Differential enrichment of TTF-I and Tip5 in the T-like promoter structures of the rDNA contribute to the epigenetic response of *Cyprinus carpio* during environmental adaptation.

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Differential enrichment of TTF-I and Tip5 in the T-like promoter structures of the rDNA contribute to the epigenetic response of *Cyprinus carpio* during environmental adaptation

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Abstract

To ensure homeostasis, ectothermic organisms adapt to environmental variations through molecular mechanisms. We previously reported that during the seasonal acclimatization of the common carp *Cyprinus carpio*, molecular and cellular functions are reprogrammed, resulting in distinctive traits. Importantly, the carp undergoes a drastic rearrangement of nucleolar components during adaptation. This ultrastructural feature reflects a fine modulation of rRNA gene transcription. Specifically, we identified the involvement of the transcription termination factor I (TTF-I) and Tip-5 (member of nucleolar remodeling complex, NoRC) in the control of rRNA transcription.

Our results suggest that differential Tip5 enrichment is essential for silencing carp ribosomal genes and that the T₀ element is key for regulating the ribosomal gene during the acclimatization process. Interestingly, the expression and content of Tip5 were significantly higher in winter than in summer. Since carp ribosomal gene expression is lower in the winter than in summer, and considering that expression concomitantly occurs with nucleolar ultrastructural changes of the acclimatization process, these results indicate that Tip5 importantly contributes to silencing the ribosomal genes.

In conclusion, the current study provides novel evidence on the contributions of TTF-I and NoRC in the environmental reprogramming of ribosomal genes during the seasonal adaptation process in carp.

Keywords: Acclimatization, homeostasis, epigenetics, ribosomal genes, Nucleolar remodeling complex.

Introduction

Ribosome-mediated protein synthesis is crucial to cell growth, proliferation, and adaptation to changing environments (Leary and Huang, 2001). Consequently, the transcription of ribosomal genes (rDNA) is a highly regulated process that responds to metabolic and environmental changes. Under these changes, the cell must finely control rDNA transcription to ensure sufficient ribosomes for protein synthesis (Grummt 2003).

Our laboratory previously reported that ribosomal RNA synthesis in the common carp *Cyprinus carpio*, a non-model organism, is reprogrammed as part of a physiological adaptation to seasonal environmental changes (Saez et al. 1984; Vera et al. 1993; Alvarez et al. 2006). This adaptation involves the phenotypic rearrangement of nucleolar components. During winter, fibrillar and granular components of the nucleolus are separated and surrounded by a thick layer of heterochromatin, and this ultrastructural feature is accompanied by a transient repression of ribosomal RNA transcription. This condition is reversed during the summer when active ribosomal biosynthesis is recovered. Therefore, the seasonal adaptation of carp involves a fine regulation of ribosomal transcription (Alvarez et al. 2006).

The rDNA genes exist in the nuclear genome as multiple copies of transcription units tandemly arrayed in clusters (Grummt 2003). In different organisms, the transcription units are flanked on both sides by terminator elements called T elements (McStay and Reeder, 1990; Evers and Grummt, 1995; Jacob 1995; Vera et al. 2003), which are recognized by the transcription terminator factor I (TTF-I). Binding of TTF-I to terminator elements downstream of the transcription unit (T₁-T₁₀) stops the transcription of RNA polymerase I

(Grummt et al. 1986). Interestingly, the binding of TTF-I to the element located immediately upstream of the ribosomal gene promoter, defined as T_0 , is a key event that facilitates the initiation of transcription for rDNA reconstituted into chromatin (Längst et al. 1997; Längst et al. 1998; Diermeier et al. 2013). Through this event, the TTF-I factor can then bind to the upstream terminator element and either activate or silence the transcription of rDNA genes by modifying the architecture of the promoter or by binding to the nucleolar remodeling complex (NoRC), respectively (Längst et al. 1998; Németh et al. 2004; McStay and Grummt, 2008).

The NoRC is a major complex involved in silencing rDNA genes through the recruitment of DNA methyltransferase and histone deacetylases to the rDNA promoter, thus promoting a closed chromatin state (Németh et al. 2004; Santoro and Grummt, 2005). The NoRC complex is formed by two components, an exclusive 205 kDa protein identified as TTF-I Interacting protein 5 (Tip5) and SNF2h, a subunit with ATPase activity. This complex has been described in mice, humans, and, recently, in carp (Strohner et al. 2001; Santoro and Grummt, 2002; Fuentes et al. 2014).

