200g and 1.0 kg trout. Bands appear in all tissues except the muscle. The positions of the 28S and 18S ribosomal bands are indicated for size markers. A photograph of the ethidium bromide-stained gel is also included to verify that approximately equal amounts of RNA were loaded per lane (Fig. 3B). The band in the liver is a 2.4 kb c-*myc* transcript that has been previously described (70). The pituitary gland shows expression of transcripts that are approximately 1.6 kb (the major band) and 3.8 kb. A transcript of about 3.8 kb also appears in the brain; the heart shows expression of a single 2.0 kb *myc*-related transcript. A densitometric scan of this blot shows that the amount of the 1.6 kb pituitary transcript, in the 200g fish, is approximately 3-fold greater than the liver transcript and nearly 8-fold greater than the heart transcript. The major *myc*-related transcripts in the PG, liver and heart appear to increase with the age of the trout. The minor transcripts, in the PG and brain are expressed at approximately the same levels in both groups.

Fig. 4A shows the expression of the 1.6 kb myc transcript in pituitary glands obtained from fish that ranged in size from 25 g to 600 g. The expression of this transcript shows a gradual increase with age, reaching a level in the 600 g fish that is more than double that observed in the 25 g fish. Fig. 4b shows a comparison between the expression level of GH and the 1.6 kb *myc* transcript in the pituitary gland. This result indicates that GH expression is much greater than that of the *myc* transcript. The control (GH expression in both lanes) indicates that each lane contains equivalent amounts of RNA in both experiments.

Figure 3. Expression of the *myc* proto-oncogene in the Rainbow trout. **(A)** The blot was probed with ***M1α***, washed at medium stringency and exposed to x-ray film for 3 days. The position of the 28S and 18S ribosomal RNA is indicated. **(B)** Photograph of the ethidium bromide stained gel showing the 28S and 18S ribosomal RNA bands. Lanes 1: 200g trout, 2: 1.0 kg trout.


PG
BRAIN
MUSCLE
LIVER
HEART
1 2
1 2
1 2
1 2
1 2
A
28S-
18S-


B

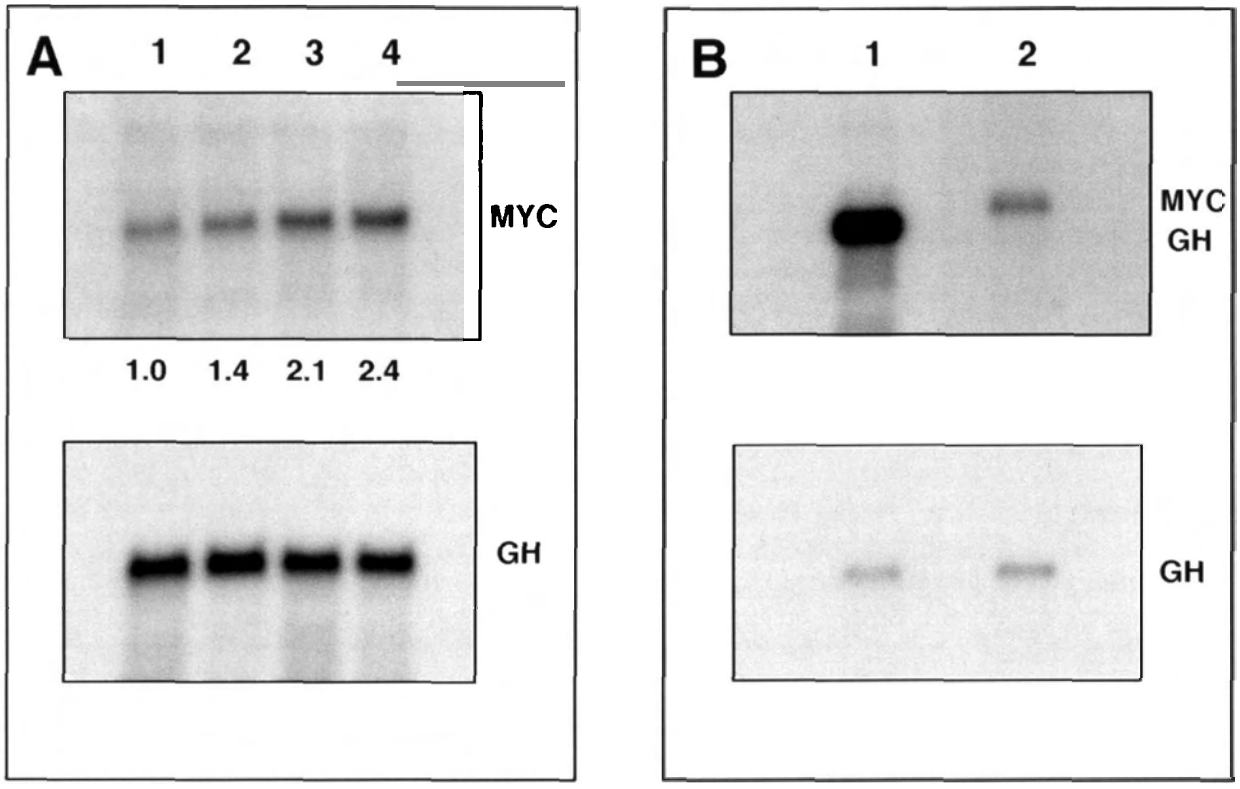
Discussion

The results in this Chapter have shown that *myc* is expressed in the pituitary gland, as well as in the brain, heart and liver of the rainbow trout. Diversification of *myc* function is apparent, in that each tissue expresses *myc*-related transcripts of different sizes. Some of these transcripts may represent different family members.

The 3.8 kb transcript that appears in the brain and pituitary gland is the same size as an L-*myc* transcript known to be expressed in rat brain (1). The identity of the heart transcript is uncertain since both c-*myc* and L-*myc* are known to have 2.0 kb transcripts. However, the identity of the various transcripts can also be inferred, to some extent, by the relative amounts of each transcript in the different tissues. Expression of c-*myc* in mammalian heart appears as a 2.4 kb transcript at a level approximately 5-fold less than that observed in mammalian liver (1). A similar trend appears in trout, since the amount of the heart transcript appears to be much less than the liver transcript, suggesting that the heart transcript is from a c-*myc* gene.

The size of the major pituitary transcript is similar to that of B-*myc*, which is expressed as a 1.3 kb transcript in the rat (46). All of the transcripts for the other *myc* family members are at least 1.9 kb in length (1,77). However, B-*myc* expression in mammals is not tissue specific, as

Figure 4. Expression of *myc* in pituitary glands from Rainbow trout of different ages. **(A)** The blot was probed with ***M1a***, washed at medium stringency, exposed to x-ray film, stripped and re-probed with ***GHa***. Lanes: 1 (25g trout); 2 (200g); 3 (400g); 4 (600g). **(B)** Total RNA (10 μg) from the pituitary gland was fractionated on a 2.5% agarose gel and transferred to Genescreen. The blot was cut down the middle and one half was probed with ***GHa*** and the other half was probed with ***M1a***. The two halfs were washed at medium stringency, aligned and exposed to the same piece of x-ray film for 4h. For a control, both pieces were stripped and re-probed with ***GHa***.


A
1
2
3
4
MYC
1.0
1.4
2.1
2.4
GH
B
1
2
MYC
GH
GH

the 1.6 kb transcript appears to be in trout. It will be necessary to clone and sequence the cDNA for this transcript in order to confirm its identity.

The fact that the expression level of the 1.6 kb pituitary *myc* transcript is much higher than that of the liver transcript is surprising and suggests a major role in pituitary function. Moreover, the level of expression increases with the attainment of sexual maturation, indicating a possible role in the growth and activity of gonadotrophs.

Interpretation of the results in this Chapter are complicated by the difficulties involved in quantifying the results from northern blot analysis. These problems are rooted in the fact that the response curve of the X-ray film is non-linear for very short (< 1.0 h) and very long (> 48 h) exposures, and is compounded by the fact that the effects of short and long exposures depends greatly on the age of the isotope used to label the probe. Short exposure non-linearity (SENL) can be dealt with by pre-flashing the X-ray film (brief exposure to very low level light source) to ensure that the response of the photographic emulsion is linear. However, the artifacts due to SENL are negligible since most exposures are long enough to ensure a linear grain response. A more serious problem involves long exposure non-linearity (LENL). In this case, the grains in the vicinity of the radioactive source have been saturated, so that the band intensity, on the X-ray film, is no longer a linear function of the radioactivity that is present.

Some workers (66,67) have attempted to deal with LENL by constructing a standard curve that gives the expected grain intensity, on an X-ray film, for various amounts of RNA in each lane on the northern blot. Such curves have shown that the response of the X-ray film is linear for exposures of up to 3 days. However, these curves are of limited usefulness since the exact age of the isotope is critical and is not likely to be the same for the standards and the experimental northern blots.

Consequently, standard curves were not used in the present study. Although northern blot analysis is extremely sensitive to variations in RNA quantities, the effects of LENL make an exact comparison between RNA transcript levels difficult. As a result, the transcript levels in this, and later Chapters, are presented as approximations and reference to specific levels (i.e. 2.5-fold) are given for clarity of presentation and not for numerical or statistical analysis.

Chapter III

Cloning of *myc* family members from the pituitary gland of rainbow trout

Introduction

Since the original isolation of v-myc in 1983 (47) the cellular homologue of this gene (c-*myc*) has been cloned from a variety of organisms, ranging from fish to human (1). The majority of these clones have been isolated from genomic libraries in order to obtain the regulatory sequences at the 5'-end of the gene and information regarding the exon-intron structure.

Few attempts have been made to clone these genes from cDNA libraries, in order to study transcript variability from one tissue type to the other. This is due, in part, to the fact that c-*myc* was shown to be expressed in mouse and human tissues as a single 2.4 kb transcript (58). Interest in cloning these genes for expression studies has increased since the discovery of L-*myc*, and the realization that this gene produces a wide variety of transcripts (48). Moreover, expression studies of *myc* genes during embryonic development showed striking differences in the spatial and temporal expression of these genes (1,66,67). Post-embryonic expression surveys of *myc* expression in mice have indicated a diversification in the function of the various family members. Some members, like c-*myc* are widely expressed in mitotic and post-mitotic tissues, whereas, L-*myc*

is more restricted, appearing primarily in post-mitotic tissues such as lung, kidney or brain (1). The case of L-*myc* is interesting, in that it is restricted to relatively few tissue types, but produces the widest variety of transcripts (48). This diversity is likely the result of being expressed in a complex tissue such as the brain where different transcripts may be produced to suite specific neurons.

The expression pattern of c-*myc* in trout appears to be more complex than it is in higher vertebrates, with different sized transcripts appearing in most of the tissues that have been examined (Chapter II). Diversification of c-*myc* expression is also apparent within a single trout tissue since the pituitary gland expresses 1.6 kb and 3.8 kb transcripts. The 1.6 kb transcript is unusual in that all c-*myc* transcripts described to date are at least 1.9 kb, although B-*myc* is known to produce a 1.3 kb transcript. This could mean that the 1.6 kb transcript in the gland is produced from exon 2, as is the case for the B-*myc* transcript, or that it is a c-*myc* with an unusually short 5' or 3' untranslated region.

This Chapter describes the cloning, expression and characterization of two *myc* genes from the pituitary gland of rainbow trout. One of these clones is the cDNA for the 1.6 kb transcript (tmyc3) and shows extensive homology to tmyc1. The second clone (tmyc2) is more divergent from tmyc1 and has an expression pattern distinct from that of tmyc1 or tmyc3. Portions of this Chapter have been published (78).

Materials and Methods

Library Construction

Total RNA was extracted from the pituitary glands of ten 400 g Rainbow trout, using guanidinium thiocyanate (72). Ten μg of this RNA were fractionated on a 1.2% MOPS-Formaldehyde gel to confirm the undegraded state of the 28S and 18S ribosomal bands. Poly $(A)^+$ RNA was extracted from the remaining sample using oligo-dT push columns (Stratagene). The cDNA library was produced using the λ-zap cDNA kit, including Gigapack II Gold packaging extract (Stratagene). Complementary DNA (cDNA) was synthesized using Moloney-Murine leukemia virus reverse transcriptase and cloned into the EcoRI (5') - XhoI (3') site of λ-zap according to the manufacturer's protocol.

The library was amplified once to a titer of 3.5×10^{10} pfu/ml before being transferred to Genescreen Plus (DuPont) at a density of 50,000 plaques/filter according to the manufacturer's protocol. The filters were probed with ***M1a*** (Chapter II, 70). The probe was labeled to high specific activity (10^9 dpm/μg) using an Oligolabelling kit (75, Pharmacia). Hybridization, stringency washes, and film exposures were as previously described (Chapter II).

A total of 5.0×10^5 clones were screened, out of which 12 positives were picked. A 1.6 kb (tmyc3) and a 1.8 kb clone (tmyc2) were selected for sequencing based on previous northern blot analysis (Chapter II) and on

restriction mapping. Sequencing was carried out manually and on an Applied BioSystems (ABS) Inc. automated sequencer (373A) according to the manufacturer's protocol. Preparation of the plasmid was carried out using an alkaline-lysis/PEG precipitation procedure supplied by ABS. Sequencing was begun at the 5' and 3' ends of the clone using the Reverse and M13-20 primers. Additional primers were synthesized as sequence data became available.

Sequence analysis

DNA and amino acid sequences were analyzed using a FORTRAN program called LARK (written for the Macintosh at SFU). Sequence data was aligned initially with LARK, but the final alignment was by visual inspection. The final alignments were used by LARK to determine percent homologies.

RNA blots

Northern blot analysis of tmyc2 and tmyc3 expression was carried out using Poly $(A)^+$ RNA isolated from the pituitary gland, brain, liver and heart of 600 g trout as described for library construction. The RNA (2 µg/ sample), containing 1 µg of ethidium bromide, was fractionated on a 1.5% MOPS-formaldehyde gel. A lane containing 5 µg of total RNA was included to provide the ribosomal RNA size markers. A second marker lane contained 5 µg of an RNA size marker (Boehringer-Mannheim, cat. # 1062-611). The gel was transferred to Genescreen Plus and probed sequentially with the following fragments: a 450 bp PstI-PvuII fragment from the middle

of Tmyc2 (probe ***M2a***), a 467 bp EcoRI-BamHI fragment from the 5' end of Tmyc3 (probe ***M3a***) and a 200 bp EcoRI-PstI fragment of the translation elongation factor 2 (EF) cDNA from rainbow trout (probe ***EFa***), which served as a loading control (This clone was isolated as a false positive in the screening experiments). The blot was washed at high stringency (0.1X SSPE, at 50°C for 20 minutes) sealed in a plastic bag and exposed to X-ray film for 3 days at -80°C with an intensifying screen. Hybridization conditions, sequential probing procedures, standardization of the data and estimation of transcript sizes were described in Chapter II.

Southern Blots

DNA was extracted from the brain of a single trout by powdering the entire brain (260 mg) on dry ice before adding it to 30 ml TNS (50mM Tris, 100 mM NaCl, 20 mM EDTA, 0.5% SDS). RNase IA (Pharmacia) was added to a final concentration of 200 mg/ml and incubated at 37°C for 1 h. Proteinase K was added to a final concentration of 0.5 mg/ml and the incubation at 37°C was continued overnight. The mixture was extracted twice with 0.5 volume phenol-chloroform (1:1) and once with 1.0 volume chloroform. 2.5 volumes of ice cold 95% ethanol was added to the recovered aqueous phase and the precipitated DNA was spooled out of the mixture using a glass rod. The DNA was washed twice with 70% ethanol and dissolved in 1.0 ml TE (pH 8.0) overnight at room temperature (79). The DNA was digested with EcoRI, BamHI, PstI and combinations of these

three enzymes (20 μg DNA/reaction); extracted with 1.0 volume phenol-chloroform, precipitated and dissolved in TE (pH 8.0).

DNA from each digest (10 μg) was fractionated on a 0.8% agarose gel. DNA size markers (1.0 kb DNA ladder, GIBCO-BRL, 2 μg/lane) were loaded into the first and last lane. After electrophoresis the gel was depurinated in 5M HCl (7 minutes), denatured in 10N NaOH-1.5M NaCl (20 minutes), neutralized in 0.2M Tris-1.5M NaCl (pH 7.5, 15 minutes), soaked in 10X SSPE (10 minutes) and transferred to Genescreen Plus overnight. After transfer the blot was denatured (2 minutes), neutralized (2 minutes) and baked for 30 minutes at 80°C (74,80). The blot was hybridized sequentially, using the ***M2a*** and ***M3a*** probes, described above. Hybridization conditions, buffers and X-ray exposures were as described in Chapter II. The blot was probed first with ***M3a***, washed at moderate stringency (1X SSPE, 50°C) and exposed to X-ray film for 3 days. It was washed again at high stringency (0.1X SSPE, 50°C), exposed to X-ray film (3 d), then stripped before being reprobed with ***M2a***. The blot was given a single high stringency wash and exposed to X-ray film (3 d). Estimation of DNA fragment sizes was carried out as described in Chapter II.

Results

Fig. 5 shows the restriction maps and sequencing strategies for tmyc2 and tmyc3. The Xho I site was introduced as part of the cloning strategy. Important areas of the Myc protein, described in Fig. 1, are also

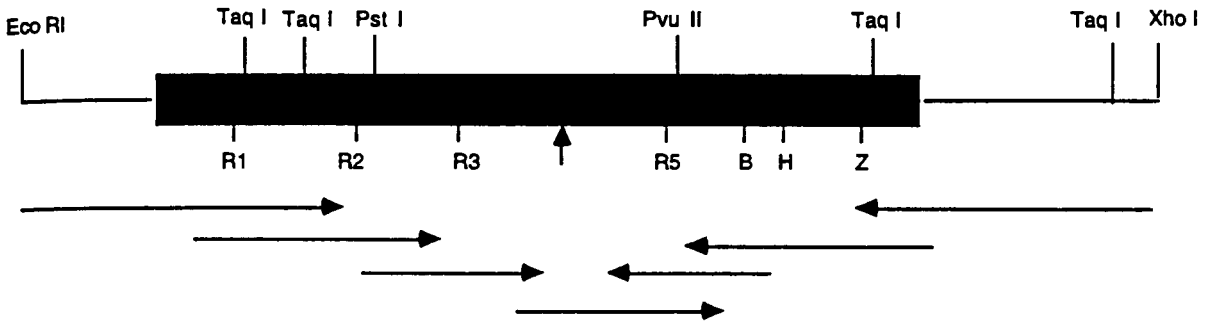
indicated. These include the transactivation domains (R1, R2), the DNA-binding basic region (B) and the HLH (H) and leucine zipper (Z) dimerization motifs. The border between exons 2 and 3 is indicated with an arrow. Tmyc2 is 1761 bp and contains an open reading frame beginning with a start codon located at position 202 and terminating with a TGA stop codon located at position 1396. The reading frame codes for a protein with 398 amino acids. This clone contains a 201 bp 5' untranslated region and a 366 bp 3' untranslated region (Table 3). Tmyc3 consists of 1586 bp with a 398 bp 3' untranslated region. A 5' untranslated region is missing, as well as 18 bp from the beginning of the coding region. An open reading frame is terminated at position 1185 by a TGA stop codon. Assuming the start codon is located at -18 (Table 3), the reading frame will generate a protein containing 401 amino acids. Table 3 also includes data on tmyc1 for comparison.

The complete DNA sequences, and the deduced amino acid sequences, for tmyc2 and tmyc3 are shown in Figures 6 and 7. Both of these clones contain the transactivation domains (regions 1 and 2), the DNA binding region (BR) and the dimerization motifs (helix 1-loop-helix2, leucine zipper) that are characteristic of all *myc* family members. The stop codon (TGA), for both clones, is indicated by the asterisks and the border between exon 2 and 3 is indicated with an arrow.

A comparison between the DNA sequences of tmyc2 and tmyc3 is shown in Fig. 8. Overall, the homology between these two clones is 73%.

