"Epigenetic regulation of *Cyprinus carpio* ribosomal cistron during the acclimatization process"

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Gino Alberto Nardocci Valenzuela
aus Santiago de Chile, Chile

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Dr. Marco Álvarez

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"Epigenetic regulation of *Cyprinus carpio* ribosomal cistron during the acclimatization process"

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“Si no conozco una cosa, la investigaré.”

Louis Pasteur

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3 LIST OF ABBREVIATIONS

aa	Amino acid
ACF	ATP-utilizing chromatin assembly and remodeling factor
ATP	Adenosine-5`-triphosphate
BAZ	Bromodomain adjacent to Zinc finger domain
bp	Base pairs
BSA	Bovine serum albumin
C-terminal	Carboxy-terminal
CHD	Chromodomain
ChIP	Chromatin Immunoprecipitation
CpG	Cytosine-phosphatidyl-guanosine
DBD	DNA binding domain
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonucleosidase I
DNMT	DNA methyl transferase
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis-(β-aminoethyl ether)-N,N,N`,N`-tetraacetic acid
EMSA	Electrophoretic mobility shift assay
fmol	femtomole
g	Relative centrifugal force
h	Hour
H1 / H2A / H2B / H3 / H4	Histone proteins
H3Kxme3	Tri-mehtylation at lysine x histone H3
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HMG	High mobility group
HMT	Histone methyl transferase
HSP	Heat shock protein
Ig	Immunoglobulin

IGS	Intergenic spacer
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ISWI	Imitation of switch
kb	Kilo base
kDa	Kilo Daltons
LB	Luria-Bertani
M	Molar
MBD	Methyl binding domain
min	Minute(s)
mL	Mililiters
mM	Milimolar
mRNA	Messenger RNA
mut	Mutant
MW	Molecular weight
N-terminal	Amino-terminal
Ni-NTA	Nickel-nitroacetic acid
NOR	Nucleolar organizer region
NoRC	Nucleolar remodeling complex
NP-40	Nonidet P-40
NRD	Negative regulatory domain
ºC	Degree Celsius
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PHD	Plant homeodomain
PMSF	Phenylmethylsulfonyl fluoride
pre-rRNA	Precursor of ribosomal RNA
pRNA	Promoter-associated RNA
PTRF	Polymerase I transcript release factor
RACE	Rapid amplification of cDNA ends
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT	Reverse transcription

S	Svedberg unit
s	Second(s)
SDS	Sodium Dodecyl Sulfate
SNF2h	SNF2 homolog protein
SWI/SNF	Switching defective/Sucrose Non-fermenting
TAM	Tip/ARBP/MBD
Taq	Thermos aquaticus
TBE	Tris borate EDTA buffer
TBP	TATA-binding protein
TIF-IA / B / C	Transcription initiation factor for RNA polymerase I
Tip5	TTF-I interacting protein 5
Tris	Tris(hydroxymethyl)-amino-methane
Triton X-100	Octal fenoxi polietoxietanol
TTF-I	Transcription termination factor for RNA polymerase I
TTF-IΔ323	N-terminal (aa 1 to 323) truncated form of TTF-I
Tween-20	Polyoxyethylene-sorbitan monolaurate
UBF	Upstream binding factor
UCE	Upstream control element
UTR	Untranslated region
UV	Ultraviolet light
V	Volts
X-Gal	5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside

4 SUMMARY

Ribosomal RNA synthesis is the major transcriptional activity in a cell, required for ribosome biogenesis, and it is critical to ensure the supply of ribosomal RNA in order to meet the cellular needs for protein synthesis. Considerable evidence indicates that epigenetics is an important regulatory mechanism for this intricate process. In addition, cells must also have the ability to maintain their functions by constantly sensing and adapting to environmental variations (homeostasis).

Seasonal acclimatization of the fish *Cyprinus carpio* (common carp) requires the implementation of complex molecular and cellular mechanisms to coordinate "phenotypic plasticity." This process involves a reprogramming of gene expression, which in turn integrates the homeostatic response. Our laboratory has reported that in different cell types of the carp, the nucleolus undergoes a dramatic rearrangement of its molecular structure. During winter, fibrillar and granular components of the nucleolus are segregated and surrounded by a thick layer of heterochromatin, which is an ultrastructural feature accompanied by a transient repression of ribosomal RNA transcription. In contrast, this condition is reversed during the summer, and is associated with a recovery of active ribosomal biosynthesis. Thus, the process of seasonal adaptation in the carp certainly involves a fine modulation of the transcriptional activity of ribosomal genes (rDNA).

Recent studies on the regulation of ribosomal genes indicate that the numerous copies of rDNA are comprised of at least two distinct transcriptional

states: active or silenced. The two states can be differentiated by their chromatin configuration and may require specific triggers to be interconverted. Thus, the use of epigenetic mechanisms to control chromatin architecture may represent an important strategy to modulate and switch the transcriptional activity of rDNA during seasonal adaptation

In this context, the protein TTF-I has been described as playing a central role in the transcriptional modulation of ribosomal genes through its interaction with epigenetic modifiers, such as the nucleolar remodeling complex (NoRC), thus playing an active role in the silencing of rDNA transcription.

Most of the conclusions regarding regulatory mechanisms that control rRNA transcription have been proposed based on *in vitro* approaches. Thus, the natural cyclic modulation of rRNA transcriptional activity observed during carp acclimatization emerges as a valuable model towards studying the overall phenomenon in a living organism.

Therefore, the general purpose of this thesis consists in studying the epigenetic regulation of the ribosomal cistron during the acclimatization process of the *Cyprinus carpio.* Consequently, our hypothesis proposes that "The factor TTF-I and the chromatin remodeling complex NoRC contribute significantly to the negative regulation of transcriptional expression of rRNAs during the seasonal adaptation of *Cyprinus carpio*."

Thus our results represent a first approach in epigenetic control involved in the expression of ribosomal genes in a natural context, where the factor TTF-I and NoRC complex play a fundamental role during the carp acclimatization process.

5 INTRODUCTION

5.1 *Cyprinus carpio* as a model for the study of seasonal acclimatization

Living organisms are constantly exposed to a wide range of environmental variations, which generate selective demands on them. These organisms can generally respond via three strategies (Holt 1990). The first strategy is to avoid environmental pressures, perhaps by migrating to regions with conditions more appropriated to their survival. The second strategy consists in the development of reversible adaptive responses according to environmental variations, in which the phenotype is modified to allow maintenance of cell function (homeostasis), a condition called phenotypic plasticity (Venkatesh 2003). The final strategy is a long-term response, in which changes occur at the genetic level through the process of evolution.

The mechanisms of adaptation to environmental pressures are undoubtedly of particular interest because organisms must be able to develop physiological and molecular responses to achieve successful adaptation.

Several physiological mechanisms of adaption have been described in many organisms. For instance, plants exposed to UV-B generate physiological responses that include growth reduction, leaf expansion, and the synthesis of secondary metabolites, such as UV protectors, via flavonoids (Brown and Jenkins 2008; Jenkins 2009). In yeast, exposure to osmotic shock causes a flow of water towards the outside of the cell, resulting in an increase in the concentration of all

cellular components, which triggers signals that lead to a process of osmoregulation (Hohmann 2002). In 1969, Somero and his team showed that the pyruvate kinase enzyme of *Paralithodes camtschatica* exists in two forms, and which have a different affinity for the substrate depending on the temperature, a mechanism in which the polypeptide sequence is able to adopt different structures depending upon environmental variations (Somero 1969).

In addition, general mechanisms of adaptation exist that are shared by a wide range of organisms, which generate global responses to environmental stress (Lopez-Maury et al. 2008). For instance, heat shock proteins (HSPs) are found in virtually all living organisms, from bacteria to humans, and their function is to protect the translation of messenger RNA, increasing the degradation of abnormal proteins and decreasing protein translation, when cells are exposed to elevated temperatures or other stress (Rylander et al. 2005).

The different animal models used for the study of adaptation mechanisms allow for answers as to how these organisms interact and respond to their environments. In particular, aquatic organisms undergo variations of external stimuli imposed by the environment, such as temperature, photoperiod, water salinity, food availability, concentration of oxygen, etc., and are thus forced to implement cellular and molecular strategies that allow them to adjust their physiology. Ectothermal fish are particularly interesting in this context because they are unable to regulate their internal temperature. Thus, they have developed a number of strategies to keep homeostasis under natural environmental conditions

(Cossins and Crawford 2005; Cossins et al. 2006). This adaptive process is called seasonal acclimatization.

With the aim of studying this complex process, we have focused our interest in *Cyprinus carpio*, a eurythermal-ectotherm fish, which is able to adapt to a wide range of habitats and environmental conditions, and consequently generating molecular responses to adjust to the seasonal changes.

In Chile, the carp was introduced from Germany around 1875 (Wolfarth 1995), and it has successfully colonized lakes and rivers throughout nearly the entire country. In these ecosystems, the fish is exposed to continuous fluctuations of natural water. For instance, in central Chile the water temperature varies between 20 and 25ºC during the summer and has a photoperiod of 14/10 h day/night. Conversely, in winter the water temperature varies between 10-15ºC, with a photoperiod of 10/14 h day/night. A number of studies have established that carp have cellular and molecular adaptation mechanisms that incorporate the reprogramming of gene expression as a central strategy to surmount these changing environmental conditions (Polley et al. 2003; Schulte 2004; Cossins et al. 2006; Ladurner 2006; Lopez-Maury et al. 2008).

In our laboratory, we are interested in understanding and elucidating the kinds of molecular mechanisms displayed in the regulation of the seasonal acclimatization process for carp. We showed that exist gene-reprogramming responses concurrent to seasonal environmental variations. Thus, we reported that several genes are differentially regulated between contrasting seasons, i.e. winter

and summer (Figueroa et al. 1994; Alvarez et al. 2001; Molina et al. 2002; Alvarez et al. 2003; San Martin et al. 2007). For instance, we have observed that expression of the *Prl* gene (which codes for the hormone prolactin) during the summer season is strongly increased (Figueroa et al. 1994). In fish, this hormone is important for osmoregulation as it regulates the flow of water and salts through the gills and kidney by modifying membrane permeability (Forsyth and Wallis 2002). Furthermore, a high expression of the transcription factor Pit-1 (involved in the regulation of the expression of prolactin, GH, and other pituitary hormones) is consistent with this increase (Kausel et al. 1999). In contrast, during the winter season there is an increase of expression of the *Cds2* gene (Polley et al. 2003), which encodes a liver isoform of Δ9-acyl-CoA desaturase. This enzyme is closely related to the metabolism of fatty acids and, in particular, for the regulation of membrane fluidity.

Additionally, during the carp's acclimatization, some components involved in ribosome biogenesis are also differentially expressed. For example, the structural protein of ribosome L41 (Molina et al. 2002), snoRNA U3, and the beta CK2b protein (Alvarez et al. 2001) increases its transcription in summer. On the contrary, gene coding nucleolin, one of the most abundant non-ribosomal proteins in the nucleolus, increase its expression in winter (Alvarez et al. 2003). Similarly, other research groups have also reported a differential regulation of transcription in carp. A transcriptomic analysis of carp acclimated to low temperatures demonstrated that at least 3,400 cDNAs (from a population of 13,440 different probes used) are differentially regulated by adaptation to cold temperatures (Gracey et al. 2004).

Additionally, our group has reported that the most striking phenotypic characteristic associated with seasonal adaptation is the reorganization of nucleolar components in different tissues (Figure 1) (Saez et al. 1984; Vera et al. 1993; Alvarez et al. 2006). During the summer, nucleolar components are entire intermingled, consistent with a state of high synthesis of ribosomal genes (rRNA), indicating active ribosomal biogenesis (Figure 1A) (Vera et al. 1993). Conversely, in cells of acclimatized carps to winter, the fibrillar and granular components of the nucleolus are separated and surrounded by a thick layer of chromatin (Figure 1B). A molecular distribution comparable to this last nucleolar structure is acquired when carp cells in primary culture are treated with Actinomycin D (an inhibitor of RNA polymerase I), suggesting that the altered distribution results from a transient repression of ribosomal RNA (rRNA) synthesis (Alvarez et al. 2006). These findings indicate a delicate process of transcriptional regulation of ribosomal genes during the seasonal adaptive process of the carp.

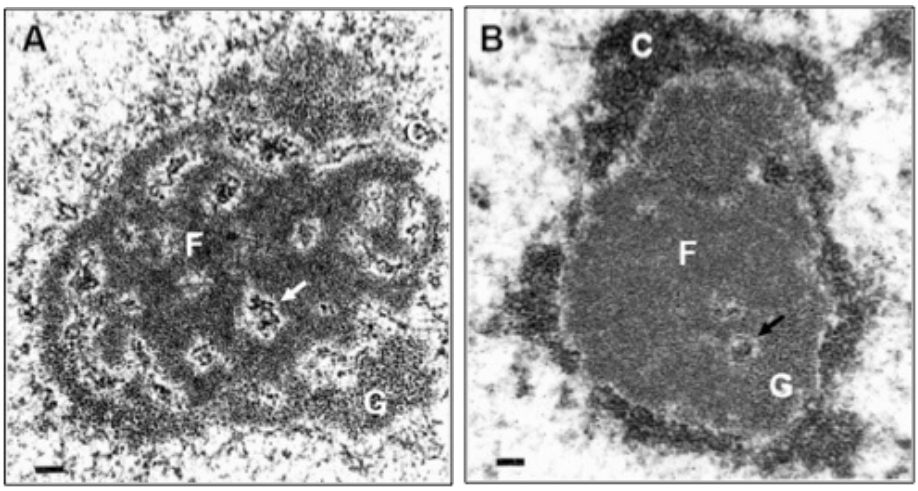

Figure 1. Nucleolar ultrastructure of acclimatized *C. carpio*

Ultrastructural rearrangements of nucleolus in hepatocytes from summer (A) and winter (B) adapted carps. During the summer season, the nucleolar components appear entirely intermingled, and in winter, the components are separated. F, Fibrillar components; G, granular components; C, condensed chromatin. Clusters of condensed chromatin are indicated with arrows. Scale bar 0.2 μm. Figure from Alvarez et al. 2006.

5.2 Structure and function of nucleolus

The nucleolus is the most prominent domain within the cell nucleus. This domain was first described in 1781, by Fontana as an ovoid body within the nucleus (Schwarzacher and Wachtler 1983). At this site, the precursor rRNA is synthesized, processed, and then assembled into ribosome subunits (Leary and Huang 2001).

In the nucleolus, three distinct morphological subcompartments have been described by electron microscopy: the fibrillar centers (fc), the dense fibrillar

components (df) and the granular components (g) (Figure 2) (Schwarzacher and Wachtler 1983; Paule 1998; Scheer and Hock 1999).

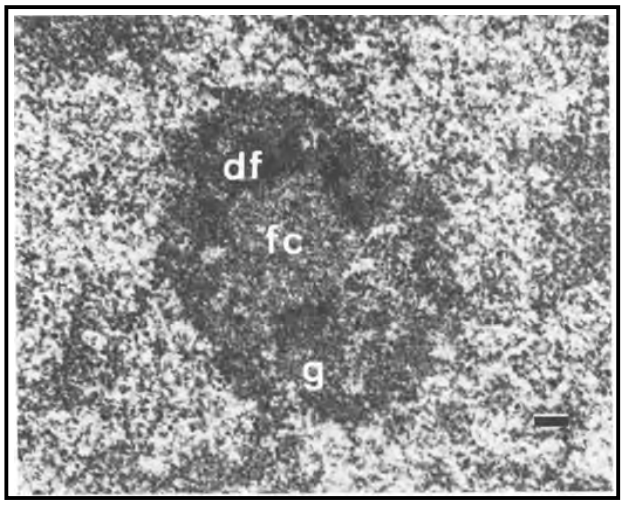

Figure 2. Nucleolar architecture

Nucleolar organization viewed by electron microscopy of human lymphocyte from peripheral blood. Three morphological structures can be distinguished: the fibrillar center (fc), the dense fibrillar component (df), and the granular component (g). Scale bar 0.1 µm. Figure modified from Schwarzacher and Wachtler 1983.

The ribosomal DNA transcription occurs at the periphery of the fibrillar center, while transitory accumulation, modification, and processing of primary rRNA transcripts occurs in the dense fibrillar component. Later, processing and rRNA assembly into ribosomal subunits occurs in the granular component (Lazdins et al. 1997; Mosgoeller et al. 2001; Andersen et al. 2002). Studies have revealed that the nucleolus has additional functions, such as the synthesis of other ribonucleoprotein particles, e.g. the signal recognition particle, pre-tRNA

processing, and a prominent role in cell senescence (Pederson 1998; Comai 1999; Olson et al. 2000).

In addition to its role in the synthesis of ribosomal genes, in recent years the nucleolus has also been associated with additional functions such as acting as a sensor of environmental stimuli. In 2003, Rubbi and Milner demonstrated that exposure of cells to agents that cause stress, such as hypoxia, heat, UV radiation, and others, generates a disorder of the nucleolus, which resulted in the stabilization of factor p53, a transcription factor that has the ability to induce cell arrest or apoptosis when the cell is exposed to a diverse cellular stress (Rubbi and Milner 2003). This evidence, combined with that obtained previously in our laboratory, allows us to postulate that the nucleolus, and particularly the regulation of ribosomal biogenesis, should play a fundamental role in the adaptive process of the carp during seasonal acclimatization.

5.3 The organization of rDNA genes

Ribosomal genes are unique in several aspects as compared with the majority of the genes transcribed by either RNA polymerase II or III. For instance, rDNA is one of the most repetitive genes founded in eukaryotic genomes, ranging from hundreds in mammals to thousands in plants, and these repetitions are spread over multiple chromosomes located on the short arms of acrocentric chromosomes. In addition, an exclusive RNA polymerase (RNA polymerase I, RNA Pol I) is responsible for the transcription of ribosomal RNAs. They are organized in

tandem and grouped in clusters called Nucleolus Organizer Regions (NORs) (Paule 1998).

In terms of gene structure (Figure 3), the ribosomal cistron (rDNA) contains an intergenic spacer (IGS) of variable size (2-20 Kb depending on species) that separates the transcriptional units of rRNA genes. This sequence includes a large number of repeated elements that regulates transcription of the rRNAs both positively and negatively (enhancers and terminators). On the other hand, the coding region contains the transcribed sequences for 18S, 5.8S and 28S, separated by internal spacers (ITS1, ITS2) and flanked by 2 external spacers (5'-ETS, 3'-ETS) (Sylvester et al. 2004). In this way, ribosomal biogenesis takes place in the framework structure provided by the nucleolus, in a time- space coordinated process (Fischer et al. 1991). This begins with the transcription of a precursor 45S rRNA by the RNA polymerase I. This pre-rRNA transcript contains the sequences of the mature 18S rRNA, 5.8S, and 28S, and is accompanied by two sequence denominated external transcribed spacers (5'-ETS and 3'-ETS) and two internal sequences (ITS1 and ITS2). They are processed during pre-rRNA maturation. A fourth component is the ribosomal 5S rRNA, which is synthesized outside the nucleolus by RNA polymerase III in a separate transcriptional unit. Finally, rRNAs are associated with approximately 80 specific ribosomal proteins in order to form the ribosomal subunits (small and large) and then transport them to the cytoplasm (Scheer and Weisenberger 1994). This complex process is finely coordinated and regulated at every stage by cellular machinery.

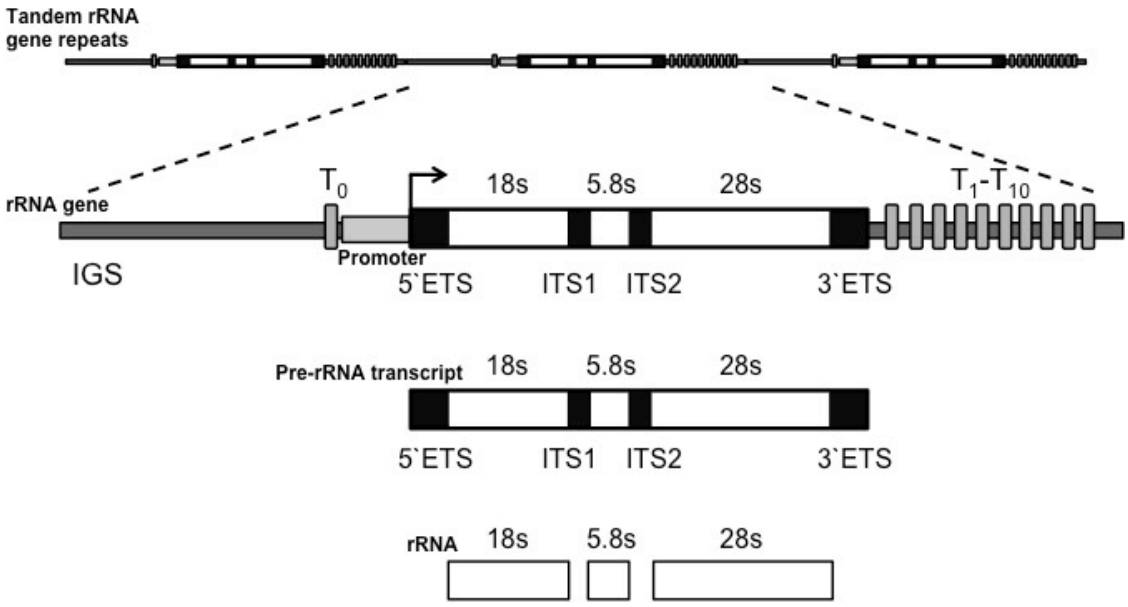

Figure 3. General scheme of ribosomal genes and their transcripts.

