



A comparative expression analysis of gene transcripts in brain tissue of non-transgenic and GH-transgenic zebrafish (*Danio rerio*) using a DDRT-PCR approach

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ABSTRACT

The presence of higher level of exogenous growth hormone (GH) in transgenic animals could lead to several physiological alterations. A GH transgenic zebrafish (*Danio rerio*) line was compared to non-transgenic (NT) samples of the species through a DDRT-PCR approach, with the goal of identifying candidate differentially expressed transcripts in brain tissues that could be involved in GH overexpression. Densitometric analyses of two selected amplification products, *p300* and *ADCY2*, pointed to a significant lower gene expression in the transgenic zebrafish (104.02 ± 57.71 ; 224.10 ± 91.73) when compared to NT samples (249.75 ± 30.08 ; 342.95 ± 65.19). The present data indicate that *p300* and *ADCY2* are involved in a regulation system for GH when high circulating levels of this hormone are found in zebrafishes.

Key words: differential display, GH, growth hormone, transgenic, zebrafish.

INTRODUCTION

The growth hormone gene (GH) is primarily responsible for the regulation of processes related to somatic growth in animals. However, GH may also affect the metabolism of fats, proteins and carbohydrates, the maintenance of the immune system and stress response, regulating the expression of a number of genes involved in

these processes. However, there is few data on the GH effects in brain. In this study, brain tissue from a transgenic line of *Danio rerio* (strain F0104) to the growth hormone gene (GH) were used for the identification and characterization of DETs (Differential Expressed Transcripts), through comparisons with brain tissue from a non-transgenic strain of this species. This study opens up important perspectives for the elucidation of mechanisms used by GH to control the expression of genes related to brain functions.

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RESULTS

The growth hormone (GH) is a multiple-function hormone whose excess can cause several physiological and behavioral collateral effects in the organism (Bartke et al. 1998, Cook et al. 2000, Brown-Borg et al. 2001, Rosa et al. 2008). Therefore, the presence of a higher level of exogenous GH in transgenic animals could lead to metabolic alterations that can influence not only the animal growth, but also several other traits.

During the last decade, the interest on GH overexpression effects has increased significantly, and several transgenic animal models overexpressing GH have been developed, including mammals (mouse and rat) and some teleost fishes (*Oncorhynchus mykiss*, *Danio rerio*) (Ikeda et al. 1998, Devlin et al. 2001, Lall et al. 2004, Figueiredo et al. 2007). However, there is few data on the GH overexpression effects in brain, a feature that has only been clearly analyzed using mouse GH transgenic models (Lall et al. 2004, Bohlooly-Y et al. 2005). As the GH secretion in the brain is regulated through a complex neuroendocrine control system that is modulated by hypothalamic peptides and neurotransmitter networks (Müller et al. 1991), it would be interesting to determine which genes in this tissue could be associated with GH overexpression.

GH transgenic zebrafish (*Danio rerio*) represents one of the most explored species lines with an exogenous gene. However, the effects of growth hormone overexpression in the brain of this transgenic animal were not clarified so far. Therefore, the present study intended to identify candidate DETs (Differentially Expressed Transcripts) in brain samples of GH transgenic and non-transgenic zebrafishes using a DDRT-PCR (Differential Display Reverse Transcription - Polymerase Chain Reaction) approach due to its fast, reproducible and technically easier methodology. Moreover, DDRT can detect low abundance mRNA species (Liang 2002, Pardee and McClelland 1999).

Six adult zebrafishes - three non-transgenic (NT) and three GH transgenic - were obtained from Universidade Federal do Rio Grande (Rio Grande, RS, Brazil). The GH transgenic zebrafish belong to the F0104 line that has a carp (*Cyprinus carpio*) β -actin promoter driving the expression of two exogenous genes - the growth hormone gene from the marine silverside fish (msGH) *Odonthestes argentinensis* and the jellyfish (*Aequorea victoria*) GFP (Green Fluorescent Protein) as a reporter gene to monitor for transformed zygotes (Figueiredo et al. 2007). Transgenic and NT genotypes were determined through UV fluorescence analysis with an epifluorescence microscope (excitation=485 nm; emission=520 nm) through the presence or absence of a fluorescence level related to the GFP expression. All procedures were carried out according to the international practices for animal use and care under the control of an internal committee of the Universidade Federal do Rio Grande (FURG), Rio Grande, RS, Brazil.