Figure 5. Restriction maps and sequencing strategies for two myc cDNA clones, tmyc2 **(A)** and tmyc3 **(B)**, isolated from rainbow trout. The 5' end of each clone is on the left (EcoRI site) and the coding region is indicated by the solid rectangle. Important areas of the *myc* protein, described in Figure 1, are also indicated. These include the transactivation domains (R1, R2), the DNA-binding basic region (B) and the HLH (H) and leucine zipper (Z) dimerization motifs. R3 and R5 are regions that show interesting differences (or similarities) between tMyc2 and tMyc3. A fourth region (R4), which is unique to tMyc2, is located at the exon 2/3 border (arrow).

A


Eco RI
Taq I
Taq I
Pst I
Pvu II
Taq I
Taq I
Xho I
R1
R2
R3
R5
B
H
Z

B

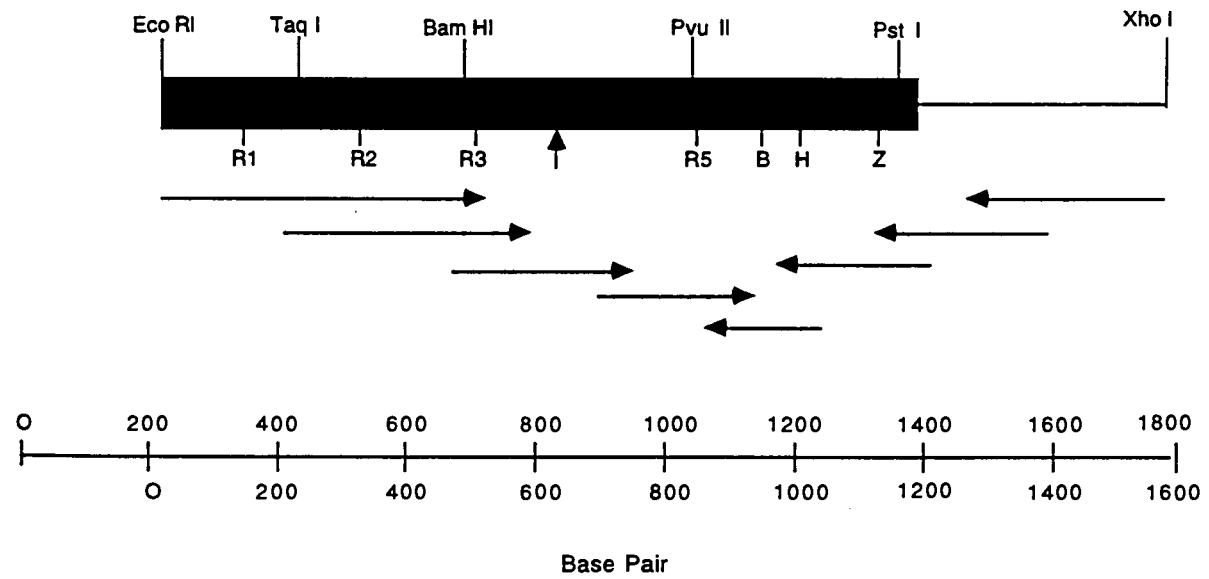

Eco RI
Taq I
Bam HI
Pvu II
Pst I
Xho I
R1
R2
R3
R5
B
H
Z
0
200
400
600
800
1000
1200
1400
1600
1800
0
200
400
600
800
1000
1200
1400
1600
Base Pair

Table 3

Numerical descriptions of three *myc* cDNA clones from rainbow trout.

GENE	5'	START	STOP	CODING	AA	3'	TOTAL
tmyc1	0	-9	1243	1251	417	47	1292
tmyc2	201	202	1396	1194	398	366	1761
tmyc3	0	-18	1185	1203	401	398	1586

Table values are in base pairs. 5' and 3' refers to untranslated regions at each end of the clone. The locations for the start (ATG) and stop (TGA) codons mark the positions of the first base in the triplet. The length of the coding region (CODING) and the number of amino acids in the protein (AA) are also indicated. The AA value shown for tmyc3 assumes the start codon is located 18 bp beyond the 5' end of the clone. The same adjustment was made for tmyc1.

The homology was also determined for three major areas of the DNA sequence. The first area, defined by the EcoRI-BamHI fragment of tmyc3, is 87% homologous; the second area (PstI-PvuII fragment of tmyc2), is 62% homologous; and the third area (PvuII-end) is 70% homologous. The EcoRI-BamHI fragment was used as a probe for tmyc3 (***M3a***) and the PstI-PvuII fragment was used as a probe for tmyc2 (***M2a***). The transactivation domains and the dimerization motifs are located in the first and third areas, respectively.

The low homology over the third area is due primarily to divergence in the 3' untranslated region of tmyc2. A comparison of the available 3' untranslated region from tmyc1 was compared to tmyc2 and tmyc3 (Fig. 9). Tmyc1 is missing a 15 bp sequence, present in tmyc2 and tmyc3, adjacent to the stop codon.

The protein sequences for tMyc2 and tMyc3 are compared to other Myc family members in Figs. 10 and 11, respectively. These comparisons indicate that both tMyc2 and tMyc3 are much more closely related to c-Myc than they are to the other family members. Although tMyc2 and tMyc3 have diverged considerably from L-myc and N-myc (Figs. 10A,B and Figs. 11A,B) there are four regions that show extensive homology. This is seen most clearly for tMyc3 (Figs. 11A, B). These regions correspond to the transactivation domains, an area near the exon 2/3 border and the dimerization motifs. By contrast, both tMyc2 and tMyc3 show extensive

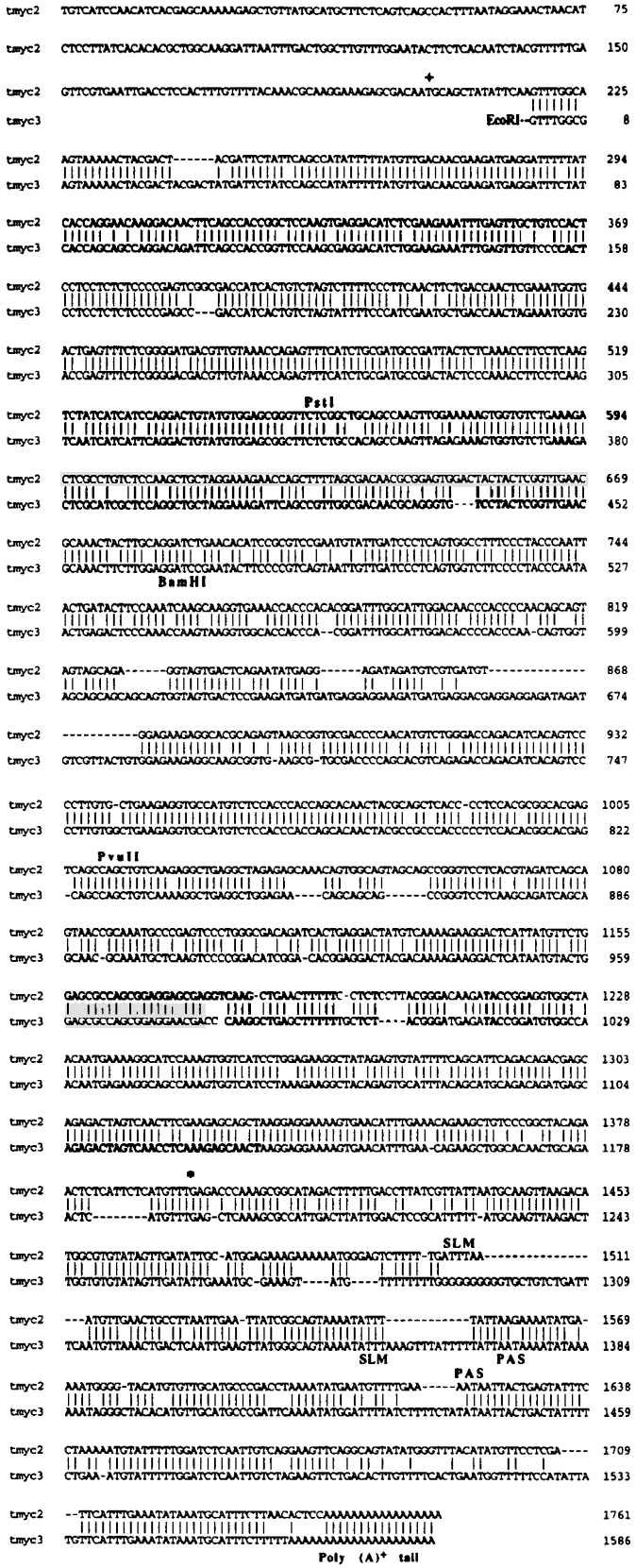
Figure 6. DNA and deduced amino acid sequence for tmyc2. The transactivation domains are indicated as regions 1 and 2. The DNA binding basic region, helix-loop-helix and leucine zipper (plus signs) are also inciated. The arrow marks the exon 2/3 border and the asterisks mark the location of the stop codon.

```
                                                                    TGTCATCCAACATCACGAGCAAAAAGAGCTGTTATGCATGCTT    43

CTCAGTCAGCCACTTTAATAGGAAACTAACATCTCCTTATCACACACGCTGGCAAGGATTAATTTGACTGGCTTGTTTG   122

GAATACTTCTCACAATCTACGTTTTTGAGTTCGTGAATTGACCTCCACTTTGTTTTACAAACGCAAGGAAAGAGCGACA   201

ATG CAG CTA TAT TCA AGT TTG GCA AGT AAA AAC TAC GAC TAC GAT TCT ATT CAG CCA TAT   261
MET GLN LEU TYR SER SER LEU ALA SER LYS ASN TYR ASP TYR ASP SER ILE GLN PRO TYR

TTT TAT GTT GAC AAC GAA GAT GAG GAT TTT TAT CAC CAG GAA CAA GGA CAA CTT CAG CCA   321
PHE TYR VAL ASP ASN GLU ASP GLU ASP PHE TYR HIS GLN GLU GLN GLY GLN LEU GLN PRO

                                  Region 1
                    ----------------------------------------------------------

CCG GCT CCA AGT GAG GAC ATC TCG AAG AAA TTT GAG TTG CTG TCC ACT CCT CCT CTC TCC   381
PRO ALA PRO SER GLU ASP ILE SER LYS LYS PHE GLU LEU LEU SER THR PRO PRO LEU SER

---------

CCG AGT CGG CGA CCA TCA CTG TCT AGT CTT TTC CCT TCA ACT TCT GAC CAA CTC GAA ATG   441
PRO SER ARG ARG PRO SER LEU SER SER LEU PHE PRO SER THR SER ASP GLN LEU GLU MET

GTG ACT GAG TTT CTC GGG GAT GAC GTT GTA AAC CAG AGT TTC ATC TGC GAT GCC GAT TAC   501
VAL THR GLU PHE LEU GLY ASP ASP VAL VAL ASN GLN SER PHE ILE CYS ASP ALA ASP TYR

                                  Region 2
                    ----------------------------------------------------------

TCT CAA ACC TTC CTC AAG TCT ATC ATC ATC CAG GAC TGT ATG TGG AGC GGG TTC TCG GCT   561
SER GLN THR PHE LEU LYS SER ILE ILE ILE GLN ASP CYS MET TRP SER GLY PHE SER ALA

-----------------

GCA GCC AAG TTG GAA AAA GTG GTG TCT GAA AGA CTC GCC TGT CTC CAA GCT GCT AGG AAA   621
ALA ALA LYS LEU GLU LYS VAL VAL SER GLU ARG LEU ALA CYS LEU GLN ALA ALA ARG LYS

GAA CCA GCT TTT AGC GAC AAC GCG GAG TGG ACT ACT ACT CGG TTG AAC GCA AAC TAC TTG   681
GLU PRO ALA PHE SER ASP ASN ALA GLU TRP THR THR THR ARG LEU ASN ALA ASN TYR LEU

CAG GAT CTG AAC ACA TCC GCG TCC GAA TGT ATT GAT CCC TCA GTG GCC TTT CCC TAC CCA   741
GLN ASP LEU ASN THR SER ALA SER GLU CYS ILE ASP PRO SER VAL ALA PHE PRO TYR PRO

ATT ACT GAT ACT TCC AAA TCA AGC AAG GTG AAA CCA CCC ACA CGG ATT TGG CAT TGG ACA   801
ILE THR ASP THR SER LYS SER SER LYS VAL LYS PRO PRO THR ARG ILE TRP HIS TRP THR

                                  ▼
ACC CAC CCC AAC AGC AGT AGT AGC AGA GGT AGT GAC TCA GAA TAT GAG GAG ATA GAT GTC   861
THR HIS PRO ASN SER SER SER SER ARG GLY SER ASP SER GLU TYR GLU GLU ILE ASP VAL

GTG ATG TGG AGA AGA GGC ACG CAG AGT AAG CGG TGC GAC CCC AAC ATG TCT GGG ACC AGA   921
VAL MET TRP ARG ARG GLY THR GLN SER LYS ARG CYS ASP PRO ASN MET SER GLY THR ARG

CAT CAC AGT CCC CTT GTG CTG AAG AGG TGC CAT GTC TCC ACC CAC CAG CAC AAC TAC GCA   981
HIS HIS SER PRO LEU VAL LEU LYS ARG CYS HIS VAL SER THR HIS GLN HIS ASN TYR ALA

GCT CAC CCC TCC ACG CGG CAC GAG TCA GCC AGC TGT CAA GAG GCT GAG GCT AGA GAG CAA  1041
ALA HIS PRO SER THR ARG HIS GLU SER ALA SER CYS GLN GLU ALA GLU ALA ARG GLU GLN

ACA GTG GCA GTA GCA GCC GGG TCC TCA CGT AGA TCA GCA GTA ACC GCA AAT GCC CGA GTC  1101
THR VAL ALA VAL ALA ALA GLY SER SER ARG ARG SER ALA VAL THR ALA ASN ALA ARG VAL

                                                  Basic Region
                                    ----------------------------

CCT GGG CGA CAG ATC ACT GAG GAC TAT GTC AAA AGA AGG ACT CAT TAT GTT CTG GAG CGC  1161
PRO GLY ARG GLN ILE THR GLU ASP TYR VAL LYS ARG ARG THR HIS TYR VAL LEU GLU ARG

                                  Helix 1
                    ----------|----------------------------------------|----------

CAG CGG AGG AGC GAG GTC AAG CTG AAC TTT TTC CTC TCC TTA CGG GAC AAG ATA CCG GAG  1221
GLN ARG ARG SER GLU VAL LYS LEU ASN PHE PHE LEU SER LEU ARG ASP LYS ILE PRO GLU

                Loop                              Helix 2
                    ----------|----------------------------------------

GTG GCT AAC AAT GAA AAG GCA TCC AAA GTG GTC ATC CTG GAG AAG GCT ATA GAG TGT ATT  1281
VAL ALA ASN ASN GLU LYS ALA SER LYS VAL VAL ILE LEU GLU LYS ALA ILE GLU CYS ILE

                                  +                   +
---------------------

TTC AGC ATT CAG ACA GAC GAG CAG AGA CTA GTC AAC TTC GAA GAG CAG CTA AGG AGG AAA  1341
PHE SER ILE GLN THR ASP GLU GLN ARG LEU VAL ASN PHE GLU GLU GLN LEU ARG ARG LYS

          +                         +
AGT GAA CAT TTG AAA CAG AAG CTG TCC CGG CTA CAG AAC TCT CAT TCT CAT GTT TGA GAC  1401
SER GLU HIS LEU LYS GLN LYS LEU SER ARG LEU GLN ASN SER HIS SER HIS VAL ***

CCAAAGCGGCATAGACTTTTTGACCTTATCGTTATTAATGCAAGTTAAGACATGGCGTGTATAGTTGATATTGCATGGA  1480

GAAAGAAAAAATGGGAGTCTTTTTGATTTAAATGTTGAACTGCCTTAATTGAATTATCGGCAGTAAAATATTTTATTAA  1559

GAAAATATGAAAATGGGGTACATGTGTTGCATGCCCGACCTAAAATATGAATGTTTTGAAAATAATTACTGAGTATTTC  1638

CTAAAAATGTATTTTTGGATCTCAATTGTCAGGAAGTTCAGGCAGTATATGGGTTTACATATGTTCCTCGATTCATTTG  1717

AAATATAAATGCATTTCTTAACACTCCAAAAAAAAAAAAAAAAA                                      1761
```

Figure 7. DNA and deduced amino acid sequence for tmyc3. The transactivation domains are indicated as regions 1 and 2. The DNA binding basic region, helix-loop-helix and leucine zipper (plus signs) are also inciated. The arrow marks the exon 2/3 border and three asterisks mark the location of the stop codon. The start codon is not present in this clone.

```
TTG GCG AGT AAA AAC TAC GAC TAC GAC TAT GAT TCT ATC CAG CCA TAT TTT TAT GTT GAC    62
LEU ALA SER LYS ASN TYR ASP TYR ASP TYR ASP SER ILE GLN PRO TYR PHE TYR VAL ASP

                                                                  ————
AAC GAA GAT GAG GAT TTC TAT CAC CAG CAG CCA GGA CAG ATT CAG CCA CCG GTT CCA AGC   122
ASN GLU ASP GLU ASP PHE TYR HIS GLN GLN PRO GLY GLN ILE GLN PRO PRO VAL PRO SER

                          Region 1
————————————————————————————————————————————————————————————————————————————
GAG GAC ATC TGG AAG AAA TTT GAG TTG TTC CCC ACT CCT CCT CTC TCC CCG AGC CGA CCA   182
GLU ASP ILE TRP LYS LYS PHE GLU LEU PHE PRO THR PRO PRO LEU SER PRO SER ARG PRO

TCA CTG TCT AGT ATT TTC CCA TCG AAT GCT GAC CAA CTA GAA ATG GTG ACC GAG TTT CTC   242
SER LEU SER SER ILE PHE PRO SER ASN ALA ASP GLN LEU GLU MET VAL THR GLU PHE LEU

GGG GAC GAC GTT GTA AAC CAG AGT TTC ATC TGC GAT GCC GAC TAC TCC CAA ACC TTC CTC   302
GLY ASP ASP VAL VAL ASN GLN SER PHE ILE CYS ASP ALA ASP TYR SER GLN THR PHE LEU

                          Region 2
————————————————————————————————————————————————————————————————————————————
AAG TCA ATC ATC ATT CAG GAC TGT ATG TGG AGC GGC TTC TCT GCC ACA GCC AAG TTA GAG   362
LYS SER ILE ILE ILE GLN ASP CYS MET TRP SER GLY PHE SER ALA THR ALA LYS LEU GLU

—
AAA GTG GTG TCT GAA AGA CTC GCA TCG CTC CAG GCT GCT AGG AAA GAT TCA GCC GTT GGC   422
LYS VAL VAL SER GLU ARG LEU ALA SER LEU GLN ALA ALA ARG LYS ASP SER ALA VAL GLY

GAC AAC GCA GGG TGT CCT ACT CGG TTG AAC GCA AAC TTC TTG GAG GAT CCG AAT ACT TCC   482
ASP ASN ALA GLY CYS PRO THR ARG LEU ASN ALA ASN PHE LEU GLU ASP PRO ASN THR SER

CCG TCA GTA ATT GTT GAT CCC TCA GTG GTC TTC CCC TAC CCA ATA ACT GAG ACT CCC AAA   542
PRO SER VAL ILE VAL ASP PRO SER VAL VAL PHE PRO TYR PRO ILE THR GLU THR PRO LYS

CCA AGT AAG GTG GCA CCA CCC ACG GAT TTG GCA TTG GAC ACC CCA CCC AAC AGT GGT AGC   602
PRO SER LYS VAL ALA PRO PRO THR ASP LEU ALA LEU ASP THR PRO PRO ASN SER GLY SER

                          ▼
AGC AGC AGC AGT GGT AGT GAC TCC GAA GAT GAT GAT GAG GAG GAA GAT GAT GAG GAC GAG   662
SER SER SER SER GLY SER ASP SER GLU ASP ASP ASP GLU GLU GLU ASP ASP GLU ASP GLU

GAG GAG ATA GAT GTC GTT ACT GTG GAG AAG AGG CAA GCG GTG AAG CGT GCG ACC CCA GCA   722
GLU GLU ILE ASP VAL VAL THR VAL GLU LYS ARG GLN ALA VAL LYS ARG ALA THR PRO ALA

CGT CAG AGA CCA GAC ATC ACA GTC CCC TTG TGG CTG AAG AGG TGC CAT GTC TCC ACC CAC   782
ARG GLN ARG PRO ASP ILE THR VAL PRO LEU TRP LEU LYS ARG CYS HIS VAL SER THR HIS

CAG CAC AAC TAC GCC GCC CAC CCC TCC ACA CGG CAC GAG CAG CCA GCT GTC AAA AGG CTG   842
GLN HIS ASN TYR ALA ALA HIS PRO SER THR ARG HIS GLU GLN PRO ALA VAL LYS ARG LEU

AGG CTG GAG AAC AGC AGC AGC CGG GTC CTC AAG CAG ATC AGC AGC ACA CGC AAA TGC TCA   902
ARG LEU GLU ASN SER SER SER ARG VAL LEU LYS GLN ILE SER SER THR ARG LYS CYS SER

                                                  Basic Region
                                                  ————————————
AGT CCC CGG ACA TCG GAC ACG GAG GAC TAC GAC AAA AGA AGG ACT CAT AAT GTA CTG GAG   962
SER PRO ARG THR SER ASP THR GLU ASP TYR ASP LYS ARG ARG THR HIS ASN VAL LEU GLU

                          Helix 1
                          ———————
CGC CAG CGG AGG AAC GAC CCA AGG CTG AGC TTT TTT GCT CTA CGG GAT GAG ATA CCG GAT  1022
ARG GLN ARG ARG ASN ASP PRO ARG LEU SER PHE PHE ALA LEU ARG ASP GLU ILE PRO ASP

          Loop                            Helix 2
          ————                            ———————
GTG GCC AAC AAT GAG AAG GCA GCC AAA GTG GTC ATC CTA AAG AAG GCT ACA GAG TGC ATT  1082
VAL ALA ASN ASN GLU LYS ALA ALA LYS VAL VAL ILE LEU LYS LYS ALA THR GLU CYS ILE

————————————————                  +                               +
TAC AGC ATG CAG ACA GAT GAG CAG AGA CTA GTC AAC CTC AAA GAG CAA CTA AGG AGG AAA  1142
TYR SER MET GLN THR ASP GLU GLN ARG LEU VAL ASN LEU LYS GLU GLN LEU ARG ARG LYS

          +
AGT GAA CAT TTG AAC AGA AGC TGG CAC AAC TGC AGA ACT CAT GTT TGA GCT CAA AGC GCC  1202
SER GLU HIS LEU ASN ARG SER TRP HIS ASN CYS ARG THR HIS VAL ***

ATTGACTTATTGGACTCCGCATTTTTATGCAAGTTAAGACTTGGTGTGTATAGTTGATATTGAAATGCGAAAGTATGTT  1281

TTTTTTGGGGGGGGGGTGCTGTCTGATTTCAATGTTAAACTGACTCAATTGAAGTTATGGGCAGTAAAATATTTAAAGT  1360

TTATTTTTATTAATAAAATATAAAAAATAGGGCTACACATGTTGCATGCCCGATTCAAAATATGGATTTTATCTTTTCT  1439

ATATAATTACTGACTATTTTCTGAAATGTATTTTTGGATCTCAATTGTCTAGAAGTTCTGACACTTGTTTTCACTGAAT  1518

GGTTTTTCCATATTATGTTCATTTGAAATATAAATGCATTTCTTTTTAAAAAAAAAAAAAAAAAAAAA              1586
```