Association of Tip5 with TTF-I leads to the recruitment of NoRC to the rDNA promoter, specifically in the T_0 element, thereby silencing ribosomal genes (Németh et al. 2004; McStay and Grummt, 2008; Strohner et al. 2004). Moreover, participation of TTF-I in the recruitment of NoRC indicates that this factor can coordinate an epigenetic regulation of ribosomal RNA transcription (McStay and Grummt, 2008). Recently, our group became the first to identify TTF-I and Tip5 in carp muscle tissue, in addition to characterizing seasonal transcriptional regulation in this fish. This finding suggests that these factors

could be directly involved in the compensatory response of carp seasonal acclimatization (Fuentes et al. 2014).

Previously, we described that the carp presents T-like elements, both in the promoter and terminal regions of the ribosomal cistron (Vera et al. 2003). Interestingly, upstream of the transcription initiation site, two potentially regulatory elements, termed T_0 and T_0' , were identified. The latter (T_0') appears to be characteristic of the carp ribosomal cistron as this element has not been described in rDNA genes from other species. Moreover, the function of T_0' is still unknown.

Considering the importance of the T elements in the transcriptional regulation of rDNA genes, this study evaluated the interaction between the T-like elements and TTF-I in carp to determine possible functions during seasonal acclimatization. Novel evidence was found that these factors are involved in the compensatory response of carp to seasonal acclimatization. Therefore, this work adds to understandings on how epigenetic mechanisms are involved in the fine regulation of ribosomal genes during a natural adaptive response.

Materials and Methods

Animals and tissues: Adult male carp (1000-2000 g) were maintained under natural seasonal conditions as previously described (Araya et al. 2010). The fish were sacrificed, and liver samples were obtained, washed with PBS pH 7.4 (137 mM NaCl; 2.7 mM KCl; 10 mM Na₂HPO₄; 2 mM KH₂PO₄), and immediately processed or stored at -80°C until required. This study adhered to animal welfare procedures and was approved by the Bioethical Committee of the Universidad Andrés Bello.

RT-qPCRs: Total RNA was purified from the liver using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The quality of RNA was evaluated by electrophoresis and spectrophotometrically quantified. The cDNA was synthesized from 2 µg of total RNA using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) and random primers according to the manufacturer's instructions. The resulting cDNAs were PCR-amplified in the GoTaq Green Master Mix (Promega, Madison, WI, USA) using the oligonucleotides shown in Table S1.

The RT-qPCR was performed with the appropriate oligonucleotides and the Brilliant SYBR Master Mix (Stratagene, Agilent Technologies, Santa Clara, CA, USA) in a final volume of 20 µL and on an Mx3000P Real-Time PCR System (Stratagene, Agilent Technologies, Santa Clara, CA, USA). The average seasonal ratio of mRNA levels was calculated using the Pfaffl method (Pfaffl 2001), and carp mitochondrial 16S rRNA as a seasonally-constitutive gene expression.

Chromatin immunoprecipitation (ChIP): ChIP assays were performed as previously described (Araya et al. 2010). For each assay, approximately 3×10^7 nuclei were isolated from hepatocytes of winter- and summer-acclimatized carp. Immunoprecipitations were performed with polyclonal anti-H3K9me3 (Abcam, Cambridge, UK), anti-H3K4me3 (Abcam, Cambridge, UK), anti-Tip5 (kindly provided by Dr. Längst), and anti-TTF-I (synthesized in this study). The immunoprecipitated DNA was amplified by qPCR on an Mx3000P Real-Time PCR System (Stratagene, Agilent Technologies, Santa Clara, CA, USA) using oligonucleotides (Table S1) corresponding to the T_0 and T_0' regions of carp rDNA (GeneBank Accession N° AF133089.2). Real-time quantification was performed using the Brilliant SYBR Master Mix (Stratagene, Agilent Technologies, Santa Clara, CA, USA). The occupancy ratios of the T_0 and T_0' regions were calculated with the following formula:

$$\text{Occupancy ratio} = ((X_a - X_{\text{IgG}})/X_{\text{input}}) \times 100$$

X corresponds to:

$$X = \text{Log}_{10} ((C_t - C_{t0})/-s)$$

where "s" is the slope obtained for PCR efficiency in the calibration curve and C_{t0} is the averaged C_t obtained in the calibration curve for each oligonucleotide (Németh and Längst, 2008).