The diagram shows the arrangement of tandemly repeated rRNA genes and their organization. The site of transcription initiation of 47S pre-rRNA (black arrow) is indicated. Terminator elements are located downstream of the transcription unit (T_1-T_{10}), and upstream of the gene promoter (T_0) (light gray boxes). The different products of rDNA are indicated (pre-rRNA and rRNA) (below). Figure modified from Raska et al. 2004.

The transcriptional modulation of ribosomal genes is reached by *cis* and *trans* factors capable of interacting with specific DNA elements located in the IGS (Jacob 1995). These elements include the promoter of rDNA (rDNA promoter), spacer promoter, repetitive enhancer elements, and terminator elements. The rDNA promoter has a bipartite structure that consists of a core promoter adjacent to the initiation site of rRNA transcription and an upstream control element (UCE) located approximately 100 bp upstream (Haltiner et al. 1986; Learned et al. 1986). Additionally, the eukaryotic ribosomal genes are flanked on both sides by transcription terminator elements called “T”, listed by number and that are

characterized by consensus sequence of 18 bp Sal box type (AGGTCGACCAGA/TT/ANTCCG), whose function is to mediate transcription termination (Grummt et al. 1986; Bartsch et al. 1988). However, an element of similar characteristics is located immediately upstream of the rDNA promoter and has been defined as "T_0" (Grummt et al. 1986; Henderson and Sollner-Webb 1986). Surprisingly, this sequence element has been described as playing a role in the activation of rRNA transcription (Längst et al. 1997; Längst et al. 1998).

In carp, we have studied in detail the organization of the gene ribosomal cistron (Vera et al. 2003). In general, the organization of carp rDNA gene (Figure 4) is similar to those described above. The IGS is 8.3 kb and contains a series of repetitive elements that vary in size and position throughout the IGS. The transcribed region covers 6.7 kb and contains 18S, 5.8S, and 28S rRNA coding sequences. Downstream of the transcription unit, the presence of five repeated elements were detected which display a sequence similar to the "Sal-box". In this context, it was suggested that these elements potentially correspond to transcription termination elements in carp rDNA (identified as T_1 to T_5). Both interestingly and unusually, upstream of the transcription initiation site, it was possible to observe two regulatory elements (T_0 and T_0'). This feature appears to be characteristic of the carp ribosomal cistron, because according to our knowledge, this feature has not been described in rDNA genes from other species.

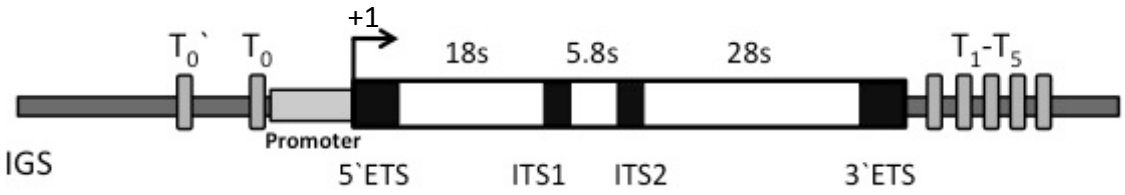

Figure 4. Structure of ribosomal cistron of *C. carpio*

The diagram shows a representation of carp ribosomal gene. The termination elements are located downstream of the transcription unit ($T_{1\text{-}5}$) and upstream of the gene promoter (T_0 and T_0`). Site of the transcription initiation is indicated by black arrow. The internal (ITS) and external (ETS) transcribed spacers are indicated (black).

5.4 Regulation of ribosomal RNA transcription

Transcription of the rDNA genes is performed by the RNA Pol I, a multiprotein complex of 14 subunits and a set of auxiliary factors for initiation, elongation, and termination (Paule 1998; Grummt 1999; Paule and White 2000; Grummt 2003). At least four known basal transcription initiation factors are implicated in specific initiation of mammalian rDNA transcription: TIF-IA / Rrn3, TIF-IB / SL1, TIF-IC, and UBF (Upstream Binding Factor) (Figure 5) (Drygin et al. 2010).

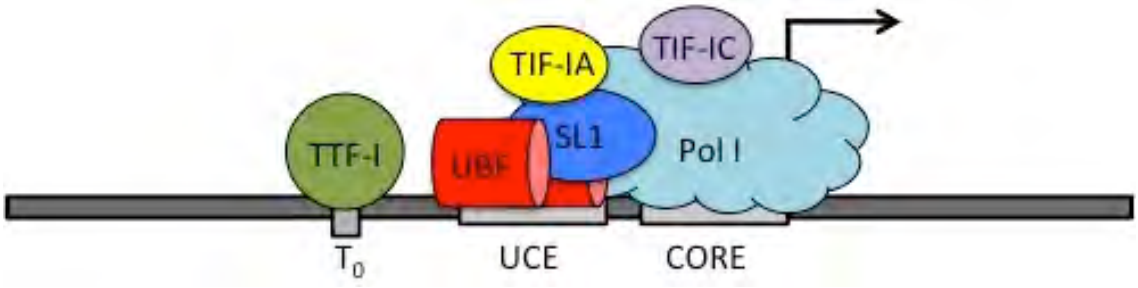

Figure 5. Basal RNA Pol I transcription factors

The scheme shows factors that are associated with the rDNA promoter and Pol I. TTF-I is associated with the upstream terminator T_0. Binding of UBF and TIF-IB/SL1 to the rDNA promoter is fundamental for the recruitment of RNA polymerase I, together with multiple Pol I-associated factors- to the transcription star site to initiate pre-rRNA transcription. Figure modified from Drygin et al. 2010.

The promoter selectivity factor TIF-IB / SL-I was shown to bind to the rDNA promoter and to confer promoter selectivity (Heix and Grummt 1995; Hannan et al. 1998). This binding is stabilized by the synergistic action of UBF (Kuhn and Grummt 1992). TIF-IB / SL1 is a complex of four polypeptides, the TATA-box binding protein (TBP) and the three Pol I specific TBP Associated Factors (TAFs), TAFI95/110, TAFI68, and TAFI48 (Paule 1998; Grummt and Pikaard 2003). In contrast to Pol II transcription, promoter recognition is carried out by the TAFIs and not by TBP.

The UBF factor is an essential component of basal transcription machinery. Functionally, it presents an exclusive role linked to RNA polymerase I activity (Moss et al. 2007). It is present not only at the promoter region, but surprisingly is

also abundant throughout the rDNA cluster (O'Sullivan et al. 2002; Mais et al. 2005). This factor interacts with the minor groove of DNA and binds to structured nucleic acids (Copenhaver et al. 1994; Kuhn et al. 1994). Through the binding and bending of DNA, dimers of UBF are able to form a loop of almost 360° once every 140 bp, creating a structure called the 'enhancesome' (Bazett-Jones et al. 1994). It is thought that the binding properties of UBF bring the CP and UCE into close proximity, and this aids TIF-IB / SL1 in recognizing the promoter. In mammals, UBF is ubiquitously expressed in two variant forms, UBF-1 and UBF-2, where UBF-2 is a product of an alternative splicing which lacks 37 amino acid residues in the second of six HMGB- boxes (O'Mahony and Rothblum 1991). UBF variants are able to associate as hetero- and homodimer for binding to the rDNA promoter, however it has been demonstrated that UBF-2 is five fold less transcriptionally active than UBF-1 (Hannan et al. 1996).

TIF-IA / Rrn3 is a key factor in growth-dependent regulation of rDNA transcription (Buttgereit et al. 1985; Schnapp et al. 1990; Milkereit and Tschochner 1998; Bodem et al. 2000). TIF-IA facilitates preinitiation complex formation and has been shown to interact directly with RNA Pol I and TIF-IB / SL1, thereby linking both protein complexes (Miller et al. 2001; Yuan et al. 2002).

TIF-IC is required for the assembly of the initiation complex, contributing to chain lengthening by stimulating elongation and suppressing RNA polymerase I pausing. This factor also inhibits nonspecific initiation and supports the synthesis of full-length, run-off transcripts (Schnapp et al. 1994; Paule 1998).

Moreover, it was demonstrated that the basal Pol II transcription factor TFII-H and CSB (Cockayne's syndrome B) are components of RNA Pol I transcription machinery, and they play important roles in mediating ribosomal RNA synthesis. In particular, CSB was shown to promote efficient rRNA synthesis *in vitro,* and TFII-H is required for the processivity of rDNA transcription, implying a post-initiation role in transcription (Bradsher et al. 2002; Iben et al. 2002).

Stepwise association of the four basal transcription factors and RNA Pol I to the promoter, mediated by protein-protein and DNA-protein interactions, leads to Pol I transcription initiation complex formation (Paule 1998). As a first step, TIFI-B and UBF bind cooperatively to the rRNA gene promoter, a process assisted by UBF; next RNA Pol I is recruited and TIF-IA and TIF-IC associate, hence forming the transcription initiation complex (Schnapp and Grummt 1991). In contrast to the stepwise assembly of the preinitiation complex, it is suggested that the transcription-initiation competent RNA Pol I exists as a preassembled complex, a 'holoenzyme', that is recruited to the rDNA promoter. In a variety of organisms, large complexes have been identified that contain RNA Pol I and most, if not all, components are required for specific rDNA transcription (Saez-Vasquez and Pikaard 1997; Seither et al. 1998; Albert et al. 1999; Hannan et al. 1999; Fath et al. 2000).

Transcription of rRNA genes is a highly regulated process for adapting cellular rRNA synthesis requirements to cell metabolism. Almost every protein required for RNA Pol I transcription is a target in a regulatory pathway. For instance, TIF-IA is a target of the TOR and MAP kinase signaling pathways.

Reversible phosphorylation of TIF-IA modulates its activity, thereby regulating its association with Pol I and controlling rDNA transcription (Grummt 2003). Acetylation of the TIF-IB subunit TAFI68 by PCAF enhances its DNA binding activity and increase RNA Pol I transcription, while deacetylation by Sir2 represses transcription (Muth et al. 2001). Phosphorylation and acetylation of UBF regulates its activity throughout the cell cycle (Voit et al. 1995).

5.5 Termination of rDNA transcription

The rDNA transcription is specifically completed at the terminator sites, which are located approximately 13 kb downstream of the initiation site. The terminator sites are called T_{1-10} or “Sal-box” elements, as mentioned above (Grummt et al. 1985; Grummt et al. 1986). Transcription termination is a multistep process, involving RNA Pol I pausing, release of both the pre- rRNA and RNA Pol I from the template, and 3´-end processing of the primary transcript (Grummt, 1999; Paule, 1998). The Transcription Termination Factor for RNA Pol I (TTF-I) and the Transcript Release Factor (PTRF) are important players in this process (Bartsch et al. 1988; Evers et al. 1995; Jansa et al. 1998). TTF-I binds specifically to the downstream terminators and this leads to pausing of the elongating RNA Pol I. PTRF interacts with both TTF-I and RNA Pol I. This interaction catalyzes the dissociation of the ternary complexes, thus finishing the termination of pre-rRNA synthesis (Jansa et al. 1998; Jansa and Grummt 1999).

5.6 The function of TTF-I at the rDNA promoter

TTF-I is a multifunctional factor that terminates RNA Pol I transcription, but also mediates replication fork arrest, exhibits contra-helicase activity, and regulates RNA Pol I transcription on chromatin (Bartsch et al. 1988; Gerber et al. 1997; Längst et al. 1997; Längst et al. 1998; Putter and Grummt 2002). It is a protein of 105 kDa that recognizes the terminator sequences (T_1-T_{10}) located downstream of the transcriptional unit of ribosomal genes (Grummt et al. 1986; Bartsch et al. 1988), as well as T_0 located within the rDNA promoter (Grummt et al. 1986). TTF-I exhibits a modular structure, consisting of a C-terminal DNA-binding domain (DBD) and a central domain that is required for transcription termination (Evers and Grummt 1995), transcriptional activation (Längst et al. 1997; Längst et al. 1998), and replication fork arrest (Putter and Grummt 2002). The N-terminal domain contains a negative regulatory domain (NRD) that manages interaction of TTF-I with DNA *in vitro* (Sander et al. 1996).

Early studies suggested that the binding of TTF-I to the T_0 element alters the local structure of chromatin, affecting nucleosomal positioning and thus facilitating the initiation of transcription, allowing a better access of the RNA Pol I to the transcription initiation region (Längst et al. 1997; Längst et al. 1998). Interestingly, some years later it was shown that TTF-I interacts with Tip5 protein (TTF-I Interacting protein 5) through the NRD domain (Strohner et al. 2001). The Tip5 protein together with SNF2h subunit forms the nucleolar chromatin remodeling complex NoRC, a chromatin remodeling complex that silencing rDNA. The association of Tip5 with NRD facilitates DNA binding of TTF-I and consequently

leads to the recruitment of NoRC to the rDNA promoter, thus silencing ribosomal genes (Nemeth et al. 2004). Taking into account this fact, TTF-I plays a dual role in rDNA transcription regulation in chromatin; TTF- I is involved in both activation and silencing of rDNA transcription.

Recent studies on the regulation of rRNA transcription show that epigenetic modifications are correlated with the 3D topology of the rRNA genes. In this situation, it is now speculated that the factor TTF-I mediates the formation of loops that bring transcription termination elements and the basal promoter into the rRNA genes, and which is also a structure that would facilitate a more efficient restarting of rRNA transcription (Németh et al. 2008). Clearly, this assumption is based on the fact that ribosomal cistron possesses elements recognized by TTF-I (T elements) in both 5' and 3' of the transcriptional unit. In this context, TTF-I has been proposed as playing a central role in the control of the transcriptional activity of ribosomal genes (McStay and Grummt 2008).

5.7 Chromatin structure of ribosomal genes

Different studies have shown that chromatin architecture and epigenetic mechanisms are major actors in the establishment and maintenance of transcriptional activity or inactivity of the ribosomal genes (Birch and Zomerdijk 2008).

The chromatin states of genes transcribed by RNA Pol II are generally summarized as being in two basic states: transcriptional active (euchromatin) or transcriptional silenced chromatin (heterochromatin). It is generally accepted that both chromatin states refer to the packaging of genes within nucleosomal structure. However, increasing evidence suggests that chromatin containing rRNA genes can exist in three different states. The rDNA can be in an inactive state, like heterochromatin, or in two euchromatin states that include transcriptional facultative chromatin (but not active) and transcriptional active state (Moss 2004; Cavanaugh et al. 2008). These different rDNA compaction states were earlier observed by Conconi *et al.* by means of psoralen photocrosslinking experiments in mouse cells, and in which compact nucleosomal forms of rDNA cross-linked with psoralen migrate differently than uncrosslinked nucleosomal DNA on agarose gels (Conconi et al. 1989).

The active chromatin of the ribosomal genes is transiently erased during replication when the newly synthesized daughter strands are re-packaged into nucleosomes (Lucchini and Sogo 1995). Regeneration of the active chromatin structure along the coding region occurs after replication and involves the disruption of pre-formed nucleosomes. Studies in yeast with an RNA Pol I deficient strain indicate that the establishment of the open chromatin conformation on the activated gene copies requires transcribing polymerase molecules (Dammann et al. 1995).

The active and inactive rRNA gene copies are randomly distributed rather than organized or grouped within a NOR (Dammann et al. 1995; French et al.

2003). Analysis of a single, tagged transcription unit within the tandem array in yeast revealed a random distribution of active and inactive copies throughout the ribosomal rRNA gene locus (Dammann et al. 1995).

Recently, studies concerning the chromatin state of rRNA genes have provided evidence indicating that “epigenetic” strategies could be playing a crucial role in this process (McStay and Grummt 2008).

5.8 Epigenetic Mechanisms

The term “epigenetic,” which means “outside conventional genetics,” is actually used to describe the study of stable alteration in gene expression that arises during developmental and cell proliferation (Jaenisch and Bird 2003).

Epigenetic mechanisms show that proteins or protein complexes are intimately associated with the DNA molecule, and they can act as gene transcription regulators. Additionally, it has been proposed that external influences have an effect on epigenetic transcriptional regulation, and moreover, this could be extremely important on long-term diseases such as cancer. Thus, epigenetic mechanisms seem to allow an organism to respond to the environment through changes in gene expression (Jaenisch and Bird 2003).

Perhaps the best-known specialized proteins associated with DNA are the histones, which interact with DNA to form the complex defined as chromatin. The histones allow for the compaction of DNA into a basic unit called the nucleosome,

which corresponds to the first level of packaging in the formation of the chromatin structure. Specifically, the nucleosomal particle is structured as an octamer through a combination of two copies each of the histones H2A, H2B, H3, and H4, each with a section of DNA of 146 bp (Luger et al. 1997). Thus, this basic unit of chromatin plays a crucial role in nuclear processes such as replication, transcription, recombination, and DNA repair. Consequently, the cell is able to modulate these processes through "epigenetic" mechanisms of regulation such as the incorporation of histone variants to the nucleosome particle, post-translational modifications of histones, methylation of DNA, and the recruitment of chromatin remodeling complexes (Turner 2007).

Despite the stability of nucleosomes and the high chromatin compaction in the nucleus, chromatin is surprisingly dynamic (Kimura 2005). In this context, the incorporation of histone variants can generate a chromatin that could display a singular local or global architecture and functionality. Histone variants are encoded separately from the canonical forms, and they differ in their sequences (Malik and Henikoff 2003). Moreover, in contrast to the canonical histones, these variants are synthesized and assembled in nucleosomes independently of DNA replication (Jin et al. 2005). In addition, some variants display profound differences from their canonical histones either by the mechanism through which they are deposited into nucleosomes or by their function after being deposited in the chromatin (Henikoff and Ahmad 2005). Several histone variants are enriched in specific chromosomal locations; for example, centromeric chromatin contains the histone H3 variant CENP-A (centromer protein A), which is essential for centromer structure and

function (Palmer et al. 1991; Ahmad and Henikoff 2001). MacroH2A, a histone H2A variant, is enriched in the nucleosomes of inactive X chromosomes (Costanzi and Pehrson 1998). The histone H3 variant H3.3 accumulates on highly transcribed regions (Ahmad and Henikoff 2002).

On the other hand, post-translational modifications of histones consist of covalent modifications at both N-or C-terminal ends of the four histones. A rich variety of post-traslational modifications exist, and which are acetylation, phosphorylation, ADP-ribosylation, methylation, and ubiquitination (Strahl and Allis 2000; Fischle et al. 2003; Vaquero et al. 2003). The combination of different post-translational modifications can be associated specifically with a transcriptional activation or repression. The impacts of certain modifications on gene regulation and the discovery of factors mediating histone modifications ('histone modifying proteins') have emerged through the last decade (Vaquero et al. 2003). Most covalent modifications are reversible, except for methylation. The most prominent and well studied are acetylation and methylation of lysine residues in the highly conserved amino termini of histone H3 and H4. At present, these post-translational modification profiles have led to the establishment of a new concept termed "the histone code" (Strahl and Allis 2000).

Another epigenetic mechanism is the methylation of DNA. This occurs in the CpG dinucleotide at C-5 of the cytosine ring (5mC), which eventually occurs in almost all eukaryotic organisms. Because this modification is widely distributed in the genomes of vertebrates, each organism displays a characteristic pattern of methylation (Bird 2002). Methylation occurs in several promoter regions of genes

and is similarly found in the promoter of the rDNA gene. DNA methylation plays an important role in gene silencing (Bird and Wolffe 1999). In particular, this epigenetic marker is recognized by methyl CpG binding proteins (MBD) that bind methylated promoter regions, thus preventing binding of transcription factors necessary for transcription initiation (Wade 2001). Interestingly, silenced rDNA genes have been shown to be methylated (Grummt and Pikaard 2003); this methylation modification occurs at -133 CpG in the rDNA promoter and has been observed to reduce UBF binding to the UCE (Santoro and Grummt 2001). Consequently, this epigenetic marker should lead to the inhibition of the formation of the pre-transcriptional initiation complex.

An alternative epigenetic mechanism is performed through the recruitment of chromatin remodeling complexes. Because the chromatin needs to be fluid to enable DNA-dependent processes, a broad group of enzymes exist that use the energy of ATP hydrolysis to alter histone-DNA interactions within the nucleosome (Becker and Horz 2002). The process of catalyzing these chromatin dynamics is known as 'ATP-dependent nucleosome remodeling.' The subsequent biochemical reactions lead to the mobilization of histone octamers and to their exact positioning to DNA regulatory elements, providing or restricting regulatory factors access to their sites.