Brain samples were collected from the animals and 100mg of each tissue were mechanically homogenized with 1mL of TRizol Reagent (Invitrogen Life Technologies), following the manufacturer's instructions. Total RNA samples were incubated with DNase I and eluted in RNase-free water. The samples were quantified (Thermo Scientific NanoDrop 1000 Spectrophotometer) by measuring the optical density (OD) at 260nm. RNA purity was ensured by obtaining a 260/280nm OD ratio ≥ 1.80 . Approximately 5 μ g of RNA samples were reverse transcribed (RT-PCR) with the commercial kit SuperScript III RT (Invitrogen Life Technologies) using the oligonucleotide AP (5'-GGCCACGCGTCGACTAGTAC(T)₁₇-3') according to the manufacturer's instructions.

Each cDNA amplification reaction consisted of 1 μ L of cDNA, 0.2mM of primer (designed based on VNTR - Variable Number of Tandem Repeats - core sequences) (Table I), 1x 25mM MgCl₂ PCR buffer, 0.2mM of dNTPs, and 0.2 unit

TABLE I
Oligonucleotides that were designed based on VNTR core sequences and used as single primers for the amplification of the cDNA samples of *Danio rerio*.

Primer	Sequence (5' - 3')	Reference
INS	ACAGGGGTGTGGGG	Nakamura et al. (1987)
YNZ22	CTCTGGGTGTCGTGC	Nakamura et al. (1987)
HBV5	GGTGTAGAGAGGGGT	Nakamura et al. (1987)
HBV3	GGTGAAGCACAGGTG	Nakamura et al. (1987)
FvIIex8	ATGCACACACACAGG	Murray et al. (1988)
EMBL	AGAGCTTCAGGCTGGGCAGCTAAG	Harris and Wright (1995)

of Platinum *Taq* DNA polymerase (Invitrogen Life Technologies) in a final volume of 25 μ L. Reactions were carried out with an initial denaturation step at 95°C for 2 minutes, followed by 40 cycles at 94°C for 50 seconds, 55°C for 2 minutes, and 72°C for 50 seconds, with an additional extension step at 72°C for 5 minutes. Amplification products (10 μ L) were fractionated on 6% polyacrylamide gel, stained with 0.17% silver nitrate (AgNO₃) (Sambrook and Russel 2001) and visualized under white light. The molecular weight of the amplified fragments was assigned through comparison with a 1Kb DNA ladder (Invitrogen Life Technologies).

The amplified fragments corresponding to putative differentially expressed transcripts were quantified by densitometry as Integrated Optical Density (IOD) using Imaging Master VDS Software version 3.2 (GE Healthcare Life Sciences). Mean and standard deviations (SD) were determined using the Microsoft Office Excel Software. The data were submitted to statistical analysis using Student's unpaired t-test ($p < 0.05$) to normal distribution (Zar 1999). The previous selected fragments were reamplified in order to obtain a higher DNA content to be used on cloning and nucleotide sequencing. The fragments were cut from the polyacrylamide matrix and used directly on reamplification reactions, as described in Dakis and Kouretas (2002). Reamplification PCR conditions and primers were the same as described above. PCR

products were cloned into pGEM-T (Promega) vector and used to transform competent cells of the *E. coli* strain DH5 α (Invitrogen Life Technologies), following the manufacturer's instructions. A total of 12 clones (6 clones of each selected fragment) were submitted to automated sequencing on an ABI 377 Automated DNA Sequencer (Applied Biosystems) with a DYEnamic ET Terminator Cycle Sequencing kit (GE Healthcare Life Sciences) following the manufacturer's instructions and using primers complementary to vector arms. Nucleic acid sequence database searches were performed using BLAST/N (Altschul et al. 1990) at the National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov/blast>). Sequence alignments were obtained by Clustal-W function (Thompson et al. 1994).

cDNA amplification using primer INS led to the identification of an intensely stained fragment of approximately 1,000 base pairs (bp), present in NT and in GH-transgenic zebrafish (Figure 1a), which seemed to correspond to a transcript with differential gene expression among different samples (Figure 1a). Densitometric analyses of this amplified product, through IOD values, evidenced a significant difference between transgenic and non-transgenic samples ($t = 0.0061$), suggesting a lower gene expression level in the transgenic (104.02 \pm 57.71) when compared to NT animals (249.75 \pm 30.08) (Figure 2).

