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REGULATION OF GONADOTROPIN RELEASING HORMONE 1 GENE TRANSCRIPTION BY MEMBERS OF THE PURINE-RICH ELEMENT BINDING PROTEIN FAMILY

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Abstract

Gonadotropin releasing hormone 1 (GnRH1) controls reproduction by stimulating the release of gonadotropins from the pituitary. To characterize regulatory factors governing GnRH1 gene expression, we employed biochemical and bioinformatics techniques to identify novel GnRH1 promoter binding proteins from the brain of the cichlid fish, *Astatotilapia burtoni*. Using an *in vitro* DNA-binding assay followed by mass spectrometric peptide mapping we identified two members of Purine-rich element binding protein family, Pur-alpha (Pur-A) and Pur-beta (Pur-B), as candidates for GnRH1 promoter binding and regulation. We found that transcripts for both Pur-A and Pur-B co-localize in GnRH1 expressing neurons in the pre-optic area of the hypothalamus in *A. burtoni* brain. Furthermore, we confirmed *in vivo* binding of endogenous Pur-A and Pur-B to the upstream region of the GnRH1 gene in *A. burtoni* brain and mouse neuronal GT1-7 cells. Consistent with the relative promoter occupancy exhibited by endogenous Pur proteins, overexpression of Pur-B, but not Pur-A, significantly down regulated GnRH1 mRNA levels in transiently transfected GT1-7 cells, suggesting that Pur-B acts as a repressor of GnRH1 gene transcription.

Introduction

In all vertebrates, the brain controls reproduction via the hypothalamic-pituitary-gonadal (HPG) axis (12, 23, 27, 59). GnRH1 is an essential signaling peptide delivered from hypothalamic GnRH1 neurons to the pituitary where it regulates gonadotropin release (80). Generally, sequence-specific DNA-binding proteins or transcription factors respond to extracellular signals to regulate GnRH1 gene expression via interaction with cognate cis-elements and other regulatory proteins. Yet little is known about the molecular details of GnRH1 gene regulation in cold-blooded vertebrates such as *A. burtoni*.

To understand how transcription of the GnRH1 gene is regulated at the molecular level, we analyzed GnRH1 upstream binding proteins in *A. burtoni*. Here we identify two protein members of the Pur family, which specifically interact with GnRH1 upstream regulatory sequences. The Pur protein family in mammalian cells comprises four members: Pur-A, Pur-B (41), and two isoforms of Pur-gamma (Pur-G) (58). All Pur proteins contain a conserved nucleic acid-binding domain composed of three basic / aromatic and two acidic / leucine–rich modular repeats (4, 36). Pur-A and Pur-B are highly homologous proteins that interact with the purine-rich strand of polypurine-polypyrimidine elements of the general form, (GGN)n (3, 4, 28, 53), as homo- or heterodimers (40). Pur-A functions in diverse aspects of nucleic acid metabolism, including DNA replication, gene transcription, RNA transport, mRNA translation (36), and cell cycle control (35, 77). Deletions of Pur-A and Pur-B have been associated with acute myelogenous leukemia (57). Genetic inactivation of the Pur-A gene in the mouse causes neonatal fatality with a multiplicity of tissue defects including brain malformation (44). Pur-A was first identified as sequence-specific ssDNA-binding protein based on its affinity for a purine-rich sequence (so called PUR element) in the upstream region of the human c-Myc gene (3, 4). Pur-A has also been reported to bind double-stranded DNA (dsDNA) under certain experimental conditions (83). Pur-A plays an important role in modulating the transcription of a growing list of genes (31, 33, 40, 51, 64, 73, 74, 85). In the case of the HIV-1 genome (13) and the mouse brain cytoplasmic RNA 1 (BC1 RNA) gene (51), Pur-A stimulates transcription and then associates with stem-and-loop structures in the resulting RNA transcript. As a ssDNA-binding protein, Pur-A is apparently able to unwind a short DNA duplex annealed to a larger

ssDNA circle in an ATP independent manner (16). In terms of transcriptional regulation, Pur-A and Pur-B have been reported to interact with a PUR-like element in the 5'- flanking region of the mouse smooth muscle α-Actin gene (6). This element exhibits a high degree of polypurine-polypyrimidine asymmetry and a theoretical propensity to form a non-B-DNA structure in a partially unpaired format (6). Several reports have suggested that binding of Pur-A and Pur-B to the purine-rich strand of this regulatory element together with MSY1/YB-1 binding to the opposing pyrimidine-rich strand results in transient disruption or de-stabilization of base-pairing, which can inhibit the interaction of other canonical dsDNA-binding activators resulting in gene repression (6, 40, 41, 50).

The data presented here suggest that while both Pur-A and Pur-B can bind to the upstream region of the GnRH1 gene, Pur-B plays the dominant inhibitory role on gene transcription

Materials and Methods

Animals and materials

We used tissue from an African cichlid fish, *Astatotilapia* (*Haplochromis*) *burtoni*, bred from wild-caught stock (24, 25) and raised in laboratory aquaria. Animals were maintained under conditions that mimicked those of the natural habitat (27°C; 12:12 light/dark cycle with full spectrum lights; pH 7.6-8.0) and were fed daily (Wardleys, Secaucus, NJ) (24). All fish used in this study were sexually mature with body sizes about 7-9 cm and body weights about 12-18 g. Fish were killed by rapid cervical transection and tissue was immediately collected for analysis. All procedures were in accordance with the National Institutes of Health protocol for animal experimentation and approved by the Animal Care and Use Committee of Stanford University.

Nucleotide primers were purchased from Invitrogen (Carlsbad, CA) (Supplemental table 1) and all other reagents were ordered from Sigma-Aldrich (St. Louis, MO) if not otherwise specified.

DNA / Protein in vitro binding assay

The protocol for identifying the GnRH1 upstream binding proteins has been previously described

(87). Briefly, crude brain lysate was balanced in large-volume low salt (LVLS) binding buffer (20 mM HEPES, pH 7.9, 50 mM KCl, 1 mM $MgCl_2$, 10% glycerol) with 1 mM DTT and 0.1 mg/ml poly-d(IC)). The lysate was then pre-cleared with 1 mg Dynabeads (Invitrogen, Carlsbad, CA) without immobilized DNA probe for 2 hours at 4°C with slow rotation. Biotinylated double-stranded GnRH1 upstream DNA fragments and a control DNA fragment from the cDNA coding sequence of proliferating cellular nuclear antigen (PCNA) were generated by PCR in which the sense primers were 5' biotin labeled. These double-stranded DNA fragments (150 μg DNA / mg beads) were then immobilized on Dynal M-280 streptavidin-coated magnetic beads (Invitrogen) following the manufacturer's instructions. The pre-cleared supernatant was then combined and incubated with the immobilized dsDNA fragments (approximately 200 μg protein per 1 mg beads) at 4°C overnight on a rotator. After magnetic separation, the beads were washed six times with 500 μl washing buffer (1 X LVLS buffer with 2 mM PMSF, 0.5 mM DTT, and 0.1 mg/ml poly-d(IC)). The captured proteins were analyzed by SDS-PAGE followed by SilverSNAP Stain for Mass spectrometry (Pierce, Rockford, IL).