Figure 8. Comparison of the tmyc2 and tmyc3 DNA sequences. The 5' end of each clone begins at upper left. Four restriction sites are indicated. The EcoRI-BamHI fragment was used as a probe for tmyc3 ***(M3a)*** and the PstI-PvuII fragment was used as a probe for Tmyc2 ***(M2a)***. The plus sign marks the location of the start codon (ATG) on tmyc2 and the asterisks marks the location of the stop codon (TGA) for both clones. The poly adenylation signal (PAS) and the signal for marking an RNA for a short half life (SLM) are also indicated.


tmyc2 TGTCATCCAACATCACGAGCAAAAAGAGCTGTTATGCATGCTTCTCAGTCAGCCACTTTAATAGGAAACTAACAT 75
tmyc2 CTCCTTATCACACACGCTGGCAAGGATTAATTTGACTGGCTTGTTTGGAATACTTCTCACAATCTACGTTTTTGA 150
tmyc2 GTTCGTGAATTGACCTCCACTTTGTTTTACAAACGCAAGGAAAGAGCGACAATGCAGCTATATTCAAGTTTGGCA 225
tmyc3 EcoRI--GTTTGGCG 8
tmyc2 AGTAAAAACTACGACT------ACGATTCTATTCAGCCATATTTTTATGTTGACAACGAAGATGAGGATTTTTAT 294
tmyc3 AGTAAAAACTACGACTACGACTATGATTCTATCCAGCCATATTTTTATGTTGACAACGAAGATGAGGATTTCTAT 83
tmyc2 CACCAGGAACAAGGACAACTTCAGCCACCGGCTCCAAGTGAGGACATCTCGAAGAAATTTGAGTTGCTGTCCACT 369
tmyc3 CACCAGCAGCCAGGACAGATTCAGCCACCGGTTCCAAGCGAGGACATCTGGAAGAAATTTGAGTTGTTCCCCACT 158
tmyc2 CCTCCTCTCTCCCCGAGTCGGCGACCATCACTGTCTAGTCTTTTCCCTTCAACTTCTGACCAACTCGAAATGGTG 444
tmyc3 CCTCCTCTCTCCCCGAGCC---GACCATCACTGTCTAGTATTTTCCCATCGAATGCTGACCAACTAGAAATGGTG 230
tmyc2 ACTGAGTTTCTCGGGGATGACGTTGTAAACCAGAGTTTCATCTGCGATGCCGATTACTCTCAAACCTTCCTCAAG 519
tmyc3 ACCGAGTTTCTCGGGGACGACGTTGTAAACCAGAGTTTCATCTGCGATGCCGACTACTCCCAAACCTTCCTCAAG 305
PstI
tmyc2 TCTATCATCATCCAGGACTGTATGTGGAGCGGGTTCTCGGCTGCAGCCAAGTTGGAAAAAGTGGTGTCTGAAAGA 594
tmyc3 TCAATCATCATTCAGGACTGTATGTGGAGCGGCTTCTCTGCCACAGCCAAGTTAGAGAAAGTGGTGTCTGAAAGA 380
tmyc2 CTCGCCTGTCTCCAAGCTGCTAGGAAAGAACCAGCTTTTAGCGACAACGCGGAGTGGACTACTACTCGGTTGAAC 669
tmyc3 CTCGCATCGCTCCAGGCTGCTAGGAAAGATTCAGCCGTTGGCGACAACGCAGGGTG---TCCTACTCGGTTGAAC 452
tmyc2 GCAAACTACTTGCAGGATCTGAACACATCCGCGTCCGAATGTATTGATCCCTCAGTGGCCTTTCCCTACCCAATT 744
tmyc3 GCAAACTTCTTGGAGGATCCGAATACTTCCCCGTCAGTAATTGTTGATCCCTCAGTGGTCTTCCCCTACCCAATA 527
BamHI
tmyc2 ACTGATACTTCCAAATCAAGCAAGGTGAAACCACCCACACGGATTTGGCATTGGACAACCCACCCCAACAGCAGT 819
tmyc3 ACTGAGACTCCCAAACCAAGTAAGGTGGCACCACCCA--CGGATTTGGCATTGGACACCCCACCCAA-CAGTGGT 599
tmyc2 AGTAGCAGA------GGTAGTGACTCAGAATATGAGG------AGATAGATGTCGTGATGT---------- 868
tmyc3 AGCAGCAGCAGCAGTGGTAGTGACTCCGAAGATGATGATGAGGAGGAAGATGATGAGGACGAGGAGGAGATAGAT 674
tmyc2 ----------GGAGAAGAGGCACGCAGAGTAAGCGGTGCGACCCCAACATGTCTGGGACCAGACATCACAGTCC 932
tmyc3 GTCGTTACTGTGGAGAAGAGGCAAGCGGTG-AAGCG-TGCGACCCCAGCACGTCAGAGACCAGACATCACAGTCC 747
tmyc2 CCTTGTG-CTGAAGAGGTGCCATGTCTCCACCCACCAGCACAACTACGCAGCTCACC-CCTCCACGCGGCACGAG 1005
tmyc3 CCTTGTGGCTGAAGAGGTGCCATGTCTCCACCCACCAGCACAACTACGCCGCCCACCCCCTCCACACGGCACGAG 822
PvuII
tmyc2 TCAGCCAGCTGTCAAGAGGCTGAGGCTAGAGAGCAAACAGTGGCAGTAGCAGCCGGGTCCTCACGTAGATCAGCA 1080
tmyc3 -CAGCCAGCTGTCAAAAGGCTGAGGCTGGAGAA----CAGCAGCAG------CCGGGTCCTCAAGCAGATCAGCA 886
tmyc2 GTAACCGCAAATGCCCGAGTCCCTGGGCGACAGATCACTGAGGACTATGTCAAAAGAAGGACTCATTATGTTCTG 1155
tmyc3 GCAAC-GCAAATGCTCAAGTCCCCGGACATCGGA-CACGGAGGACTACGACAAAAGAAGGACTCATAATGTACTG 959
tmyc2 GAGCGCCAGCGGAGGAGCGAGGTCAAG-CTGAACTTTTTC-CTCTCCTTACGGGACAAGATACCGGAGGTGGCTA 1228
tmyc3 GAGCGCCAGCGGAGGAACGACC CAAGGCTGAGCTTTTTGCTCT----ACGGGATGAGATACCGGATGTGGCCA 1029
tmyc2 ACAATGAAAAGGCATCCAAAGTGGTCATCCTGGAGAAGGCTATAGAGTGTATTTTCAGCATTCAGACAGACGAGC 1303
tmyc3 ACAATGAGAAGGCAGCCAAAGTGGTCATCCTAAAGAAGGCTACAGAGTGCATTTACAGCATGCAGACAGATGAGC 1104
tmyc2 AGAGACTAGTCAACTTCGAAGAGCAGCTAAGGAGGAAAAGTGAACATTTGAAACAGAAGCTGTCCCGGCTACAGA 1378
tmyc3 AGAGACTAGTCAACCTCAAAGAGCAACTAAGGAGGAAAAGTGAACATTTGAA-CAGAAGCTGGCACAACTGCAGA 1178
tmyc2 ACTCTCATTCTCATGTTTGAGACCCAAAGCGGCATAGACTTTTTGACCTTATCGTTATTAATGCAAGTTAAGACA 1453
tmyc3 ACTC--------ATGTTTGAG-CTCAAAGCGCCATTGACTTATTGGACTCCGCATTTTT-ATGCAAGTTAAGACT 1243
SLM
tmyc2 TGGCGTGTATAGTTGATATTGC-ATGGAGAAAGAAAAAATGGGAGTCTTTT-TGATTTAA---------- 1511
tmyc3 TGGTGTGTATAGTTGATATTGAAATGC-GAAAGT----ATG----TTTTTTTTGGGGGGGGGGTGCTGTCTGATT 1309
tmyc2 ---ATGTTGAACTGCCTTAATTGAA-TTATCGGCAGTAAAATATTT----------TATTAAGAAAATATGA- 1569
tmyc3 TCAATGTTAAACTGACTCAATTGAAGTTATGGGCAGTAAAATATTTAAAGTTTATTTTTATTAATAAAATATAAA 1384
SLM PAS
PAS
tmyc2 AAATGGGG-TACATGTGTTGCATGCCCGACCTAAAATATGAATGTTTTGAA-----AATAATTACTGAGTATTTC 1638
tmyc3 AAATAGGGCTACACATGTTGCATGCCCGATTCAAAATATGGATTTTATCTTTTCTATATAATTACTGACTATTTT 1459
tmyc2 CTAAAAATGTATTTTTGGATCTCAATTGTCAGGAAGTTCAGGCAGTATATGGGTTTACATATGTTCCTCGA---- 1709
tmyc3 CTGAA-ATGTATTTTTGGATCTCAATTGTCTAGAAGTTCTGACACTTGTTTTCACTGAATGGTTTTTCCATATTA 1533
tmyc2 --TTCATTTGAAATATAAATGCATTTCTTTAACACTCCAAAAAAAAAAAAAAAAA 1761
tmyc3 TGTTCATTTGAAATATAAATGCATTTCTTTTTAAAAAAAAAAAAAAAAAAAA 1586
Poly (A)+ tail

homology to human c-Myc throughout the protein sequence (Figs. 10C and 11C), although tMyc2 shows more divergence in the middle portion of the protein.

Fig. 12 shows a comparison between the amino acid sequences of tMyc1, tMyc2 and tMyc3. Important areas of the Myc protein, described in Figs. 1 and 5 are shown. the leucine zipper is indicated with plus signs and the border between exon 2 and 3 is marked with an arrow. The overall homology between these three proteins is 65%, whereas the similarity is 76%.

A summary of comparisons of the three trout Myc proteins is shown in Table 4. In general, tMyc3 shows extensive homology to tMyc1 whereas tMyc2 shows more divergence. Regions 1 and 2 are highly conserved between all three proteins. The basic region (BR) is also highly conserved, as is the HLH domain, between tMyc1 and tMyc3. However, there is extensive divergence in this region between tMyc1 and tMyc2 (74% homology) as well as between tMyc2 and tMyc3 (68% homologous). The leucine zipper shows even greater divergence between tMyc2 and tMyc3 with only 55% homology and between tMyc1 and tMyc3 with 62%. Regions 3, 4 and 5 define additional differences between these three proteins. R3 is part of a tandem repeat, present in tMyc1, which is not present in tMyc2 and tMyc3. R4 defines a second region that is partially deleted in tMyc2 but is conserved in tmyc1 and tMyc3. 45% of the residues in this region are acidic in tMyc2, as compared to 76% in tmyc1 and tmyc3. R5 is an area that contains many substitutions in tMyc2, with the carboxyl end containing five

Figure 9. A comparison between the 3' untranslated region of tmyc1 with tmyc2 **(A)** and tmyc3 **(B)** indicates that tmyc1 is missing a 15 bp sequence adjacent to the stop codon (TGA) that is present in both tmyc2 and tmyc3.

A

```
tmyc2   TGAGACCCAAAGCGGCATAGACTTTTTGACCTTATCGTTATTAATGCAAGTTAAGACATGGCGTGTATAG   70
        |||                  |||   |  ||   | || ||| |||||||||||||| ||| ||||||||
tmyc1   TGA------------------CTTACCGGACTCCGCATTGTTA-TGCAAGTTAAGACTTGGTGTGTATA   50
```

B

```
tmyc3   TGAGCTCAAAGCGCCATTGACTTATTGGACTCCGCATTTTTATGCAAGTTAAGACTTGGTGTGTATAGTT   70
        |||                  |||||  |||||||||||| ||||||||||||||||||||||||||||
tmyc1   TGA------------------CTTACCGGACTCCGCATTGTTATGCAAGTTAAGACTTGGTGTGTATA   50
```

Figure 10. Comparisons between the tMyc2 protein sequence and human Myc family members. The sequences were aligned in pairs and the homology determined at intervals of 10 amino acid residues, beginning at the amino terminus of each protein. Tmyc2 was compared to L-Myc **(A)**, N-Myc **(B)** and c-Myc **(C)**.

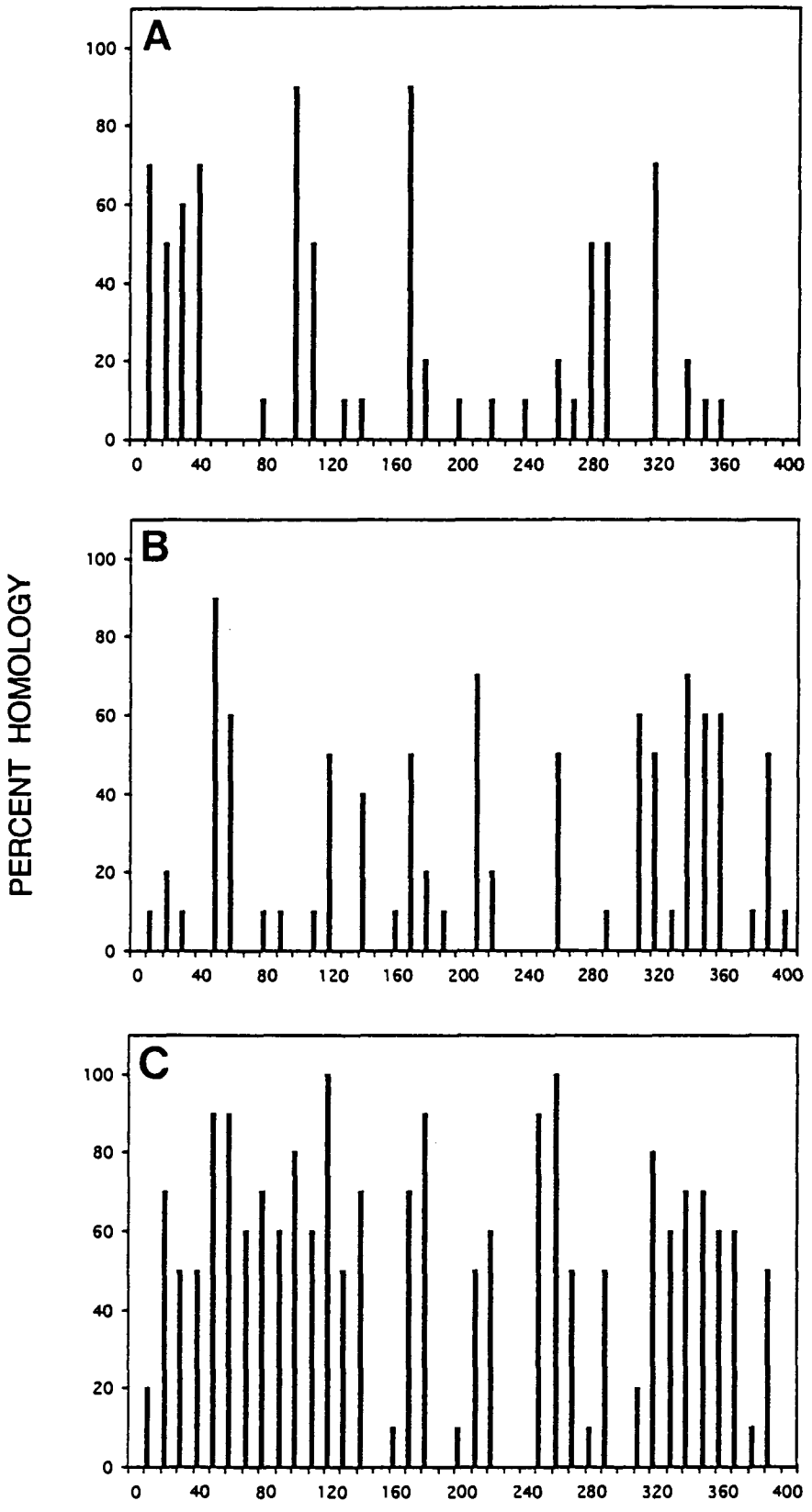

A
B
C
PERCENT HOMOLOGY
0
20
40
60
80
100
0
40
80
120
160
200
240
280
320
360
400
AMINO ACID RESIDUES

Figure 11. Comparisons between the tMyc3 protein sequence and human Myc family members. The sequences were aligned in pairs and the homology determined at intervals of 10 amino acid residues, beginning at the amino terminus of each protein. Tmyc3 was compared to L-Myc **(A)**, N-Myc **(B)** and c-Myc **(C)**.