Nuclear extract and protein quantification: Nuclear extracts were prepared from liver tissue lysates in an H buffer (250 mM sucrose, 3 mM CaCl_2 , and 1 mM phenyl-methylsulfonyl fluoride in 20 mM Tris pH 7.4). The subsequent procedures were performed as previously described (Araya et al. 2010).

Nuclear proteins were quantified using the DC Universal Protein Assay Kit (Bio-Rad, Hercules, CA, USA), and protein integrity was confirmed by SDS-PAGE.

Electrophoretic Mobility Shift Assays: Reactions (10 μ l) contained 500 fmol of IR700- or IR800-labelled oligonucleotides (Table S1), electrophoretic mobility shift assay (EMSA) buffer (20 mM Tris-HCl pH 7.0, 80 mM KCl, 5 mM MgCl₂, 0.2 mM EDTA, 10% glycerol, and 0.2 mM DTT), and 250-1000 fmol of purified carp TTF-I. After incubation for 10 min at 30°C, protein-DNA complexes were separated by electrophoresis on native 4.8% polyacrylamide gels and visualized with the Odysseys Infrared Imaging System (LI-COR, Lincoln, NE, USA).

Expression and purification of recombinant carp TTF-I: Primers were designed from the sequence previously obtained in our lab.²⁰ The PCR product was cloned into the pET-15b expression vector (Novagen, Merck Millipore, Darmstadt, Germany). Rosetta 2 (DE3) pLys-competent cells were transformed with a pET-15b-TTF-I clone. Bacteria containing this clone were grown at 37°C with agitation in a minimal medium plus 50 mg/mL ampicillin and 33 mg/ml chloramphenicol until the log-phase. The cultures were subsequently induced with 1 mM IPTG for 4 h. The recombinant protein was purified with Ni-NTA according to the manufacturer's instructions (Qiagen, Germantown, MD, USA).

Western blot: For immunodetection, the polyclonal α -Tip5 (1:500), α -TTF-I (1:100), and α -H2B (Abcam, Cambridge, UK) (1:250) antibodies were used. Peroxidase-conjugated anti-rabbit IgG (H+L) secondary antibodies were

obtained from KPL (Gaithersburg, MD, USA). Protein-antibody complexes were visualized through the Enhanced Chemiluminescence (ECL) Detection System (Amersham Biosciences, Amersham, UK).

Preparation of TTF-I polyclonal antibodies: Specific carp TTF-I antibodies were prepared by BiosChile S.A. (Santiago, Chile) by immunizing two rabbits with the recombinant TTF-I protein.

Statistical Analysis: Each assay was performed in triplicate and repeated with three independent samples. Results are expressed as the mean $\pm$ standard deviation (SD) of the mean for three different experiments. Statistical differences were assessed by an unpaired Student's *t*-test, with significance established at $p < 0.05$. Statistical analyses were performed and graphs were constructed with the GraphPad Prism v.5 software (GraphPad Software Inc., La Jolla, CA, USA).

Results

Expression of *C. carpio* TTF-I during acclimatization.

To assess the gene expression of TTF-I during seasonal acclimatization, qRT-PCR assays were performed on carp liver cells. TTF-I transcripts were detected in both seasons, with no significant differences between winter and summer (Fig. 1A). Likewise, TTF-I protein levels did not seasonally vary (Fig. 1B).

Considering the important role of Tip5 in ribosomal gene regulation (Németh et al. 2004; Strohner et al. 2004), the transcriptional expression of Tip5 was also evaluated in acclimatized carps. Tip5 expression significantly increased during the winter (Fig. 1A), a result supported by Western blot assays (Fig 1B). As a control, the expression of rDNA was analyzed during the process of seasonal acclimatization. In the summer, rRNA expression was significantly higher compared to winter. These results are consistent with our previous research (Vera et al. 1993; Alvarez et al. 2004; Alvarez et al. 2006).

In vitro interaction of recombinant TTF-I with *C. carpio* T₀ and T₀' elements.

Our group previously studied the organization of the carp rDNA gene in detail (Vera et al. 2003). To investigate whether carp TTF-I has the ability to bind to the T₀ and T₀' elements, the recombinant protein was purified and EMSAs were performed (Fig. 2B). The probes for both carp T elements were able to bind with carp TTF-I. However, in mutant T elements, the electrophoretic mobility shift was abolished, thus demonstrating the binding specificity of this factor.