Mass spectrometric peptide mapping based on homology

Novel bands were extracted from the silver stained NuPAGE Novex 4-12% Bis-Tris gel (Invitrogen) and submitted for trypsin enzymatic digestion and mass mapping (Pan Facility, Stanford, CA). Mass spectrometric peptide mapping data were collected and analyzed on an Applied Biosystems 4700 Proteomics Analyzer. Mass spectrometry (MS) and MS/MS data were analyzed with the use of the Mascot server and the NCBInt database (www.matrixscience.com). In Mascot results, the "ion score" for an MS/MS match is -10Log(P) where P is the absolute probability that the observed match between the experimental data and the database sequence is a random event. The protein score is derived from the ion scores. Protein scores greater than the threshold (=78) are significant (p<0.05) (68). Candidate proteins identified in the NCBInt database with the highest protein score were then considered as the best match to the unknown DNA-binding proteins resolved by electrophoresis. If the candidate proteins were not known *A. burtoni* proteins, degenerate primers based on conserved sequences across different species were used

in PCR to clone the corresponding gene from *A. burtoni*. The predicted *A. burtoni* protein sequences from the cloned cDNA were then submitted to GenBank so that the updated NCBInt database included these new sequences. The original MS and MS/MS data were then analyzed again using the Mascot server with the updated NCBInt database to verify whether the newly cloned *A. burtoni* protein could also be found in the resulting matches with a significant protein score.

Molecular cloning of A. burtoni Pur-A and Pur-B

Fish brains were homogenized in 1 ml Trizol (Invitrogen, Carlsbad, CA) followed by 250 μl chloroform to isolate RNA. Rapid amplification of cDNA ends (RACE) from total brain RNA was performed (SMART RACE cDNA Amplification Kit, Clontech, Palo Alto, CA). Primers for Pur-A and Pur-B were designed based on the conserved region of zebrafish Pur-B (GenBank Accession No. BC056517). Based on the resultant partial sequence from the PCR using zebrafish primers, RACE PCR primers used for both 3' and 5' ends of Pur-A and Pur-B cDNAs were then designed. All primers were designed with 60°C melting temperature. Touchdown PCR was used for cloning experiments in this study: 3 minutes 95°C initial denaturing followed by 16 touchdown cycles from 68°C to 60°C (annealing temperature, decrease 0.5°C every cycle) and continued for another 25 cycles with 60°C annealing temperature. The resulting sequences for *A. burtoni* Pur-A and Pur-B cDNA containing the complete coding sequences were verified by sequencing in both directions (Sequetech, Mountain View, CA). Multiple alignment analysis of Pur-A and Pur-B from *A. burtoni* compared to Pur proteins from other species was performed (Vector-NT software, Invitrogen). The phylogenetic tree for the Pur proteins across various species was generated by Mega 3.1 (52) using neighbor-joining and bootstrap tests.

mRNA expression analysis

Reverse transcription PCR was performed on various tissues from adult *A. burtoni* (spinal cord, brain, retina pituitary gland, muscle, gill, spleen, stomach, gut, liver, kidney, ovary, testicle, and heart). Tissue was collected and homogenized before extraction of total RNA (RNeasy Micro Kit, Qiagen Inc., Valencia,

CA). 3'-RACE cDNA for each tissue was synthesized (SMART cDNA synthesis kit, Clontech Laboratories Inc., Palo Alto, CA). Touchdown PCR was then conducted using specific primers of *A. burtoni* Pur-A and Pur-B.

In situ hybridization

To co-localize Pur proteins and GnRH1 expression, double *in situ* hybridization was used. Methods developed in our laboratory (8) were used with minor modifications. Animals were killed by rapid cervical transection and the brains were immediately embedded in OCT Compound (Tissue-Tek, Torrence, CA), flash frozen, sectioned at 14 μm using a cryostat (Microm, Zeiss, Thornwood, NY), and thaw mounted onto slides (Superfrost, Fisher, Santa Clara, CA). Templates for radioactively labeled RNA probes specific for Pur-A and Pur-B were generated by PCR. In the PCR reaction, one of the primers was designed to contain an additional T7 promoter sequence on its 5' end so that the PCR product could be used as a reverse transcription template for making an RNA probe from the end with the T7 promoter. RNA probes were then synthesized by T7 transcriptionase (Ambion, Austin, TX) in the presence of ^{35}S labeled UTP (Amersham Biosciences, Piscataway, NJ). Brain slices were hybridized with the ^{35}S labeled sense or antisense probes and dipped in nuclear emulsion (NBT-2; Kodak, Rochester, NY) and exposed for ~one month at -20°C. To identify GnRH1, probe was labeled with digoxigenin (DIG) coupled nucleotide triphosphates (NTPs) (Roche Applied Science, Indianapolis, IN) and visualized by 3,3'-Diaminobenzidine (DAB) staining using anti-DIG-peroxidase primary antibody (Roche) and Tyramide Signal Amplification kit (NEN Life Sciences, Boston, MA). Cresyl violet staining was used to visualize cell bodies. Photomicrographs were acquired (Axioscope, Zeiss). *In situ* hybridization signals were photographed under both bright field and dark field illumination and images were captured digitally by Spot camera (Diagnostic Instruments, Sterling Heights, MI).

Cell culture

A mouse hypothalamic cell derived GnRH-secreting cell line, GT-1 was donated by Dr. P.L.

Mellon and Dr. R.I. Weiner (60). All cell culture reagents were supplied by Invitrogen. GT1-7 cells were cultured in 100-mm master plate in Dulbecco's Modified Eagle's Medium (DMEM, CA#11995-065) and supplemented with 10% fetal calf serum (FCS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin in a humidified atmosphere of 5% CO2 at 37°C. Media was replaced every 2-3 days until confluence was reached. For experiments, cells were seeded into 24-well plates and cultured under the same condition. Cells utilized for chromatin isolation and transient transfection were of similar passage (passage 6-15) and confluence (~90%).

Chromatin immunoprecipitation

To isolate specific nucleoprotein complexes from tissue of cells, a previously described chromatin immunoprecipitation (ChIP) protocol was used (87). Briefly, one adult fish brain was homogenized in PBS on ice followed by addition of formaldehyde to a final concentration of 1%. For cultured GT1-7 cells, 1% formaldehyde in PBS was added to a 100 mm culture dish directly. Cross-linking reactions were stopped after 10 minutes by adding glycine to a final concentration of 0.125 M. After lysis, chromatin was fragmented by sonicating the lysate five times for 10 seconds (Power level 3, Branson Sonifier 250, VWR International, West Chester, PA). Pre-cleared chromatin solution was then incubated with or without antibody (1 μg/ml) for 1 hour at 4°C. The preadsorbed protein A-magnetic beads were added to precipitate the immune complexes overnight at 4°C. The beads were washed extensively before eluting the immune complexes. Eluted immune complexes were incubated at 67°C for 4 hours to reverse the formaldehyde cross-linking. Genomic DNA was then purified by phenol-chloroform extraction. To detect the presence of the upstream promoter region of the GnRH1 gene, PCR amplification was performed using primers specific to GnRH1 upstream region (Supplemental table 1).

Overexpression and transcriptional regulation in GT1-7 cells

Cloned full-length *A. burtoni* Pur-A and Pur-B were subcloned into pcDNA3.1(+)-Myc-His-B mammalian expression plasmids (Invitrogen) between EcoRI and BstBI. Expression plasmids encoding

His-tagged mouse Pur-A and Pur-B were described previously (6). Mouse GT1-7 cells were transfected with 1 μg plasmid and 3 μl TransFast reagent (Promega, Madison, WI) in a 24-well plate according to manufacturer's protocol. Total RNA from these cells was purified (RNeasy Mini-plus Kit, Qiagen) after 48 hours of transfection. Real-time PCR was then performed and the raw fluorescent data were analyzed using the Real-time PCR Miner program (86). Transfected cells were also analyzed by western blotting using antibodies specific to Pur-A and Pur-B to confirm the overexpression of the transfected genes (40). Mouse beta-Actin was used as an internal control for both Real-time PCR and western blot.