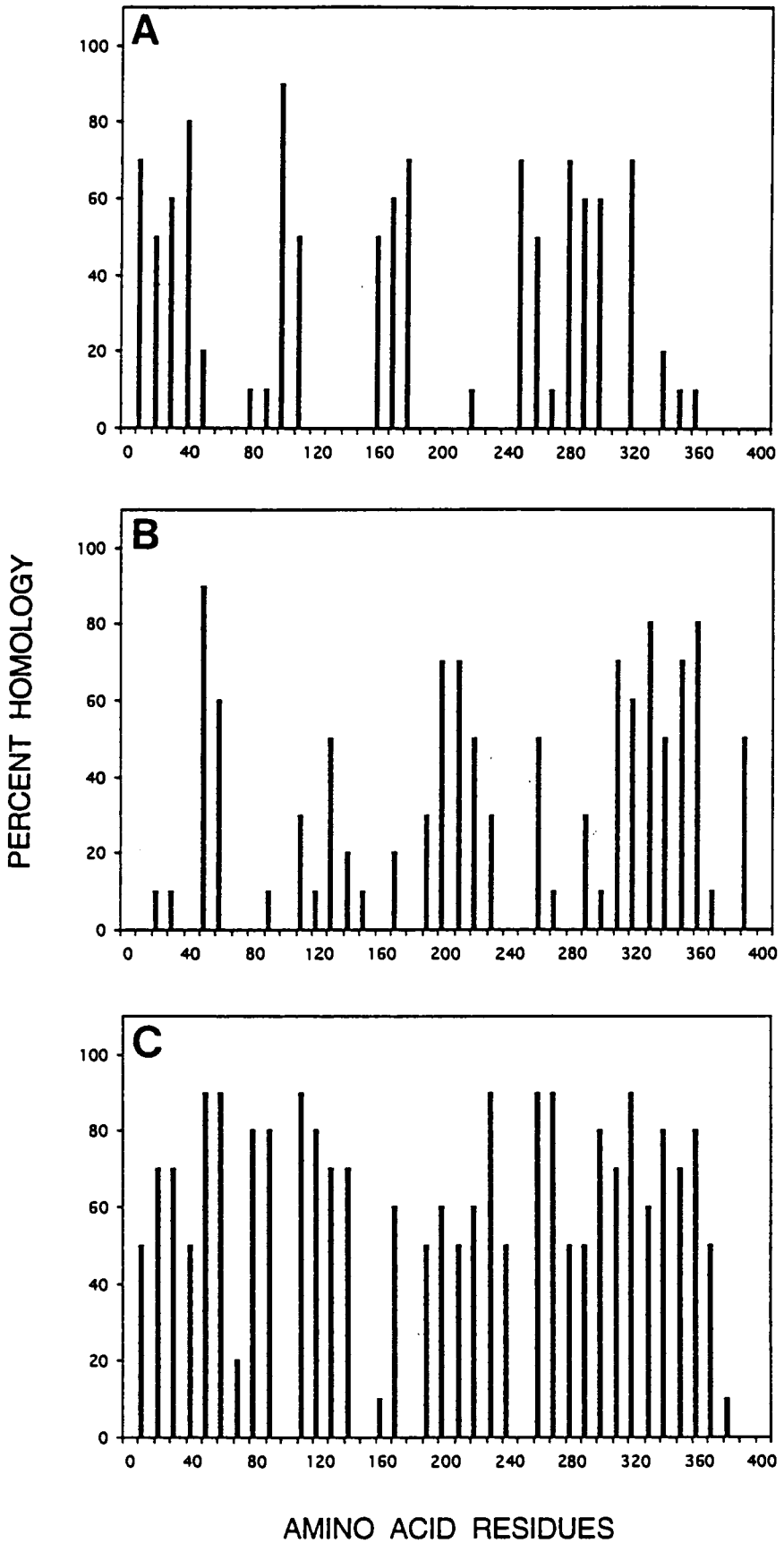

A
B
C
100
80
60
40
20
0
0
40
80
120
160
200
240
280
320
360
400
PERCENT HOMOLOGY
AMINO ACID RESIDUES

Figure 12. A comparison between the amino acid sequences of three myc proteins (tMyc1, tMyc2 and tMyc3) from rainbow trout. The asterisks indicate positions where all three proteins have the same amino acid. The dots indicate positions where the amino acids are of the same type: acidic, basic, polar uncharged and nonpolar residues. The transactivation domains, located at the amino terminus of all *myc* family members, are shown as regions 1 and 2 (R1, R2). The locations of the two dimerization motifs are also shown. The first is the BR-HLH domain and the second is the leucine zipper, which is indicated with plus signs. The border between exon 2 and 3 is marked with an arrow.

```
                                                                  R1
tMyc2   MQLYSSLASKNYDYD--SIQPYFYVDNEDEDFYHQEQGQLQPPAPSEDISKKFELLSTPPLSPSRRPS   66
tMyc3   ------LASKNYDYDYDSIQPYFYVDNEDEDFYHQQPGQIQPPVPSEDIWKKFELFPTPPLSPSR-PS   61
tMyc1   ---NSSLASKNYDYDYDSIQPYFYVDNEDEDFYHQQPGQLQPPAPSEDIWKKFELLPTPPLSPSR-PS   64
        *********  ******************  **.***.***** *****. ******** **

                                                  R2
tMyc2   LSSLFPSTSDQLEMVTEFLGDDVVNQSFICDADYSQTFLKSIIIQDCMWSGFSAAAKLEKVVSERLAC   134
tMyc3   LSSIFPSNADQLEMVTEFLGDDVVNQSFICDADYSQTFLKSIIIQDCMWSGFSATAKLEKVVSERLAS   129
tMyc1   LSSIFPSTADQLEMVTEFLGDDVVTQSFICDADYSQTFLKSIIIQDCMWSGFSATAKLEKVVSERLAS   132
        ***.***. ***************.******************************** ************

                                          R3
tMyc2   LQAARKEPAFSDNAEWITTTRLNANYLQDLNTSASEC----------IDPSVAFPYPITDTSKSSKVKP   192
tMyc3   LQAARKDSAVGDNAGCP-TRLNANFLEDPNSSPSVI----------VDPSVVFPYPITETPKPSKVAP   186
tMyc1   LQTARKDSAVGDNAACP-TRLNANYLQDPNTSASECIGPNTSASECIDPSVVFPYPITETPKPSKVAP   199
        ** ***. *. *** .  ****** * *.*.*.* .          .****.******.* * *** *

                                  R4
tMyc2   PTRIWHWITHPN--SSSSRGSDSEY----------EEIDVVMWRRGTQSKRCDPNMSGTRHHSPLVLK   248
tMyc3   PTDLALD-TPPNSGSSSSSGSDSEDDDEEEDDEDEEEIDVVTVEKRQAVKRATPARQRPDITVPLWLK   253
tMyc1   PTDLALD-TPPNSGSSSSSGSDSEDDDEEEDDEDEEEIDVVTVEKRQAVKRCDPSTSETRHHSPLVLK   266
        ** ..   * **  **** *****           ****** . . .  **. *  .      **.**

                                          R5                          BR
tMyc2   RCHVSTHQHNYAAHPSTRHESASCQEAEAREQTVAVAAGSSRRSAVTANARVPGRQITEDYVKRRTHY   316
tMyc3   RCHVSTHQHNYAAHPSTRHEQPAVKRLRLENSSSRVLKQISSTRKCSSPRTSD----TEDYDKRRTHN   317
tMyc1   RCHVSTHQHNYAAHPSTRHEQPAVKRLRLENSSSRVLKQISSNRKCSSPRTSD----TEDYDKRRTHN   330
        ********************.. . . . ..  *.   *    ..          **** *****.

          BR       HELIX 1        LOOP          HELIX 2
        ———|———————|———————|————————————| +     +     +
tMyc2   VLERQRRSEVKLNFFLSLRDKIPEVANNEKASKVVILEKAIECIFSIQTDEQRLVNFEEQLRRKSEHL   384
tMyc3   VLERQRRNDPRLSFFA-LRDEIPDVANNEKAAKVVILKKATECIYSMQTDEQRLVNLKEQLRRKSEHL   384
tMyc1   VLERQRRNELKLSFFA-LRDEIPDVANNEKAAKVVILKKATECIYSMQTDEQRLVNLKEQLRRKSEHL   397
        *******....*.**. *** **.******* ***** ** *** *.*********. **********

            +
tMyc2   KQKLSRLQNSHSHV                                                        398
tMyc3   NRSWHNCRTHV                                                          395
tMyc1   KQKLAQLQNSCLSSKRH                                                    414
           . . .
```

additional amino acids. The ratio of acidic/basic residues in this region increases from 0.2 in tMyc1 to 0.6 in tMyc2. TMyc3 is nearly identical to tMyc1 over this region.

Table 4 also shows a comparison between tMyc2 and human L-Myc. Overall, the homology is low (30%) with a 49% similarity. This is reflected in the homologies over several regions, except for region 4, which is 91% homologous. A more detailed comparison of this region, between tMyc2 and other Myc proteins, is shown in Fig. 13. Region 4 appears as a highly extended acidic domain, consisting primarily of glutamic and aspartic acid residues, in all Myc proteins examined, except for tMyc2 and L-Myc. The tyrosine (Y) to asparagine (N) substitution that occurs in L-Myc is conservative.

Northern blot analysis of tmyc2 expression is shown in Fig. 14A. This blot is probed with ***M2a***, a fragment that corresponds to an area of low homology (62%) between tmyc2 and tmyc3 (see Fig. 8). Tmyc2 is expressed as a transcript of 1.9 kb in the pituitary gland, and 2.0 kb transcripts in the brain and heart. Two minor bands also appear in the pituitary gland and brain at approximately 3.8 kb. The expression pattern of tmyc3, using ***M3a*** at moderate stringency (Fig 14B), is similar to that obtained with ***M1a***, as described in Chapter II. A major band appears in the pituitary gland at 1.6 kb with less abundant transcripts appearing in the liver (2.4 kb) and in the heart (2.0 kb). At high stringency, tmyc3 appears only in the pituitary gland, and at a much higher level than is apparent for tmyc2 (Fig. 14C). Signals from tmyc2 were not completely re-

moved from the previous hybridization and are evident in this figure. The 3.8 kb transcript, clearly detected with the tmyc2 probe (Fig. 14A), appears very faint and only in the pituitary gland. Fig. 14D, probed for the expression of the elongation factor, indicates approximately equal amounts of RNA in each lane.

Southern blot analysis, with ***M3a*** at moderate stringency, indicates the existence of multiple *myc*-related loci in the trout genome (Fig. 15A). Washing the blot at high stringency reduces the number of bands to two per lane (Fig. 15B). When the blot from Fig 15B is stripped and hybridized with ***M2a***, a different pattern emerges consisting of two bands per lane (Fig. 15C).

The high stringency results, for the Southern blot analysis, were used to construct restriction maps for tmyc2 (Fig. 16A) and tmyc3 loci (Fig. 16B). These maps account for all of the bands shown in Figs. 15B and 15C. Inspection of both maps shows that the band with the highest optical density (OD), in each lane of Fig. 10B, is from tmyc3 whereas the low density band is from tmyc2. Similarly, the high density bands in Fig. 15C are from tmyc2, whereas the low density bands are from tmyc3. Lane 3 of Fig. 15C shows a single band because both genes generate a 3 kb-PstI fragment and, accordingly, the bands OD is higher than the others. The OD of the two bands shown in Fig. 15C, lane 2 are very similar. However, comparing the density of the 15 kb band with that obtained with the tmyc3 probe (Fig. 15B, lane 2) indicates that this fragment is from tmyc2.

Table 4

Comparisons between several Myc proteins over regions that are crucial to the normal function of the protein or that are unique to some of the proteins shown.

Comparison	R1	R2	R3	R4	R5	BR	HLH	LZ	Total
M1, M2	90	95	45	48	24	86	74	83	72 (80)
M1, M3	100	100	50	100	97	100	93	62	88 (93)
M2, M3	90	95	50	48	17	86	68	55	64 (76)
M2, L-Myc	67	70	-	91	24	50	34	22	30 (49)

Table values are percent homologies, except the values in parentheses, which are percent similarities. Table headings are as defined in the text and include regions 1 to 5 (R1-R5), the basic region (BR), the helix-loop-helix (HLH) and leucine zipper (LZ) dimerization motifs. The three trout Myc proteins are abbreviated as M1 (tMyc1), M2 (tMyc2) and M3 (tMyc3). A comparison between tMyc2 and the human L-Myc protein is also shown.

Figure 13. A detailed comparison of region 4, at the exon 2/3 border, between tMyc2 and other Myc proteins.

Trout tMyc2	SDSEYEEIDVV
Human L-Myc	SDSENEEIDVV
Xenopus L-Myc	SDSEDDEIDVV
Human c-Myc	SDSEEEQEDEEEIDVV
Mouse c-Myc	SDSEEEQEDEEEIDVV
Human N-Myc	SDSDDEDDEEEDEEEEIDVV
Trout tMyc1	SDSEDDDEEEDDEDEEEIDVV
Trout tMyc3	SDSEDDDEEEDDEDEEEIDVV
Zebra fish c-Myc	SDSEDEEEEDEEEEEEEEEEEEEEEIDVV

Figure 14. Northern blot analysis of tmyc2 and tmyc3 expression in the rainbow trout. A blot, containing Poly $(A)^+$ RNA was probed with ***M2a*** and washed at high stringency **(A)**. The blot was stripped and re-probed with ***M3a*** and washed at moderate stringency **(B)** and at high stringency **(C).** Finally, the blot was stripped and re-probed for the expression of the elongation factor **(D).** Lanes: 1; pituitary gland, 2; brain, 3; liver, 4; heart.

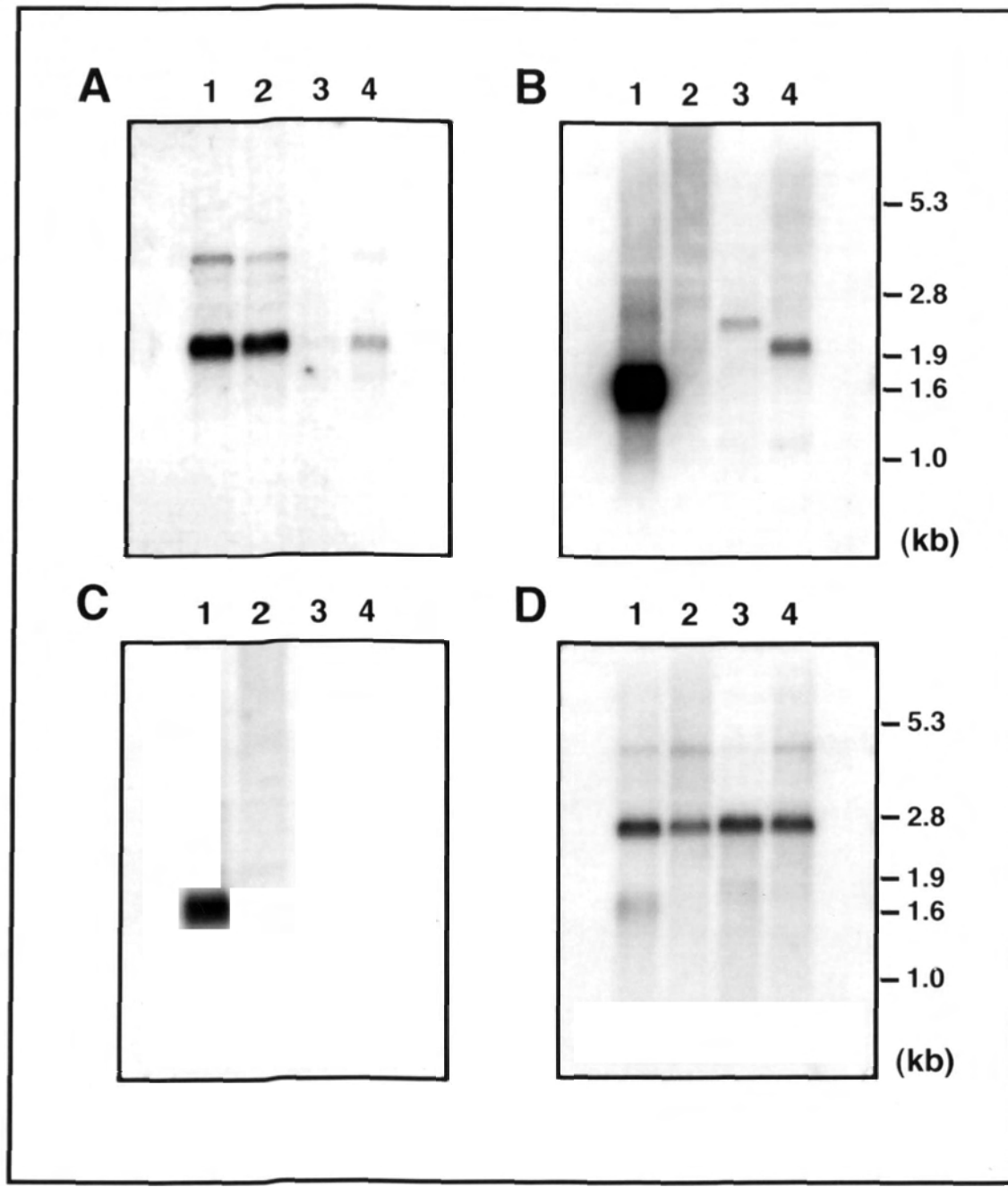

A
1 2 3 4
B
1 2 3 4
5.3
2.8
1.9
1.6
1.0
(kb)
C
1 2 3 4
D
1 2 3 4
5.3
2.8
1.9
1.6
1.0
(kb)

Figure 15. Southern blot analysis of c-*myc* loci in the rainbow trout. The blot was probed with ***M3a*** and washed at medium stringency **(A)** and high stringency **(B).** The blot was stripped and re-hybridized with ***M2a*** and washed at high stringency **(C)**. DNA, from a single individual, was digested with BamHI (lane 1), EcoRI (lane 2), PstI (lane 3), BamHI-EcoRI (lane 4), BamHI-PstI (lane 5) and PstI-EcoRI (lane 6).