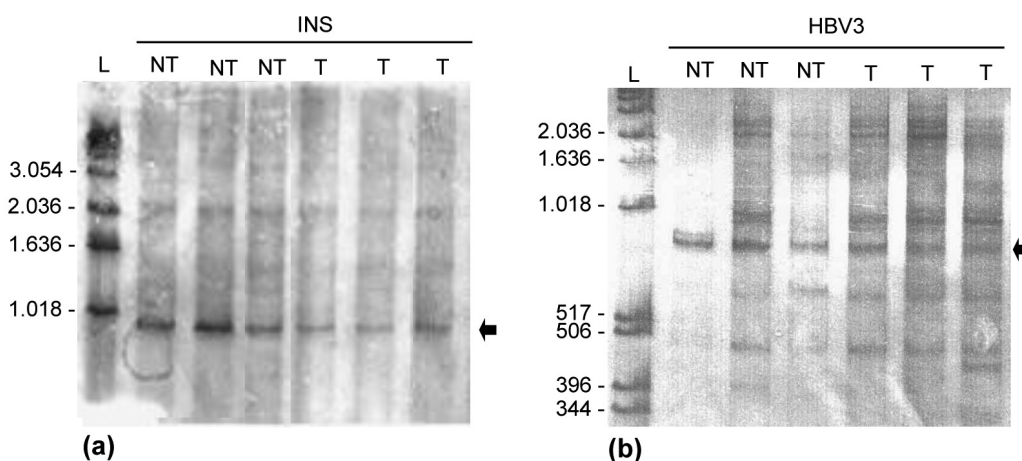


Figure 1: DDRT-PCR patterns of non-transgenic (NT) and GH-transgenic (T) zebrafish F0104 line, visualized through 6% polyacrilamide gel electrophoresis. **(a)** brain cDNA amplification using primer INS; **(b)** brain cDNA amplification using primer HBV3. L, 1 Kb DNA molecular marker. Arrows indicate putative differentially expressed transcripts between the samples.

Cloning and sequencing of this transcript permitted the characterization of a 941 bp DNA fragment. Database nucleotide searches indicated a 95% identity level with a partial region of the gene that codes for the p300 protein of *Danio rerio* (accession number XM_001332682, NM_001510744) and also a high identity with the p300 gene of several other vertebrates, as *Ctenopharyngodon idella* (81%), *Gallus gallus* (81%) and *Mus musculus* (78%).

The use of the primer HBV3 to amplify the cDNA samples of *Danio rerio* resulted on several faint and diffuse bands (Figure 1b). However, a fragment of approximately 900 bp was clearly visible in both NT and GH-transgenic samples (Figure 1b). The densitometric analyses of this transcript suggested a significant ($t=0.0115$) higher expression level in NT animals (342.95 ± 65.19) than in GH-transgenic fishes (224.10 ± 91.73) (Figure 2). Nucleotide sequencing analysis evidenced a DNA fragment with 831 bp, and BLAST database searches revealed a high identity level (94%) with the 3' end of the gene that codes for the protein adenylate cyclase 2 (ADCY2) found in the brain tissue of *Danio rerio* (accession numbers CR759914 and GI6270118).

The p300, also known as E1A binding protein p300, is involved in several processes, as cellular differentiation and proliferation, cycle cell regulation, growth, apoptosis and histone acetylation (Huret 2000, Vleugel et al. 2006). Tan et al. (2009) suggested that this protein protects neuron from neurologic damages by inducing an increase of histone acetylation and preventing neuronal degeneration. Several studies have evidenced that the p300 is a transcriptional coactivator that interacts with other proteins, leading to the activation of the transcriptional process of several genes (Huret 2000, Tu and Luo 2007), including few genes related to cancer (Vleugel et al. 2006, Zhao et al. 2006, Chen et al. 2009) and neural pathologies development (Renoult et al. 2007, Francis et al. 2007). The relationship between p300 and GH was evidenced through an analysis of the *c-fos* proto oncogene expression - the GH activates the *c-fos* through a stimulation of the p300 protein, which acts as a transcription factor, to occupy the enhancer/promoter region of the gene (Cui et al. 2005).