Results

To identify the DNA-binding proteins that interact with the upstream region of the *A. burtoni* GnRH1 gene, we combined mass spectrometry (MS)-assisted peptide mapping with classic homology based molecular cloning and functional tests.

Mapping the DNA / Protein binding sites upstream of the GnRH1 gene

Double-stranded biotinylated DNA fragments (~1150 bps) of the GnRH1 upstream region were generated by PCR. These fragments covered 3489 bps with 500~600 bps overlap (Figure 1B, designated G1 to G5). Since multiple transcription initiation sites of GnRH1 have been found in other species (18, 19, 43, 69, 78), there might be other transcription initiation sites in addition to the predicted sites in *A. burtoni* (79). To avoid missing any upstream sequence, we included all the sequence corresponding to the GnRH1 mRNA 5' untranslated region up to the translation start codon (+165) in the last fragment (G5). Using mass spectrometry compatible silver staining, GnRH1 upstream binding proteins were visualized by SDS-PAGE. At least seven novel bands could be unambiguously identified in a whole gel (87). In this study, two bands with molecular weight (MW) around 41 KDa and 38 KDa were analyzed further (Figure 1A). Based on their apparent MW and preferential association with probe G3, we designated these binding proteins as G3-41 and G3-38. The hypothetical DNA-binding sites of G3-41 and G3-38 in GnRH1 upstream sequence were then deduced by comparing the overlapping regions of each fragment

(Supplemental table 2 and Figure 1). For example, a complete binding site for G3-41 and G3-38 in the -996 ~ +164 region was ruled out due to their absence in lane G5. The fact that lane G3 had stronger signals for both G3-41 and G3-38 than the other lanes suggested that there might be multiple recognition sites in the G3 fragment, or alternatively, the binding sites in G3 might have a higher affinity for these proteins.

Mass spectrometric peptide mapping of the binding proteins

To characterize these binding proteins further, we performed mass spectrometric peptide mapping of G3-41 and G3-38. Both MS and MS/MS data were submitted to the Mascot sever using NCBInt database, which contains all known proteins to date from all species. Band G3-41 matched a member of the purine-rich element binding protein family, Pur-B (gi|45768686) from zebrafish (*Danio rerio*) with the highest protein score of 264, which was much greater than the threshold of 78 ($p<0.05$). There were 9 MS matches for the G3-41 band of which 4 were confirmed by MS/MS peptide sequencing (Supplemental table 3). The G3-38 band matched best to frog Pur-A protein (Supplemental table 4) with a highly significant protein score of 319. Eight MS matches were found for the G3-38 band of which 4 were confirmed by MS/MS peptide sequencing (Supplemental table 4). Since Pur protein family members have been reported to function as transcriptional regulators in other species (6, 10, 11, 30, 31, 33, 51, 73, 74), we chose to clone the cDNAs encoding these candidate binding proteins from *A. burtoni* and to perform further functional analyses.

Molecular cloning of Pur-A and Pur-B

From *A. burtoni*, we cloned the full-length 858-bp coding sequence (CDS) of Pur-A (GenBank Accession No. DQ630740), which predicted a protein of 285 amino acids. The full-length 891-bp CDS of Pur-B (GenBank Accession No. DQ630741), which predicted a protein of 296 amino acids, was also cloned. Multiple alignments (Figure 2A and 2B) and phylogenetic tests (Figure 2C) were performed for these two Pur proteins and their homologs from other species. Identical branching patterns in the

phylogenetic trees were generated regardless of which method was used to produce the tree (Data not shown). Analysis across species proved that the Pur proteins are conserved from fish to humans (Figure 2A and 2B): Identities = 224/237 (94%), Positives = 229/237 (96%) for Pur-A; Identities = 227/280 (81%), Positives = 242/280 (86%) for Pur-B. Pur-A and Pur-B are highly conserved in their central ssDNA/RNA-binding domain but are more variable in N- and C-terminal sequences. Phylogenetic analysis reveals that the cichlid sequences are closer to *Tetraodon nigroviridis* than *Danio rerio*.

Confirmation of the mass mapping results by cloned A. burtoni sequences of Pur-A and Pur-B

Following submission of the cloned sequence of Pur-A and Pur-B to GenBank, we conducted a new Mascot search with the original mass mapping data against the updated NCBInt database. As expected, the *A. burtoni* Pur-A and Pur-B sequences also resulted in high protein scores and the major MS peaks were found to be matched (Supplemental table 5 and 6). These results confirmed that band G3-41 and G3-38 are indeed *A. burtoni* Pur-B and Pur-A respectively.

Tissue distribution of Pur-A and Pur-B mRNA

To discover where Pur-A and Pur-B are expressed in *A. burtoni*, we used reverse transcription PCR in a variety of tissues. These data showed that both Pur-A and Pur-B are widely expressed in most tissues sampled (Figure 3A). Pur-A is highly expressed in brain, gill, gut, kidney, and testicle, with reduced expression in retina, pituitary, muscle, spleen, liver, ovary, and heart, and no expression in spinal cord and stomach. Pur-B is expressed abundantly in most tested tissues but is absent in stomach.

To test if Pur-A and Pur-B are co-expressed in *A. burtoni* brain, we performed *in situ* hybridization. We detected Pur-A and Pur-B mRNA in the pre-optic area (Figure 3B) where GnRH1 neurons reside. Double *in situ* hybridization results clearly demonstrated co-localization of Pur-A and Pur-B mRNA in GnRH1 neurons and nearby cells. In addition, using PCR, we found that mouse Pur-A (mPur-A) and mPur-B are also expressed in mouse GT1-7 neuronal cells (Data not shown).

To confirm the molecular size of *A. burtoni* Pur-A (aPur-A) and Pur-B (aPur-B), fish retina and brain

lysate were probed via western blotting using specific antibodies against Pur-A (A149) and Pur-B (B302) (40). In both tissues, the endogenous aPur-A and aPur-B proteins exhibited apparent molecular weights of ~38 KDa and 41 KDa, respectively (Figure 3C, left panel), which were consistent with the molecular weights of the GnRH1 promoter binding proteins found in DNA / protein binding assay (G3-38 for aPur-A and G3-41 for aPur-B). However, endogenous aPur-A is smaller than the endogenous mPur-A (~42 KDa) detected GT1-7 cells while aPur-B is larger than mPur-B (~39 KDa) (Figure 3C, left panel). When expressed in GT1-7 cells, the apparent molecular weights of Myc-His tagged aPur-A and Myc-His tagged aPur-B increased to 42 KDa and 40 KDa, respectively (Figure 3C, right panel). While this change is consistent with the addition of the Myc-His tag, a larger than expected increase in the apparent molecular weight of Myc-His tagged aPur-A compared to Myc-His tagged aPur-B in GT1-7 cells was observed (from 38 KDa to 42 KDa for aPur-A versus from 39 KDa to 40 KDa for aPur-B). This finding implied that different post-translational modifications, such as post-translational processing (37), N-glycosylation, and/or phosphorylation (38), might distinguish aPur-A from aPur-B in *A. burtoni* and mouse cells.