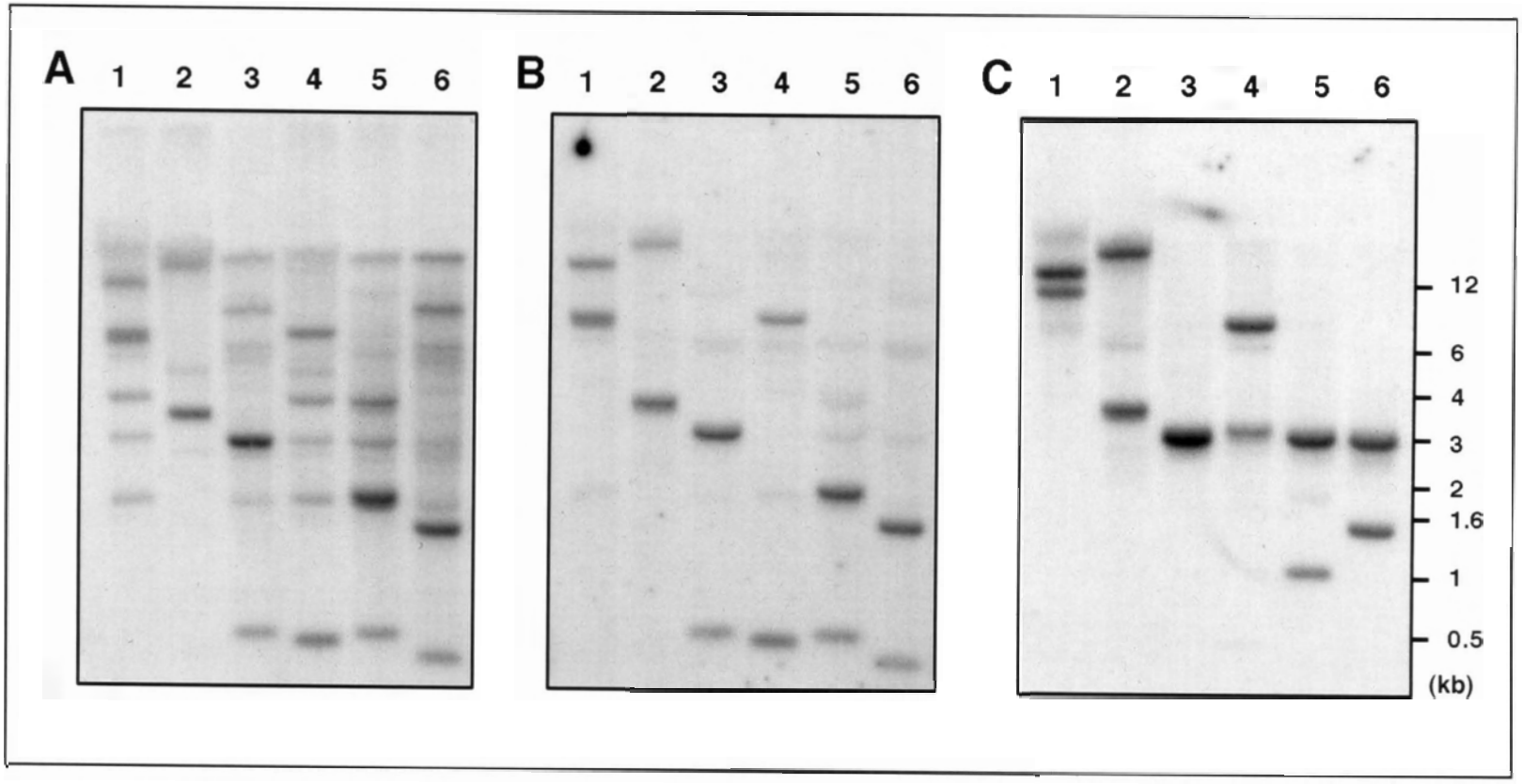
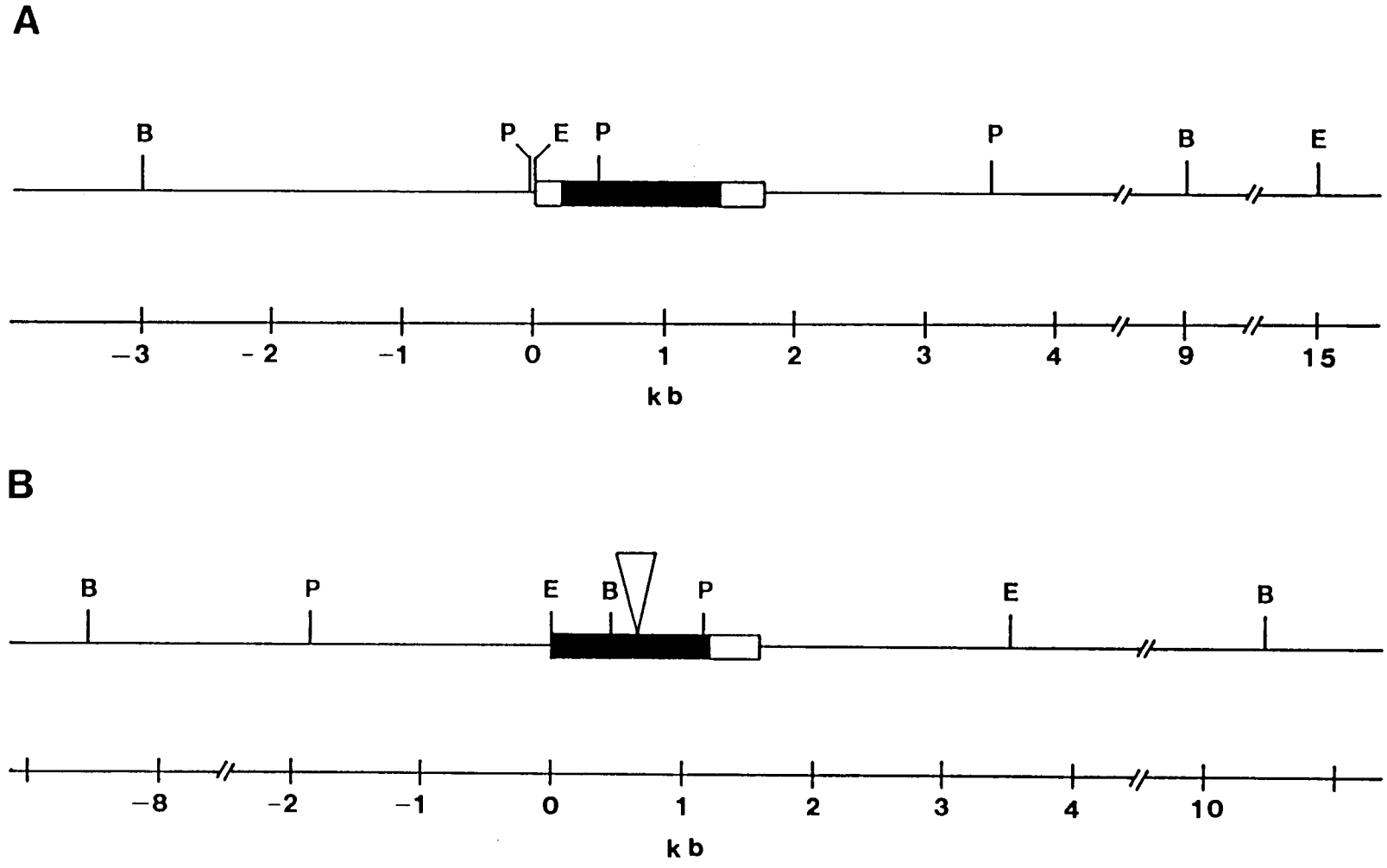

A
1
2
3
4
5
6
B
1
2
3
4
5
6
C
1
2
3
4
5
6
12
6
4
3
2
1.6
1
0.5
(kb)

Figure 16. Genomic maps arround the tmyc2 and tmyc3 loci. Southern blot analysis was used to construct restriction maps for tmyc2 **(A)** and tmyc3 loci **(B)**. The 5' end of each clone is located at left. The coding regions for each clone are indicated by the solid rectangle, the 5' and 3' untranslated regions are indicated by open rectangles. The position, and approximate size, of the tmyc3 intron is indicated by an arrow. Restriction sites: BamHI (B), EcoRI (E), PstI (P).


A
B
P
E
P
P
B
E
−3
−2
−1
0
1
2
3
4
9
15
kb
B
B
P
E
B
P
E
B
−8
−2
−1
0
1
2
3
4
10
kb

The genomic maps indicate several differences in the area surrounding each locus. There is an EcoRI site located 15 kb downstream (towards the 3' end of the clone) from the 5' end of tmyc2. There is an EcoRI site downstream from tmyc3 as well but it is only 3.5 kb from the 5' end of this clone. Tmyc2 has a downstream PstI site whereas tmyc3 does not, and the upstream PstI site in tmyc2 is very near the 5' end of the gene, whereas the upstream PstI site in tmyc3 is nearly 2 kb distant. The length of intron 2 in tmyc3 was estimated to be 295 bp by comparing the size of the low density BamHI-PstI band (1100 bp, Fig 15C, lane 5) with the known length of this fragment in the Tmyc3 clone (705 bp, Fig. 5). The position of the intron is indicated by the arrow.

Discussion

Myc genes were cloned from the pituitary gland to determine the identity of a c-*myc*-related transcript that appears in the gland, but not in other tissues (Chapter II). The cloning experiments yielded two distinct c-*myc* genes, tmyc2 and tmyc3, that have an overall homology of 73%. The deduced amino acid sequences of these clones (tMyc2, tMyc3) contain the transactivation domains and dimerization motifs that are characteristic of all *myc* family members. A comparison of tMyc2 and tMyc3 with other *myc* genes indicates that they are both more similar to c-Myc than they are to other family members. TMyc2 and tMyc3 are of similar size, but are shorter than tMyc1 (414 amino acids, 62) and c-Myc proteins from higher vertebrates, which can be as long as 439 amino acids (1,46). In

general, tMyc3 shows extensive homology to tMyc1 whereas tMyc2 is more divergent.

The divergence of tMyc2, from tMyc1 and tMyc3, involved several areas including R3, R4, R5, the HLH and the leucine zipper. Homologies over these areas ranged from 17% in R5 to 83% in the zipper region. Perhaps the most interesting difference between tMyc2 and the other Myc proteins is the loss of an extensive acidic domain on the carboxyl side of the exon 2/3 border (R4). This region consists of numerous acidic residues in c- N- and L-Myc proteins (43, 44, 45, 81, 82, 83), but the human (45) and Xenopus L-Myc proteins (77) are nearly identical to tMyc2 in this region. Region 4 is believed to be part of a nonspecific DNA-binding domain that may enhance specific, BR-HLH mediated DNA binding. Deletion of the acidic residues at the exon 2/3 border reduces the efficiency of chicken hematopoietic cell transformation (2). This may explain, in part, why the transforming capability of human L-Myc is the lowest among the known Myc family members (1). This could mean that tMyc2 is not oncogenic and that, functionally, it is more closely related to L-Myc than it is to c-Myc.

Northern and Southern blot analysis indicates that Tmyc3 is the cDNA for the 1.6 kb transcript described in Chapter II. TMyc3 shows extensive homology to tMyc1, although there are two areas that have diverged. One is adjacent to the trans-activation domains (R3), where the homology is 50%, and the other is the leucine zipper, where the homology is 62%. Divergence is also apparent between tmyc3 and tmyc1 in the 3'

untranslated region, where tmyc1 is lacking a 15 bp sequence adjacent to the stop codon that is present in tmyc3.

Northern blot analysis demonstrated a distinct difference in the size, amounts and expression patterns of tmyc2 and tmyc3 transcripts. Tmyc2 transcripts are between 1.9-2.0 kb and appear at low levels in several tissues, whereas tmyc3 is expressed as a single 1.6 kb transcript in the pituitary gland but not in the other tissues examined. However, at moderate stringency tmyc3-related transcripts appear in the liver and in the heart.

The expression pattern for tmyc2 is unusual for a c-*myc* gene, in that all c-*myc* transcripts that have been described appear in the liver at levels 2 to 5 times higher than they do in the brain. By contrast, N-*myc* and L-*myc* expression is 5 to 20 times greater in the brain than it is in the liver (1). Although tmyc2 is clearly a c-*myc* family member, its expression level in the brain, compared to the liver or heart, is more similar to that of L-*myc* or N-*myc*.

The expression pattern of tmyc3 is also unusual in that c-myc family members are generally expressed in a wide variety of tissues. This suggests that tmyc3, like tmyc2, has an expression pattern more like that of L- or N-myc family members, which are usually restricted to one or two cell types. However, it is possible that tmyc3 represents a pituitary gland-specific *myc* gene that is common to many vertebrate groups. At present there is no way to confirm this conclusion since this thesis represents the

first attempt to characterize the expression of these genes in the pituitary gland.

Southern blot analysis indicate that tmyc2 and tmyc3 represent distinct loci. Each of the restriction enzymes used, either singly or in pairs, produced two bands per lane when the blot was probed for either tmyc2 or tmyc3. Moreover, a genomic restriction map showed several differences in the neighborhood of each locus. The analysis of Southern blots also confirms the transcript identities of tmyc2 and tmyc3. At high stringency, all fragments on the Southern blot were identified as coming from tmyc2 or tmyc3. Consequently, it is unlikely that any other tmyc2- or tmyc3-related transcripts exist.

No attempt was made to distinguish between tmyc1 and tmyc3 in the Southern blot analysis since the tmyc3 probe is 90% homologous to tmyc1. However, the divergence within the coding region and a small section of the 3' non-coding region, suggests that tmyc1 and tmyc3 are also distinct loci. Confirmation will require additional sequence data from the 5' and 3' untranslated regions of both clones and from intron 2.

The somatic cells of the rainbow trout are known to be tetraploid although for many of the loci they are considered to be functionally diploid (84). This may account for the presence of two c-*myc* loci, and the fact that other genes have been isolated in pairs from salmonids, including gonadotropin, insulin-like growth factor, GH and vasotocin (85, 86, 87, 88, 89, 90). In some cases duplicated genes are functionally identical.

However, the two gonadotropin genes (GTH I and II) have diverged to such an extent that they are not only controlled separately but they appear to be expressed in different subpopulations of gonadotrophs (26). Tmyc2 and tmyc3 may also be functionally distinct loci since they appear to have very different expression patterns, whether they are compared between different tissues or within the pituitary gland, where the transcript level of tmyc3 is much higher than that of tmyc2. Moreover, the divergence between tmyc2 and tmyc3 suggests that these two genes are expressed in different cells, or regions of the pituitary gland.

Chapter IV
Location and regulation of *myc* expression in the pituitary gland of rainbow trout

Introduction

The cloning experiments in Chapter III have shown that the trout pituitary gland expresses two distinct forms of the c-*myc* proto-oncogene. One of these, tmyc3, is expressed at much higher levels than the other (tmyc2) and the expression level appears to increase as the fish approach sexual maturity. This suggests that tmyc3 is expressed primarily in the gonadotrophs, since the activity of these cells is known to increase during this period (26). However, some of the other cells within the gland, such as the somatotrophs and somatolactotrophs, also become hypertrophic as the fish approaches sexual maturity (64,65,91).

This chapter describes the use of *in situ* hybridization and northern blot analysis in order to localize the expression of tmyc2 and tmyc3 to specific regions of the gland, and a series of *in vitro* stimulation experiments that tested the ability of certain releasing factors and neurotransmitters to influence the expression of these genes. The results show that tmyc3 is expressed primarily in the intermediate lobe and that its expression level is stimulated 2 to 5-fold by dopamine and norepinephrine.

Materials and Methods

Fish Rearing Conditions

Rainbow trout were obtained from a local fish farm and kept in indoor, circular tanks (4800 liter capacity) with a constant flow of de-chlorinated water that had an average temperature of 15°C. The fish were not fed for several days after arrival and were given one week to acclimatize to the tank before any experiments were begun. Standard fish pellets were given at 1% body weight/day, five days /week. The number of fish kept in the tank varied with size; usually this meant 30 fish weighing 200-400 g or 15 fish weighing 750-800 g. A part of the tank was covered with black plastic to provide shade.

In Situ hybridization

A) Tissue preparation

Pituitary glands from five rainbow trout (750 g) were dissected out into ice cold 4% paraformaldehyde (phosphate buffered, pH 7.0). After all the glands were collected they were left in the fixative for 20 h at room temperature. The glands were washed four times (15 min each) in water, dehydrated through an ethanol series prior to embedding in Paraplast. Sections were cut at 7 µm, mounted on slides that were coated with 2% binding silane (3-aminopropyl-triethoxysilane) and baked at 62°C for 1h. The slides were de-waxed in xylene for 10 min (2 times), rinsed in 100% ethanol for 5 min (2 times) and air dried.

B) Pre-treatment

The sections were digested with proteinase K at 10 µg/ml (dissolved in 50 mM Tris,10 mM EDTA and 10 mM NaCl, pH 7.5) for 10 min at 37°C. The reaction was stopped by immersing the slides in cold (4°C) 4% paraformaldehyde (5 min), followed by three washes (5 min each) in distilled water and dehydration through an ethanol series.

C) Pre-hybridization

The sections were prehybridized at 37°C for 1h in a buffer consisting of 5X SSC (0.75 M NaCl, 75 mM sodium citrate), 5X Denhardt's (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, fraction V) and 30% formamide. Prehybridization was in a volume of 50 ml under a plastic coverslip or in 10 ml in a slide mailer.

D) Probe description and preparation

Three probes were prepared: ***M2a***; ***M3b*** (a 200 bp EcoRI-TaqI fragment from the 5' end of tmyc3, shown in Fig. 5) and ***GHa***. These fragments were labeled with digoxigenin (DIG) using a random primer labeling kit that included DIG-UTP, from Boehringer-Mannheim (B-M). The labeling reaction was set up as described by the manufacturer using 400 ng of DNA, a reaction volume of 20 µl, and incubating at 37°C overnight. The reaction was precipitated with 2.5 µl of 4.0 M LiCl and 75 µl of 100% ethanol. The dried pellet was either dissolved in hybridization buffer or in TE (pH 8.0).

E) Hybridization

Hybridization was carried out in the same buffer used for the pre-hybridization. Labeled probe was added to the buffer to a final concentration of 2-5 ng/µl. Pre-hybridized slides were drained thoroughly before adding 10-12 µl of hybridization buffer containing labeled probe and covered with a silanized (dimethyldichlorosilane) coverslip (22 X 22 mm). The coverslip was sealed with rubber cement and the slide incubated in a humidified chamber at 37°C overnight.

F) Post-hybridization washes

The rubber cement was removed and the slides were soaked in 1X SSC for 10 min. to allow a gentle detachment of the coverslips. The slides were soaked at room temperature (RT) in 1X SSC for 5 min. (3 times) and at 48°C for 20 min. (2 times) followed by a 5 minute rinse in 1X SSC at RT.

G) Detection

Detection was according to the manufacturers protocol, using alkaline phosphate-DIG antibody conjugate and a color substrate consisting of X-phosphate (5-bromo-4-chloro-3-indolyl-phosphate) and NBT (nitro-blue-tetrazolium). The activity of the DIG-antibody in the presence of this substrate generates a purple precipitate. Slides were rinsed in 40 ml of Buffer 1 (B-M) for 2 min. (twice) followed by 30 min. in Buffer 2 (B-M) before incubating in DIG antibody (1:500 in Buffer 2) for 1h at RT.

The slides were washed in Buffer1 for 5 min (3 times) and in Buffer 3 (B-M) for 5 min (2 times). The substrate-color reagent was applied and the slides were incubated overnight at 4°C in the dark. The reaction was stopped by dipping the slides in TE (pH 8.0) and washing several times in water, after which the sections were mounted in 50% glycerol and photographed.

The various procedures described above were adapted from several sources (92, 93, 94, 95).

Northern blot analysis

Four pituitary glands were collected from 750 g trout and placed in chilled phosphate-buffered saline (0.1M phosphate, 0.8% NaCl, pH 7.4) and separated into distal and intermediate lobes. Total RNA was extracted from the lobes, fractionated on a 1.2% agarose gel and transferred to Genescreen as described (Chapter II). The blot was probed sequentially with ***M2a***, ***M3a***, ***GHa*** and ***EFa***. Hybridization procedures were described in Chapter III, standardization of transcript levels was described in Chapter II.

***In Vitro* Stimulation**

A) Culture Medium

All experiments were carried out using RPMI 1640 (Gibco-BRL, cat#22400-048) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and fungizone (0.25 µg/ml), pH 7.4, osmolarity 300 mOsm as

described (61, 62, 63). Glands were stimulated in this medium supplemented with 10^{-4} M vitamin C, but without the antibiotics.

B) Stimulation Compounds

The compounds used in these experiments included carp growth hormone releasing factor (GRF, 1-29), thyroid stimulating hormone releasing factor (TRF, Sigma), dopamine (DA, Sigma, H8502), norepinephrine (NE, Sigma, A9512), γ-aminobutyric acid (GABA, Sigma A2129) and serotonin (ST, Sigma H9523). Stock solutions of GRF and TRF were made, by dilution, at 10^{-6} M in RPMI. On the day of stimulation 100 ml of the stock was added to 10 ml of the stimulation medium for a final concentration of 10^{-8} M. Neurotransmitter stocks (20 mM) were made in a vehicle consisting of 10^{-4} M vitamin C and 0.2 μM HCl. On the day of stimulation 10 ml of the stock was added to 10 ml of stimulation medium for a final concentration of 10^{-5} M.

C) Fish

Different sized fish were used for the various experiments: 200 g trout for GRF, 350 g for TRF, 312 g for a null control, 800 g for a dopamine time course and 400g for a neurotransmitter survey.

D) Culture

Pituitary glands were collected aseptically into chilled RPMI, partially bisected along the sagittal axis and transferred to 24-well plates

containing 500 µl RPMI at one gland per well. Each treatment group consisted of 3 glands for each of 5-10 groups. The plates were incubated at 15-18°C under a humidified atmosphere containing 5% CO_2/95% O_2 for 4 days, as described (63). On the fourth day the glands were rinsed twice in 500 µl stimulation medium and allowed to incubate for 1h in the final change. Test compounds were added to the wells for the stimulation groups and the controls received vehicle only. In a null experiment all groups received vehicle. The glands were collected at the appropriate times and frozen immediately on dry ice.