The molecular machines that rearrange the nucleosome structure are called ATP-dependent chromatin remodeling factors. All ATP-dependent chromatin remodeling factors identified so far are multiprotein complexes consisting of 2 to 12 subunits and contain a related motor protein that belongs to the SNF2 family of

ATPases (Eisen et al. 1995). Members of the chromatin remodeling enzymes are found in all eukaryotes where they participate in many DNA-mediated processes like transcriptional regulation, DNA repair, homologous recombination, and chromatin assembly (Tsukiyama 2002; Lusser and Kadonaga 2003). They are classified according to protein domains outside of the ATPase region. At least four major classes of catalytic subunits of chromatin remodeling complexes belonging to the family SNF2 are distinguished: the Swi/Snf family, the Mi-2 / CHD family, the ISWI class, and the Ino80 group (Kwon et al. 1994; Wade et al. 1998; Deuring et al. 2000).

5.9 Epigenetic control of ribosomal genes

An increasing number of studies seem to demonstrate that different epigenetic mechanisms can act coordinately to regulate transcription of rDNA genes (Hirschler-Laszkiewicz et al. 2001; Santoro and Grummt 2001; Lawrence and Pikaard 2004; Grummt 2007; McStay and Grummt 2008). In this context, the chromatin remodeling complex NoRC (nucleolar remodeling complex) can silence rDNA genes through the recruitment of DNA methyltransferase and histone deacetylases to the rDNA promoter, and thus contribute to the establishment of a closed chromatin state (Santoro and Grummt 2005). In particular, NoRC was demonstrated to induce DNA methylation of a CpG dinucleotide at position -133 in the core region of the rDNA gene promoter, an epigenetic mark that has been implicated in the silencing of murine rRNA genes (Santoro and Grummt 2001).

However, this epigenetic mechanism seems to be used for long-term rDNA gene silencing. This is corroborated by a recent study where depletion of UBF-1 (but not UBF-2) results in a stable silencing of rRNA genes independently of CpG methylation, and thus becoming reversible (Sanij et al. 2008). Restoring UBF levels rescues the wild-type ratio of active to inactive genes.

Another example of epigenetic control of rDNA genes can be mediated by the TTF-I factor. As mentioned above, this protein recognizes the “T” elements in the ribosomal cistron. Binding of TTF-I in the vicinity of the proximal promoter of rDNA is a key step in the activation of the transcription start (McStay and Grummt 2008). Moreover, the fact that there are TTF-I binding elements located at each end of the rRNA gene suggests a functional link between transcription initiation and termination (Németh et al. 2008). In this context, it has been proposed that TTF-I coordinates the recruitment of the NoRC remodeling complex for rDNA silencing, as well as with the activation of ribosomal transcription (Bradsher et al. 2002; Németh et al. 2008; Nemeth and Langst 2008).

Among epigenetic mechanisms associated with the regulation of active and silent ribosomal genes, chromatin-remodeling complexes seem to contribute significantly to the regulation of rRNA transcription. In this context, a major complex involved in the silencing of rDNA was identified as NoRC (Strohner et al. 2001). The NoRC complex is a member of the family of ATP-dependent remodeling factors ISWI. It is composed of two subunits, the ATPase SNF2h and a 205 kDa protein termed Tip5 (TTF-I Interacting protein 5). This complex has been described only in the mouse and human (Santoro and Grummt 2001; Strohner et al. 2001),

but according to their functionality, this should be present in all eukaryotes as an important element of the transcriptional control of ribosomal genes (Strohner et al. 2004). The Tip 5 subunit is restricted for the NoRC complex, and instead, SNF2h is one of the best ATPase subunits studied which is also present in other remodeling complexes (Vignali et al. 2000). The Tip5 protein is organized in a large number of protein domains, similar to other proteins belonging to remodeling complexes, such as ACF, WCRF, CHRAC, and WICH (Clapier and Cairns 2009). Within these domains, we can find the TAM domain (a methyl binding domain that binds to DNA), AT hooks (that interact with TTF-I as well as with the DNA), BAZ1 and BAZ2 motifs (that interact with the SNF2h subunit), WAKZ motif, a Plant Homeodomain (PHD), a C-terminal bromodomain, and a DDT domain (a DNA binding domain) (Strohner et al. 2001).

In terms of function, NoRC interacts with TTF-I through the Tip5 subunit, which leads to its recruitment in the rDNA promoter (Nemeth et al. 2004; Strohner 2004). This binding allows NoRC to inhibit the transcription of RNA Pol I (Santoro et al. 2002). Concordantly, a Tip5 overexpression in human cells establishes heterochromatic features and silences rRNA transcription (Santoro and Grummt 2005). Additionally, it has been observed that rRNA transcription repression by NoRC does not occur in the presence of inhibitors of DNA methyl transferase (DNMT) and histone deacetylase, respectively. This feature suggests that NoRC exerts its function by promoting DNA methylation and deacetylation of histones. In the same way, it was reported that NoRC physically interacts with DNMT1 and DNMT3, as well as with Sin3 (a co-repressor complex), which contains the histone

deacetylase HDAC1 and HDAC2 (Santoro et al. 2002; Zhou et al. 2002). In summary, these findings seem to indicate that NoRC serves as a coordinator of the recruitment of macromolecular complexes that modify histones, methylate DNA, and determine a closed chromatin state.

On other hand, non-coding RNA (ncRNA) has received increasing attention because it has a diverse range of functions and participates in many biological pathways (Moulton 2005). Recent analyses of mammalian transcriptomes have revealed that the majority of the genomes of mammals and other complex organisms are transcribed into ncRNAs. These RNAs have an important role in epigenetic control and in the modulation of gene expression, tissue-specific patterning, and cell fate specification (McStay and Grummt 2008). A large number of transcripts are outside any known gene regions (Kapranov et al. 2002; Kampa et al. 2004), which implies that ncRNA genes are widely distributed in the genome. In the epigenetic field, non-coding RNAs have been involved in the formation of specialized chromatin domains in processes as diverse as dosage compensation, RNA interference-mediated heterochromatin assembly and gene silencing, and programmed DNA elimination (Bernstein and Allis 2005). In mouse cells, it has been observed that long transcripts of IGS which originated 2 kb upstream of the initiation site of the transcription of rRNAs plays an important role in heterochromatin formation and rDNA silencing (Mayer et al. 2006). These transcripts are synthesized by RNA Pol I and usually do not accumulate *in vivo* (Morgan et al. 1983; Kuhn and Grummt 1987; Paalman et al. 1995). Most probably, these transcripts are processed into small pieces that are either rapidly degraded

or protected from degradation by binding to proteins. A segment of the IGS transcripts of about 100 nucleotides, and whose sequence matches with rDNA promoter sequence, have been called “promoter associated RNA” (pRNA). This pRNA is stabilized by binding to the subunit Tip5 of the remodeling complex NoRC, which recognizes a stem loop structure of the pRNA, and this interaction results as crucial for the function of NoRC (Mayer et al. 2008).

In summary, the epigenetic processes involved in the regulation of rDNA genes may function as a key strategy to implement “phenotypic plasticity,” whereby organisms can respond to the environment via changes in gene expression (Hochachka and Somero 2002; Cossins and Crawford 2005).

Therefore, this thesis provides new evidence for elucidating the molecular mechanisms involved in the complex process of seasonal acclimatization.

6 HYPOTHESIS AND OBJETIVES

6.1 Hypothesis

The transcriptional regulation of ribosomal genes remains a central question in terms of the molecular mechanisms that underlie the seasonal adaptation of *Cyprinus carpio*. Recent evidence suggests that epigenetic mechanisms are the basis of transcriptional control for the rRNAs, and particularly those associated with the remodeling of chromatin. The active participation of specific remodeling complexes in the rDNA gene (such as NoRC) for maintaining the balance between active or silent rDNA genes is emerging as one of the central mechanisms of regulation. However, this remodeling complex is coordinated by an additional molecular factor, factor TTF-I.

While understanding the regulatory mechanisms of rDNA gene expression is not fully elucidated, the epigenetic study of these mechanisms in the context of the natural genetic reprogramming process of the carp represents an excellent opportunity to deepen the scope of understanding. Thus, the hypothesis of this thesis postulates that **"The factor TTF-I and the chromatin remodeling complex NoRC contribute significantly to the negative regulation of transcriptional expression of rRNAs during the seasonal adaptation of *Cyprinus carpio***."

6.2 Objectives

The aim of this doctoral thesis was to study the epigenetic regulation of ribosomal cistron in the process of acclimatization of *Cyprinus carpio*.

6.2.1 Specific Objectives

I. - Isolate and characterize the cDNA sequence encoding carp factor TTF-I.

II. - Analyze the seasonal expression of factor TTF-I.

III. - Evaluate the seasonal expression of Tip5.

IV. - Evaluate the interaction between the cis elements for termination of transcription T_0 and T_0' with factor TTF-I.

V. - Isolate and characterize the sequence encoding the pRNA of *C. carpio*.

VI. - Evaluate the expression of the pRNA transcript during acclimatization.

7 MATERIALS AND METHODS

7.1 Materials

7.1.1 Specimens

Male carp fish with a weight of about 1-2 kg and a size around 40-50 cm were caught during the winter and summer seasons. They were held under natural conditions of acclimatization (Colina, Santiago) for at least four weeks before experimental procedures. To acclimatize the carp, they were kept in a temperature range of 6-8 ºC and 18-25 °C for winter and summer, respectively. Samples of liver tissue from acclimatized fish were dissected, cleaned, and frozen at -80 ° C until use. All fish manipulation protocols were approved by the Bioethical Committee of the Universidad Andrés Bello.

7.1.2 Chemicals and biochemical Reagents

Unless otherwise stated, all common chemicals and materials were purchased from Merck (Germany), Invitrogen (USA), Sigma Aldrich (USA), and Winkler (Chile).

Table I. Overview of standard chemicals and biochemical used

Acrilamide/Bis-acrilamide	Bio-Rad
Agarose	Winkler
Ampicilin	Sigma Aldrich
Basic Phenol/Chloroform	Winkler
Boric Acid	Winkler
Bromophenol blue	Merck
BSA	Sigma Aldrich
Coomasie Blue	Sigma Aldrich
DEPC water	Winkler
DNA Ladder GeneRuler 1kb Plus	Fermentas
dNTPs	Bioline

DTT	Bio-Rad
EDTA	Sigma Aldrich
EGTA	Sigma Aldrich
Ethidium Bromide	Merck
Formaldehyde	Merck
Formamide	Merck
Glicine	Winkler
Glycerol	Merck
Glycogen	Merck
HEPES	Winkler
Imidazole	Bio-Rad
IPTG	Sigma Aldrich
Ni-NTA Agarose	Qiagen
Nonidet P-40	Sigma Aldrich
Nuclease free water	Winkler
Oligo (dT)	Promega
Page Ruler Prestained Protein Ladder	Fermentas
PMSF	Roche
Protein A agarose	Santa Cruz Biotechnology
SDS	Sigma Aldrich
Sodium Deoxycolate	Sigma Aldrich
TEMED	Sigma Aldrich
TRIS	Winkler
Triton X-100	Sigma Aldrich
Trizol®	Invitrogen
Trypan blue	Invitrogen
Tween-20	Sigma Aldrich
UREA	Sigma Aldrich
X-Gal	Sigma Aldrich
Yeast tRNA	Sigma Aldrich
β-Mercaptoethanol	Merck

7.1.3 Enzymes

Table II. List of recombinant enzymes

Brilliant SYBR Green QPCR Master Mix	Stratagene
DNAse I	Sigma Aldich
Go Taq® DNA Polymerase	Promega
AMV Reverse Transcriptase	Promega
Paq5000 polymerase	Stratagene
Proteinase K	Sigma Aldrich
Restriction Endonucleases	New England Biolabs, Promega, Fermentas
RNAsa A	Sigma Aldrich
T4 DNA Ligase	Promega
Taq DNA polymerase	Promega

7.1.4 Blotting materials

Table III. Materials used in blotting experiments

Nitrocellulose Membrane	Schleicher & Schuell
Whatman 3MM paper	Whatman

7.1.5 Antibodies

Table IV. List of used antibodies

α-H2B	Abcam
α-H3K4me3	Abcam
α-H3K9me3	Abcam
α-Rabbit IgG (H+L), Peroxidase labeled	KPL
α-Tip5 (mouse)	Non commercial (Dr. Längst)
α-TTF-I (carp)	Non commercial

7.1.6 Plasmids

Table V. List of plasmids used for experiments

pGEM-T TTF-I
pGEM-T TTF-I C terminal
pGEM-T Tip5 partial
pET15b TTF-I peptide
pET15b TTF-I

7.1.7 Kits

Table VI. Kits for standard applications

DC protein assay	Bio-Rad
Enhanced Chemi-Luminescence (ECL)	Amersham
FirstChoice® RLM-RACE Kit	Ambion
Plasmid isolation Kit	Qiagen, Invitrogen
Plasmid purification Kit	Qiagen
Wizard SV Gel and PCR Clean-Up System	Promega

7.1.8 Oligonucleotides

The oligonucleotides used in PCR reactions were derived from *Danio rerio* sequences because the genome of *Cyprinus carpio* is not still available. All oligonucleotides were purchased from IDT (USA) and Eurofins MWG Operon (Germany), and diluted to final solutions of 100 μM with ddH2O.

Table VII. List of synthesized oligonucleotides

I3F	TGAGCTGACTGGTCGAAGTGC
135pRNA Fow	GCGATTTTGGCGAACGCGTCATTT
167pRNA Fow	CCCGAGAGGGGATGGCGTTTTTAC
16s Fow	GGGGTTTACGACCTCGATGTT
16s Rev	GCTTTAAGTATGGGCCCCCCT
78pRNA Fow	AACGGATCGATTGGAACGCGT
E3F	AAAGGATCGCGGA AGGAATTCCC
E5R	TCTGCGTCAGAGTATCTTCCCA
I5R	CTGCGTCAGAGTATCTTCCCAT
pre-RNA Fow	GGTACAGTGAAACTGCGAATGGCT
pre-RNA Rev	CCCGGATGGGTTTTGGATCTGATA
pRNA Rev	TAGGTGCACCCGCCCACAGC
qPCR Tip5 Fow	GAAACCAGCCTGACCTCACATACT
qPCR Tip5 Rev	CTGCAGAAGTCGTTCCCTCATAGT
qPCR TTF-I Fow	AAAGGATCGCGGA AGGAATTCCC
qPCR TTF-I Rev	TAAGCACTTCGACCAGTCAGCTCA
T0 Fow	AGGGGCCGTACTGAGAGATG
T0 Fow probe IR700	TCCGTTCACGGGGTGACCAGTGGCAGGTCG
T0 mut Fow probe IR800	TCCGTTCACGGGGTTTTTTTTGGCAGGTCG
T0 mut Rev probe	CGACCTGCCAAAAAAAACCCCGTGAACGGA
T0 prima Fow	AGATGGCGCCCTACTACTCC
T0 prima Rev	CCCCTTTCCGACTTAGTCATT
T0 Rev	GATAGGCCTCTCGCCTCATA
T0 Rev probe	CGACCTGCCACTGGTCACCCCGTGAACGGA

T0p Fow probe IR700	TCCGTTCGCGGGTTGACCAGTGGCAGGTCG
T0p mut Fow probe IR800	TCCGTTCGCGGGTTTTTTTTTGGCAGGTCG
T0p mut Rev probe	CGACCTGCCAAAAAAAAACCCGCGAACGGA
T0p Rev probe	CGACCTGCCACTGGTCAACCCGCGAACGGA
T1 Fow probe	GTCGATGGGGGGTGACCAGCGGCGGGGAAG
T1 Rev probe	CTTCCCCGCCGCTGGTCACCCCCCATCGAC
Tip5Fow	AGGTCCAGCTCAAGTACTTCAC
Tip5Rev	CTATTTGTCTTTGTTGTCGTAAAACTCC
TTF-I BamHI	TCTCCCGGATCCTCAAGAGGAGTTGTCATCTTCTTTCTGG
TTF-I BamHI pept.	TCTCCCGGATCCATGGTCACGCACAGCCCTTAAAAGTC
TTF-I NdeI	TCTCCCCATATGTTAGATGAAATGCAGTCAGATTCGC
TTF-I NdeI pept.	TCTCCCCATATGTTTCATCCCAAACGGTTCCCAAAAG

7.1.9 Bacteria

Table VIII. Bacterial strains used for plasmid propagation

BL21 (DE3)	Protein Expression
DH5α	General DNA plasmid propagation
JM109	General DNA plasmid propagation
Rosetta 2(DE3) pLyss	Protein Expression

7.1.10 Standard solutions

Stock solutions and buffers were made according to standard protocols. (Sambrook and Russell 2001; Roche 2010) Protease Inhibitors (either Complete® EDTA-free (Roche, USA) or mix Leupeptin 0.5 µg/ml, Pepstatin 1 µg/ml, Aprotinin 1 µg/ml, and PMSF 0.5 mM) were freshly added. Common solutions are listed below. Special buffers are described in the individual method sections.

Table IX. Standard buffers and solutions

Buffer	Composition
Phosphate Buffered Saline (PBS)	140 mM NaCl 2.7 mM KCl 8.1 mM Na_2HPO_4 1.5 mM KH_2PO_4 pH adjusted to 7.4 with HCl
TBE-buffer	90 mM Tris 90 mM Boric acid 2 mM EDTA
DNA sample buffer (10x)	50 % glycerol 50 mM Tris-HCl pH 7.6 10 mM EDTA 0.05% (w/v) bromophenol blue, xylene cyanol and Orange G
Orange G loading dye (10x)	50 % glycerin 10 mM EDTA 0.05 % (w/v) Orange G
SDS-protein loading buffer (5x)	300 mM Tris-HCl pH 6.8 10 % (w/v) SDS 50 % glycerol 5 % β-Mercaptoethanol 0.2 % (w/v) bromphenol blue
SDS-PAGE stacking buffer (4x)	0.5 M Tris-HCl 0.4 % SDS pH 6.8 with HCl
SDS-PAGE separating buffer (4x)	1.5M Tris-HCl 0.4 % SDS, adjust to pH 8.8 with HCl
SDS-PAGE running buffer	192 mM glycine 25 mM Tris 0.1 % (w/v) SDS
Coomassie staining solution	45 % water 45 % methanol 10 % acetate acid 0.1 % (w/v) coomassie blue R250
Coomassie destain solution	45 % water 45 % methanol 10 % acetate acid

7.2 Methods

7.2.1 Hepatocyte nuclei isolation

3 g of liver tissue per sample were homogenized in 10 mL of H buffer (20 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 3 mM $CaCl_2$, 0.1 mM PMSF) using a mechanical homogenizer. Then, the homogenate was filtered through sterile gauze and was centrifuged at 228 g for 5 min at 4 ° C. The pellet obtained was washed in PBS at least three times. To confirm the purity and integrity of nuclei, we proceeded to stain it with Trypan blue. When the samples were used in immunoprecipitation assays, quantified the nuclei in a Neubauer chamber. However, if the samples were used in Western blot analysis, the nuclei were sonicated (Misonix XL 2020, USA) and then the nuclear protein content was quantified. For the preservation and storage of nuclei, they were resuspended in a volume of 2 M sucrose solution and cooled to -20 ºC.

7.2.2 Nuclear protein quantification

Nuclear proteins were quantified using the DC Protein Assay Kit (Bio-Rad, USA) according to manufacturer's instructions. The samples were measured in triplicates at an absorbance of 630 nm in a microplate reader, Model ELX 800 (BioTek Instruments, Inc., USA).

7.2.3 SDS-PAGE

15-20 µg of nuclear proteins were mixed with SDS-protein loading buffer and heated for 5 min at 100 °C. Proteins were fractionated on a polyacrylamide gel prepared at denaturing conditions. The percentage of gels varied depending on the experiments performed. The electrophoretic run was performed for 2 h at 100V with SDS-PAGE running buffer.

After electrophoresis, the gels were stained for 30 min in Coomassie staining solution. Subsequently, the gels were treated with Coomassie destain solution. The protein profiles were visualized in a white light transilluminator.

7.2.4 Western blot

Proteins were separated by SDS-PAGE and transferred to nitrocellulose filters using the Bio-Rad Western blotting system. The gel was placed onto a membrane and sandwiched between gel-sized Whatman papers soaked in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). The proteins were then transferred onto the membrane for 1 h (120 V constant) at 4 °C. The transfer reaction was cooled by the addition of an ice block into the transfer chamber. After transfer, nitrocellulose filters were incubated for 1 h in blocking solution (1x PBS, containing 5% dried milk and 0.2% Tween-20) in order to reduce the non-specific background. Filters were incubated for 1 h with an appropriate dilution of the primary antibody directed against the protein of interest (α-TTF-I, α-Tip5, α-H2B). Filters were washed three times in PBS-Tween for 10 min each and incubated for one additional hour with horseradish peroxidase-coupled secondary antibody (KPL, USA). After 3 washes (10 min each, in PBS-Tween) antigen-antibody complexes were detected using ECL kit (Amersham, UK) and autoradiography according to the instructions given. All steps were performed at room temperature.