In vivo binding of Pur proteins to the upstream region of the GnRH1 gene

Although we successfully identified Pur-A and Pur-B from a pool of *in vitro* DNA / protein complexes, it is possible that this finding could be a false positive due to the *in vitro* binding conditions. To confirm the ability of Pur-A and Pur-B to interact with the upstream region of the GnRH1 gene *in vivo*, we performed chromatin immunoprecipitation. We utilized two antibodies specifically against Pur-A (A149) and Pur-B (B302) (40) to immunoprecipitate genomic DNA / Pur protein complexes from *A. burtoni* brain (Figure 4A) and GT1-7 cells (Figure 4B). Expected PCR products were observed only from the brain samples treated with Pur-A and Pur-B antibodies. In GT1-7 cells, we also found strong binding between mPur-B and the mouse GnRH1 (mGnRH1) upstream region, while mPur-A only showed very weak binding to the -909 ~ -387 segment of the mGnRH1 gene (Figure 4B). None of the negative controls (bacteria genomic DNA or sample without antibody) produced relevant PCR products with the expected molecular weight. Thus, the ChIP results did confirm that the Pur proteins (especially Pur-B) are

able to bind to the upstream region of GnRH1 gene *in vivo*.

Overexpression of Pur-B down-regulates mouse GnRH1 in GT1-7 cells

We also investigated if Pur proteins transcriptionally regulate mGnRH1 expression in the GT1-7 cell line. Overexpression of either *A. burtoni* Pur-B (aPur-B) or mouse Pur-B (mPur-B) resulted in a significant reduction in mGnRH1 transcripts (Figure 5A). The level of mGnRH1 mRNA in aPur-B transfected cells was reduced to 79.2% of the control cells transfected with empty plasmid. Overexpression of mPur-B repressed mGnRH1 transcription to 73.8%. In both cases, the level of inhibition of mGnRH1 mRNA transcription, although statistically significant, was relatively small, indicating that overexpression of Pur-B alone was insufficient to silence the transcription of the mGnRH1 gene completely. We did not observe any change of GnRH1 expression in Pur-A transfected cells, which is consistent with the lower apparent affinity and/or stoichiometry for Pur-A to the GnRH1 upstream regulatory region observed by ChIP assay (Figure 4). Overexpression of Pur proteins in GT1-7 cells was confirmed by western blot analysis (Figure 5B and 5C).

Putative Pur protein binding sites in GnRH1 gene

Finally, we compared the upstream sequence of *A. burtoni* and the mouse GnRH1 genes to search for putative Pur protein binding sites. Beside the Pur-A binding consensus sequence, GGGAGA (83), Pur-A and Pur-B have also been found to interact with purine-rich ssDNA sequences of the general form, (GGN)n (3, 4, 28, 53), where N is A/T/C (H) and with at least two repeats minimally required for ssDNA-binding (50). We therefore searched the *A. burtoni* GnRH1 gene (GenBank Accession No. AF076961) and mouse GnRH1 gene (GenBank Accession No. NT_039606) with these putative Pur-A and Pur-B binding sequences (Figure 6). Multiple putative Pur protein binding sites are found in both the proximal promoter region and the distal enhancer region in mouse GnRH1 gene (48, 56, 65). Two GGGAGA sites are located in complementary strands in the distal enhancer region (-1600 ~ -1100 bp) of the *A. burtoni* GnRH1 gene. There is one GGGAGA site on each DNA strand in the proximal promoter region (-800 ~ -

200 bp) of the mouse GnRH1 gene. The GGHGGH sites are concentrated in -1600 ~ -400 region of the *A. burtoni* GnRH1 gene but are more uniformly distributed in the mouse GnRH1 far upstream region, which is consistent with the results from binding assays (Figure 1, Supplemental table 2, and Figure 4).

Discussion

Both a previous study (49) and our recent results (87) showed that the upstream region of *A. burtoni* GnRH1 gene contains predicted transcription factor binding motifs for various DNA-binding proteins, including nuclear receptors (estrogen receptor, ER; progesterone receptor, PR; glucocorticoid receptor, GR; and thyroid hormone receptor, TR), Sp1 and CCAAT/enhancer binding protein (C/EBP), and Oct-1. However, database searches for transcription factor binding sites based on short consensus sequence may produce false positives and novel binding sites may remain undiscovered. In cold-blooded vertebrates, none of the identified putative binding sites in the GnRH1 gene have been investigated for possible direct protein association. However, some evidence of transcriptional regulation (e.g. overexpression or down-regulation) has been reported for putative binding proteins such as the sex hormone receptors, ER (67), PR (66), TR (20, 34), and GR (26, 81). In this study, we sought direct evidence for regulation of *A. burtoni* GnRH1 expression at the level of gene transcription by identifying novel promoter binding proteins.

To understand the transcriptional regulation of GnRH1, we utilized a proteomics approach to uncover specific proteins that bind to the GnRH1 5' flanking region and then validated their putative functional role in cultured cells. We identified Pur-A and Pur-B as novel GnRH1 upstream binding proteins in both *A. burtoni* brain and mouse GT1-7 neuronal cells and subsequently confirmed that Pur-B acts as a repressor of GnRH1 gene transcription. Moreover, we also found putative Pur protein binding sites in the upstream region of GnRH1 gene from other fish species, e.g. *Oreochromis niloticus*, *Oryzias latipes*, and *Morone saxatilis* (Data not shown).

In mammals, GnRH1 is known to be controlled by multiple signals including growth factors, secondary messengers, steroid hormones, neurotransmitters, and neuropeptides (2, 32, 45, 56, 62).

Previous research defined a proximal promoter (21, 43, 48) and distal enhancer regions (42, 48, 55, 65, 82) that are critical for mammalian GnRH1 gene regulation. The proximal promoter is important for basal GnRH1 gene expression and the distal enhancer region contains an enhancer sufficient for GnRH1 neuron-specific expression. Both regions contain regulatory elements for numerous dsDNA-binding proteins. Transcription factors, including the GATA-factor families (54, 55), octamer-binding transcription factor-1 (Oct-1) (14, 22), Otx2 (39), and three-amino acid loop extension (TALE) homeodomain proteins (70) have been shown to play an important role in the transactivation of the rat GnRH1 gene. In the mouse, the neuron-specific expression of GnRH1 was found to be highly dependent on the proximal promoter region (-356 ~ -249 bp) where two Otx 2 binding sites were found (47). The MAP kinase pathway (46) and another proximal promoter region (-75 ~ -67 bp) binding protein, early growth response (Egr-1) gene (17), were found to be responsible for insulin-induced mouse GnRH1 expression. In contrast, COUP-TFI or CCAAT/enhancer binding protein-β (C/EBP-β) may be involved in the melatonin mediated repression of rat GnRH1 gene expression (29). The binding of Oct-1 to the distal negative glucocorticoid response element (nGRE) in the mouse GnRH1 gene may mediate glucocorticoid-dependent repression of transcription (7). Interaction between the proximal promoter and enhancer is also required for optimal expression of the GnRH1 gene in GT1-7 cells (63). In this regard, it is particularly intriguing that there appears to be multiple potential binding sites for Pur-B in the upstream region of the mouse GnRH1 gene. Because Pur-B functions as a transcriptional repressor in GT1-7 cells, it is reasonable to speculate that Pur-B binding may disrupt the interaction between the proximal promoter and distal enhancer region of the GnRH1 gene. Although the Pur-A protein also showed an ability to bind to a region of the GnRH1 gene co-occupied by Pur-B (Figure 4), the overexpression of Pur-A protein did not have a significant effect on GnRH1 mRNA synthesis (Figure 5).

The apparent functional differences observed between ectopically expressed Pur-A and Pur-B raise several questions for future investigation: First, does the intrinsic binding affinity and stoichiometry of Pur-A for selected elements in this region differ quantitatively from that of Pur-B? Second, is the inhibitory effect of Pur-B on GnRH1 gene transcription mediated by interaction with multiple regulatory

sites in the upstream region of GnRH1 gene? Third, do endogenous Pur-A and Pur-B synergize to elicit more complete repression of GnRH1 expression in relevant brain cell types as has been reported for the smooth muscle α-actin gene promoter in fibroblasts and smooth muscle cells (40, 50)? Finally, are other Pur family proteins, such as the two isoforms of Pur-G, also involved in the regulation of GnRH1 gene expression?