E) Northern blot analysis

Total RNA was extracted from the pituitary glands, fractionated on a 1.2% agarose gel, transferred to Genescreen and probed as described in Chapter III. The blots were probed sequentially with ***M2a***, ***M3a***, ***GHa*** and ***EFa***. Hybridization procedures were as described in Chapter III, standardization of transcript levels was described in Chapter II.

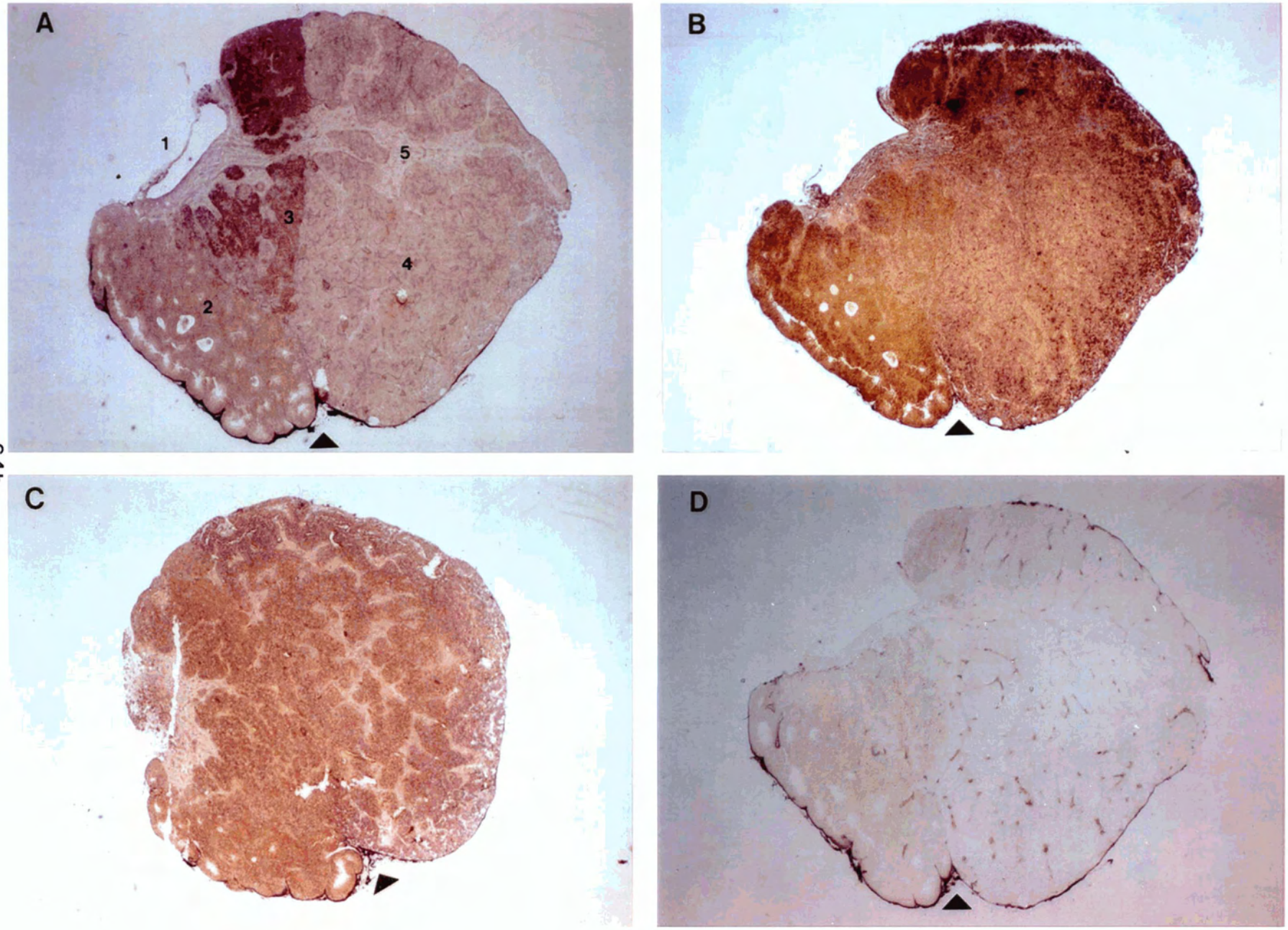
Results

Fig. 17A shows a section of the pituitary gland probed for the expression of the GH gene. The positive signal (purple color) is restricted entirely to the known location of somatotrophs (64) and serves as a positive control for the *in situ* procedure. The spatial distribution of tmyc3 expression is shown in Fig 17B where the signal is restricted almost entirely to the intermediate lobe. The results for tmyc2 are shown in

Fig. 17C. The expression pattern is inconclusive owing to the low level at which this gene is transcribed, although a very faint blue can be seen in the intermediate lobe and to some extent in the anterior lobe as well. A negative control, processed along with the other sections, but without a probe, is shown in Fig. 17D. This control indicates that the reagents alone do not contribute to the color seen in the other sections. Note that all sections are from the same gland. Those shown in A, B and D are mid-sagittal, whereas the one shown in 17C is more peripheral. This section is rotated slightly counterclockwise relative to the others.

Northern blot analysis of RNA obtained from the distal and intermediate lobes of the pituitary gland (Fig. 18) confirms the results obtained with *in situ* hybridization. Fig. 18A shows a blot probed for the expression of tmyc3 and indicates that the expression level of this gene is 8-fold greater in the intermediate lobe (lane 2) than it is in the distal lobe (lane 1). The two arrows mark the position of the 28S (top) and 18S ribosomal RNA. Expression of tmyc2 (Fig 18B) appears to be equal in both lobes of the gland. Note that there is some tmyc3 signal remaining from the previous hybridization. The band appearing just below the 28S ribosomal RNA is of unknown identity, though closely related to these *myc* genes and apparently specific to the distal lobe. Fig. 18C shows negligible contamination of the IL RNA with RNA from the distal lobe, since GH expression appears primarily in RNA from the distal lobe (lane 1). Finally, the blot was probed for the expression of elongation factor 2 (EF) and the results show that both lanes contain approximately equal amounts of

Figure 17. Digoxigenin-*in situ* hybridization of tissue sections from the pituitary gland of the rainbow trout. Sections were hybridized with ***GHa*** **(A)**, ***M3b*** **(B)**, ***M2a*** **(C)** and without probe **(D)**. All sections are from the same gland. Those shown in **A**, **B** and **D** are mid-saggital, whereas **C** is more peripheral. This section is rotated slightly counterclockwise relative to the others. Arrows mark the border between the distal and intermediate lobes. Regions indicated in **A**: Attachment site of the gland to the hypothalamus (1), rostral pars distalis (2), proximal pars distalis (3), intermediate lobe (4), neural lobe (5).



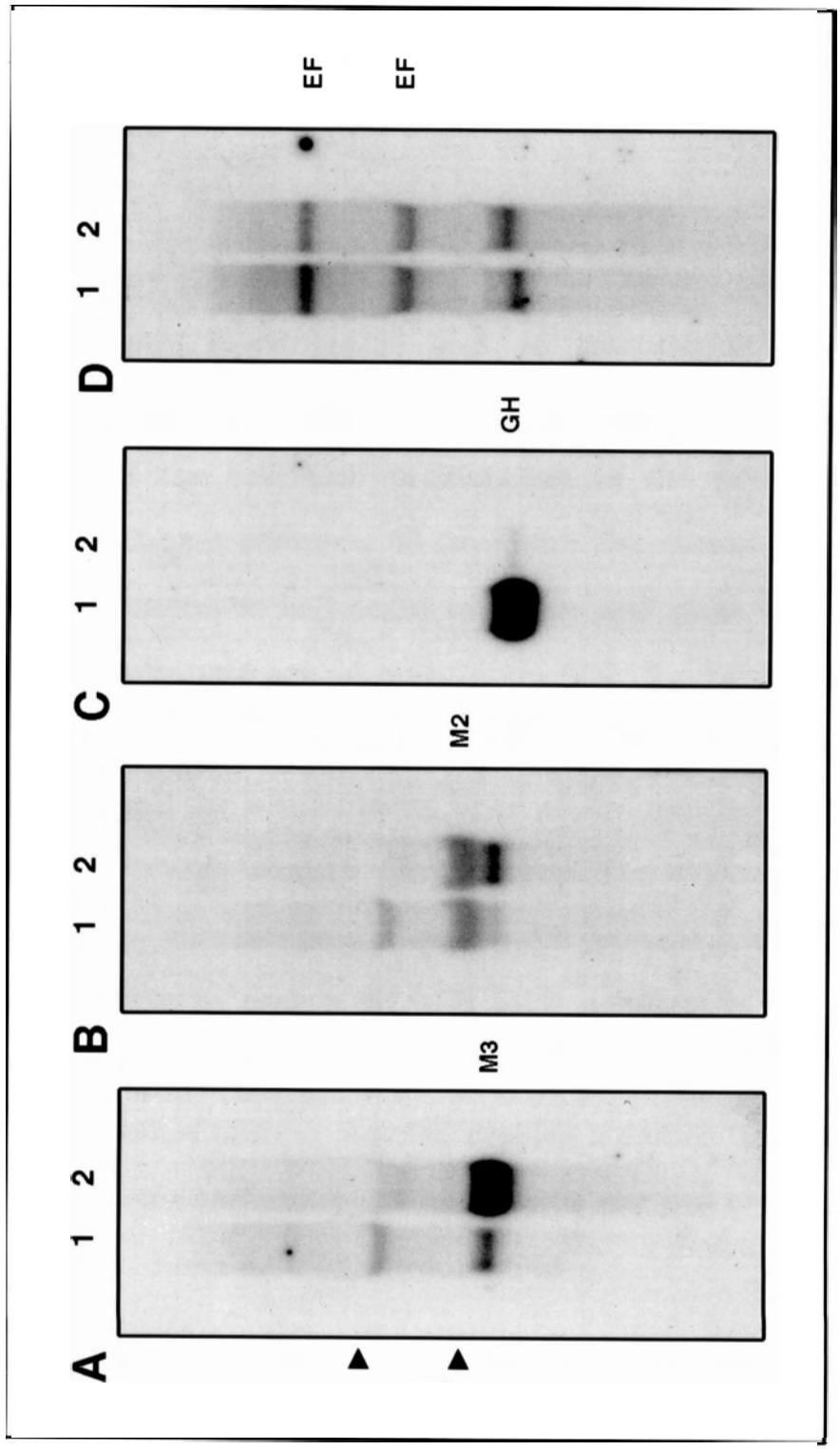
64b

RNA (Fig 18D). Residual signals from GH and tmyc3 are evident in this figure from previous hybridizations.

Exposing pituitary glands to GRF increased the expression of GH 2.6-fold over the control level without affecting the expression of tmyc2 or tmyc3 (Fig 19A) The expression level of GH dropped 2.3-fold in the *in vitro* control as compared to the *in vivo* group (Lane V). A similar drop also occurred for the expression of tmyc3. A null control experiment (Fig. 19B) indicates that the culturing system had a negligible affect on the expression of GH or tmyc3. Cross-lane standard deviations for these two transcripts are 0.05 and 0.1, respectively. The maximum difference between any two lanes is 30%. Accordingly, any substance that failed to stimulate (or inhibit) *myc* expression more than this amount was scored as having no effect. Exposure of the pituitary glands to TRF had no effect on the expression of tmyc2 or tmyc3 (Fig 19C) but exposure to dopamine increased tmyc3 transcript level 5-fold after 24h (Fig. 19D). The GRF, TRF and dopamine experiments were repeated once with similar results (Table 5). The results for the null control are from a single experiment.

In a separate experiment, dopamine and norepinephrine stimulated the expression of tmyc3 nearly two fold after a 2 h exposure, whereas GABA and serotonin had no effect (Fig. 20). After a 24 h exposure the dopamine-induced tmyc3 transcript level remained higher than the control, but the tmyc3 level, in the norepinephrine group, returned to the control level. Again GABA and serotonin had no effect. None of the neurotransmitters tested had any effect on the expression of tmyc2. This

Figure 18. Northern blot analysis of RNA obtained from the distal and intermediate lobes of the pituitary gland from rainbow trout. The blot was probed sequentially with ***M3α*** **(A)**, **M2a (B)**, ***GHα*** **(C)** and ***EFα*** **(D)**. Residual signals from tmyc3 are evident in **B** and **D** from previous hybridizations. Residual signals from GH also appear in **D**. The arrows mark the location of the 28S (top) and 18S ribosomal RNA. Lanes 1: distal lobe, 2: intermediate lobe.


A
1 2
B
1 2
M3
M2
C
1 2
GH
D
1 2
EF
EF

experiment was repeated once with similar results. Averaged values for Tmyc3 are shown in the histogram. The values obtained from both experiments are shown in Table 6.

Discussion

The pituitary gland, like all tissues, grows by two processes: an increase in cell size (hypertrophy) and an increase in cell numbers (proliferation). Hypertrophy is also associated with accelerated biosynthetic activity of the cell. Cell proliferation in the pituitary gland of rainbow trout is largely complete 30 days after the eggs have hatched, although the gonadotrophs will begin to divide and grow several months after hatching to initiate sexual maturation (26). During this time other cells of the gland are maturing, among them the somatotrophs of the distal lobe (64,91) and the somatolactotrophs of the intermediate lobe (65), both of which undergo extensive hypertrophy. The melanotrophs, of the intermediate lobe, also increase in size in the juvenile and mature adult, but this appears to be associated with color adaptation and not sexual maturation. Trout that are reared in a dark-background environment produce high levels of MSH in order to develop a darker body color. Under these conditions the melanotrophs increase in size but can regress if the fish are transferred to a light-background (96).

In Chapter II, the expression level of *myc* was shown to double in the pituitary gland of trout as they approached sexual maturity, suggesting that this gene may be expressed in the gonadotrophs. However, *in situ*

Figure 19. Northern blot analysis of *in vitro* experiments that tested the effect of GRF **(A)**, the culturing system **(B)**, TRF **(C)** and dopamine **(D)** on *myc* expression in the pituitary gland of rainbow trout. The exposure times (hours) are indicated above each lane. Lane **V** is an *in vivo* control. The blots were probed sequentially with ***GHα***, ***M2α***, ***M3α*** and ***EFα***. Abbreviations: growth hormone (GH), tmyc2 (M2), tmyc3 (M3), elongation factor (EF).

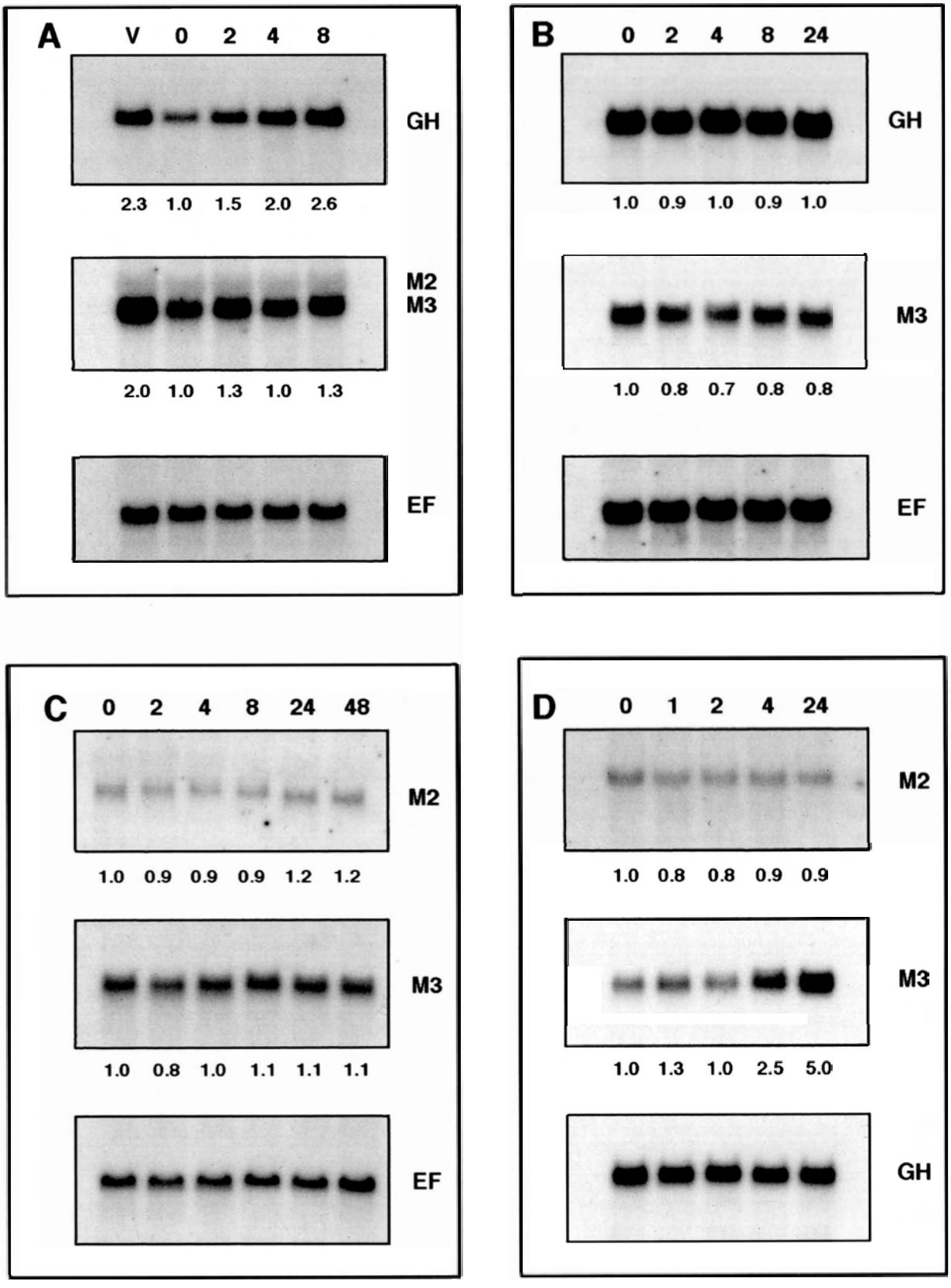

A
V 0 2 4 8
GH
2.3 1.0 1.5 2.0 2.6
M2
M3
2.0 1.0 1.3 1.0 1.3
EF
B
0 2 4 8 24
GH
1.0 0.9 1.0 0.9 1.0
M3
1.0 0.8 0.7 0.8 0.8
EF
C
0 2 4 8 24 48
M2
1.0 0.9 0.9 0.9 1.2 1.2
M3
1.0 0.8 1.0 1.1 1.1 1.1
EF
D
0 1 2 4 24
M2
1.0 0.8 0.8 0.9 0.9
M3
1.0 1.3 1.0 2.5 5.0
GH

Table 5

Results of *in vitro* experiments that tested the effect of growth hormone releasing factor (GRF), thyroid hormone releasing factor (TRF), dopamine (DA) and a null control (NC) on the expression of tmyc3 (M3) in the pituitary gland of rainbow trout.

EXP	GENE	TIME POINTS (HR)							
		V	0	1	2	4	8	24	48
GRF 1	GH	2.3	1.0	-	1.5	2.0	2.6	-	-
-	M3	2.0	1.0	-	1.3	1.0	1.3	-	-
GRF 2	GH	2.5	1.0	1.0	1.3	2.2	2.0	-	-
-	M3	2.0	1.0	1.2	1.0	1.0	1.0	-	-
NC	GH	-	1.0	-	0.9	1.0	0.9	1.0	-
-	M3	-	1.0	-	0.8	0.7	0.8	0.8	-
TRF 1	M3	-	1.0	-	1.2	0.9	0.8	1.4	1.5
TRF 2	M3	-	1.0	-	0.8	1.0	1.1	1.1	1.1
DA 1	M3	-	1.0	1.3	1.0	2.5	-	5.0	
DA 2	M3	-	1.0	1.2	1.0	1.8	-	3.5	

Table values are standardized optical densities. Values of duplicate experiments are shown for tmyc3 (M3) and GH. The null control was a single experiment. Tmyc2 expression was not affected by any of the treatments shown. The GRF experiments included an *in vivo* control (V).