7.2.5 DNA standard procedures

Preparation of competent bacteria, transformation of chemically-competent bacteria with DNA, amplification of plasmid DNA in bacteria, purification, concentration determination, restriction enzyme digestion, ligation of DNA fragments, analysis of DNA on agarose and polyacrylamide gels, and amplification

of the DNA by the polymerase chain reaction (PCR) were performed according to the standard protocols (Sambrook and Russell 2001; Roche 2010).

7.2.6 Determination of 5` and 3` extremes of carp TTF-I transcript

First, the purification of total RNA using Trizol® (Invitrogen, USA) was performed from 100 mg of liver of acclimatized carp according to manufacturer instructions. Then, RNA was resuspended in 50 µL of nuclease free water, and the total RNA concentration was measured by spectrophotometry at 260 nm. For the integrity of RNA, 5 µg of total RNA were loaded in an agarose gel (1,5% w/v) with formaldehyde (1,75% v/v).

After obtaining the total RNA, the FirstChoice® RLM-RACE kit was used, which allowed a rapid amplification of cDNA ends. The protocol was used according to manufacturer's instructions. For to determine the 5` terminal region of carp TTF-I, the cDNA obtained was used as template for PCR reaction by using as a sense, oligonucleotide 5`RACE outer primer (specific adapter) and as gene specific antisense, oligonucleotide E5R. Reactions were performed in a volume of 25 uL containing 50 mM KCL, 20 mM Tris-HCL pH 8.4, 1.5 mM $MgCl_2$, 25 mM of each dNTP`s, 0.4 µM of each oligonucleotide, 1,5 µL cDNA template, 1,25 U of Taq DNA polymerase, and 1.25 U of Paq5000 polymerase. The reactions were performed in a Eppendorf Mastercycler personal thermocycler (Eppendorf, Germany), using the following protocol: initial denaturation for 2 min at 95 ºC, denaturation 30 s at 95 ºC, annealing 45 s at 55 ºC, extension 90 s at 72 ºC for 35 cycles, and a final extension for 10 min at 72 ºC. Subsequently the nested PCR reaction was performed using the product obtained above as a template and as a sense, oligonucleotide 5`inner primer (specific adapter) and as gene specific antisense, oligonucleotide I5R. PCR conditions were the same as described above.

For to determine 3` terminal of carp TTF-I, reverse transcription used oligonucleotide complementary to the end of polyadenylated mRNA (oligo dT). The cDNA obtained was used as a template for PCR reaction using as a gene specific sense, oligonucleotide E3F and as antisense, oligonucleotide 3`RACE outer primer (specific adapter). Then, the nested PCR was performed using as a template the product obtained above and I3F and as gene specific sense, oligonucleotide and as the antisense, oligonucleotide 3`inner primer (specific adapter). The conditions and thermal profile of the PCR reaction were the same as previously used in the 5` terminal region.

7.2.7 Carp TTF-I antibody synthesis

7.2.7.1 Cloning

Using cDNA as a template, a 354 bp region corresponding to the C-terminal region of carp TTF-I was amplified through PCR. The oligonucleotides designed contain sequences that have restriction sites that facilitate cloning. The reaction mixture contained 20 pmoles of each oligonucleotide (TTF-I NdeI pept. and TTF-I BamHI pept.), 0,2 mM each dNTP, 1,5 mM $MgCl_2$, 1 U Taq DNA polymerase, and 1 µL of cDNA synthetized. The PCR conditions were an initial denaturation of 10 min at 95 ºC, denaturation 2 min at 95 ºC, annealing 45 s at 56 ºC, and extension 20 s at 72 ºC, for 30 cycles. The final extension was 10 min. at 72 ºC. The amplification reactions were performed in a Master cycler personal thermocycler (Eppendorf, Germany). The amplicon was cloned into pGEM-T easy vector (Promega, USA), and the ligation was used to transform JM109 competent cells. The insert of this clone was released and ligated in pET-15b, the expression vector (Novagen, Germany).

7.2.7.2 Protein expression and purification

BL21 (DE3) competent cells were transformed with the pET-15b TTF-I expression vector, which contains the 354 bp insert that encodes 116 residues in a peptide corresponding to the C-terminal region of carp TTF-I.

For the induction of the TTF-I, recombinant protein were inoculated in 2 L of minimum medium (200 mL of 5x M9 salt, 2 mL 1 M $MgSO_4$, 20% glucose, and 0,1 mL 1 M $CaCl_2$) with 50 mL of preculture at 37 ºC with shaking until a OD=0,6. After reaching the optimal optical density, the culture was induced with 1 mM IPTG (final concentration) for 4 h at 37 ºC (Figure 6).

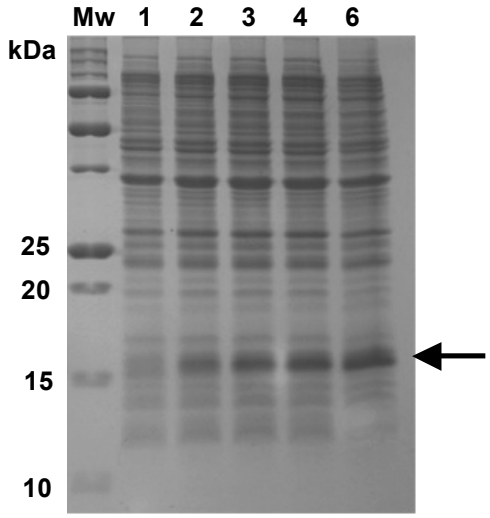

Figure 6. *C. carpio* TTF-I peptide induction

Lysed bacteria extracted to different times of induction were fractionated in a 12% SDS-PAGE gel. The first lane corresponds to the molecular weight marker (MW), Lane 1 culture without induction, Lanes 2 to 6 correspond to different aliquots of the induction kinetics (1 to 4 h), The arrow indicates the overexpression of the recombinant TTF-I carp peptide.

The culture obtained was centrifuged and the purification was performed under denaturing conditions. The pellet was resuspended in 5 mL of B Buffer pH

8.0 (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M Urea, 20 mM Imidazol) for each gram of bacterial pellet. Bacteria were lysed by sonication at 40% intensity at intervals of 30 s 25 times (Misonix 2020 XL, USA). The lysate were centrifugated at 10.000 g for 30 min at room temperature. In parallel the resin was prepared by using1 mL of Ni-NTA resin for each 4 mL of lysate. The resin was washed 3 times with B buffer. Subsequently, the supernatant (containing recombinant protein) was incubated with Ni-NTA resin for 1 h with gentle shaking at room temperature. Then, Ni-NTA resin containing TTF-I recombinant protein bound was washed 2 times with 4 mL of C buffer pH 6,3 (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M Urea, 20 mM imidazole) and eluted with D buffer pH 5.9 (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M Urea) 4 times with 0,5 mL, followed by 4 elutions with 0,5 mL of E buffer pH 4,5 (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M Urea). Purity of the recombinant protein was assessed by SDS-PAGE gels at 12% (Figure 7).

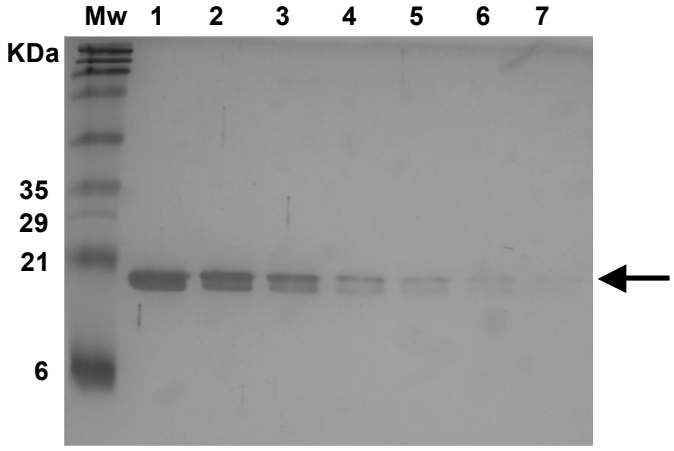

Figure 7. Purification of *C. carpio* TTF-I peptide

Individual fractions eluted from the Ni-NTA column were electrophoretically fractionated on a 12% SDS-PAGE gel. MW: molecular weight marker; lanes 1 to 7 represent different fractions of the purified protein. The arrow indicates recombinant TTF-I carp peptide.

The purified carp TTF-I recombinant protein was used as an antigen to generate an antibody. The generation of this antibody was through the service offered by GrupoBios S.A. (Chile).

7.2.8 Expression and purification of recombinant carp TTF-I

7.2.8.1 Cloning

For isolating the complete coding sequence of carp TTF-I, primer from the sequence obtained in the RLM-RACE protocol were designed. The PCR reaction mixture was the same used above in the protocol of cloning of carp TTF-I peptide. The oligonucleotides used were NdeI TTF-I and BamHI TTF-I. The PCR conditions were initial denaturation 10 min at 95 ºC, denaturation 2 min at 95 ºC, annealing 45 s at 59 ºC, extension 100 s at 72ºC, for 30 cycles. The final extension was 10 min at 72 ºC. The amplicon was cloned into pGEM-T easy vector (Promega, USA), and the ligation was used to transform DH5α competent bacteria.

The insertion was released and ligated into pET-15b expression vector (Novagen, Germany). Rosseta 2 (DE3) pLys competent cells were transformed with this vector that contain the complete coding sequence of carp TTF-I.

7.2.8.2 Expression

For the induction of TTF-I complete sequence, 1 L of LB medium were inoculated with 100 mL of preculture at 37 ºC with shaking until OD=0,6. Then the culture was induced with 1 mM IPTG. Subsequently the culture was incubated for 4 h at 24 ºC. Finally, the culture obtained was centrifuged and the pellet was frozen in liquid nitrogen and stored at -80 until the purification.

7.2.8.3 Purification

The pellet was resuspended in 100 mL pre-cooled lysis buffer pH 8.0 (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM Imidazole) with 250 mM PMSF, added fresh. Then, sample was sonicated on ice with intensity 5 during 10 s on/10 s off, 50% duty cycle (Branson 250 Sonifier Cell Disruptor, USA). Subsequently, the lysate was centrifuged for 45 min at 4.000 g at 4 ºC. The supernatant was transferred to a new tube containing 4 mL of Ni-NTA resin and incubated for 1 h at 4 ºC with gentle shaking. Then, Ni-NTA resin containing the protein bound was centrifuged and

washed 3 times with pre-cooled lysis buffer. Finally, the protein was eluted with the elution buffer pH 8.0 (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM Imidazole). After purification, the protein purity was verified by SDS-PAGE gels.

7.2.9 T_0 and T_0` elements

From the sequence of the carp ribosomal cistron isolated by Vera et al. (2003), primers were designed corresponding to the sequences of T_0 and T_0` elements. Both forward and reverse oligonucleotides belonged to the same position, and the annealing of a double-stranded oligonucleotide was possible (see annealing protocol). Also, primers with mutations were designed in the T_0 and T_0´ elements for evaluating the interaction between these elements and TTF-I.

7.2.10 Chromatin Immunoprecipitation (ChIP)

The protocol described below corresponds to a modification of that described by Abcam (USA).

7.2.10.1 Chromatin cross-linking

Approximately 30 million nuclei were resuspended in 500 µL of ChIP lysis buffer (50 mM Hepes-KOH pH 7.5; 140 mM NaCl; 1mM EDTA; 1% Triton X-100; 0,1% sodium deoxycolate, and protease inhibitors) and 1% (v/v) formaldehyde final concentration. The samples were incubated for 10 min with gentle shaking at room temperature and centrifuged for 5 min at 1.960 g at 4 ºC. The pellet was washed 2 times with 500 µL PBS pH 7.4 cooled and supplemented with protease inhibitors. Finally, the pellet was resuspended in 700 µL of ChIP lysis buffer.

7.2.10.2 Chromatin preparation

The samples obtained in the previous step were sonicated 28 times for 15 s with intervals of 2 min on ice. From the DNA quantity, aliquots of 70 µL for each sample were taken, 130 µL of ChIP lysis buffer (without protease inhibitors) was added, and a digestion with proteinase K at 65 ºC was performed over night (the rest of samples were frozen at -20 ºC). Then, 10 µg of RNAse A was added and incubated for 30 min at 37 ºC. Subsequently, the proteins of the samples were extracted with a volume of basic phenol/chloroform, and were centrifuged to 24.100 g for 10 min at room temperature. The obtained DNA were precipitated with two volume of cold 100% ethanol and 0,1 volume of 3 M sodium acetate pH 5.2 and 50 µg of glycogen at -20 ºC for 30 min. Afterward, the samples were centrifuged to 24.100 g during 20 min at room temperature, and the pellets were resuspended in 100 µL nuclease free water. The DNA concentration was determined by spectrophotometry at 260 nm and the fragment size was analyzed on a 1,5% (w/v) agarose gel.

7.2.10.3 Resin blocking

10 µL of protein A agarose were taken for each sample and had added 100 µg of BSA, 5 µg of plasmid DNA, 100 µg of yeast tRNA, and the corresponding antibody for the immunoprecipitation. Finally ChIP lysis buffer was added until a complete 70 µL final volume per reaction. The volumes of antibodies used were 1 µL in commercial antibodies and 5 µL or 20 µL for antibodies against Tip5 and TTF-I, respectively. In addition, for each sample of cross-linked chromatin, 1 µL of anti-rabbit IgG was included separately as a non-specific control. The blocking mixture was incubated with gentle shaking at 4 ºC overnight.

7.2.10.4 Antibody-Protein-DNA complex

Aliquots of 5 µg each were taken of samples of cross-linked chromatin, which were diluted in a 1/10 ratio in dilution buffer (1% Triton X-100; 2 mM EDTA; 150 mM NaCl; 20 mM Tris-HCl pH 8.0; protease inhibitors), for each antibody used

(α-TTF-I, α-Tip5, α-K4H3me3, α-K9H3me3). 70 µL of protein A agarose was added pre-incubed with the respective antibody and incubated at 4 ºC with gentle agitation overnight. Subsequently, the samples were centrifuged at 1.500 g for 2 min at 4 ºC. The resin was washed 4 times with 1 mL of cold wash buffer (20 mM Tris-HCl, pH 8.0; 0,1% SDS; 1% Triton X-100; 2 mM EDTA; 1 M NaCl) and was centrifuged for 2 min at 1.500 g. In parallel, 5 µg of DNA cross-linked from each sample were sonicated and purified. The purified DNA was used as "Input" for quantitative PCR assays.

7.2.10.5 DNA elution and purification

For the elution of the antibody-protein-DNA complex, the supernatant of the last wash was removed and 450 µL of elution buffer (1% SDS; 100 mM NaHCO3) was added. Then, the samples were incubated for 15 min at room temperature with gentle shaking and then centrifuged for 2 min at 1.960 g. The supernatant corresponded to the immunoprecipitate. Subsequently, the cross-linking was reversed by adding 100 µg of proteinase K at 65 ºC overnight. Finally, the DNA was purified for use as a template for the successive quantitative PCR.

7.2.10.6 Real-time PCR

The enrichment of TTF-I and Tip5 on the T_0 and T_0` elements was quantified from the carp's ribosomal cistron. For this quantification, samples of acclimatized carp from winter and summer were used and treated with the process described above.

First, purification was evaluated by a dissociation curve. Following this was the calculation of PCR efficiency for each amplicon (Table X)

Table X. Properties of the oligonucleotides employed in the qPCR.

Oligonucleotide	Efficiency	Ct averange
T_0 Fow	99,3	14,3
T_0 Rev		
T_0 prima Fow	111,2	12,8
T_0 prima Rev		

The quantification was performed in triplicate. The reaction mixture for the qPCR contained 2,4 pmoles of each oligonucleotides (sense and antisense), Sybr Green Master Mix 1x, 700 pmoles of ROX, and 1 µL of DNA template in a final volume of 20 µL. The PCR reactions were performed in the thermocycler MxPro 3000p (Stratagene, USA) associated to specific software. The thermal profile of the PCR reaction was: initial denaturation for 10 min at 95 ºC, denaturation for 2 min at 95 ºC, annealing 45 s at 55 ºC, extension for 20 s at 72 ºC for 30 cycles.

For the quantification of each immunoprecipitated sample, the computer calculated Ct values (Cycle threshold). These values were averaged. After obtaining these values, of the occupancy ratio (OR) on the T_0 and T_0` regions was calculated by the following formula:

$$Or = ((X_a - X_{IgG})/X_{input}) \times 100$$

X correspond to:

$$X = Log_{10} ((Ct - C_{t0})/-s$$

Where "s" is the slope obtained in the efficiency of PCR in the calibration curve and Ct_0 is the average of the Ct obtained in the calibration curve for each oligonucleotide used (Németh et al. 2008).

7.2.11 Carp Tip5 isolation

cDNA was used as a template for isolating the Tip5 sequence through PCR. The PCR reaction contained 20 pmoles of each oligonucleotide (Tip5Fow and Tip5Rev), 0,2 mM each dNTP, 1,5 mM $MgCl_2$, 1 U Taq DNA polymerase, and 1 µL of cDNA synthetized. The amplification conditions were initial denaturation 10 min at 95 ºC, denaturation 2 min at 95 ºC, annealing 45 s at 52 ºC, extension 90 s at 72 ºC, for 30 cycles. The final extension was 10 min at 72 ºC. The amplicon obtained was cloned into pGEM-T easy vector (Promega, USA), and the ligation was used to transform JM109 competent cells. A positive clone was sequenced, and a sequence of approximately 1.530 bp corresponding to 3`end region of carp Tip5 was obtained.

7.2.12 RT-qPCR assays

The cDNA synthesis was performed from 2 µg of total RNA using AMV reverse transcriptase (Promega, USA) according to manufacturer's instructions.

The real time PCR assays were performed using Brilliant SYBR Green qPCR Master Mix (Stratagene, USA). The reaction mixture was 1 pmol of corresponding qPCR oligonucleotides (see Table VII), 1 µL cDNA, 0,14 µL of normalizer of fluorescence reference Rox (1:200), and 10 µL Sybr Green kit in a final volume of 20 µL. The PCR reactions were performed in the thermocycler MxPro 3000p (Stratagene, USA). The thermal profile of PCR reaction for TTF-I was: initial denaturation 10 min at 95 ºC, denaturation for 2 min at 95 ºC, annealing 45 s at 56 ºC, extension 20 s at 72 ºC for 30 cycles. The conditions for Tip5 were:

initial denaturation for 10 min at 95 ºC, denaturation 2 min at 95 ºC, annealing 45 s at 54 ºC, extension 20 s at 72 ºC for 30 cycles.

The comparative quantification of the transcripts with their respective internal control (normalizer) was analyzed in triplicate according to the Pfaffl formula:

$$R: \frac{(E_{TTF\text{-}I})^{\,Ct\,(TTF\text{-}I)}}{(E_{16s})^{\,Ct\,(16S)}}$$

Where "R" is the ratio of the relative expression of the corresponding transcript with respect to 16s. Ct values (Cycle threshold) correspond to the average of Ct values obtained in the efficiency curve of each sample in triplicate. The normalizer used in this experiment was the carp mitochondrial 16s rRNA.

The efficiency of PCR for each gene was calculated by the following formula:

$$E: [10(-1/s)] -1$$

The “s” values correspond to the slope obtained in the efficiency of the PCR for the corresponding oligonucleotide.

7.2.13 Infrared electrophoretic mobility shift assay (EMSA)

Non-radioactive labeled DNA (IR700 or IR800) was used to study protein-DNA interactions. Similar quantities of complementary single strand oligonucleotides were mixed in EX-50 buffer (20 mM Tris-HCl pH 7.6, 1,5 mM $MgCl_2$, 0,5 mM EGTA, 10% glycerol and 50 mM KCl), denatured in a thermoblock (95°C for 5 min), and slowly (1-2 h) chilled to room temperature (by switching the thermoblock off) to allow complete oligonucleotide annealing.

The reactions were analyzed by electrophoretic mobility shift assays (EMSA). Typical reactions contained 500 fmol of DNA in a total volume of 10 µL and 250-1000 fmol for purified carp TTF-I. Mouse TTF-IΔ323 protein (provided by Dr. Längst) was used as a control. These reactions were typically performed in EMSA buffer (20 mM Tris-HCl pH 7.0, 80 mM KCl, 0,2 mM EDTA, 5 mM $MgCl_2$, 10% glycerol, and 2 mM DTT).

The incubation time was 10 min at 30 ºC and then protein-DNA complexes were separated from free DNA by native gel electrophoresis. The reactions were loaded on pre-electrophoresed 4.8% native polyacrylamide gels in 0.4x TBE and run for 1 h at 100 V at 4 ºC. The gels were visualized with the Odysseys® Infrared Imaging System.