It is important to note that functionally relevant interactions among Pur-A, Pur-B, and other ssDNA/RNA-binding proteins has been observed in a number of different genes. For example, in the smooth muscle α-actin gene, Pur-A and Pur-B appear to collaborate with the pyrimidine-rich strand binding protein, YB-1/MSY1, to repress MCAT enhancer activity (6). In the rat aldolase B gene, where the transcriptional promoter overlaps an origin of DNA replication, there are distinct ssDNA recognition elements for Pur proteins (75) and hnRNP-A/B (71, 84). These elements appear to function in concert with a nearby A/T-rich sequence to initiate DNA replication (61, 88). In the case of Pur-A and Pur-B, evidence suggests that these proteins may help stabilize the single-stranded state during replication initiation (75). Curiously, coordinate down-regulation of both hnRNP-A/Bs and Pur-A has been demonstrated in the anterior pituitary gland of ovariectomized rats by addition of estrogen (5). Beside hnRNP-A/B, hnRNP-K has also been reported to act together with Pur-A to transcriptionally repress the promoter of the CD43 gene (15). Interestingly, in an earlier study, we identified several other ssDNA/RNA binding proteins, namely hnRNP-A/B and hnRNP-G (87), as participating in the transcriptional repression of the GnRH1 gene. Since hnRNP proteins have been shown to play important roles in hormone-related signaling by androgen (72), and estrogen (1, 9, 76), it is tempting to speculate that certain members of the Pur and hnRNP family of single-stranded nucleic acid binding proteins function in a collaborative manner to co-repress of GnRH1 gene expression in hypothalamic-pituitary-gonadal axis.

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Figure Legends

Figure 1. Mapping DNA / Protein binding sites in the GnRH1 upstream region by capturing nucleoprotein complexes with selected DNA fragments immobilized on magnetic beads.

A: Mass spectrometry compatible silver stain of SDS-PAGE resolved DNA-binding proteins captured from *A. burtoni* brain lysate. G1 to G5, upstream DNA fragments of GnRH1 gene sequence; Ctr, a control fragment from the cDNA coding sequence of PCNA. Compared with the control, distinct 41 KDa (black arrows) and 38 KDa (white arrows) bands were observed in the gel. SB, SeeBlue pre-stained protein ladder; SB+, SeeBlue-plus pre-stained protein ladder (Invitrogen). B: Schematic representation of the GnRH1 upstream fragments (gray lines) and the deduced binding sites of the 41 KDa and 38 KDa binding proteins.

Figure 2. Molecular cloning of Pur-A and Pur-B from *A. burtoni*.

Vector-NT software (Invitrogen) was used to generate multiple alignments of predicted protein sequences for *A. burtoni* Pur-A (A) or Pur-B (B) with corresponding sequences from other fish species, mouse, and human. Conserved sequences required for DNA/RNA-binding are indicated by an open box and peptides matched in mass mapping are underlined. C: The phylogenetic tree for Pur proteins was generated by Mega 3.1 using neighbor-joining and bootstrap test. Note that the *A. burtoni* sequence co-segregates with sequences from other fish species.

Figure 3. Localization of Pur-A and Pur-B mRNA in *A. burtoni*.

mRNA of Pur-A and Pur-B in various *A. burtoni* tissues (A). SC, spinal cord; Br, brain; Re, retina; Pit, Pituitary gland; Mu, muscle; Gill, gill; Sp, spleen; St, stomach; Gut, gut; Li, liver; Ki, kidney; Ov, ovary; Te, testicle; He, heart; and Ctr, control (water). Beta-Actin was used as an internal control. *In situ* hybridization of Pur-A or Pur-B mRNA and GnRH1 mRNA in the pre-optic area of the *A. burtoni* brain (B). GnRH1 releasing neurons were stained by DAB (brown in

upper panel, bright field) and Pur-A or Pur-B mRNA were visualized by silver grains developed in the emulsion (black dots in upper panel, bright field, or white dots in lower panel, dark field). Cresyl violet staining (blue in upper panel) was used to visualize cell bodies. Cofirmation of the molecular weights of Pur proteins (C). The apparent molecular weights of Pur proteins detected on western blots are comparable to the 41 KDa and 38 KDa GnRH1 upstream binding proteins identified in the *in vitro* binding assay (Figure 1). Bands corresponding to endogenous *A. burtoni* Pur-A (aPur-A) and *A. burtoni* Pur-B (aPur-B) in fish retina (Re) and brain (Br) are shown in the left panel while Myc-His tagged aPur-A and Myc-His tagged aPur-B overexpressed in GT1-7 cells are highlighted in the right panel. The sizes of endogenous mouse Pur-A (mPur-A) and mouse Pur-B (mPur-B) from GT1-7 cells (Gt) were compared with the fish Pur proteins side by side. M, protein molecular weight marker; Re, retina; Br, brain; and Gt, GT1-7 cells, Oe, overexpressed. αA, antibody against Pur-A (A149) and αB, antibody against Pur-B (B302).

Figure 4. *In vivo* binding of Pur-A and Pur-B to the upstream region of the GnRH1 gene.

After ChIP, PCR was performed using specific primers against *A. burtoni* (A) or mouse (B) GnRH1 upstream sequence. GnRH1 DNA fragments were amplified from the immuno-complex pulled down by antibody against Pur-A and Pur-B. PCR products were visualized by electrophoresis (arrows). Bg, bacteria genomic DNA; NA, sample without antibody; αA, sample with antibody against Pur-A (A149); αB, sample with antibody against Pur-B (B302); and MK, 1 KB plus DNA Marker (Invitrogen).

Figure 5. Repression of mouse GnRH1 gene transcription by overexpressing Pur-A and Pur-B in GnRH releasing mouse neuronal GT1-7 cells.

Control empty His plasmid (Ctr), *A. burtoni* Pur-A (aPur-A), *A. burtoni* Pur-B (aPur-B), mouse Pur-A (mPur-A), and mouse Pur-B (mPur-B) plasmids were transfected into GT1-7 cells for 48 hours. A: mGnRH1 mRNA level was analyzed by real-time PCR and normalized using

mouse beta-Actin mRNA (n=5). Two-tail homoscedastic *t*-test (two sample assuming equal variance) was used for statistical analysis. ** indicates that a significant difference was found between the experimental group and control group ($p<0.01$). Western blots were performed to verify the overexpression of Pur-A (B) and Pur-B (C). Mouse beta-Actin was used as an internal control. αActin, antibody against mouse beta-Actin; αA, antibody against Pur-A (A149), and αB, antibody against Pur-B (B302).

Figure 6. Schematic representation of *A. burtoni* and mouse GnRH1 gene with putative Pur proteins binding sites.

GnRH1 genes were analyzed and putative Pur-A and Pur-B binding sites (GGGAGA and GGHGGH) were plotted. Symbols for binding sites on the upper side of the gene indicate that they are on the sense strand. Lower side indicates the antisense strand. Double-stranded DNA fragments used for the *in vitro* DNA / protein binding assay are indicated by the gray lines and the possible binding sites for Pur-A and Pur-B were deduced from the *in vitro* DNA / protein binding assay (Supplemental table 2 and Figure 1).

Supplemental table 1. Primers used in this study. Asterisk designates biotin labeling. T7 stands for the T7 promoter sequence: TAATA-CGACT-CACTA-TAGGG-AGA.