Evaluation of TTF-I enrichment in the T_0 and T_0' regions of *C. carpio* ribosomal genes during acclimatization.

To evaluate the enrichment of TTF-I in carp T elements during the acclimatization process, ChIP assays were performed (Fig. 3). TTF-I was significantly enriched in both the T_0 and T_0' elements during the winter. Interestingly, this enrichment was clearly higher in T_0 (Fig. 3A).

In turn, Tip5 enrichment did not change in the T_0' element between winter and summer. However, there was significantly higher Tip5 enrichment in the T_0 element during winter compared to summer (Fig. 3B).

To evaluate the chromatin state in carp T elements during the acclimatization process, ChIP assays were performed using antibodies against specific post-translational histone modifications associated with activation (H3K4me3) or repression (H3K9me3) (Fig. 3C and 3D, respectively) (Bernstein and Hake, 2006). The euchromatin mark, H3K4me3, was more abundant in both T elements during the summer than in the winter. Notably, this mark was more present in T_0 than T_0' . On the other hand, H3K9me3 was significantly higher during the winter for both T_0 and T_0' .

Discussion

An increasing number of studies indicate that different epigenetic mechanisms can coordinately act to regulate rDNA gene transcription (Hirschler-Laszkiewicz et al. 2001; Santoro and Grummt, 2001; Lawrence 2004; Grummt 2007; McStay and Grummt, 2008). However, few studies have explored the role of these mechanisms in a context influenced by natural environmental conditions (Araya et al. 2010; Simonet et al. 2013; Baulcombe and Dean, 2014).

Previous research by our group found that during the seasonal acclimatization process, *C. carpio* can modulate the expression of different genes (Kausel et al. 1999; Alvarez et al. 2001; Molina et al. 2002; Pinto et al. 2005). Specifically, carp can regulate the expression of ribosomal genes as part of a compensatory response to environmental stimuli.

Moreover, we reported that the carp ribosomal cistron has a T_0 element approximately 414 base pairs upstream of the transcription start site. Interestingly, we also identified another T element approximately 615 base pairs upstream of T_0 . This element is termed T_0' , and its role remains unclear (Vera et al. 2003). In the present report, we evaluated the interaction of these T elements with TTF-I to study possible functions during the seasonal acclimatization of the carp. Through EMSA, the specificity of carp TTF-I protein binding to both the T_0 and T_0' sequences was highlighted. When the T probes were mutated in the specific nucleotides that contained the sequences of these elements, the binding was abolished, thus confirming specificity. These results support that both the T_0 and T_0' elements are capable of binding with recombinant TTF-I.

On the other hand, ChIP assays for TTF-I in the T elements during the adaptation process showed a differential enrichment of the TTF-I ribosomal cistron. In particular, the enrichment of carp TTF-I varied between winter and summer for T_0 and T_0' . In both cases, TTF-I was significantly more enriched during winter, but 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 in the T_0 element, which is in agreement with its function in mice (Längst et al. 1998; Németh and Längst, 2004; McStay and Grummt, 2008). Regarding TTF-I expression and content, recent studies have shown that TTF-I levels are essential in the cell for efficient ribosomal biogenesis (Lessard et al. 2012). Carp TTF-I showed similar transcriptional expression and protein contents in winter- and summer-acclimatized fish. This result suggests that for correct cellular homeostasis during seasonal adaptation, TTF-I levels remain constant independent of the environmental stimuli. Therefore, the differential expression of ribosomal genes between winter and summer appears to occur independent of TTF-I levels. Consequently, TTF-I may play a role as a scaffold or pivot for other proteins that control rDNA expression during acclimatization.