7.2.14 pRNA characterization

cDNA was used as template for pRNA characterization. The oligonucleotides (pRNA primers) were derived from the sequence of the carp ribosomal cistron (Vera et al. 2003). In the different experiments the same primer reverse (pRNA reverse) was used. The PCR reaction contained 20 pmoles of each oligonucleotide, 0,2 mM each dNTP, 1,5 mM $MgCl_2$, 1 U Taq DNA polymerase, and 1 µL of cDNA synthetized. The amplification conditions were initial denaturation 10 min at 95 ºC, denaturation 2 min at 95 ºC, annealing 45 s at 58 ºC, extension 12 s at 72 ºC, for 30 cycles. The product was sequenced and run in a gel. The quantification was performed by relative intensity of the samples obtained from acclimatized carp.

8 RESULTS

8.1 Identification of *Cyprinus carpio* TTF-I coding sequence

As the sequence of TTF-I is only described in mammals, we first searched to determine if this sequence factor exists in fish. For this, we used the mouse TTF-I sequence described in GeneBank (Evers et al. 1995), which was then aligned in the Zfin database (zfin.org), and as a result, we obtained a hypothetical protein (XP_002662223.1), which was used to work with. The bioinformatics approximation was performed in the *Danio rerio* database (zebrafish) by the close phylogenetic proximity between carp and zebrafish and with the knowledge of the complete genome of the latter fish.

From this sequence, heterologous oligonucleotides that amplify the sequence encoding carp TTF-I were designed. The PCR reaction performed amplified a 354 bp amplicon (Figure 8).

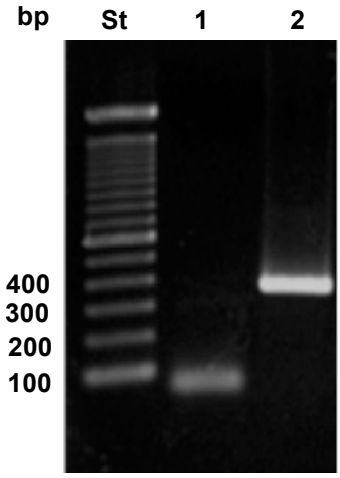

Figure 8. Partial amplification of *C. carpio* TTF-I

Amplicon of 354 bp corresponding to carp TTF-I (lane 2) was obtained from liver cDNA by RT-PCR. In lane 1, the negative control of the PCR reaction is shown (PCR reaction mixture without cDNA). Lane M indicates the DNA ladder. The RT-PCR products were visualized in a 1.8% agarose gel and ethidium bromide staining.

From this incomplete sequence, we proceeded to explore both the 5` and 3`ends in order to complete the carp TTF-I sequence (Figure 9A). For the 5`end, specific primers to this region were designed, and together with the FirstChoice® RLM-RACE kit (PROMEGA, USA), it was possible to obtain a product of approximately 1.5 kb, which was cloned and sequenced. The sequence resulted in the TTF-I 5` end together with an untranslated region (UTR) (Figure 9).

Also from the incomplete sequence, it was possible to obtain the carp TTF-I 3` end. Using oligo dT primers, together with specific primers derived from the predetermined sequence, a product was obtained which was cloned and

sequenced. The sequence obtained resulted in a 1 kb amplicon that was comprised of a 3` end and 3`UTR (Figure 9B).

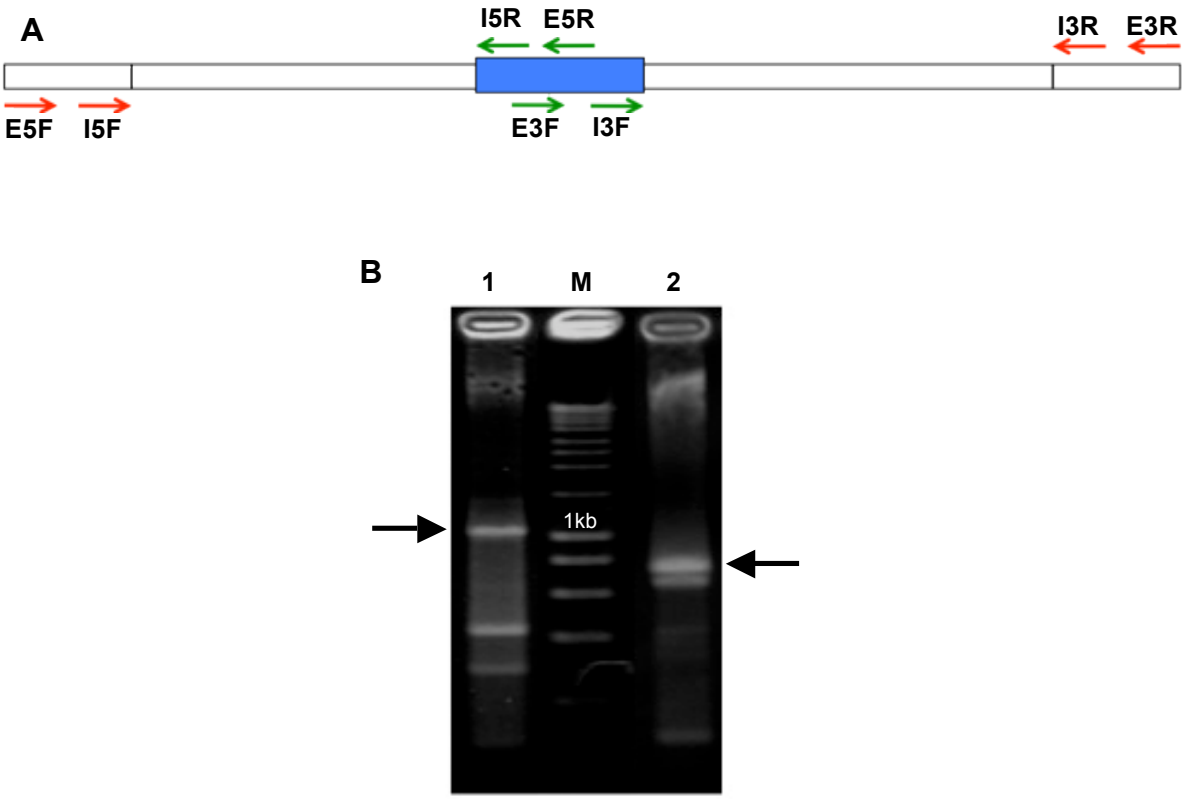

Figure 9. 5` and 3` end amplification of *C. carpio* TTF-I complete sequence by RLM-RACE

(A) Amplification scheme. Green arrows correspond to the oligonucleotides deduced from partial carp TTF-I (blue box). Red arrows correspond to the oligonucleotides (specific adapters) provided by FirstChoice® RLM-RACE kit. (B) Gel electrophoresis of the results of RLM-RACE. The procedure first involved a PCR reaction using external oligonugleotides (E) and then a second PCR reaction using the first PCR product and internal primers as a template (I). The arrows indicate the fragment cloned and sequenced for the 5` end (lane 1) and the 3` end (lane 2). Lane M: 1 kb DNA ladder.

Once the sequences were obtained, a bioinformatic analysis was performed, resulting in a sequence of 2.159 bp. This segment has 5` UTR of 284 bp, an open reading frame (ORF) of 1.677 bp, and a 3` UTR of 198 bp (Figure 10).

TCCCGTCTTAATTCTTTTGCTGTTCTTCAGAAATCACAATATGCGATGATGACGTGATATACTAACGAC
ACGCGTGCAAGGCTAACGTGACCAGACTCGAGTTGGATTTCGTGTTTGTTGGTGAAAGGCACGTTGTGC
TGTCTTTTTCTGATTTTTGATTTTCGATTTTCAAAGAATGTTAGATGAAATGCAGTCAGATTCGCGTGA
TGACATAATGGGCCGCGTTAATGGAGAAACGGTGAAGAAGAAGAGAAAGAAAAGTGAAACTCCAGAGCA
GCACGAACTCACTCAGTGTCCAAAGGTGCAGACTGAGAAGCAACGTGAAAAGGTGCAGAAGAAGAAGAA
GAAGAAGAAGGAATTGGAGAATATAAGTCTGCAGACACCTACAGACAAAAAGAAAAAAAGAAAACTGAA
TGAAGGTGTGGAGGTTGTCACAAGCCTGCATAAAGTAAAAGACAAGACAGCAGAGTCACAAGAGCCGCA
GGGTCCAGGGGAACAGAAGCATGCTAAGAAAAAGAAAAAGAGAGCAAATGTGACACAGGCAGACTGTGA
TGAGATGACACAAGGGGAACAGCCTGTCCTGATGACAGAACACGAGGAAGTCCATCACAAGAAGAAAGA
TCAGGAGATGGACTTGCCTGATGTATTAATTGGTACTAGAAAGCGCAAAAGAAAGCGAGCGCTGTTGGA
TGAACCAGAAGTTGACCCCGATCTCTTGAATGAACTGAAAGAATTTTGTCCTAAAATAGAGTCCAGAAG
CTCACATGAAATCAATAAAATGATCATGTATGATCTGCCGAGCTTCAAGGAATTCAGAAAGCAAGGTAT
CATATTGAGGCATGGAAGATACTCTGACGCAGAAAATGAGAGGTTAAGACAGAACGTTAGAGATTTTCT
TGCTCTCACAGGAGTGAAAGATGCTATCAAGCTCTTTCATCCAAAGCGTTTCCCAGAAGAGACACAGGA
ATTGACAAAGCTGAAAAAGGTCTACAAGTTTTTTGAAAGGATCGCGGAAGGAATTCCCAGGCCTTGCCA
TGATGTTTTTAGCCGTGGAAGGAAAGTTTTTGATGGTGGAAACTATAAGGGAAGGTTTACAGAAGAAGA
AGTCAAATCATTACTTAAGTATCATTCACTACATGGCAATAACTGGCAAAAGATTTCTGAGCTGACTGG
TCGAAGTGCTTACTCTCTTGAGAAACGTTTTACCCAGCTCAATACTGCTAGAAAAAGTGGGCCGTGGTC
AGCAAAAGAGGTGCAGAGACTTTTGAGGGCTGTGCAAGACCATATTGTAACGGTGCTGAAATCTGAGTC
CCCTAATAAAACAACACCGAAAAGAGTCAGTAGAGAAATACTGTACCGAAAATTGCCCTGGTTCAATAT
TTCTCTGAAGGTGAAAACTCGATGTTGGACCAAATGCAGAGAGAAATGGATGTCCATCCTCGCTGTGCG
GATGTCTTCAGGGACTTGTACAGGAAGGAAAGCTCAGGAGTCCAAAATCAGACTCATTAAAGTAATGTA
TCAAATGCAAGTGGAGGATGTTACGGATGTCAACTGGGATGATCTCACAGCTGTTTTCGGGGATGTTCC
TCCAGCCTATGTGCAAGCAAAGTGGCACCAGCTTAAAGTTTGCTATGTGCCCAATTGGAAGACCAAGTG
TTTTGGAGACATTGTTGACTTCCTCTATGAGAAAGTCTTGCCAGGGATGGTGAAAGACTGTGAAGACCT
TGATGACAATGAGCTGAAGGTTGACCAGAAGCAGAGCTTCCTTCTATCTGACATTTTTAAAGACATTGA
GGATCATTCCGATGACAGTGATGAGAAGAGTGGCCAGAAAGAAGATGACAACTCCTCTTGAACTATTAT
CACTTGTCAATCAGACCTTAATGATTTCTGTAAAAATCACATTAAATCATACCCCAACTAAAGAAAAAT
ACATTGTCGAGATGTATATGTAAAAGATGCATGATAATTTTGTTTTATTCTTGTTATTCCATTTATTTT
TTTTATCAAGAATTTAATAAACAAGATTTGTATTGTGAAAAAAAAAAAA

Figure 10. Complete mRNA sequence coding for *C. carpio* TTF-I

The complete mRNA sequence coding for carp TTF-I. The 5` UTR and 3` UTR are depicted in green and red, respectively. Not highlighted region corresponds to the open reading frame of the carp TTF-I gene.

8.2 Characterization of *C. carpio* TTF-I protein

A detailed *in silico* analysis of the open reading frame sequence of carp TTF-I shows that it contains 559 amino acids (Figure 11). A multiple alignment analysis of this protein with heterologous sequences deposited in the NCBI database revealed a 61% identity with hypothetical *Danio rerio* TTF-I (XP_002662216.1), 39% identity with *Mus musculus* TTF-I (NP_033468.2), and 38% identity with *Homo sapiens* TTF-I (NP_031370.2) (Figure 12).

In addition, the multiple alignment analysis shows that fish TTF-I are shorter than the other proteins described, being shorter than in the N-terminus. In particular, carp TTF-I contains 274 amino acids less than compared with mouse TTF-I. On the other hand, the carboxyl terminus of carp TTF-I shares a 35% identity with others TTF-I proteins described (Figure 12).

On the basis of the evolutionary distances, a phylogenetic tree was constructed by Neighbor-Joining method (Saitou and Nei 1987). This showed that the carp TTF-I have a close proximity with the mammalian clade, compared to other proteins with similar properties as Reb1p (Morrow et al. 1993) (Figure 13).

In the C-terminus region of mouse TTF-I, domains exist that are involved in DNA binding domain (DBD) (Evers et al. 1995) (Figure 14A). Through bioinformatics analysis, it was possible to describe similar domains in carp TTF-I (Figure 14B). The DBD 1 and 2 of carp TTF-1 have a 45.9% and 34.3% identity, respectively, when compared with similar domains in mouse TTF-I (Figure 14C).

```
atgttagatgaaatgcagtcagattcgcgtgatgacataatgggccgcgttaatggagaa
 M  L  D  E  M  Q  S  D  S  R  D  D  I  M  G  R  V  N  G  E
acggtgaagaagaagagaaagaaaagtgaaactccagagcagcacgaactcactcagtgt
 T  V  K  K  K  R  K  K  S  E  T  P  E  Q  H  E  L  T  Q  C
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 P  K  V  Q  T  E  K  Q  R  E  K  V  Q  K  K  K  K  K  K  K
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 E  L  E  N  I  S  L  Q  T  P  T  D  K  K  K  K  R  K  L  N
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 D  H  S  D  D  S  D  E  K  S  G  Q  K  E  D  D  N  S  S
```

Figure 11. *C. carpio* TTF-I amino acid sequence

The derived amino acid sequence of carp TTF-I is show according to the standard genetic code. This analysis was performed using the EXPASY database tools (expasy.org/tools/).

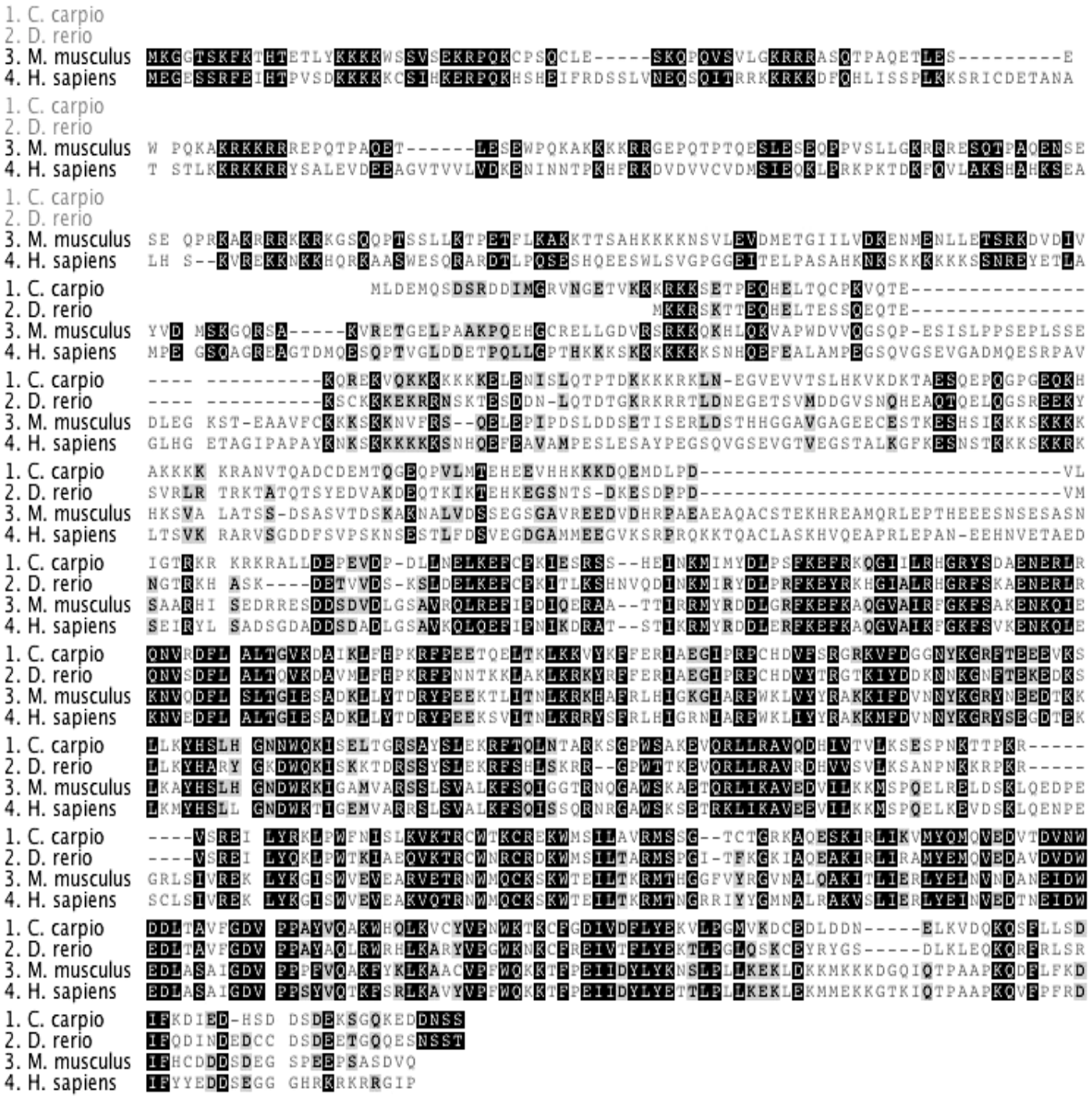


Figure 12. Multiple alignments between *C. carpio* TTF-I and TTF-I from other species

TTF-I protein sequences from *Cyprinus carpio*, *Mus musculus*, *Homo sapiens* and *Danio rerio*) are aligned. Identical residues in all four proteins are indicated by black boxes, and gaps are indicated by hyphens. The gray boxes indicate similarity between residues. The alignment was performed using ClustalW software.

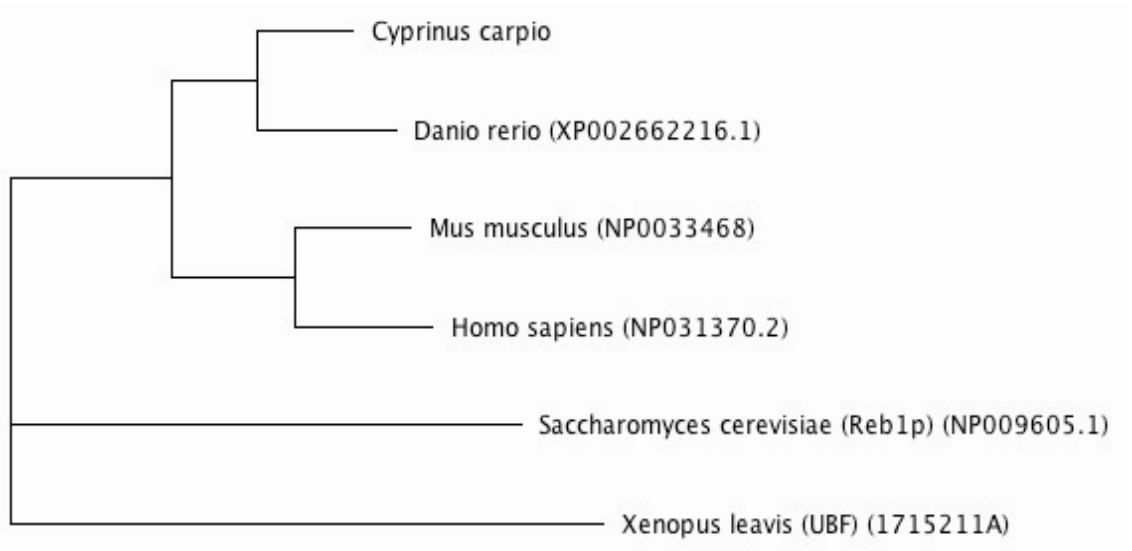
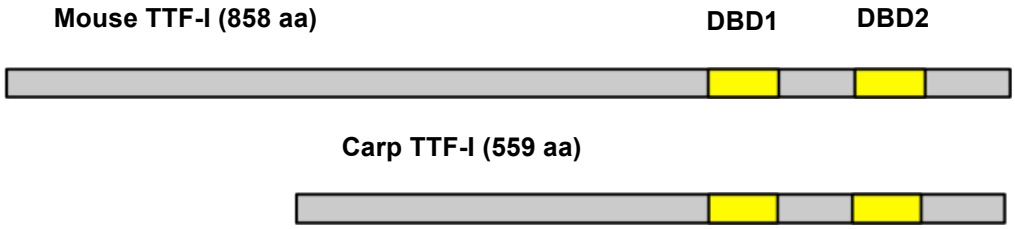


Figure 13. Phylogenetic analysis of TTF-I from different species.