The Adenylate cyclase (AC) catalyses the conversion of ATP into cAMP, which, in turn, acts to regulate a wide variety of cellular processes.

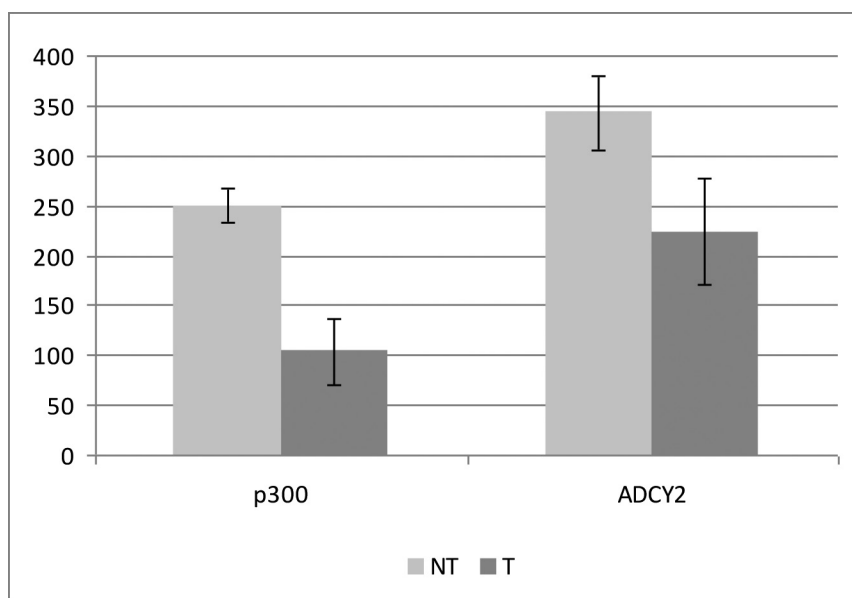


Figure 2: Densitometric analyses (Integrated Optical Density-IOD) of a fragment amplified with the primer INS that corresponds to the gene that codes for protein p300 (E1A binding protein p300) and of a fragment amplified with the primer HBV3 that corresponds to the gene that codes for protein ADCY2 (adenylate cyclase 2). These fragments correspond to putative differentially expressed transcripts identified through DDRT-PCR in non-transgenic (NT) and GH-transgenic (T) zebrafish F0104 line.

It was evidenced that the neuropeptide PACAP (pituitary adenylate cyclase-activating polypeptide) can modulate the AC activity, leading not only to the formation of cAMP, but also playing a role in mediating the growth hormone release in the pituitary gland of several vertebrates (Anderson et al. 2004, Mitchell et al. 2008), including some fishes as grass carp (Wong et al. 2005), common carp (Xiao et al. 2002), sockeye salmon (Parker et al. 1997) and European eel (Montero et al. 1998). Likewise, increased concentrations of PACAP and/or higher concentrations of cAMP can effectively induce GH release (Anderson et al. 2004, Wong et al. 2005).

The present data indicate that the p300 and ADCY2 are involved in a regulation system for GH in zebrafishes in the presence of high circulating levels of this hormone. This study is the first step on the characterization of differentially expressed transcripts in brain tissues of GH transgenic and NT zebrafishes, and can lead to the development of new analyses related to the expression and regulation of these genes.

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RESUMO

A presença de níveis mais elevados do hormônio de crescimento (GH) em animais transgênicos poderia levar a várias alterações fisiológicas. Uma linhagem transgênica de paulistinha (*Danio rerio*) para o GH foi comparada com amostras não transgênicas (NT) desta espécie, através de uma abordagem de DDRT-PCR, com o objetivo de identificar transcritos candidatos diferencialmente expressos em tecido cerebral que poderiam estar envolvidos na superexpressão de GH. Análises densitométricas de dois produtos de amplificação selecionados, *p300* e *ADCY2*, apontaram uma expressão gênica significativamente menor nas amostras transgênicas de paulistinha (104.02 ± 57.71 ; 224.10 ± 91.73), quando comparadas com as amostras

NT (249.75±30.08; 342.95±65.19). Os presentes dados indicam que p300 e ADCY2 estão envolvidos em um sistema de regulação do GH, quando altos níveis circulantes desse hormônio são encontrados em paulistinha.

Palavras-chave: display diferencial, GH, hormônio de crescimento, transgênico, paulistinha.

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