Protein binding assay	**Fragment**	**Sense Primer**	**Antisense Primer**
	G1	*CCCTGGCATTCCACCAATATTATGTTAGC	GAAATACACATTATGATTGCCTTCATCATTC
	G2	*GAGGTGTGAGTGATCCACAACTGCA	ACACTACCACCCTGGTGCTAATAAGGTG
	G3	*ACCCTGAGTGTGAAAACCAATGGAAG	TGGTTGCTCATGCAGCAGACATGGCCA
	G4	*CGCACTGTTGCAGATTTGTTGGCT	AACAACCAGGGTATGCAGAAGAACATC
	G5	*TCACCTGCCATATCGCCTTCTTCT	TCTGAGGATAAAGCTTCACATTTTGCACG
PCR using zebrafish primers	**Related gene**	**Sense Primer**	**Antisense Primer**
	Pur-A	CGCTTCTTCTTCGACGTGGGCTCC	TCGCTCCTGGATCTCCTTCATCTCC
	Pur-B	TCGACCTGAAGGAGAACCAGCG	
RACE PCR	**Pur-A**	GGGCTCGGCCTCCGGGCTCCA	CAATCGTCTGGCCTTGCGTGAA
	Pur-B	GGTCCAACAAATACGGCGTGTTCCTG	CCTCCCCCGCTGGTTCTCTTTC
mRNA localization in organs	**Actin**	CATTTGCCTGAAACCGTTTCCCT	TCTTCTCCATGTCATCCCAGTTG
	Pur-A	GAAAAGGCAGGCTGGCAGCATC	CGTTTGTTGTCCACAGTCAACGATGAC
	Pur-B	ATACGCAGGAGAGAGGGGCAGAAG	CCACCGTCTTCACCCGCGGTAG
***In situ* template**	**Pur-A**	TTATGGTGTAGAGGACGAACCTGCAGA	T7-TTGCTGTCTATATCAATCCTCGTCTCCAT
	Pur-B	AGATGACCGGGGGCATGGCC	T7-CGCAAAATAACCCTATAGTGTAGATCTGCCA
ChIP	**aGnRH1**	TGGACATGATACAAGGCATGATGCA	TCTGAGGATAAAGCTTCACATTTTGCACG
	mGnRH1	TAGCCAAACACCACAGTCTTCTCTTGA	ACCATCCTTTAGCAGATGCTGCCTC
		CAACCACATGGTGGCTCACAACCA	ATTCTATCCCAGGGTCCCACAGG
		AAGACTATGGGCTGTGCTGCAACTGT	GCCAGCCTGGTCTACAGAGTGAGTTC
		CAGAAATTCGCCTGCCTCTGCCT	GTCCACTCTAAGGGACATCAAGACACAG

Supplemental table 2. Summary of the GnRH1 upstream binding proteins deduced by the *in vitro* DNA / protein binding assay. Identified binding proteins G3-41 and G3-38 matched to Pur-B and Pur-A respectively. There are at least three binding sites for G3-41 and G3-38 protein in the *A. burtoni* GnRH1 upstream region.

Upstream Regions	G1	G2	G3	G4	G5
G3-41	Mixed band	Weak	Strongest	Strong	No band
G3-38	Mixed band	Weak	Strongest	Strong	No band
Position	-3325 ~ -2133	-2692 ~ -1537	-2132 ~ -997	-1536 ~ -383	-996 ~ +164
Size	1193 bps	1156 bps	1136 bps	1154 bps	1160 bps
Protein ID	**Matched Protein**	**Known as a transcription Activator**	**Known as a transcription repressor**	**Deduced minimum sites**	**Deduced binding Regions**
G3-41	Pur-B	NA	(6, 30, 50)	2	-2132 ~ -997
G3-38	Pur-A	(10, 11, 31, 33, 51, 74)	(6, 30, 50, 73)	2	-2132 ~ -997

Supplemental table 3. Mass spectrometric peptide mapping of the 41 KDa band (Pur-B).

Highlighted peptides were confirmed by MS/MS peptide sequencing.

gi|45768686: Pur-B protein [*Danio rerio*]								
Mass: 32455		Protein Score: 264			Sequence Coverage: 21%			
Observed	**Mr(expt)**	**Mr(calc)**	**Delta**	**Start**	**End**	**Miss**	**Ions**	**Peptide**
1060.461	1059.453	1059.503	-0.049	223	231	0	----	FFFDVGSNK
1064.524	1063.517	1063.566	-0.0492	238	246	0	35	VSEVKPSYR
1216.559	1215.551	1215.604	-0.0524	222	231	1	47	RFFFDVGSNK
1318.605	1317.598	1317.652	-0.0548	22	32	1	----	EQETQELASKR
1324.623	1323.616	1323.686	-0.0696	67	78	0	----	LTLSMSVAAEFR
1340.622	1339.614	1339.681	-0.0662	67	78	0	----	LTLSMSVAAEFR Oxidation (M)
1341.621	1340.613	1340.672	-0.059	126	135	1	73	YYLDLKENQR
1425.607	1424.6	1424.661	-0.0609	266	276	1	70	YAEEMKEIQER
1441.604	1440.597	1440.655	-0.0589	266	276	1	----	YAEEMKEIQER Oxidation (M)

Supplemental table 4. Mass spectrometric peptide mapping of the 38 KDa band (Pur-A).

Highlighted peptides were confirmed by MS/MS peptide sequencing.

gi|50603800: Pura-prov protein [*Xenopus laevis*]								
Mass: 31537		Protein Score: 319			Sequence Coverage: 27%			
Observed	**Mr(expt)**	**Mr(calc)**	**Delta**	**Start**	**End**	**Miss**	**Ions**	**Peptide**
1060.455	1059.448	1059.503	-0.0543	207	215	0	63	FFFDVGSNK
1078.539	1077.531	1077.582	-0.0505	222	230	0	43	VSEVKPTYR
1216.554	1215.547	1215.604	-0.0569	206	215	1	----	RFFFDVGSNK
1359.572	1358.565	1358.629	-0.0643	131	140	1	60	YYMDLKENQR
1375.563	1374.556	1374.624	-0.0681	131	140	1	----	YYMDLKENQR Oxidation (M)
1831.792	1830.785	1830.945	-0.1604	216	230	1	----	YGVFMRVSEVKPTYR
1875.766	1874.759	1874.704	0.0553	266	282	0	----	ASEQPQQQQEGDDGEDD
2875.235	2874.228	2874.377	-0.1492	181	206	1	110	LIDDYGVDEEPAELPEGTSLTVDNKR

Supplemental table 5. Confirmation of mass mapping result after cloning *A. burtoni* Pur-A.

Highlighted peptides were confirmed by MS/MS peptide sequencing.