Phyogenetic analysis based upon the alignment of amino acids sequences of TTF-I from *C. carpio*, *D. rerio*, *M. musculus* and *H. sapiens*. The Reb1p is a protein from *S. cerevisiae* with similar characteristics and functions as TTF-I. UBF from *X. leavis* was used as an outgroup. The phyilogenetic tree was constructed by the Neighbor-Joining method provided by the ClustalW software. The GenBank accession numbers are showed between parentheses.

A



B

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C

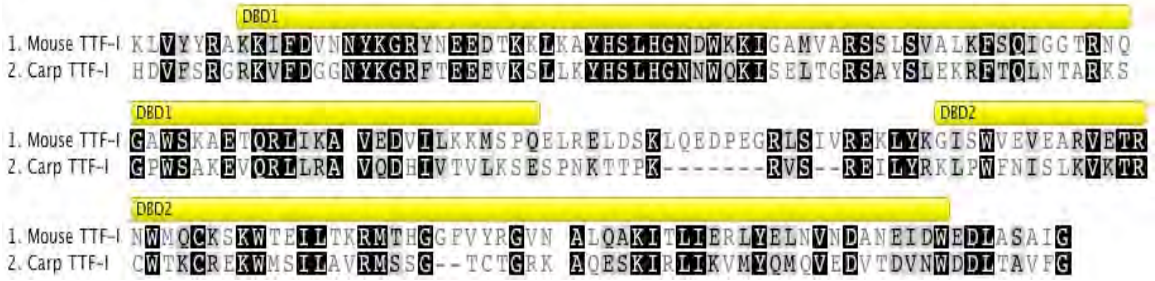


Figure 14. DNA binding domains in the C-terminal region of *C. carpio* TTF-I

The figure shows the comparison and distribution of DNA Binding Domains (DBD) between Mouse and carp TTF-I. (A) Scheme of carp and mouse TTF-I, where DBD are shown in yellow boxes. (B) Complete amino acids sequence of carp TTF-I. DNA Binding Domains are highlighted in yellow. (C) Alignment between mouse TTF-I DBD and carp TTF-I DBD; the black boxes indicate identical amino acids, and grey boxes indicate similarity between residues. The alignment was performed using ClustalW software.

8.3 Expression analysis of *C. carpio* TTF-I during the acclimatization process

8.3.1 Transcriptional expression of carp TTF-I

Acclimatization involves a seasonal reprogramming of molecular and cellular functions (Gracey et al. 2004; Pinto et al. 2005). To confirm if carp TTF-I modifies its expression during seasonal acclimatization, Quantitative Real-Time RT-PCR (RT-qPCR) was performed.

cDNA from liver tissues from carp acclimatized to winter and summer was used as template for the quantification of TTF-I. The results show that no significant differences exist between winter and summer for the expression of carp TTF-I transcripts (Figure 15A). The pre-rRNA was used as a control, and it was observed more highly expressed in the summer (Figure 15B).

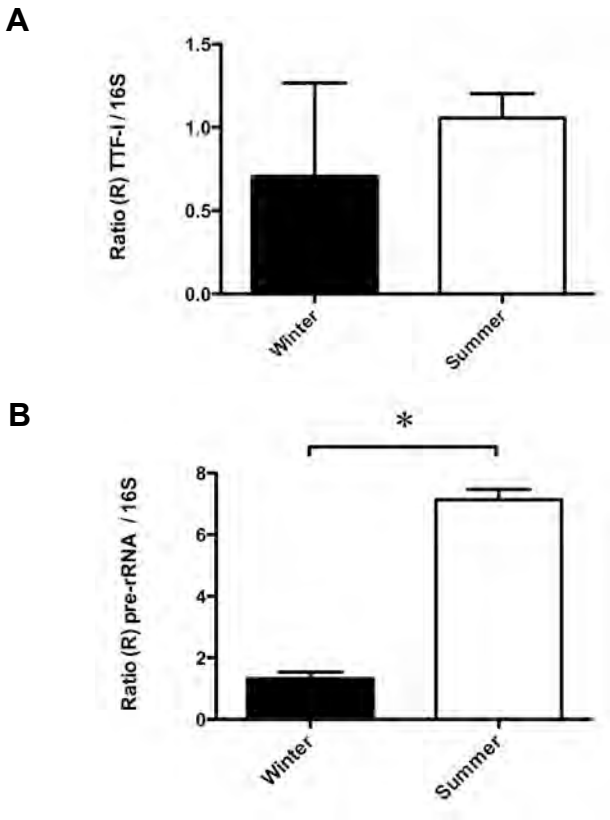


Figure 15. Seasonal expression of *C. carpio* TTF-I transcript

Real time qRT-PCR quantification of carp TTF-I (A) and pre-rRNA (B) expression during the acclimatization process. The black and white columns indicate winter and summer seasons respectively. The graphs show the average expression of three independent experiments quantified in triplicate. Standard deviation (±SD) is shown. The analyses rendered significant differences with Student's t test (∗= $p < 0.05$).

8.3.2 Carp TTF-I protein content

To determine if the levels of TTF-I mRNA were associated with protein levels, a *Western blot* analysis was performed on nuclear protein extracts from the liver cells of carp acclimatized to winter and summer. The results show that the protein content of TTF-I does not vary between winter- and summer- seasons (Figure 16). The apparent molecular weight of the carp TTF-I is consistent with the bioinformatics approximation (around 65 kDa). However, it was possible visualize a second band below of the strong one. This band is not described in any previous report for TTF-I, but also may be due to cross-reaction of the serum.

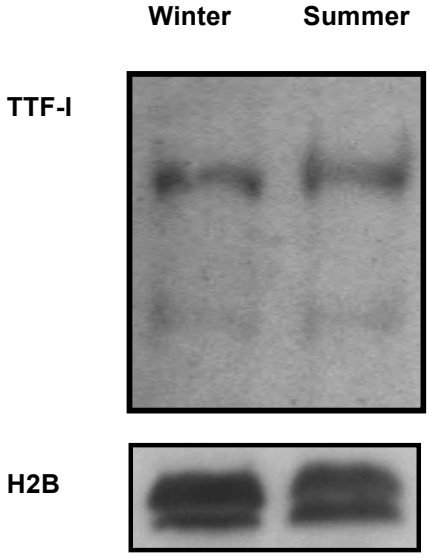


Figure 16. *C. carpio* TTF-I protein content

Western blot analysis of carp TTF-I. Nuclear proteins from liver tissue of winter- and summer-acclimatized carp were used to evaluate the TTF-I content. The same blot was then probed with antibodies against H2B histone, which was used as a loading control.

8.4 Identification of *C. carpio* Tip5

Tip5 is a protein member of the nucleolar remodeling complex (NoRC) which is characterized by controlling the activity of the ribosomal genes (Strohner 2004). Due to its regulatory role, we first proceeded to identify if this protein is present in the carp.

Tip5 has only been described in mammals. For that, the mouse Tip5 sequence (AJ309544) was used to perform bioinformatics searches of this protein in the databases of the *Danio rerio*. The result obtained was a hypothetical protein (CR450824.9).

From this protein, the nucleotide sequence was deduced. Heterologous oligonucleotides were designed to amplify the sequence of carp Tip5. The PCR reaction performed amplified a 722 bp amplicon (Figure 17).

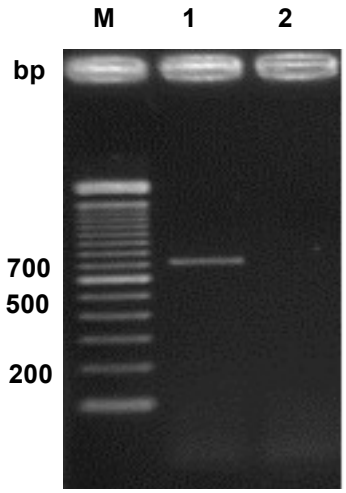


Figure 17. Partial amplification of *C. carpio* Tip5

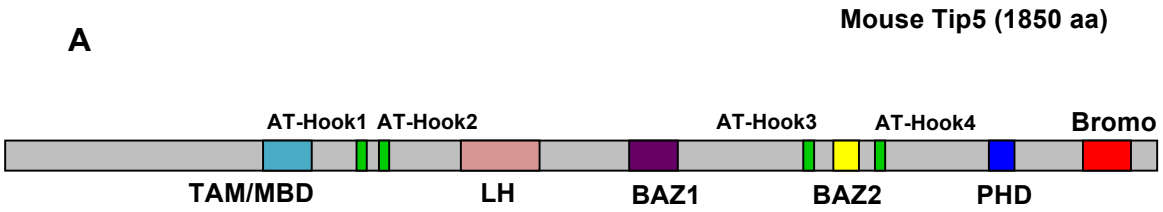
Figure shows the electrophoretic pattern of the obtained amplicon by RT-PCR (Lane 1). Amplicon size was around 700 bp. Line 2: negative control of the reaction (reaction mixture without template). M: DNA ladder. The RT- PCR product was analyzed on a 1.5% agarose gel and revealed by ethidium bromide staining.

From this amplicon, new primers were designed for obtaining a complete sequence of Tip5. Due to the large size of the Tip5 sequence (approximately 5500 bp in a mouse) and not having other bioinformatics tools to design new primers, it was not possible to complete the entire sequence of Tip5. Finally, a unique sequence of 1530 bp was obtained.

8.5 Characterization of a partial sequence of *C. carpio* Tip5

A bioinformatic analysis of the partial sequence of carp Tip5 was performed from the 1530 bp sequence. This analysis resulted in a sequence of 496 amino acids residues.

In the analysis of the carp Tip5 polypeptide, it was observed that this corresponds to the C-terminal region of the protein because its similarity to this region in mouse Tip5 (Figure 18A). The carp Tip5 polypeptide shares a 52.5% identity with this region (Figure 18B), where it was possible to observe that in fish, similar domains exist with a high identity as described in mouse Tip5. The domains found are: bromodomain, PHD finger, and the AT-hook 4, with a 77.6%, 70.6% and 69.2% identity, respectively, when compared with similar domains in mouse Tip5 (Figure 18C).



o **B**

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C

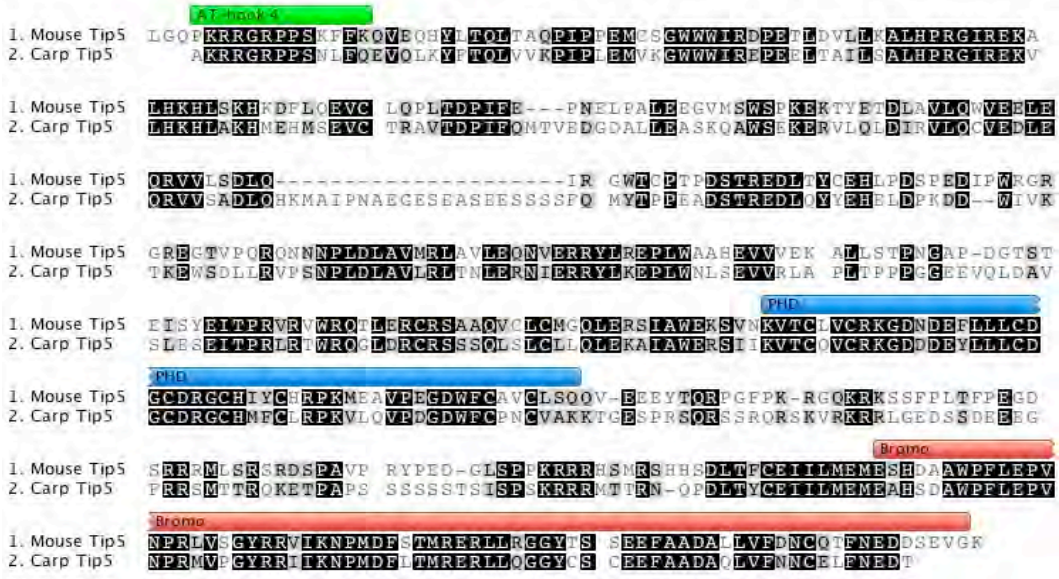

Figure 18. C-terminal region of *C. carpio* Tip5

(A) Organization and domains of mouse Tip5 protein. Schematic organization and localization of sequence motifs in Tip5 and which have been associated with functions in chromatin structure are represented. (modified from McStay and Grummt 2008). (B) C-terminal sequence of carp Tip5. Highlighted in colored boxes are C-terminal domains: AT-hook 4 (green), PHD (blue), and bromodomain (red). (C) Alignment between the C-terminal sequences of mouse and carp Tip5. Black and grey boxes indicate identities.

8.6 *C. carpio* Tip5 expression during seasonal adaptation

8.6.1 Transcriptional expression of carp Tip5 transcript

Because of the important role of Tip5 in ribosomal gene regulation (Santoro et al. 2002; Strohner 2004), we first proceeded to evaluate the transcript levels in acclimatized carps. The transcript expression of carp Tip5 was measured by RT-qPCR using the same procedure described before. The expression of this protein was significantly increased during the winter, consistent with a repressor function (McStay and Grummt 2008) (Figure 19A). As an expression control, the pre-rRNA was used, and it was observed being significantly expressed in summer (Figure 19B).

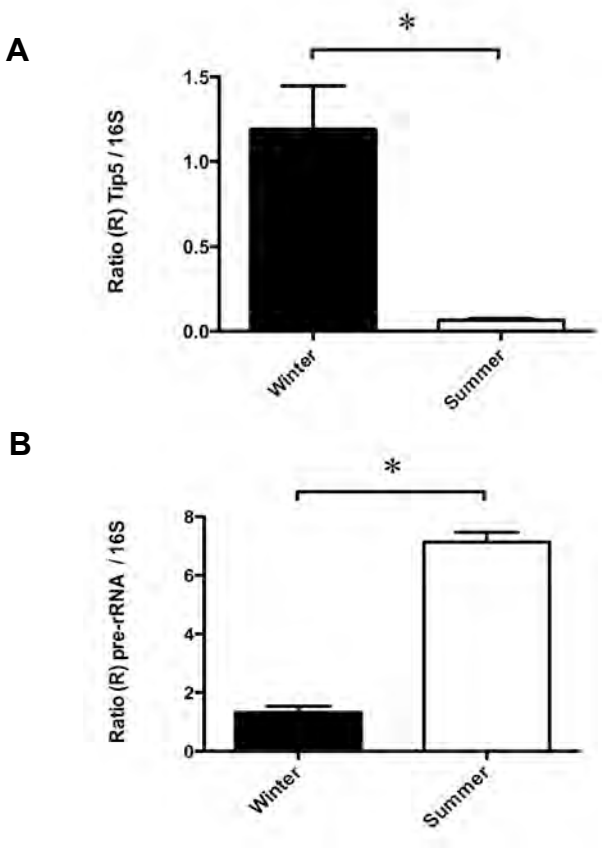

Figure 19. Expression of *C. carpio* Tip5 transcript during the acclimatization process

The seasonal quantification of carp Tip5 (A) and pre-rRNA (B) are showed. The black and the white columns represent winter and summer seasons respectively. For both experiments, the average expression of three independent experiments were graphed and quantified in triplicate. Standard deviation (±SD) is shown. The Statistical analyses were performed using Student's t test (∗= $p < 0.05$).

8.6.2 Detection of carp Tip5 protein

To establish if the protein content of Tip5 coincides with the transcriptional expression Western blot assays were performed. Nuclear proteins extracts from liver fish undergoing seasonal adaptations were used to measure the Tip5 content. The results showed that Tip5 levels have differences between winter and summer. This protein is significantly more expressed in winter that in summer, suggesting its repressive action (Figure 20).

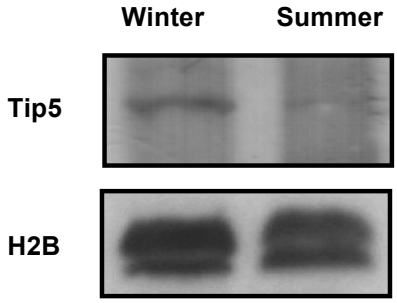

Figure 20. *C. carpio* Tip5 protein content

Nuclear proteins from liver tissue from winter- and summer- acclimatized carp were used to determine the Tip5 content. The same blot was probed with H2B antibody; which was used as loading control.

8.7 Purification of recombinant *C. carpio* TTF-I

TTF-I is a factor playing an important role in the regulation of rDNA transcription chromatin (Längst et al. 1997; Längst et al. 1998). After obtain the complete sequence of carp TTF-I, this was cloned in an expression vector, and the transformed bacteria were induced with 1 mM IPTG (Figure 21A). After checking if the induction worked, the biochemical purification of carp TTF-I was continued by affinity chromatography (see methods 7.2.8.3), obtained different elution with the purified protein (Figure 21B). The recombinant purified protein has a molecular weight of approximately 70 kDa.

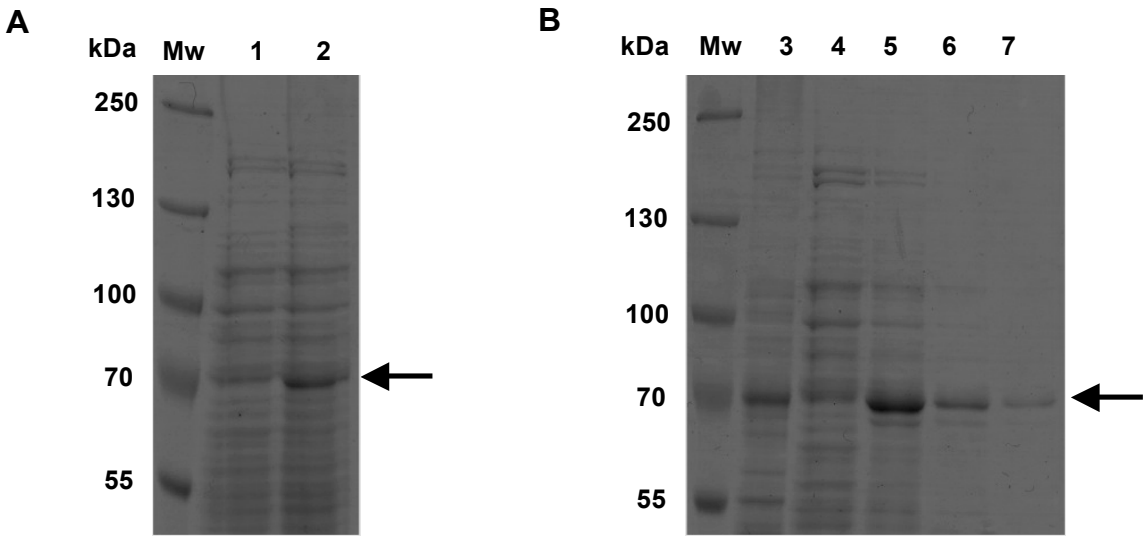

Figure 21. Induction and purification of *C. carpio* TTF-I

(A) Bacteria induction at start (lane 1) and 4 h after the induction of IPTG (lane 2). (B) Carp TTF-I purification. Recombinant carp TTF-I was expressed in Rosetta 2 bacteria and purified by Ni-NTA resin. Lane 3: pellet; lane 4: supernatant; lane 5: Elution 1 (Imidazole 500 mM); lane 6: Elution 2 (Imidazole 250 mM); lane 7: Elution 3 (Imidazole 250 mM). For A and B, the 8% SDS-PAGE and stain with Coomassie blue are presented. The band corresponding to the recombinant TTF-I protein is highlighted with arrows.

8.8 *In vitro* interaction of recombinant TTF-I with *C. carpio* T_0 and T_0` elements

To assess the ability of TTF-I to bind to the T_0 and T_0` elements, electrophoretic mobility shift assays (EMSA) were performed. For this assay, the recombinant carp TTF-I was used, and as control the N-terminal truncated mouse TTF-I (mTTF-I Δ323) was used. This mouse protein has the specific DNA binding domains for mouse T_0 element.

The EMSA assays demonstrate that the probe containing the carp T_0 and T_0` elements are capable of binding with both carp TTF-I (Figure 22) and mTTF-IΔ323 recombinant proteins (Figure 23).

In addition, to determine the specificity of the DNA-protein complex, probes with 1 and 2 nucleotide mutated within T elements were designed. Under these conditions, the electrophoretic mobility shift is abolished.