Figure 20. Northern blot analysis of *in vitro* experiments that tested the effect of several neurotransmitters on *myc* expression in the pituitary gland of rainbow trout. Results are shown for the control **(C)**, dopamine **(D)**, norepinephrine **(N)**, GABA **(G)** and serotonin **(S)**. The glands were exposed to the neurotransmitters for 2h (first set) and 24h (second set). Expression is shown for tmyc2 (M2), tmyc3 (M3) and growth hormone (GH). The histogram shows standardized optical densities (ranging between 1 and 2) for tmyc3. The values were averaged from two replicate blots.

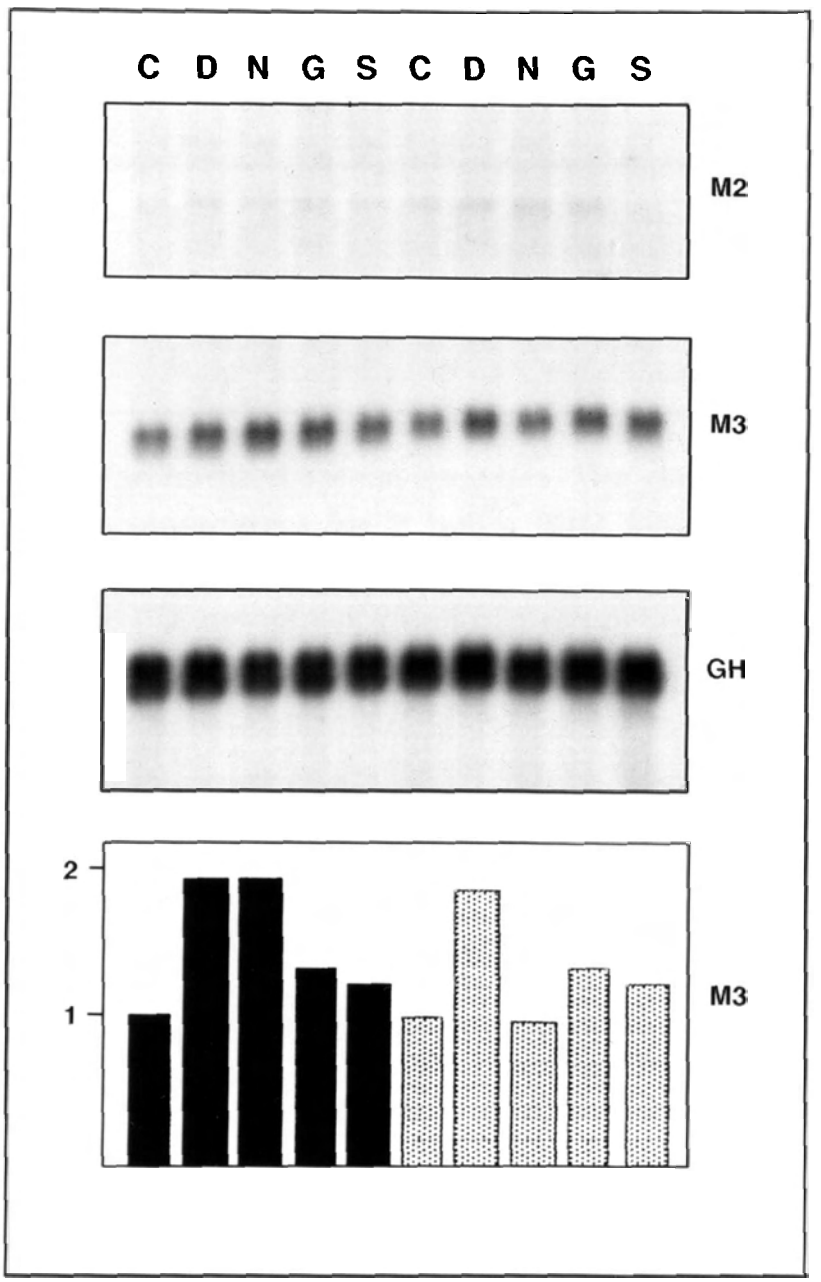

C D N G S C D N G S
M2
M3
GH
2
1
M3

Table 6

Results of *in vitro* experiments that tested the effect of several neurotransmitters on tmyc3 expression in the pituitary gland of rainbow trout.

EXPERIMENT	C	D	N	G	S	C	D	N	G	S
1	1.0	1.6	1.8	1.5	1.2	1.0	1.9	1.1	1.5	1.4
2	1.0	1.9	1.7	1.2	1.2	1.0	1.6	0.8	1.2	1.1
MEAN	-	1.8	1.8	1.3	1.2	-	1.7	0.9	1.3	1.2

Table values are standardized optical densities. The glands were exposed to different neurotransmitters for 2 hours (first set) or for 24 hours (second set). Each set has a control (C). Neurotransmitters tested included dopamine (D), norepinephrine (N), γ-aminobutyric acid (G) and serotonin (S).

hybridization and northern blot analysis of RNA from the distal and intermediate lobes, described in this chapter, have shown that the major pituitary *myc* transcript (tmyc3) is expressed primarily in the intermediate lobe. Therefor, tmyc3 is not likely to be involved in the proliferation or maturation of gonadotrophs. Moreover, the results of the *in situ* hybridization show that tmyc3 is not expressed throughout the intermediate lobe but appears to have a peripheral distribution. This suggests that tmyc3 is expressed primarily in somatolactotrophs, as the melanotrophs are known to be distributed throughout the IL, whereas the somatolactotrophs are restricted to areas that border the neurohypophysial tissue (97).

The pituitary gland is controlled by neurosecretions from the hypothalamus (13,14,15). The results of *in vitro* experiments show that dopamine and norepinephrine stimulate the expression of tmyc3 two- to five-fold over control levels, whereas GABA and serotonin have no effect. In addition, hypothalamic releasing factors (GRF, TRF) that control cells in the distal lobe of the pituitary, have no effect on tmyc3 expression. Dopamine and norepinephrine, at the concentrations used in the experiments (10^{-5} M), are known to inhibit the release of MSH from melanotrophs (101). If tmyc3 is expressed in these cells it cannot be directly involved in regulating MSH transcription. It is possible that it could be involved in maintaining the cAMP or IP_3 pathways, since the replacement rate of the various components will have to increase, with constant

stimulation of the receptor, regardless of whether MSH release is up- or down-regulated.

However, it is more likely that the results of the *in vitro* experiments are another indication that tmyc3 is expressed primarily in the somatolactotrophs, where it may initiate hypertrophy of these cells, as the trout approaches sexual maturity. A detailed comparison of the SL cells in immature and mature salmon has shown that, in addition to the greatly enlarged cytoplasm, the cell nucleus increases in size and changes from an ovoid to a circular shape (65). This could mean that the DNA content is increasing, since endo-polyploidization has been observed in other hypertrophic cells (98).

The expression of c-*myc* is known to be high in proliferating cells, during embryogenesis, and in actively dividing cells of an adult. This association has lead to the realization that the Myc protein can stimulate replication as well as transcription. *In vitro* experiments have demonstrated that exposure of quiescent cells to mitogenic stimuli leads to a rapid induction of *myc* expression (99). A similar induction of *myc* expression has been observed *in vivo* during synchronized cell growth in the regenerating liver (100). However, *myc* is expressed in post-mitotic cells as well, where it is believed to function as a transcription factor (1,2). In the special case of cellular hypertrophy, involving endo-polyploidization, *myc* may function both as a replication and transcription factor, even though the cell does not divide. Norepinephrine is known to induce hypertrophy in cultured myocytes, and this is associated with the stimulation of c-*myc*

expression (56). It is usually assumed that the primary role of this neurotransmitter in the pituitary gland is to inhibit the release of MSH from the intermediate lobe (21,101), but it may also act as a trophic factor to stimulate growth of somatolactotrophs or to maintain these cells in an active state.

Dopamine's role is more problematic. The *in situ* results described in this chapter show that this neurotransmitter can stimulate tmyc3 expression 5-fold, but there is no evidence linking the activity of this neurotransmitter with cellular hypertrophy. However, it is known that somatolactin and GH are closely related (65,102) and it is likely that these two genes are controlled through the same signaling pathway, involving similar ligands (103). Dopamine has recently been shown to stimulate the release of GH through a cAMP-dependent pathway (16), suggesting that this neurotransmitter may also be involved in stimulating the release of somatolactin. If this is true, tMyc3 would have a dual role involving activation of the somatolactin gene and induction of somatolactotroph hypertrophy.

Chapter V
General Discussion

Although the basic function of the *myc* proto-oncogene is understood to involve regulation of transcription and replication (1,2), its interaction with signaling pathways is still unclear. The motivation for examining *myc* expression in the trout is to clarify the expression pattern of this gene family in the hope that it will provide some clues to its function in normal cellular and physiological processes. C-*myc* is often found expressed in proliferating tissue such as the liver, whereas L-*myc* and N-*myc* expression occurs predominantly in post-mitotic neural tissue (67). These observations suggest the possibility that some of the *myc* family members have become specialized as transcription factors whereas others function primarily during replication.

Several studies have shown that *myc* expression is associated with the activation of signaling pathways (33, 37, 104), and that it will co-operate with other oncogenes, such as *ras*, to effect the transformation of a cell (105). Observation of *myc* expression in the pituitary gland (PG), which depends on at least two signaling pathways (15,29) could provide many clues to the physiological conditions and factors that contribute to the control of this gene family. Some of the factors controlling *myc* may activate its role as both transcription and replication factor, in order to regulate the hypertrophy that is known to occur in some of the PG cells during sexual maturation (38,64,91).

The results in Chapter II showed that The *myc* proto-oncogene is expressed in the pituitary gland, brain and heart of the Rainbow trout. Moreover, the amount of the major pituitary transcript (1.6 kb) more than doubles as the fish approach sexual maturity, suggesting that this gene may be expressed preferentially in gonadotrophs. In addition, the transcript sizes varied between the tissues examined suggesting that there is some diversification in *myc* function in trout, as observed for c-*myc* and L-*myc* in higher vertebrates. Based on transcript sizes, it was concluded that the pituitary gland expresses B-*myc*, L-*myc* or an unusual c-*myc*; the brain expresses L-*myc* and the heart expresses either c-*myc* or L-*myc*.

Cloning experiments were carried out in order to confirm the identity of the major *myc*-related transcript that was observed in the pituitary gland (Chapter III). Two c-*myc* related cDNA clones were isolated and sequenced. One of these (tmyc2) is expressed as 1.9-2.0 kb transcripts in the PG, brain and heart but is not expressed in the liver. The expression of the second clone (tmyc3) is restricted to the PG (1.6 kb), although at moderate stringency, transcripts appear in the liver and heart. The tMyc3 protein shows extensive homology to tMyc1 (88%) whereas tMyc2 is more divergent (72%). Tmyc2 contains three highly modified areas within the coding sequence, one of which shows an extensive loss of acidic residues that is uncommon in c-*myc* family members but appears in human and Xenopus L-*myc*. Southern and northern blot analysis showed that tmyc2 and tmyc3 are functionally distinct c-*myc* genes.

By combining *in situ* hybridization and northern blot analysis it was possible to show that tmyc3 is expressed primarily, if not exclusively, in the somatolactotrophs of the intermediate lobe. The expression of tmyc2, by contrast, appeared in both the distal and intermediate lobes of the gland (Chapter IV). The results of *in vitro* experiments indicated that the expression of tmyc3 can be stimulated with dopamine and norepinephrine but not with GABA, serotonin or hormone releasing factors that are known to control cells in the distal lobe (Chapter IV). These results suggested a dual role for tmyc3 involving stimulation of somatolactotroph hypertrophy and activation of the somatolactin gene.

The implications of these results involves the presumed role of somatolactin in trout physiology and the mechanisms by which the *myc* proto-oncogene is converted to oncogene status, thereby gaining the ability to transform cells.

Release of somatolactin, in salmonids, has been shown to increase with attainment of sexual maturation (38,106,107), exhaustive exercise (108) and with stress (108,109). Expression of *myc* was observed to increase in the pituitary gland of trout as they approach sexual maturity (Chapter II), suggesting a correlation with somatolactin release, consistent with the results in Chapter IV. This conclusion is complicated by the fact that somatolactin levels also increase as part of the stress response. Consequently, it is not clear whether the increase in *myc* expression, observed in Chapter II, is due solely to maturation or a combination of stress and maturation. The release of SL during sexual maturation, and as part of

the stress response, has been reconciled by the suggestion that SL is required for gluconeogenesis, in order to meet the energy requirements of both processes, and may play a role in energy metabolism similar to that of cortisol (107,109,110).

The trout that were used for the expression survey (Chapter II) were allowed to acclimate for a week before the tissues were collected. This assumes that small trout (25g) have the same stress threshold as do larger trout (600g), and this may not be the case. However, the observed increase in SL levels in stressed trout is extremely rapid, beginning within minutes following the onset of stress and reaching peak levels within 1-2 hours (109). The *in vitro* experiments in Chapter IV indicate that the expression level of tmyc3, under the influence of dopamine or norepinephrine, increases at a more leisurely rate, reaching maximal levels after 2-4 hours. This suggests that the pathway controlling SL release during maturation is separate from the pathway controlling its release during the stress response. Alternatively, the increase in tmyc3 expression level may lag behind the release of SL, but is sufficient to stimulate transcription of the Sl gene, in order to replenish the level of stored SL in the cell.

The known rapid release of SL in stressed fish, has led to the suggestion that release of this protein is a result of direct hypothalamic stimulation, rather than a positive feedback stimulation involving cortisol or vasopressin (109). The results of Chapter IV indicate that the release is mediated by dopamine or norepinephrine but does not rule out the possibility of feedback stimulation acting at the level of the hypothalamus.

These results could be used to further clarify the role of somatolactin in the stress response, and during sexual maturation, by determining dopamine, norepinephrine, *myc* expression level and somatolactin release in trout, of various ages, that are subjected to acute stress.

As described in Chapter I, the *myc* proto-oncogene is converted to an oncogene through overexpression. This occurs primarily through insertional mutagenesis or translocations, which bring the *myc* gene under the control of a powerful promoter. The results in Chapter IV suggest that this may also occur as the result of a chronic elevation in dopamine or norepinephrine levels, leading to an overexpression of the *myc* oncogene. A similar mechanism is known to occur in the induction of breast cancer, where a lifelong exposure to estrogen leads to the overstimulation of the *myc* oncogene (111). Moreover, elevated levels of dopamine, leading to overexpression of *myc*, may represent a link between chronic stress and the incidence of tumor formation in the human population (112).

Conclusions

A study of *myc* expression in the rainbow trout has shown the following:

1. Rainbow trout express two, functionally distinct, c-*myc* proto-oncogenes (tmyc2, tmyc3).

2. Tmyc2 is expressed in the pituitary gland, brain and heart, but not in the liver. Expression and sequence analysis suggest that the function of tmyc2 is more like that of L-*myc*, which appears primarily in brain and lung.

3. Tmyc3 is expressed primarily in the pituitary gland but at a much higher level than tmyc2.

4. Tmyc2 is expressed throughout the pituitary gland, whereas tmyc3 appears primarily in the somatolactotrophs of the intermediate lobe.

5. Tmyc3 expression is stimulated by dopamine and norepinephrine, and may be involved in regulating hypertrophy of somatolactin cells and expression of the somatolactin gene.

6. Chronic stress, or repeated exposure to substances that elevate dopamine or norepinephrine levels, could lead to the formation of *myc*-induced cancers.

References

1. Alt F and Zimmerman BA (1990) Expression and function of *myc* family genes. Crit. Rev. in Oncogenesis 2: 75-95.

2. Dang CV (1991) C-Myc oncoprotein function. Biochemica et Biophysica Acta 1072, 103-113.

3. Rous P (1911) A sarcoma of the fowl transmissible by an agent separable from tumor cells. J. Exp. Med. 13:397-402.

4. Czernilofsky A., Levinson AD, Varmus HE and Bishop JM (1980) Nucleotide sequence of an avian sarcoma virus oncogene (*src*) and proposed amino acid sequence for gene product. Nature 287:198-205.

5. Hunter T, Sefton BM (1980) Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. 77:1311-1317.

6. Bishop JM (1987) The molecular genetics of cancer. Science 235:305-311.

7. Simon MA, Drees B, Kornberg T, Bishop JM (1985) The nucleotide sequence and tissue-specific expression of drosophila *C-src*. Cell 42:831-838.

8. Vielkind JR and Dippel E (1985) Oncogene-related sequences in xiphophorin fish prone to hereditary melanoma formation. Can. J. Genet. Cytol. 26:607-612.

9. Takeya T, Feldman RA and Hanafusa H (1982) DNA sequence of the viral and cellular *src* gene of chickens. J. of Virology 44:1-12.

10. Johnston JG and Van-Der-Kooy D (1989) Proto-oncogene expression identifies a transient columnar organization of the forebrain within the rat central nervous system. Dev. Brain Res. 45:161-170.

11. Alberts B, Bray D, Lewis J, Raff M, Roberts K and Watson JD (1994) The Molecular Biology of the Cell. Third edition, Garland Publishing, N.Y., Chapter 24.

12. Barbacid M (1986) Oncogenes and human cancer: cause or consequence? Carcinogenesis 7(7):1037-1042.

13. Bentley PJ (1976) Comparative vertebrate endocrinology. Cambridge University Press, N.Y., pp 17-21.

14. Ball JW and Baker BI (1969) The pituitary gland, anatomy and histophysiology. In: Fish Physiology, ed. Hoar WS and Randall DJ. Academic Press, N.Y., Chapter 1.

15. Lussier BT, French MB, Moor BL and Raicer J (1991) Mechanism of action of GH-releasing factor and somatostatin. Endocrinology 128:592-598.

16. Wong AO, Van der Kraak G and Chang JP (1994) cAMP mediates dopamine D1-stimulated GH release from golfish pituitary. Neuroendocrinology 60(4): 410-417.

17. Karanth S and McCann SM (1991) Anterior pituitary hormone control by interleukin 2. Proc. Natl. Acad. Sci. 88: 2961-2965.

18. Desrves L, Lamacz M, Jenks BG, Vaudry H and Tonon MC (1993) Effect of dopamine on adenylate cyclase activity, IP_3 metabolism and cytosolic calcium concentration in frog pituitary melanotrophs. J. Endocrinol. 136(3):421-429.

19. Mckeown BA, Jenks BG and van Overbeeke AP (1980) Biosynthesis and release of prolactin from the pituitary gland of the rainbow trout, Salmo gairdneri. Comp. Biochem. Physiol. 65B: 705-709.

20. Chuang TT, Caccavelli C, Kordon C and Enjalbert A (1993) PKC regulation of prolactin gene expression in lactotroph cells: involvement of dopamine inhibition. Endocrinology 132(2):832-838.

21. Lamacz M, Garnier M, Hery F, Tonon ML and Vaudry H (1995) Adrenergic control of alpha-MSH release in frog pituiatry is mediated by both beta and non-conventional alpha2-subtype of adrenoreceptors. Neuroendocrinology 61(4):430-436.

22. Derves L, Vaudry H, Lamacz M and Tonon ML (1995). Mechanism of action of GABA on frog melanotrophs. J. Mol. Endorinol. 14(1):1-12.

23. Thornton VF and Geschwind II (1975) Evidence that serotonin may be a melanocyte stimulating hormone releasing factor in the lizard, *Anolis carolinensis*. Gen. Comp. Endocrinol. 26:346-353.

24. Bates DJ, Barrett BA and McKeown BA (1989) Daily variation in plasma growth hormone of juvenile coho salmon, *Oncorhynchus kisutch*. Can. J. Zool. 67:1246-1248.