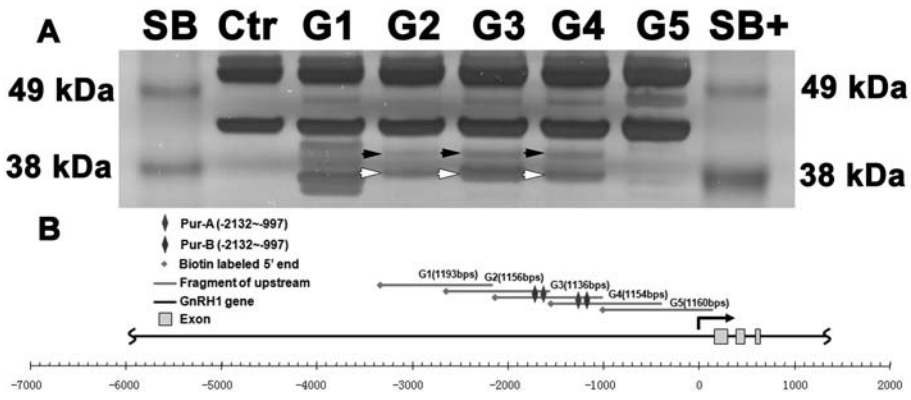
gi|108744013: Purine-rich element binding protein-alpha [*Astatotilapia burtoni*]								
Mass: 32015		Protein Score: 267			Sequence Coverage: 35%			
Observed	**Mr(expt)**	**Mr(calc)**	**Delta**	**Start**	**End**	**Miss**	**Ions**	**Peptide**
1060.4554	1059.4481	1059.5025	-0.0544	209	217	0	63	FFFDVGSNK
1078.5386	1077.5313	1077.5818	-0.0505	224	232	0	43	VSEVKPTYR
1216.554	1215.5467	1215.6036	-0.0569	208	217	1	---	RFFFDVGSNK
1359.5718	1358.5645	1358.6288	-0.0643	133	142	1	60	YYMDLKENQR
1375.5629	1374.5556	1374.6237	-0.0681	133	142	1	---	YYMDLKENQR
1831.7919	1830.7846	1830.945	-0.1604	218	232	1	---	YGVFMRVSEVKPTYR
2266.7756	2265.7683	2265.875	-0.1067	267	285	1	---	RACELQQQEEMQQDDGDED
2371.1567	2370.1494	2370.2695	-0.1201	155	177	0	46	GPGLGFTQGQTIALPAQGLIEFR
2861.2227	2860.2154	2860.3613	-0.1459	183	208	1	---	LIDDYGVEDEPAELPEGSSLTVDNKR

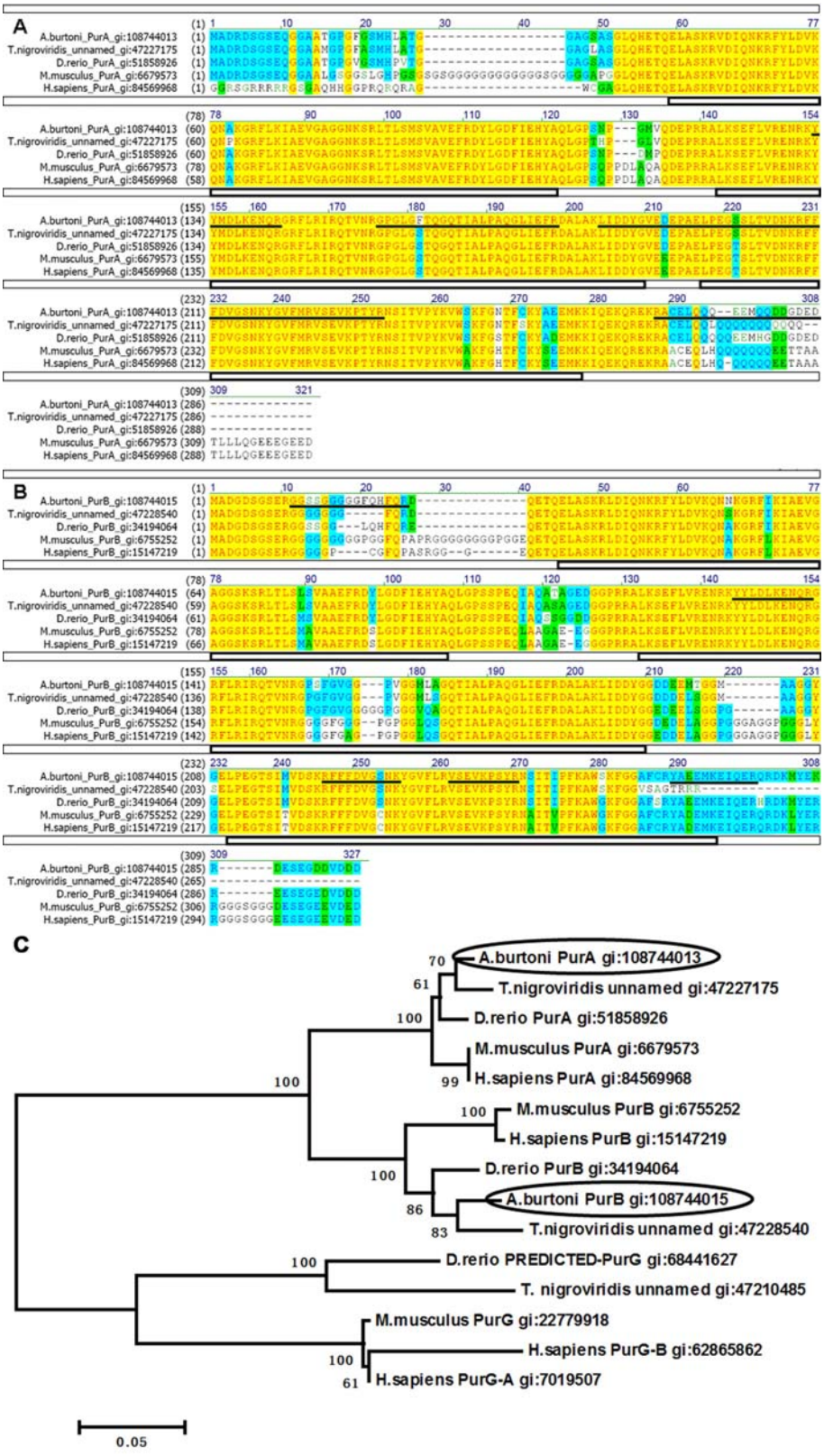
Supplemental table 6. Confirmation of mass mapping result after cloning *A. burtoni* Pur-B.

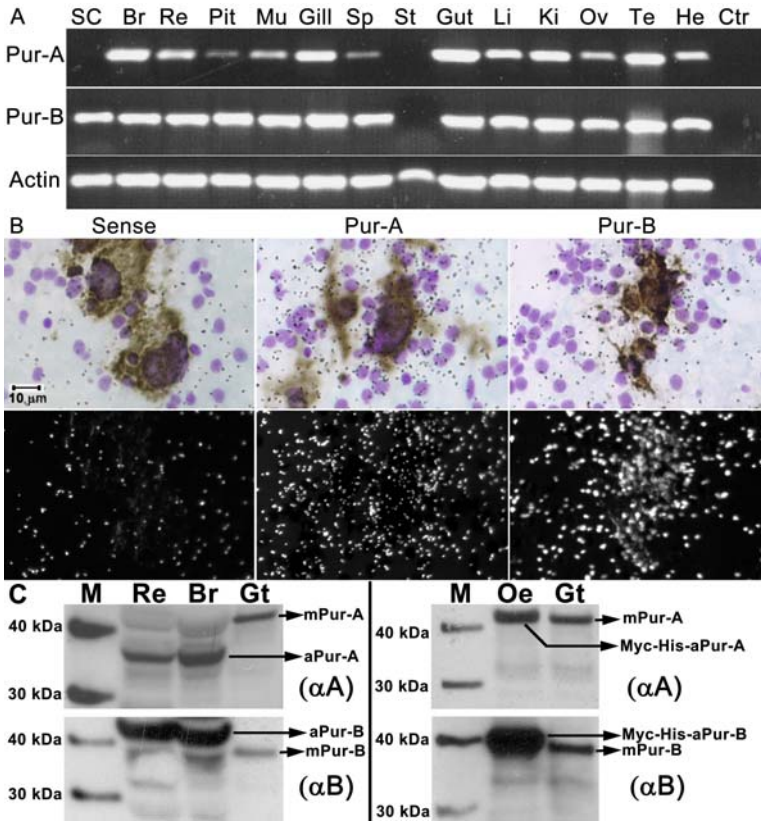
Highlighted peptides were confirmed by MS/MS peptide sequencing.