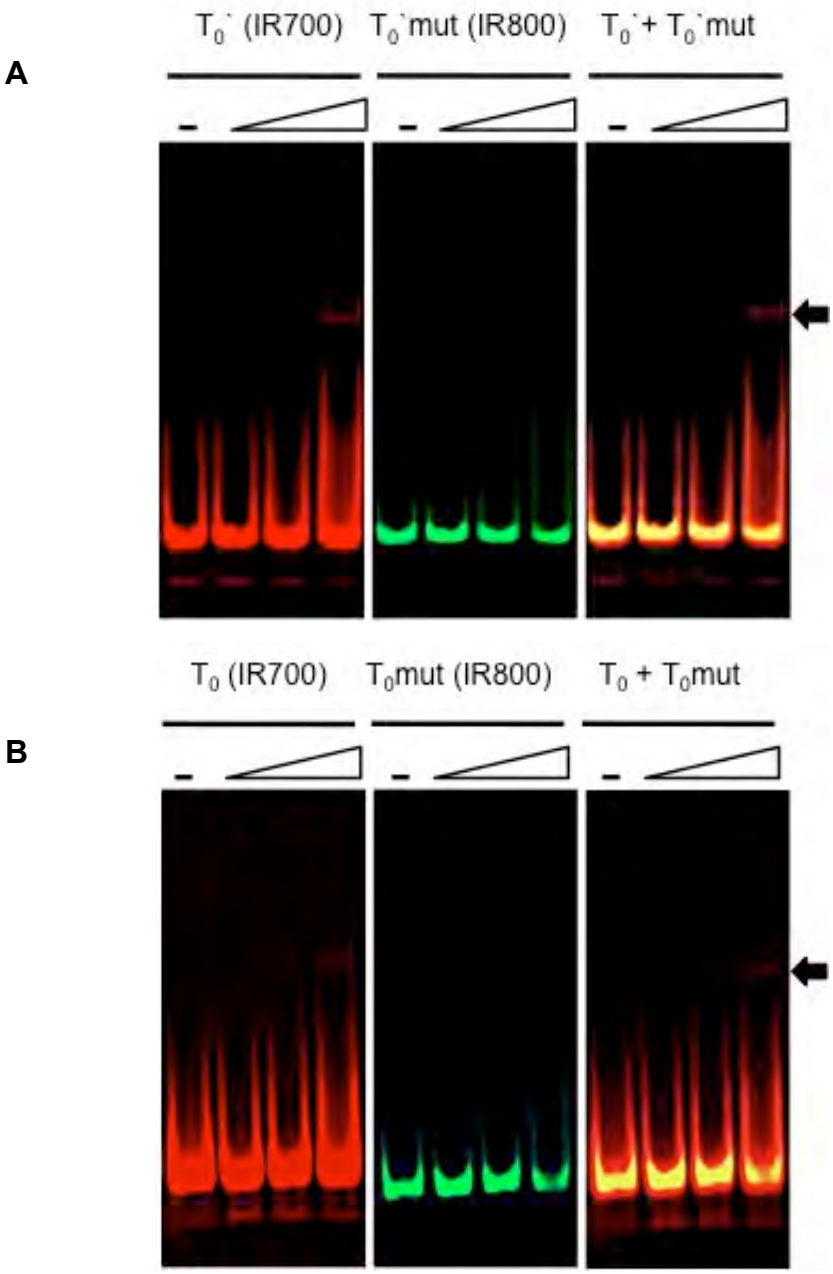

Figure 22. DNA binding properties of recombinant *C. carpio* TTF-I

Electrophoretic mobility shift assay using the carp T_0`(A) or T_0 elements (B) as probes. Increasing amounts of carp TTF-I recombinant protein (250 – 1000 fmol) were incubated with an equimolar mixture of non-mutated (IR700-labelled) or mutated (IR800-labelled) carp T elements. In yellow, the overlay of the assay is shown. The arrow identifies the DNA-protein complex. The EMSA were analyzed on 4.8% native polyacrylamide gels.

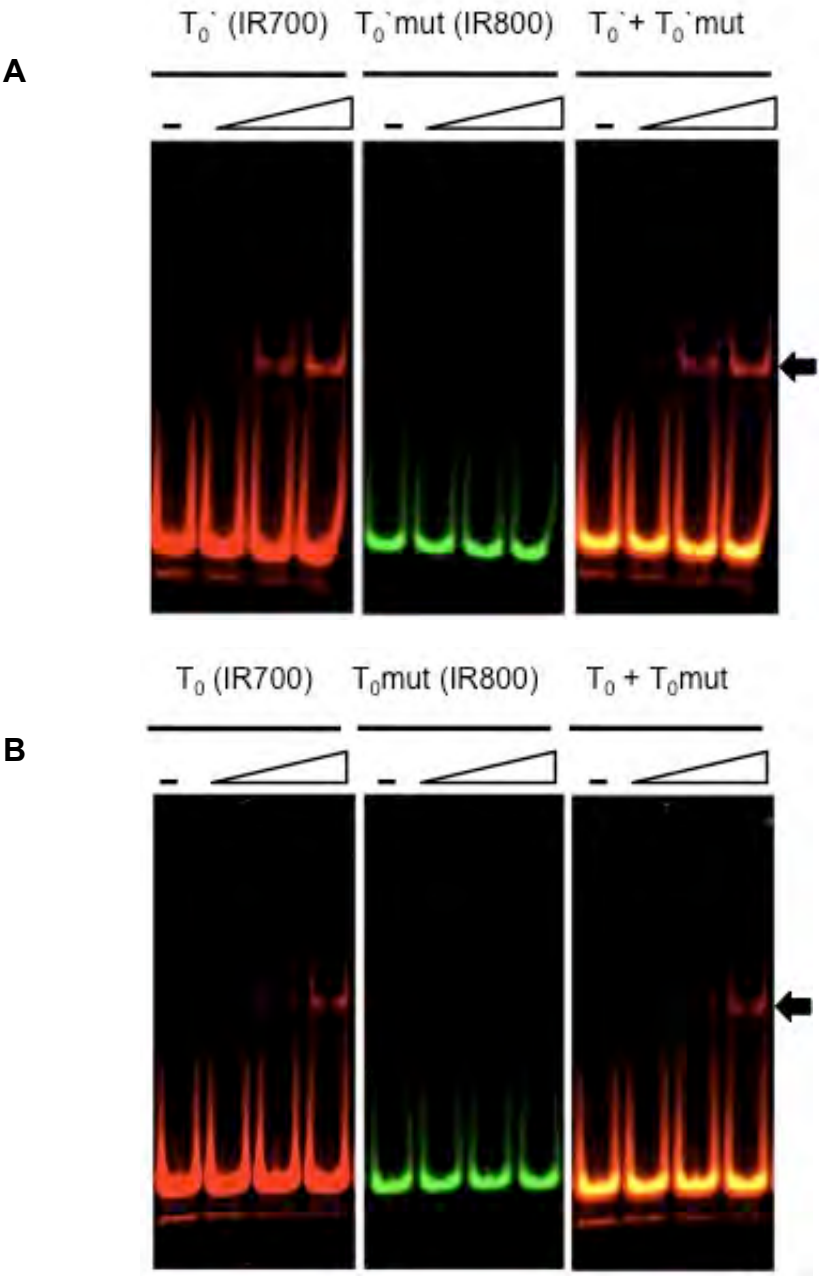

Figure 23. Binding of N-terminal truncated mouse TTF-I with *C. carpio* T elements

Electromobility shifts assay using carp T0` (A) or T0 (B) elements and increasing amounts of mTTF-I Δ323 protein (250 – 1000 fmol). An equimolar mixture of non-mutated (IR700-labelled) or mutated (IR800-labelled) carp T elements was used. In yellow, the overlay of the assay is shown. TTF-I-DNA (marked by an arrow) complexes were analyzed on 4.8% native polyacrylamide gels.

8.9 Evaluation of TTF-I and Tip5 content in the regions T_0 and T_0' of the *C. carpio* ribosomal gene during acclimatization process

Due to differential expression in the transcription of ribosomal genes in acclimatized carps (Alvarez et al. 2004), and considering the regulatory role of TTF-I in the regulation of rDNA transcription (Längst et al. 1997; Längst et al. 1998), and that the chromatin remodeling complex (NoRC) silences rDNA (McStay and Grummt 2008), it was decided to analyze the presence of TTF-I and Tip5 in the regions T_0 and T_0` of the carp ribosomal cistron (Figure 4) during seasonal acclimatization. For this, we first proceeded to evaluate the enrichment of these proteins in the carp by chromatin immunoprecipitation (ChIP) associated to real-time PCR (qPCR) on the regions contains the $T_0$ and $T_0$` elements.

The enrichment of TTF-I varies between winter and summer for both T_0 as T_0`. In both cases, this factor is significantly enriched during the cold season, but the relative enrichment was higher in T_0 (Figure 24A).

The Tip5 enrichment did not change in T_0` during the acclimatization process. Interestingly, in the T_0 element a significantly higher enrichment exists in winter, whereas no significant positioning was detected in summer (Figure 24B).

To verify if the positioning of TTF-I and Tip5 on the “T” elements, coinciding with a transcriptionally silent or active chromatin state, ChIP assays were performed using antibodies that recognize specific histone post-translational modifications associated to activation (H3K4me3) or repression (H3K9me3). The results showed that the enrichment of H3K9me3 was significantly higher in the

chromatin from carps acclimatized to winter for both T_0 and $T_0´$ (Figure 25A). On the contrary, the euchromatin marker is more abundant in the “T” elements during the summer season (Figure 25B). The relative enrichment was higher in T_0 in comparison to $T_0`$.

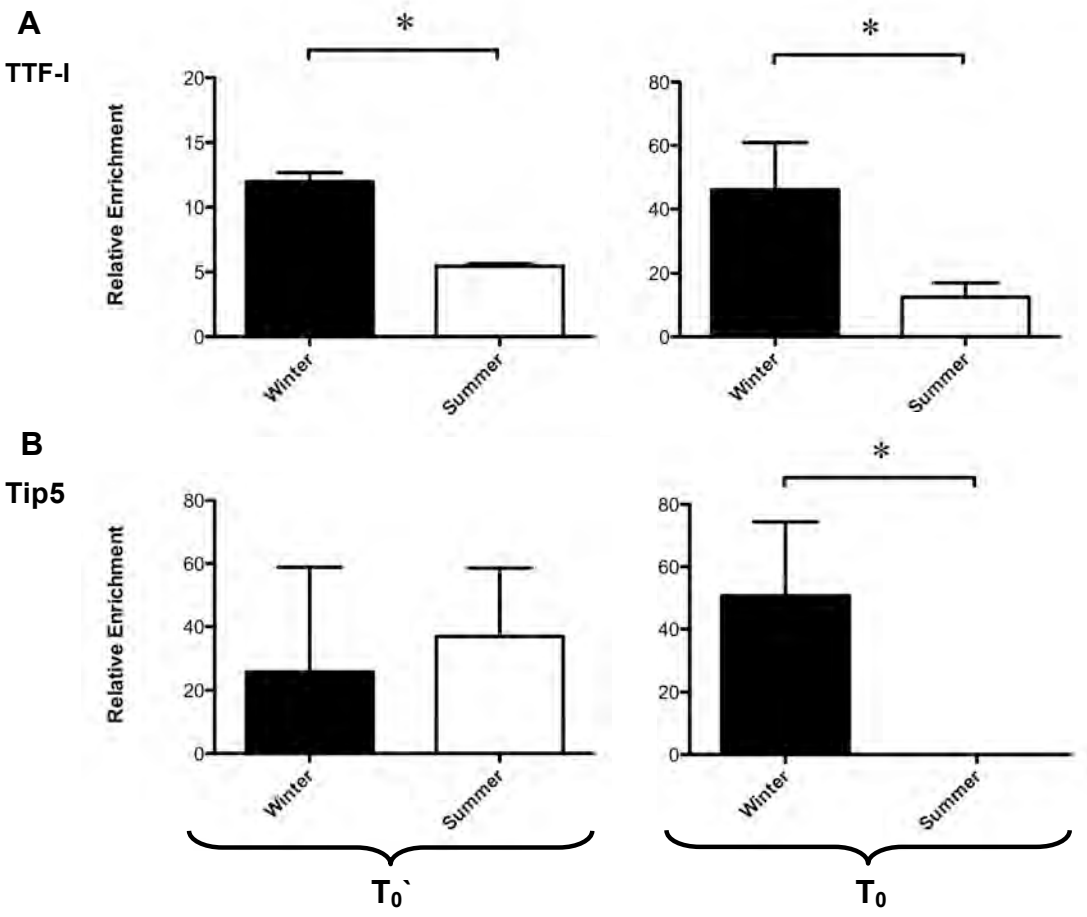

Figure 24. Evaluation of TTF-I and Tip5 enrichment on the T_0 and T_0` elements in seasonally acclimatized *C. carpio*

ChIP analysis for the binding of TTF-I (A) and Tip5 (B) on T_0 and T_0` elements. Standard deviation (±SD) is shown. The analyses rendered significant differences through Student's t test (∗= $p < 0.05$).

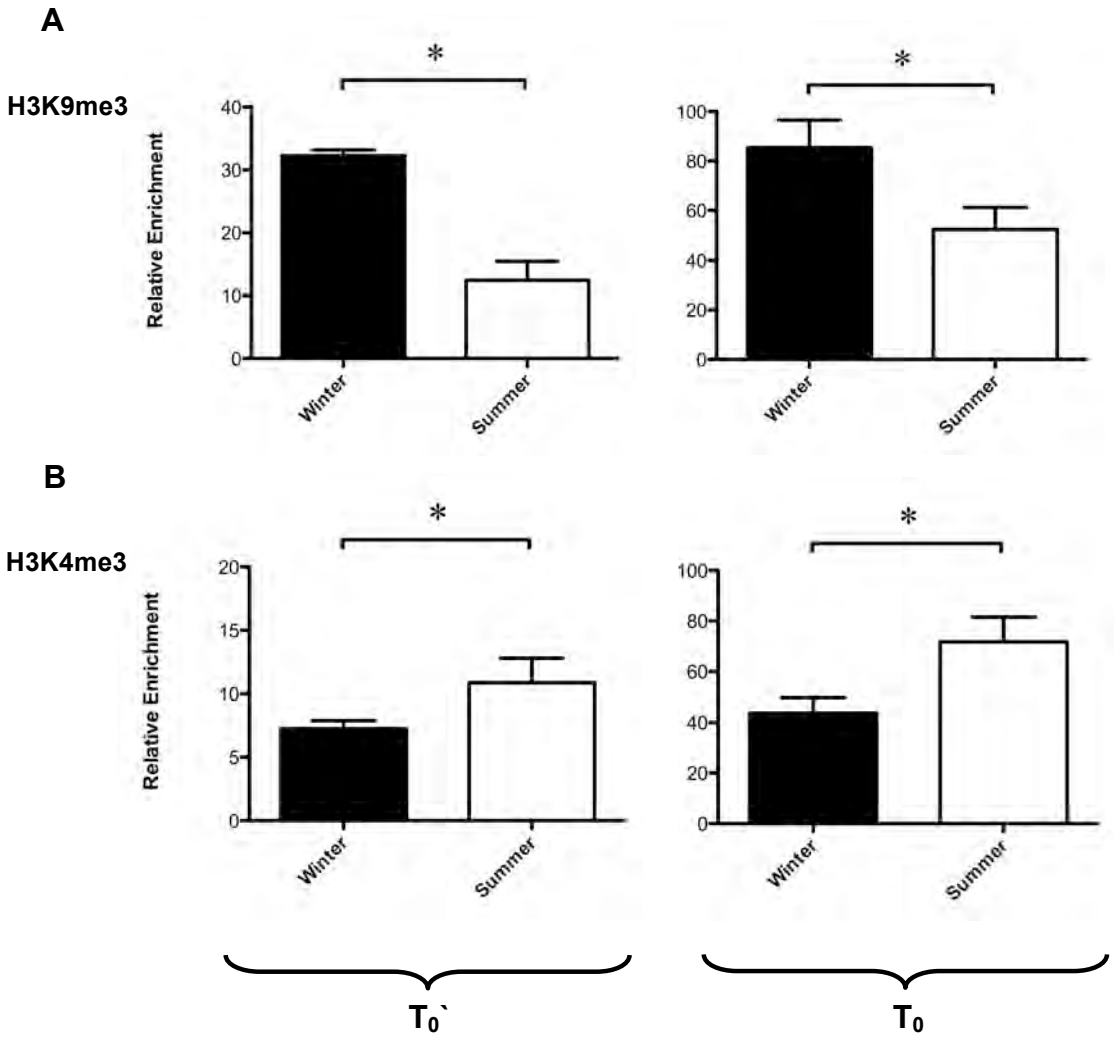

Figure 25. Evaluation of the chromatin state in the *C. carpio* T elements regions

The enrichment of H3K9me3 (heterochromatin) (A) and H3K4me3 (euchromatin) (B) to the promoter-proximal T elements was examined by ChIP and quantified by real time PCR. Standard deviation (±SD) is show. The Statistical analyses were performed using Student's t test (∗= $p < 0.05$).

8.10 pRNA expression during the acclimatization process in *C. carpio*

The noncoding RNAs (ncRNAs) have an important role in the epigenetic control and in the modulation of gene expression, tissue-specific patterning, and cell fate specification. The pRNA (promoter-associated RNA) is an novel ncRNA related with the NoRC function because it is indispensable for heterochromatin formation and rDNA silencing (McStay and Grummt 2008).

In order to assess the contribution of carp pRNA in transcriptional rRNA regulation, primers were designed from the rDNA carp promoter sequence (AF133089) (Vera et al. 2003) in the same regions described for mouse pRNA (Figure 26B). Then, RT-PCR experiments were performed using total RNA as a template from the liver tissue isolated from summer- and winter- adapted carps.

The amplicons obtained were sequenced and correspond to carp rDNA promoter sequence. RT-PCR results showed no significant differences between the seasons (Figure 26C).

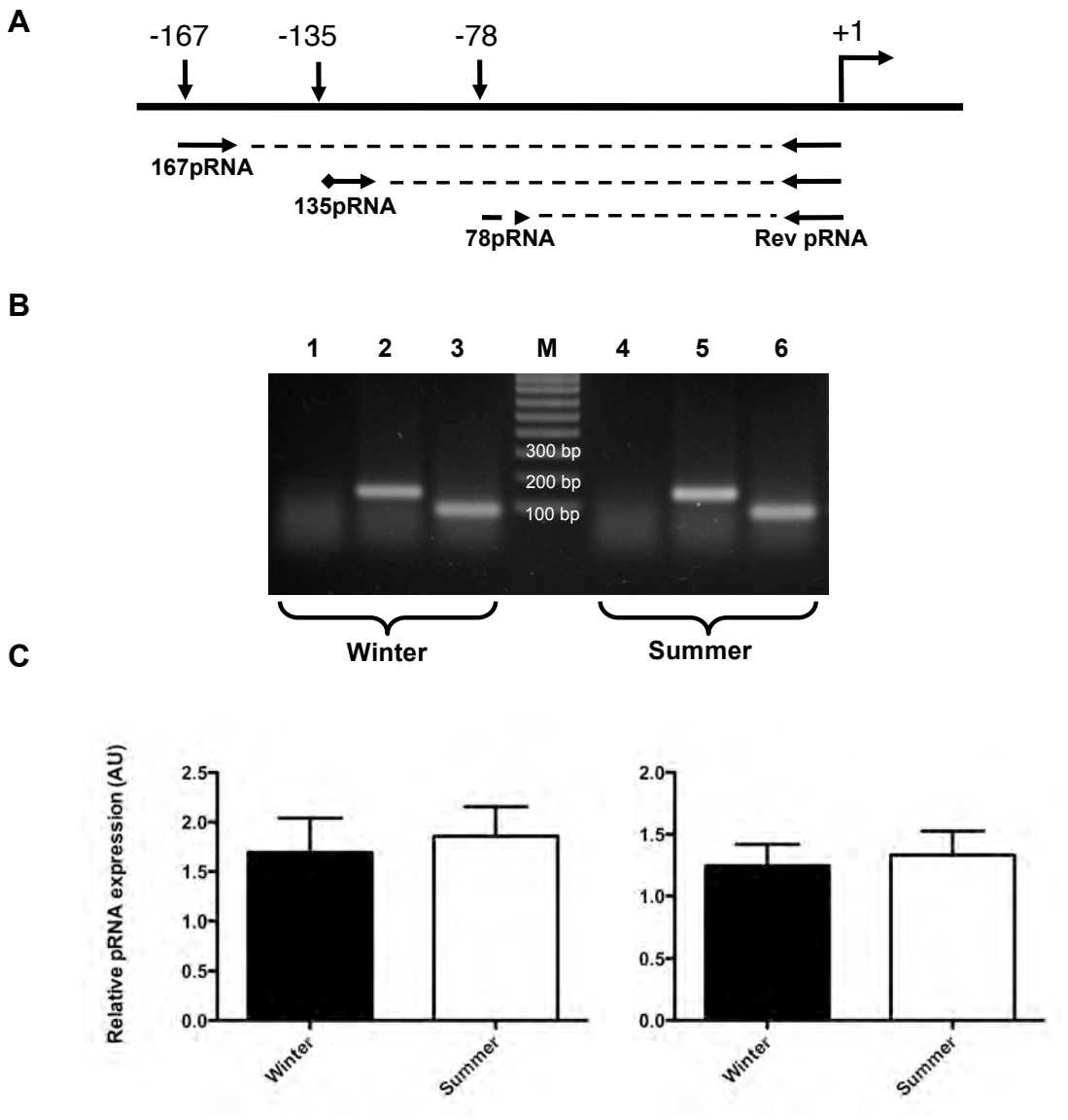

Figure 26. Assessment of *C. carpio* pRNA expression

(A) Scheme of the distribution of the primers used. (B) RT-PCR analysis of pRNA expression from cDNA from winter and summer adapted carps. M: 100 bp DNA ladder. (C) Semi-quantitative conventional RT-PCR performed from liver tissue from seasonally adapted carps (n=3). Standard deviation (±SD) is shown. The left and right graphs show the expression of the amplification used in 135pRNA and 78pRNA respectively.