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 ribosomal gene silencing (Stohner et al. 2004; Németh and Längst, 2004). Different studies show that Tip5 can repress rDNA transcription through interaction with other factors such as HDAC, DNMT, and HMT or even through the concentration of the Tip5 subunit itself. Therefore, Tip5 is essential for silencing the rDNA gene. Tip5 enrichment in carp T elements was evaluated

during seasonal adaptation, with the results showing dramatic differences between T_0 and T_0' . Tip5 enrichment did not change in T_0' during acclimatization. In contrast, there was significantly higher Tip5 enrichment in the T_0 element during winter, but no binding was detected in the summer. Differential enrichment could be due to the contiguous T element sequences that can recognize Tip5, especially considering that this factor can bind by its AT-hooks elements to DNA (Stohner et al. 2001). Similarly, an alternative explanation could be different methylation states in these regions. In this respect, NoRC-mediated rRNA gene silencing requires the methylation of CpG at position 133 (Santoro et al., 2005), and in carp, we have observed hypermethylation of the proximal promoter during winter as compared to summer (Pinto et al., 2005). Unfortunately, we do not have information about the methylation state at position -1077, which corresponds to T_0' (Vera et al., 2003). Certainly, different methylation states of the DNA regions containing T-elements could also explain the differentiated seasonal enrichments of Tip5. These results suggest that differential Tip5 enrichment is essential for silencing carp ribosomal genes and that the T_0 element is key for regulating the ribosomal gene during the acclimatization process.

The present study evaluated epigenetic modification in the rRNA gene, specifically in the T elements, during the adaptation process of carp. The epigenetic mark of euchromatin, H3K4me3, was more enriched in both T_0 and T_0' during summer. Notably, this mark was more enriched in T_0 , suggesting the importance of this element in the activation process of rDNA genes during acclimatization. Additionally, the repressive mark, H3K9me3, was more enriched during winter in both elements. These results are similar to that

reported by Simonet et al. (2013), indicating the importance of the heterochromatin state in the ribosomal promoter. Specifically in Simonet et al. (2013), H3K4me3 enrichment was similar to the present study, where the mark was more present in T₀, thus confirming the importance of this element.

All of our findings suggest that during the acclimatization process of *Cyprinus carpio*, there is an epigenetic modulation of the ribosomal genes. These genes pass from an active to repressive state during winter acclimatization, which is crucial for the homeostasis of this fish. During regulation, the epigenetic response is necessarily involved in different mechanisms, including NoRC, that play fundamental roles during the adaptation process. Furthermore, while other factors studied by our group, such as macroH2A and H2A.z, are not specific for ribosomal genes, they can act on other genes during acclimatization (Araya et al. 2010; Simonet et al. 2013)

In conclusion, our results provide novel evidence on how epigenetic mechanisms contribute to environmentally-stimulated genetic rDNA reprogramming during the seasonal adaptation of *Cyprinus carpio*. Epigenetic responses appear to be an efficient and gene-regulation mechanism for cell homeostasis. However, further studies are needed to define the molecular mechanism of these proteins in the control of rRNA synthesis during seasonal acclimatization.

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Figure legends

Figure 1. Differential expression of epigenetic factors during the acclimatization process.

Real-time qRT-PCR quantification of carp (A) TTF-I (upper), Tip5 (middle), and pre-rRNA (lower) expressions during the acclimatization process. The black and white columns indicate winter (W) and summer (S), respectively. For all experiments, the average expression of three independent experiments were graphed and quantified in triplicate. Bars indicate standard deviation ($\pm$ SD) of the mean. Student's *t*-test analyses rendered significant differences (* = $p < 0.05$). (B) Western blot analysis of the carp. Nuclear proteins from the liver tissue of winter- and summer-acclimatized carp were used to evaluate the TTF-I (upper) and Tip5 (middle) contents. The same blot was then probed with antibodies against H2B histone (lower), which was used as a loading control.

Figure 2. Electrophoretic mobility shift assay.

(A) Schematic representation of the carp cistron. The regions evaluated in the experiments are indicated by asterisks. (B) Electrophoretic mobility shift assay using the carp T_0' (left) or T_0 (right) elements as probes. Increasing amounts of recombinant carp TTF-I 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 complexes were analyzed on 4.8% native polyacrylamide gels.

Figure 3. Enrichment of epigenetic factors and marks in the ribosomal cistron of *C. carpio*.

(A) Chromatin immunoprecipitation analysis for the enrichment of TTF-I, (B) Tip5, (C) H3K4me3 (euchromatin), and (D) H3K9me27 (heterochromatin) on T₀' and T₀ elements. Bars indicate standard deviation ($\pm$ SD) of the means. The black and white columns indicate winter (W) and summer (S), respectively. The graphs show the average expression of three independent experiments quantified in triplicate. Student's *t*-test analyses rendered significant differences (* = $p < 0.05$).

Draft

A

B