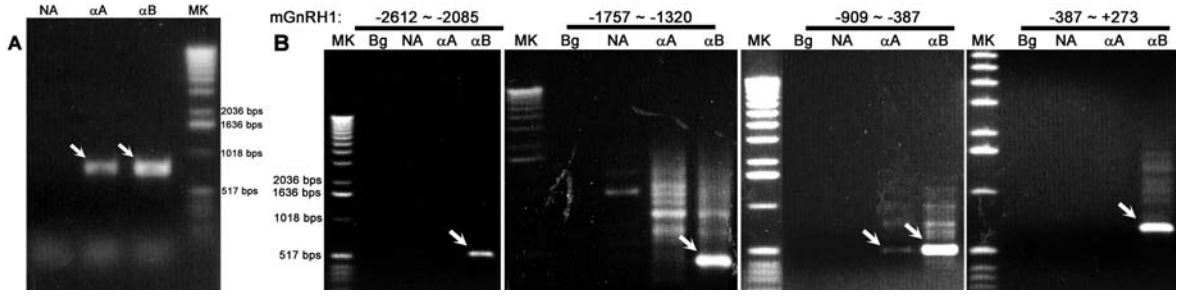
gi|108744015: Purine-rich element binding protein-beta [*Astatotilapia burtoni*]								
Mass: 32722		Protein Score: 263			Sequence Coverage: 18%			
Observed	**Mr(expt)**	**Mr(calc)**	**Delta**	**Start**	**End**	**Miss**	**Ions**	**Peptide**
1060.4607	1059.4534	1059.5025	-0.0491	223	231	0	---	FFFDVGSNK
1064.5242	1063.5169	1063.5661	-0.0492	238	246	0	35	VSEVKPSYR
1216.5585	1215.5512	1215.6036	-0.0524	222	231	1	47	RFFFDVGSNK
1341.6207	1340.6134	1340.6724	-0.0590	130	139	1	73	YYLDLKENQR
1425.6069	1424.5996	1424.6605	-0.0609	266	276	1	70	YAEEMKEIQER
1435.588	1434.5807	1434.6388	-0.0581	11	25	0	7	GGSSGGGGGFQHFQR
1441.6038	1440.5965	1440.6554	-0.0589	266	276	1	---	YAEEMKEIQER Oxidation (M)

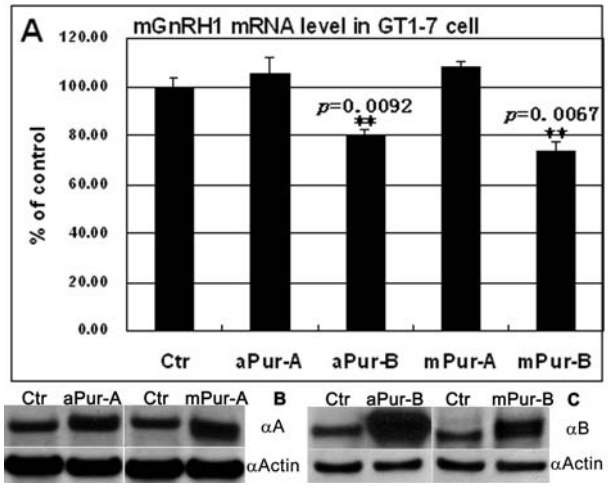
745


A
SB
Ctr
G1
G2
G3
G4
G5
SB+
49 kDa
38 kDa
49 kDa
38 kDa
B
Pur-A (-2132~-997)
Pur-B (-2132~-997)
Biotin labeled 5' end
Fragment of upstream
GnRH1 gene
Exon
G1(1193bps)
G2(1156bps)
G3(1136bps)
G4(1154bps)
G5(1160bps)
-7000
-6000
-5000
-4000
-3000
-2000
-1000
0
1000
2000


A
B
C
A.burtoni PurA gi:108744013
T.nigroviridis unnamed gi:47227175
D.rerio PurA gi:51858926
M.musculus PurA gi:6679573
H.sapiens PurA gi:84569968
A.burtoni PurB gi:108744015
T.nigroviridis unnamed gi:47228540
D.rerio PurB gi:34194064
M.musculus PurB gi:6755252
H.sapiens PurB gi:15147219
D.rerio PREDICTED-PurG gi:68441627
T. nigroviridis unnamed gi:47210485
M.musculus PurG gi:22779918
H.sapiens PurG-B gi:62865862
H.sapiens PurG-A gi:7019507
0.05


A
SC Br Re Pit Mu Gill Sp St Gut Li Ki Ov Te He Ctr
Pur-A
Pur-B
Actin
B
Sense
Pur-A
Pur-B
10 μm
C
M Re Br Gt
40 kDa
30 kDa
mPur-A
aPur-A
(αA)
aPur-B
mPur-B
(αB)
M Oe Gt
mPur-A
Myc-His-aPur-A
(αA)
Myc-His-aPur-B
mPur-B
(αB)


A
NA
αA
αB
MK
2036 bps
1636 bps
1018 bps
517 bps
B
mGnRH1:
-2612 ~ -2085
-1757 ~ -1320
-909 ~ -387
-387 ~ +273
MK
Bg
NA
αA
αB
2036 bps
1636 bps
1018 bps
517 bps


A
mGnRH1 mRNA level in GT1-7 cell
120.00
100.00
80.00
60.00
40.00
20.00
0.00
% of control
p=0. 0092
**
p=0. 0067
**
Ctr
aPur-A
aPur-B
mPur-A
mPur-B
Ctr
aPur-A
Ctr
mPur-A
B
αA
αActin
Ctr
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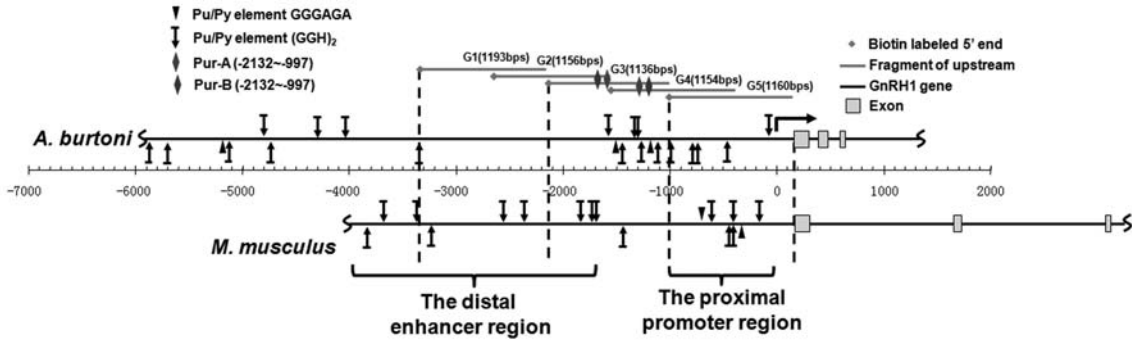
Ctr
mPur-B
C
αB
αActin


Pu/Py element GGGAGA
Pu/Py element (GGH)2
Pur-A (-2132~-997)
Pur-B (-2132~-997)
G1(1193bps)
G2(1156bps)
G3(1136bps)
G4(1154bps)
G5(1160bps)
Biotin labeled 5' end
Fragment of upstream
GnRH1 gene
Exon
A. burtoni
−7000
−6000
−5000
−4000
−3000
−2000
−1000
0
1000
2000
M. musculus
The distal enhancer region
The proximal promoter region