25. Barrett BA and Mckeown BA (1988) Sustained exercise augments long-term starvation increases in plasma growth hormone in the steelhead trout, *Salmo gairdneri*. Can. J. Zool. 66(4):853-855.

26. Saga T, Oota Y, Nozaki M and Swanson P (1993) Salmon pituitary gonadotrophs. III. Chronological appearance of GTH I and other adenohypophysial hormones in the pituitary of the developing rainbow trout *(Oncorhynchus mykiss irideus)*. Gen. Comp. Endocrinol. 92:233-241.

27. Sumpter JP, Pickering AD and Pottinger TG (1985) Stress-induced elevation of plasma alpha-MSH and endorphin in brown trout, (*Salmo trutta L.*). Gen. Comp. Endocrinol. 59:257-265.

28. Suda T, Tozawa F, ushiyama T, Tomori N, Sumtomo T, Nakagami Y, Yamada M, Demura H and Shizume K (1989) Effects of protein kinase-C- related adrenocorticotropin secretagogues and interleukin-1 on proopiomelanocortin gene expresion in rat anterior pituitary cells. Endocrinology 124:1444-1449.

29. Naor Z (1990) Signal transduction mechanisms of calcium mobilizing hormones: the case of GnRH. Endocrine reviews 11:326-330.

30. Gershengorn MC (1986) Mechanism of thyrotropin releasing hormone stimulation of pituitary gland release. Ann. Rev. Physiol. 48:515-520.

31. Taylor SS, Buechler JA and Yonemoto W (1990) cAMP-dependent protein kinase: framwork for a diverse family of regulatory enzymes. Ann. Rev. Biochem. 59:971-1005.

32. Iwaki K, Sakhatme VP, Shubeita HE and Chien KR (1990) alpha and beta-adrenergic stimulation induces distinct early gene expression in neonatal rat myocardial cells. J. Biol. Chem. 265:13809-13819.

33. Ran W, Dean, Levine RA, Henkle C and Campisi J (1986) Induction of c-*fos* and c-*myc* mRNA by epidermal growth factor or calcium ionophore is cAMP dependent. Proc. Natl. Acad. Sci. 83:8216-8220.

34. Berridge MJ and Irvine RF (1984) Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature 312:315-321.

35. Nishizuka Y (1988) The molecular heterogeneity of PKC and its implications for cellular regulation. Nature 334:661-665.

36. Dekker LV and Parker PJ (1994) Prorein kinase C- a question of specificity. Trends Biochem. Sci. 19(2):73-77.

37. Saksela K, Makela TP, Evan G and Alitalo K (1989) Rapid phosphorylation of the L-Myc protein induced by phorbol ester tumor promoters and serum. EMBO J. 8(1):149-157.

38. Olivereau M and Rand-Weaver M (1994) Immunocytochemical study of somatolactin cells in the pituitary of pacific salmon at some stages of the reproductive cycle. Gen. Comp. Endocrinol. 93(1):28-33.

39. Carbajo S, Hernandez JL and Carbajo PE (1992) Proliferative activity of cells of the intermediate lobe of the rat pituitary during the postnatal period. Tissue cell. 24(6):829-834.

40. Little CD, Nau M.M, Carney D.N, Gazdar AF and Minna JD (1983) Amplification and expression of the c-*myc* oncogene in human lung cancer cell lines. Nature 306, 194-196.

41. Schwab M, Ramsay, Alitalo K, Varmus H.E, Bishop J.M, Martinsson J, Levan G. and Levin A. (1985) Amplification and enhanced expression of the c-*myc* oncogene in mouse SEWA tumor cells. Nature 315, 345-347.

42. Dalla-Favera R, Wong-Stahl F. and Gallo R. (1982) Oncogene amplification in promyelocytic leukemia cell line HL-60 and primary leukemic cells of the same patient. Nature 298, 61-63.

43. Watt R, Stanton LW, Marcu KB, Gallo RC, Croce CM and Rovera G (1983) Nucleotide sequence of cloned cDNA of human *c-myc* oncogene. Nature 303:725-728.

44. Kohl NE, Legouy E, DePinho RA, Nisen D, Smith RK, Gee CE and Alt FW (1986) Human N-*myc* is closely related in organization and nucleotide sequence to c-*myc*. Nature 319:73-77.

45. Nau M, Brooks BJ, Battey J, Sausville E, Gazdar AF, Kirsch IE, Mcbride OW, Bertness V, Hollis GF and Minna JD (1985) L-*myc* : a new *myc*-related gene amplified and expressed in human small cell lung cancer. Nature 318:69-73.

46. Ingvarsson S., Asker C., Axelson H., Klein G. and Sumegi J. (1988) Structure and expression of B-*myc*, a new member of the *myc* gene family. Mol. Cell. Biol. 8(8): 3168-3174.

47. Colby W, Chen W, Smith D and Levinson A (1983) Identification and nucleotide sequence of a human locus homologous to the v-*myc* oncogene of avianmyelocytomatosis virus MC29. Nature 301:722-725.

48. Kaye, F., Battey, J., Nau, M., Brooks, B., Seifter, E., DeGreve, J., Birrer, M., Sausville, E., and Minna J. (1988). Structure and expression of the Human L-*myc* gene reveal a complex pattern of alternative mRNA processing. Mol. Cell. Biol. 8, 186-195.

49. Blackwood, E.M., and Eisenman, R.N. (1991). Max: A helix-loop-helix zipper protein that forms sequence-specific DNA binding complex with Myc. Science 251,1211-1217.

50. Goll-Muhle c, Nilges M and Pastore A. (1995) The leucine zippers of the HLH-LZ proteins Max and c-Myc preferentially form heterodimers. Biochemistry 34:13554-13564.

51. Ferre-D'Amare AR, Prendergast GC, Ziff EB and Burley SK (1993) Recognition by Max of its cognate DNA through a dimeric b/HLH/Z domain. Nature 363:38-41.

52. Maheswaran S, Lee H and Sonenshein GE (1994) Intracellular association of the protein product of the c-*myc* oncogene with the TATA-binding protein. Mol. Cell. Biol. 14(2):1147-1152.

53. Ptashne M and Gann AAF (1990) Activators and targets. Nature 346:329-331.

54. Buratowski S (1994) The basics of basal transcription by polymerase II. Cell 77:1-3.

55. Gu W, Cechova K, Tassi V and Dalla-Favera R (1993) Opposite regulation of gene transcription and cell proliferation by c-Myc and Max. Proc. Natl. Acad. Sci. 90:2935-2939.

56. Starksen NF, Simpson PC, Bishopric N, Coughlin SR, Lee WMF, Escobedo JA and Williams LT (1986) Cardiac myocyte hypertrophy is associated with c-*myc* protooncogene expression. Proc. Natl. Acad. Sci. 83:8348-8350.

57. Kolbeck-Ruhmkorff C, Horban A, Zimmer HG (1993) Effect of pressure and volume overload on proto-oncogene expression in the isolated working rat heart. Cardiovasc. Res. 27:1998-2004.

58. Szijan I, Parma DL and Engel NI (1992) Expression of c-*myc* and c-*fos* protooncogenes in the anterior pituitary gland of the rat. Effect of estrogen. Horm. Metab. Res. 24:154-157.

59. Barrett BA and Mckeown BA (1988) Growth hormone response and sustained swimming is exercise-acclimated steelhead trout, *Salmo gairdneri.* J. Fish Biol. 32:799-800.

60. Wagner GF and Mckeown BA (1986) Development of a salmon growth hormone radioimmunoassay. Gen. Comp. Endocrinol. 62:452-458.

61. Luo D and Mckeown BA (1991) Interaction of carp growth hormone-releasing factor and somatostatin on *in vitro* release of growth hormone in rainbow trout *(Oncorhynchus mykiss)*. Neuroendocrinology 54:359-364.

62. Luo D , Mckeown BA, Rivier J and Vale W (1990) *In vitro* responses of rainbow trout *(Oncorhynchus mykiss)* somatotrophs to carp growth hormone-releasing factor (GRF) and somatostatin. Gen. Comp. Endocrinol. 80:288-298.

63. Weil C, Hansen P, Hyam D, Le Gac F, Breton and Crim LW (1986) Use of pituitary cells in primary culture to study the regulation of GTH secretion in rainbow trout: setting up and validating the system as assessed by its responsiveness to mammalian and salmon gonadotropin releasing hormone. Gen. Comp. Endocrinol. 62:202-209.

64. Mckeown BA and van Overbeeke AP (1971) Immunohistochemical identification of pituitary hormone producing cells in the sockeye salmon (Oncorhynchus nerka, Walbaum). Z. Zellforsch. 112:350-362.

65. Rand-Weaver M, Toyohiko N, Maramoto K and Kawauchi H (1991) Isolation and charactrization of somatolactin, a new protein related to growth hormone and prolactin from atlantic cod (*Gadus morhua*) pituitary glands.Biochem. 30:1509-1515.

66. Zimmerman KA, Yancopoulos GD, Collum RG, et al. (1986) Differential expression of *myc* family genes during murine development. Nature 319, 780-783.

67. Semsei I, May S and Cutler RG (1989) Differential expression of the *myc* proto-oncogene family throughout the lifespan of the C57BL/6J mouse strain. Oncogene 4,465-470.

68. Jakobovits A, Schwab M, Bishop JM and Martin GR (1985) Expression of N-*myc* in teratocarcinoma stem cells and mouse embryos. Nature 318, 188-191.

69. Vriz S, Taylor M and Mechali M (1989) Differential expression of two Xenopus c-*myc* proto-oncogenes during development. EMBO J. 8(13):4091-4097.

70. Van Beneden RJ, Watson DK, Chen TT, Lautenberger T.A. and Papas T.S. (1986) Cellular *myc* (c-*myc*) in fish (rainbow trout). Proc. Natl. Acad. Sci. 83, 3698-3702.

71. Panno JP and McKeown BA (1993) Expression of the *myc* proto-oncogene in the rainbow trout, *Oncorhynchus mykiss*. Comp. Biochem. Physiol. 104B:649-652.

72. Chomczynski P and Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analyt. Biochem. 162, 156-159.

73. Fourney RM, Miyakoshi J, Day RR and Peterson ML (1989) Northern blotting: Eficient RNA staining and transfer. Focus 10, 5-7.

74. Sambrook J, Fritsch EF and Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York, p7.43-7.49.

75. Feinberg AP and Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Analyt. Biochem. 132, 6-13.

76. Agellon LB, Davies SL, Chen TT and Powers DA (1988) Structure of a fish (rainbow trout) growth hormone gene and its evolutionary implications. Proc. Natl. Acad. Sci. 85: 5136-5140.

77. Schreiber-Agus N, Torres R, Horner J, Lau A, Jamrich M and DePinho A (1993) Comparative analysis of the expression and oncogenic activities of *Xenopus* c-, N-, and L-*myc* homologs. Mol. Cell. Biol. 13(4):2456-2468.

78. Panno JP and Mckeown BA (1995) Cloning and expression of a *myc* family member from the pituitary gland of the Rainbow trout, *Oncorhynchus mykiss*.. Biochimica et Biophysica Acta 1264:7-11.

79. Schwab M, Alitalo K, Varmus HE, Bishop JM and George D (1983) A cellular oncogene (c-Ki-*ras*) is amplified, overexpressed, and located within karyotypic abnormalities in mouse adrenocortical tumour cells. Nature 303:497-501.

80. Southern EM (1975) Detection of specific sequences amoung DNA fragments separated by gel electrophoesis. J. Mol. Biol. 98:503-517.

81. Stanton LW, Fahrlander PD, Tesser PM and Marcu KB (1982) Nucleotide sequence comparison of normal and translocated murine *c-myc* genes. Nature 310:423-424.

82. DePinho RA, Legouy E, Feldman LB, Kohl NE, Yancopoulos and Alt, FW (1986) Structure and expression of the murine N-*myc* gene. Proc. Natl. Acad. Sci. 83:1827-1831.

83. Schreiber-Agus N, Horner J, Torres R, Chiu FC and DePinho RA (1993) Zebra fish *myc* family and *max* genes: Differential expression and oncogeneic activity throughout vertebrate evolution. Mol. Cell. Biol. 13(5): 2765-2775.

84. Ohno S (1970) Evolution by gene duplication. Springer-Verlag, N.Y.

85. Devlin RH (1993) Sequence of sockeye salmon type 1 and 2 growth hormone genes and the relationship of rainbow trout with atlantic and pacific salmon. Can. J. Fish. Aquat. Sci. 50:1738-1748.

86. Rentier-Delrue F, Swennen D, Mercier L, Lion M, Benrubi O and Martial JA (1989) Molecular cloning and characterization of two forms of trout growth hormone cDNA: Expression and secretion of tGH-II by *escherichia coli*. DNA 8(2): 109-117.

87. Devlin RH and Wallis AE (1993) Duplicate insulin-like growth factor-1 genes in salmon display alternative splicing pathways. Molec. Endorinol. 7(3):409-422.

88. Hiraoka S, Suzuki M, Yanagisawa T, Iwata M and Urano A (1993) Divergence of gene expression in neurohypophysial hormone precursors among salmonids. Gen. Comp. Endocrinol. 92:292-301.

89. Salbert G, Chauveau I, Bonnec G, Valotaire Y and Jego P (1992) One of two trout proopiomelanocortin messenger RNAs potentially encodes new peptides. Mol. Endocrionol. 6(10):1605-1613.

90. Sekine, Saito A, Itoh H, Kawauchi H and Itoh S (1989) Molecular cloning and sequence analysis of chum salmon gonadotropin cDNA. Proc. Natl. Acad. Sci. 86:8645-8649.

91. Robertson OH and Wexler BC (1962) Histological changes in the pituitary gland of rainbow trout (Salmo gairdnerii) accompanying sexual maturation and spawning. J. Morhology 110:157-185.

92. Komminoth P, Merk FB, Leav I, Wolf HJ and Roth J (1992) Comparison of S^{35} and digoxigenin-labeled RNA and oligonucleotide probes for in situ hybridization. Histochemistry 98: 217-228.

93. Hirota S, Ito A, Morii E, Wanaka A, Tohyama M, Kitamura Y and Nomura S (1992) Localization of mRNA for c-*kit* receptor and its ligand in the brain of adult rats: an analysis using in situ hybridization histochemistry. Mol. Brain. Res. 15:47-54.

94. Nonradioactive in situ hybridization application manual (1992). Boehringer Mannheim, Laval Quebec, Canada.

95. Molecular Cytogenetics: Tissue in situ hybridization. workshop manual, Oncor, Gaitherburg, MD, USA.

96. Baker BI (1963) Effect of adaptation to black and white backgrounds on the teleost pituitary. Nature 198:404.

97. Kaneko T, Kakizawa S, Yada T and Hirano T (1993) Cell expression and intracellular localization of somatolactin in the pituitary of rainbow trout. Cell. Tissue Res. 272:11-16.

98. Nair KK, Chen TT, Wyatt GR (1981) Juvenile hormone stimulated polyploidy in adult locust fat body. Dev. Biol. 81:356-360.

99. Reed J, Nowell P and Hoover R (1985) Regulation of c-*myc* mRNA levels in nornal human lymphocytes by modulators of cell proliferation. Proc. Natl. Acad. Sci. 82:4221-4225.

100. Makino R, Hayashi K and Sugimara T (1984) c-*myc* transcript is induced in rat liver at a very early stage of regeneration or by cycloheximide treatment. Nature 310:697-700.

101. Bower A, Hadley ME and Hruby VJ (1974) Biogenic amine and control of melanophore stimulating hormone release. Science 184:70-72.

102. Takayama Y, Rand-Weaver M, Kawauchi H and Ono M (1991) Gene structure of chum salmon somatolactin, a presumed pituitary hormone of the growth hormone/prolactin family. Molec. Endocrinol. 5(6):778-786.

103. Shepard AR, Zhang W and Eberhardt N (1994) Two CGTCA motifs and a Pit1 binding site mediate cAMP-dependent protein kinase A regulation of human GH gene expression in rat anterior pituitary cells. J. Biol. Chem. 269(3):1804-1814.

104. McCaffrey P, Ran W, Campisi J and Rasner M (1987) Two independent growth factor generated signals regulate c-*fos* and c-*myc* mRNA levels in Swiss 3T3 cells. Biol. Chem. 262:1442-1445.

105. Alexander WS, Adams JM and Cory S (1989) Oncogene cooperation in lymphocyte transformation: Malignant conversion of Eu-*myc* transgeneic pre-B cells *in vitro* is enhanced by v-H-*ras* or v-*raf* but not v-*abl*. Mol. Cell. Biol. 9(1): 67-73.

106. Rand-Weaver M, Swanson P, Kawauchi H and Dickhoff WW (1992) Somatolactin, a novel pituitary protein: purification and plasma levels during reproductive maturation of coho salmon. J. Endocrinol. 133:393-403.

107. Rand-Weaver M and Swanson P (1993) Plasma somatolactin levels in coho salmon (*Onchorhynchus kisutch*) during smoltification and sexual maturation.Fish Physiol. Biochem. 11:175-182.

108. Kakizawa S, Kaneko T, Hasegawa S and Hirano T (1995) Effects of feeding, fasting, background adaptation, acute stress, and exhaustive exercise on the plasma somatolactin concentrations in rainbow trout. Gen. Comp. Endocrinol. 98:137-146.

109. Rand-Weaver M, Pottinger TG and Sumpter JP (1993) Plasma somatolactin concentrations in salmonid fish are elevated by stress. J. Endocrinol. 138:509-515.

110. McKeown BA (1984) Fish Migration. Chap. 4, pp. 138-152. Timber Press, Oregon.

111. Leyque E, Gal-Winkler R, Gompel A, Louis-Sylvestre C, Soquet L, Staub S, Kutten F and Mauvais-Jarvis P (1995) Estradiol stimulates c-*myc* proto-oncogene expression in normal human breast epithelial cells in culture. J. Steroid Biochem. Mol. Biol. 52(4): 299-305.

112. Leeming RA (1994) From the common cold to cancer: how evolution and the modern lifestyle appear to have contributed to such eventualities. Med. Hypotheses43(3):145-158.

Glossary

cDNA complementary DNA, made by reverse transcribing messenger RNA.

c-*myc* Cellular *myc*, one of four known members of the *myc* oncogene family. By convention the gene name is in lower case, and frequently in italics.

Dysplasia Abnormality of development. Alteration in size, shape and organization of adult cells.

EDTA Disodium ethylenediamine acetate, a calcium chelator.

Homology As applied to DNA or protein sequence analysis, any two sequences that are identical are defined as being 100% homologous.

M1a A 475 bp EcoRI-BamHI fragment from the 5' end of tmyc1, a partial genomic clone isolated from rainbow trout.

M2a A 450 bp PstI-PvuII fragment from the middle of tmyc2, a cDNA clone isolated from the pituitary gland of rainbow trout.

M3a A 467 bp EcoRI-BamHI fragment from the 5' end of tmyc3, a cDNA clone isolated from the pituitary gland of rainbow trout

M3b A 200 bp EcoRI-TaqI fragment from the 5' end of tmyc3.

EFa A 200 bp EcoRI-XhoI fragment of the translation elongation factor 2, a cDNA isolated from rainbow trout.

myc An oncogene that is responsible for the formation of myelocytomatosis, a cancer of blood forming cells generally referred to as a leukemia.

Myc The protein coded for by *myc*. By convention the gene name is capitalized, without italics, when referring to the protein product.

MOPS (3(-N-morpholino)propanesulfonic acid, a buffer commonly used for RNA gel electrophoresis, with an optimum pH range of 6.5-7.9.

mRNA Messenger RNA.

Poly(A)$^+$ Refers to adenine repeats that occurs at the 3' end of most messenger RNA.

PEG Poly-ethylene glycol, used to precipitate RNA.

Pfu Plaque forming units.

rRNA Ribosomal RNA.

tmyc1 Trout c-*myc* gene or cDNA.

tMyc1 The protein coded for by tmyc1.

Oncogene Any gene that has the ability to transform cells to a cancerous phenotype, characterized by neoplastic growth and dysplasia.