9 DISCUSSION

Organisms are constantly exposed to environmental variations, and have to develop compensatory responses in order to adapt. The natural environment of the carp is subject to a wide range of biotic and abiotic changes that vary according seasons. Consequently, the fish must develop compensatory responses to adapt to the cyclical, seasonal habitat changes. This phenomenon is called seasonal acclimatization (Krauskopf et al. 1988; Alvarez et al. 2004).

We have observed that during the seasonal acclimatization process, *C. carpio* is capable of modulating the expression of different genes, such as mH2A, Pit-1, L41, Ck2β, and others (Kausel et al. 1999; Alvarez et al. 2001; Molina et al. 2002; Pinto et al. 2005). In particular, we have observed that carp can modulate the expression of the ribosomal genes as part of the compensatory response, a process that is concomitant with the seasonal acclimatization of this fish (Saez et al. 1984; Vera et al. 1993).

Ribosome biogenesis is one of the most important functions in the cell. The transcription of ribosomal genes comprises about 40-60% of cellular transcription, whereas rRNA represents approximately 80% of the total RNA in eukaryotic cells (Hannan et al. 1998). Ribosomal production depends directly on the transcription of the ribosomal genes. Consequently, studying the gene expression reprogramming that occurs in the nucleolus is essential for understanding the molecular mechanisms behind the seasonal acclimatization process.

In recent years, the study of the epigenetic mechanisms in transcriptional regulation has attracted the interest of the scientific community. Recent studies show that epigenetic mechanisms are emerging as ways of transducing extracellular signals, having a reversible control in genetic expression according to the requirements of an organism (Turner 2007; Boyko and Kovalchuk 2008). In this context, during the seasonal acclimatization of the carp, epigenetic mechanisms appear to be an ideal candidate for playing a key role in the gene reprogramming underlying the adaptive process.

An increasing number of studies seem to demonstrate that different epigenetic mechanisms can act coordinately to regulate transcription of rDNA genes (Hirschler-Laszkiewicz et al. 2001; Santoro and Grummt 2001; Lawrence and Pikaard 2004; Grummt 2007; McStay and Grummt 2008), however only a few have been oriented towards the study of the influence of natural environmental conditions.

9.1 Identification of epigenetic factors in *C. carpio*

The transcription terminator factor I (TTF-I), is a factor that recognizes the T elements in the ribosomal cistron and which plays a dual role in the regulation of the rDNA genes, in both the activation and silencing of rDNA transcription (Nemeth et al. 2004). Before this study, this factor was only described in mammalians, and its function in other organisms was unknown.

In this thesis, factor TTF-I in fish is described for the first time within a context of seasonal acclimatization. One of the more important features described is the length difference between mouse and carp TTF-I. Interestingly, the carp protein is 247 aa shorter (around 40% less) than mouse TTF-I in the N-terminus. This region has the negative regulatory domain (NRD) that causes oligomerization and masks the DNA binding domains located in the C-terminal region of the same protein (Smid et al. 1992; Evers et al. 1995; Sander et al. 1996; Nemeth et al. 2004). In addition, the NRD domain in the murine protein interacts with Tip5, a subunit of the nucleolar chromatin-remodeling complex (NoRC) (Strohner et al. 2001). This association facilitates the binding of TTF-I with the promoter regions of the rDNAs, producing the silencing of ribosomal gene expression (Santoro and Grummt 2005). The absence of the NRD domain in carp TTF-I could indicate that the regulation of this protein in fish is with a different mechanism than that described in mammals. The possible mechanism of control should be verified in future experiments.

Furthermore, when performing the analysis of the C-terminus of carp TTF-I, a higher degree of conservation with the mammalian TTF-I was detected. In this region, the DNA binding domains (DBD) exist with a high conservation between the mouse and human (Evers and Grummt 1995). These domains have a high homology with the Myb-type HTH domain, which is a DNA-binding, helix-turn-helix (HTH) domain of approximately 55 amino acids, and is typically found in eukaryotic transcription factors (Peters et al. 1987). The principal feature of this motif is in the degree of conservation that the tryptophan residues in the proteins have with the

DNA binding domain of Myb type (Kanei-Ishii et al. 1990). In the C-terminal of Mouse TTF-I there are two domains with striking homology to the DNA binding domains of Myb (Evers et al. 1995). In the *in silico* C-terminal analysis of carp TTF-I, it was possible to find two putative DBD, both with around 40% similarity with mouse TTF-I DBD (data not show). In relation to tryptophan residues, it was possible to locate at position 418 of carp TTF-I a tryptophan residue that is conserved with respect to tryptophan 688 in the mouse. This amino acid has been described as essential for the binding of TTF-I to the DNA (Evers et al. 1995). It is possible that the conservation between species of this residue could be indicating that it is crucial for carp TTF-I binding to the DNA. All of this data demonstrates the high conservation in the C-terminal of TTF-I between species, indicating that this protein should have similar properties in fish as those described in mice.

Phylogenetic analysis shows that the TTF-I fish clade is a separate group than that of mammal TTF-I, but both clades are more related than with other, similar proteins like Reb1p, which is described as the terminator of the RNA Pol I transcription in *C. cerevisiae* (Morrow et al. 1993). This approximation demonstrates the proximity and similarity between TTF-I from different species.

Concerning the expression and the content of TTF-I, recent studies have shown that TTF-I levels are essential in the cell for an efficient ribosomal biogenesis (Lessard et al. 2012). Carp TTF-I levels showed similar transcriptional expression and protein content in winter and summer acclimatized fish. This result suggests that for a correct cellular homeostasis during seasonal adaptation, the levels of TTF-I would remain constant independently of the environmental stimuli.

Therefore, the differential expression of the ribosomal genes between winter and summer seems does not depend directly of the TTF-I levels. Consequently, TTF-I can play a role as scaffold or pivot to other proteins that controlling the rDNA expression in the acclimatization process.

The TTF-I-interacting protein-5 (Tip5) is a subunit of the nucleolar chromatin-remodeling complex (NoRC) that silences the rDNA. This protein of 205 kDa shares a number of important protein domains. Such shared domains include AT hooks; BAZ1, BAZ2, and WAKZ motifs; a C-terminal PHD (plant homeodomain); and a bromodomain. Tip5 protein has been described only in the mouse and human (Strohner et al. 2001; Santoro et al. 2002), and in this thesis, it was possible to described for first time its presence in a fish. In a bioinformatics analysis performed in the putative carp Tip5 protein, it was possible to detect three domains located in the C-terminus with the same distribution and high identity to the mouse Tip5 protein (McStay and Grummt 2008). The domains described are the bromodomain, PHD, and AT-hook4 with approximately 70% identity each. The high conservation between species domains, essentially in the C-terminal, indicates that this protein should have similar properties to those described in mammals, principally as part of the NoRC complex in ribosomal gene silencing.

TTF-I can interact with the NoRC complex through the Tip5 subunit, leading to the recruitment of the complex to the rDNA promoter, thus allowing the silencing of the ribosomal gene (Nemeth et al. 2004; Strohner 2004). Different works show that Tip5 can repress rDNA transcription through interaction with other factors such as HDAC, DNMT, and HMT, or even by the concentration of this subunit (Santoro

et al. 2002; McStay and Grummt 2008). Therefore, Tip5 is essential for the silencing of this gene. In carp, we found a differential expression of this factor between winter and summer. In both RT-PCR and *Western blot* analyses, the expression and content of Tip5 was significantly higher in winter than in summer. Due to the expression of the ribosomal genes is lower in winter than in summer, and that this expression is concomitant with the nucleolar ultrastructural changes that occur during the acclimatization process, these results suggest us that Tip5 can play an important role in the silencing of ribosomal genes, and its expression is consistent with its repressive action.

The pRNA is a non-coding RNA with an important role for NoRC function. It has been described as essential for heterochromatin formation and rDNA silencing; principally playing a role either as a scaffold or an alosteric effector of NoRC in the epigenetic control of rDNA transcription (Mayer et al. 2006; McStay and Grummt 2008). When the content of pRNA in acclimatized carp was evaluated, the presence of RNA was detected with a sequence that matches the rDNA promoter and which has a length approximately between 135 - 160 nt. Significant differences were not found between winter and summer when was evaluated the pRNA transcript in the cDNAs from acclimatized carps. In order to describe a specific role of this ncRNA in the acclimatization process of the carp, subsequent studies should be performed.

9.2 Epigenetic factors in rDNA gene regulation during the acclimatization process

The TTF-I factor is a specific DNA binding protein that stops the elongation of Pol I and which has an important role in epigenetic regulation of rRNA genes (Grummt et al. 1985; Grummt et al. 1986; Henderson and Sollner-Webb 1986; McStay and Reeder 1986). *In vitro* studies in mouse cells have shown that TTF-I can bind to the T_0 element, located upstream to the start of transcription of ribosomal genes, and can alter the local chromatin structure which can be associated with RNA Pol I, resulting in the activation of rDNA genes (Längst et al. 1997; Längst et al. 1998). Furthermore, according to cell requirements, TTF-I can interact in the T_0 with the NoRC complex, leading to the silencing of the ribosomal genes (Santoro et al. 2002). The T_0 element has been described only in mammals, but an element with similar characteristic also has been described in frog, termed T_3 (Reeder 1999). The conservation of a binding site for a Pol I transcription terminator protein adjacent to the gene promoter suggests that TTF-I exerts an essential function in transcription control of ribosomal genes (McStay and Grummt 2008).

In our laboratory we have reported that the carp ribosomal cistron also has a T_0 element, approximately 414 bp upstream of the transcription start site. Interestingly, we have identified another T element, approximately 615 bp upstream of the T_0, which was called T_0` and whose role remains unclear (Vera et al. 2003). In this thesis we evaluated the interaction of these T elements with TTF-I, in order to study its possible function during the seasonal acclimatization of the

carp. In electrophoretic mobility shift assays (EMSA), it was possible to appreciate the specificity of carp TTF-I protein binding to both T_0 and T_0' sequences. When the T probes were mutated in the specific nucleotides that contained the sequences of these elements, the binding was abolished confirming its specificity. In the same way, mouse TTF-IΔ323 was used as a control, which like carp TTF-I does not have the NRD domain (Nemeth et al. 2004). The results were equivalent, demonstrating that both T_0 and T_0` elements are capable of binding with the recombinant TTF-I. Unexpectedly, mouse TTF-IΔ323 apparently has a higher binding to carp T elements than recombinant carp TTF-I. A possible explanation for the difference can be that fish protein requires other auxiliary components in the nuclear environment in order to allow its binding to T elements. In 1996, Sander et al. showed a similar process. By *in vitro* experiments, the complete mouse TTF-I has less binding affinity to DNA than that of the TTF-IΔ323 protein used, and its binding was dependent on other nuclear components (Sander et al. 1996).

Different studies describe TTF-I with a double function. This factor can bind to the upstream terminator and can either activate the transcription of the rDNA genes or silence them when bind to the NoRC complex (Längst et al. 1998; Nemeth et al. 2004; McStay and Grummt 2008). As described before, the carp has two T elements upstream of the transcription star site, the T_0 and T_0`(Vera et al. 2003). During the acclimatization process, the chromatin immunoprecipitation experiments showed a differential enrichment of TTF-I in the T elements. In particular, the enrichment of carp TTF-I varies between winter and summer for $T_0$ and $T_0$`. In both cases, this factor is significantly more enriched during the cold

season, but the relative enrichment was higher in T_0. These results suggest that the regulatory role of TTF-I in the ribosomal gene during the adaptation process occurs mainly on the T_0 element.

In the formation of the epigenetically silent state of rRNA genes, TTF-I binds to the T_0 element and interacts with Tip5, and this interaction targets NoRC to the rDNA promoter (Nemeth et al. 2004; Strohner 2004; McStay and Grummt 2008). When we assess the Tip5 enrichment in the carp T elements throughout the seasonal adaptation, the results showed a dramatic difference between T_0 and T_0`. The Tip5 enrichment did not change in $T_0$` during the acclimatization process. On the contrary, in the T_0 element, a significantly higher enrichment occurs in winter, whereas no positioning was detected in summer. A possible explanation to this differential enrichment can be responsibility of the contiguous sequence of the T elements that can recognize Tip5, especially considering that this factor can be binding for its AT-hooks elements to the DNA (Strohner et al. 2001). In a bioinformatics approximation of the surrounding sequences of the T elements of carp, and in comparison with the AT-hook DNA sequence for mouse Tip5 (data not shown), the T_0 elements obtained have more similarity (62%) than T_0` element (1,3%) with the mouse sequence. These results strongly suggest that the differential Tip5 enrichment is essential for the silencing of the ribosomal genes, and the T_0 element is key for the regulation of the ribosomal gene during the acclimatization process.

The epigenetic markers are universal and can be directly related with active or silent transcriptional states. Consequently, we evaluated if the seasonal

enrichment of TTF-I and Tip5 can be correlated with inactivation or activation markers, such as H3K9me3 and H3K4me3 respectively (Jaenisch and Bird 2003). The H3K9me3 was more enriched in both T_0 and T_0` in winter conditions, opposite to H3K4me3 that was more enriched in summer. These evidences show the architecture state of the chromatin in the promoter of the rDNA genes, because as markers of activation or inactivation, represent the euchromatin or heterochromatin conformation, respectively. Interestingly, both inactivation and activation markers were more enriched in T_0 than in T_0`, suggesting once again that the T_0 element plays an important role during the transcriptional modulation of the ribosomal genes.

The T_0` element may be involved in the transcription initiation of the pRNA, because different studies have described that the basal promoter (near T_0 in the mouse) is structurally similar to the spacer promoter. In fact, in the spacer promoter an element called T_{sp} exists, but its location is downstream of this promoter and can interact with TTF-I although its function in pRNA transcription is unknown (Németh et al. 2008). In a bioinformatics evaluation of sequences surrounding the T_0 and T_0` elements, around 80% similarity was shown, thus confirming its structural relationship. These approaches suggest that carp $T_0$` element can have an additional potential regulatory function, principally in the pRNA expression control.

9.3 Model Proposed

The diverse evidences reported in the literature, which is in agreement with our results, allow us to postulate an epigenetic mechanism that seems to contribute to the ribosomal gene transcription regulation during the adaptation process in the *Cyprinus carpio* (Figure 27). In a wild condition, carp must be able to respond to the biotic and abiotic conditions variations to survive, and consequently, these environmental changes affect the cell requirements of the fish. In this context, we have described the activity of the ribosomal genes and the nucleolar organization change in response to seasonal adaptation (Vera et al. 1993). These changes occur due to ribosome biosynthesis adapting rapidly to changes; therefore, rRNA synthesis is tightly regulated in response to metabolic and environmental changes (Grummt 2003; Moss et al. 2007). Because the ribosomal genes are present in multiple copies, rRNA synthesis could be modulated by varying transcription rate per gene or by varying the number of active genes (Murayama et al. 2008).

In carp acclimatization, the general activity of the ribosomal genes is lower in winter than in summer, and we postulate that one control point in this process require the NoRC complex. Thus, Tip5 (a main subunit of NoRC) is expressed higher in the cold season in carp, and its enrichment in this season on the promoter of the rDNA genes is concurrent with its function, such as silencing these genes (Figure 27A). To allow for the binding of the NoRC complex to the rDNA promoter, the interaction with TTF-I is necessary. We observed that the content of this protein in carp is constant between winter and summer, but we detect a

difference in its enrichment on the T elements during seasonal adaptation. Consequently, we speculate that in this case, TTF-I could act as scaffolding for Tip5 in winter, while in the summer, TTF-I act as a transcriptional supporter (Figure 27B).

Finally, this work provides novel evidence on how the epigenetic mechanisms contribute to the genetic reprogramming of the rDNAs caused by environmental stimuli during the *Cyprinus carpio* seasonal adaptation process. Thus, we conclude that epigenetic response appears as an efficient and rapid gene regulatory mechanism for cell homeostasis.

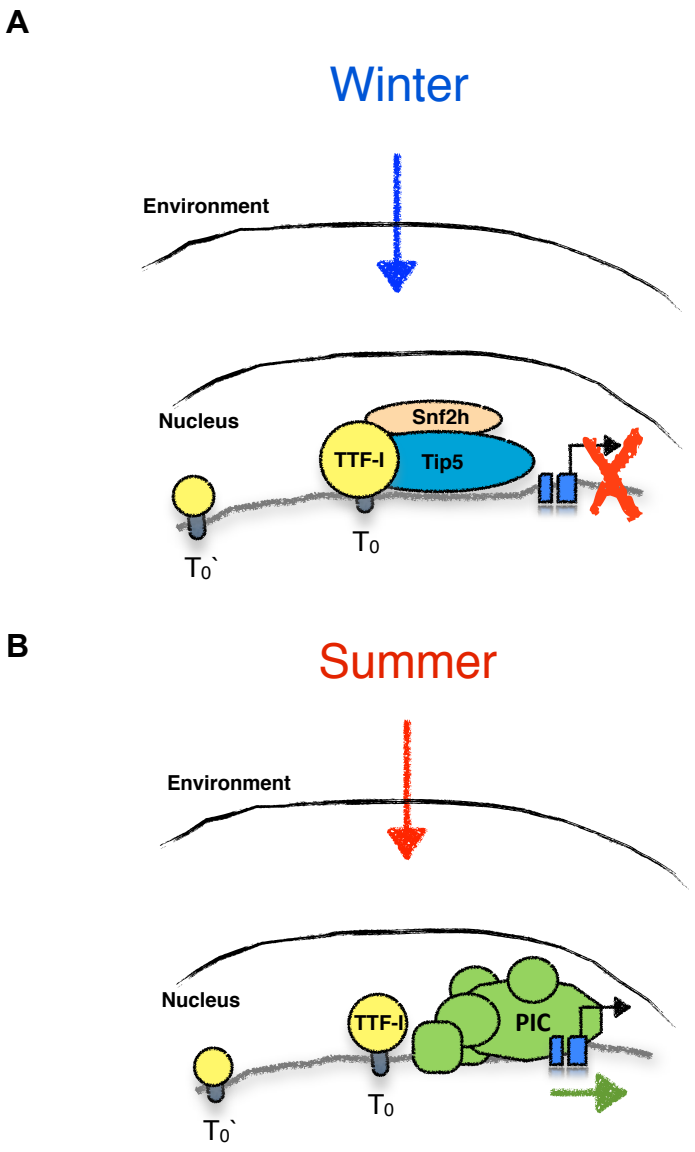

Figure 27. Model of the regulation of rDNA genes during the acclimatization process

During acclimatization process, carp must be able to adapt to environment changes. In winter (A), the NoRC complex is key for the establishment and maintenance of the silent state of the rDNA genes. The interaction with TTF-I allows binding to the promoter region, silencing the ribosomal gene. In summer (B), TTF-I allows the formation of the pre-initiation complex (PIC), promoting transcription. The TTF-I (yellow) are show in proportion with enrichment in the winter and summer. The red X indicates non-expression of the rDNA gene, and the green arrow indicates expression of the rDNA gene.

10 APPENDIXES

10.1 Curriculum Vitae

Last Name: Nardocci

First name: Gino

Nationality: Chilean

Date of Birth: November 10, 1979

Place of Birth: Santiago, Chile

Education

2009 - Present	PhD student of the German-Chilean PhD Program. Supervisor: Dr. Gernot Längst, Regensburg University, Germany.
2009	PhD Qualifying Examination. Molecular Biosciences Program. Andrés Bello University. Chile.
2006 - Present	PhD student at the Molecular Biosciences Program. Supervisor: Dr. Marco Alvarez, Andrés Bello University. Chile.
2005 - 2006	Diploma thesis, “Study of p80-coilin protein of *Cyprinus carpio* as a marker oh the Cajal bodies”. At the Laboratory of Dr. Marco Alvarez. Andrés Bello University
1999 - 2005	Degree in Biochemistry. Chemistry and Biology Faculty. University of Santiago, Chile.
1998 - 2000	Bachelor degree in Science and Humanities. University of Santiago, Chile.

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