Zhao S, Kelm RJ Jr, Fernald RD. Regulation of gonadotropin-releasing hormone-1 gene transcription by members of the purine-rich element-binding protein family. Am J Physiol Endocrinol Metab 298: E524–E533, 2010. First published December 8, 2009; doi:10.1152/ajpendo.00597.2009.—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 (A. burtoni). Using an in vitro DNA-binding assay followed by mass spectrometric peptide mapping, we identified two members of the purine-rich element-binding (Pur) protein family, Purα and Purβ, as candidates for GnRH1 promoter binding and regulation. We found that transcripts for both Purα and Purβ colocalize in GnRH1-expressing neurons in the preoptic area of the hypothalamus in A. burtoni brain. Furthermore, we confirmed in vivo binding of endogenous Purα and Purβ 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 Purβ, but not Purα, significantly downregulated GnRH1 mRNA levels in transiently transfected GT1–7 cells, suggesting that Purβ acts as a repressor of GnRH1 gene transcription.

IN ALL VERTEBRATES, the brain controls reproduction via the hypothalamic-pituitary-gonadal axis (12, 23, 27, 59). Gonadotropin-releasing hormone-1 (GnRH1) is an essential signaling molecule in the hypothalamic-pituitary-gonadal axis (12, 23, 27, 59). Gonadotropin-releasing hormone-1 (GnRH1) is an essential signaling molecule in the hypothalamic-pituitary-gonadal axis (12, 23, 27, 59). This element exhibits a high degree of polypurine-polypyrimidine asymmetry and a theoretical propensity to form non-B-DNA structures in the resulting RNA transcript. As a ssDNA-binding protein, Purα 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, Purα and Purβ 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 Purα and Purβ 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 destabilization 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, although both Purα and Purβ can bind to the upstream region of the GnRH1 gene, Purβ plays the dominant inhibitory role on gene transcription.

MATERIALS AND METHODS

Animals and materials. We used tissue from an African cichlid fish, A. (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 (Pura 1987). All fish used in this study were sexually mature with body sizes ~7–9 cm and body weights ~12–18 g. Fish were euthanized by rapid cervical transection, and tissue was collected immediately for analysis. All procedures were in accordance with the National Institutes of Health protocol for animal experimen-
tion and approved by the Animal Care and Use Committee of Stanford University.

Nucleotide primers were purchased from Invitrogen (Carlsbad, CA) (Supplemental Table S1; Supplemental Material for this article is available at the AJP-Endocrinology and Metabolism website), and all other reagents were ordered from Sigma-Aldrich (St. Louis, MO) if not specified otherwise.

**DNA/protein in vitro binding assay.** The protocol for identifying the GnRH1 upstream binding proteins has been described previously (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-l-arginine-cytidylylate (IC). The lysate was then precleared with 1 mg of Dynabeads (Invitrogen) without immobilized DNA probe for 2 h at 4°C with slow rotation. Biotinylated double-stranded GnRH1 upstream DNA fragments and a control DNA fragment from the cDNA coding sequence (CDS) 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 precleared supernatant was then combined and incubated with the immobilized dsDNA fragments (~200 μg protein/1 mg beads) at 4°C overnight on a rotator. After magnetic separation, the beads were washed six times with 500 μl of washing buffer (1× 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 NCBI database (www.matrixscience.com). In Mascot results, the “ion score” for an MS/MS match is ~10 Log(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 NCBI 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 NCBI database included these new sequences. The original MS and MS/MS data were then analyzed again using the Mascot server with the updated NCBI 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 PurA and PurB.** Fish brains were homogenized in 1 ml of Trizol (Invitrogen) followed by 250 μl of chloroform to isolate RNA. Rapid amplification of cDNA ends (RACE) from total brain RNA was performed SMART RACE cDNA Amplification Kit; Clontech Laboratories, Palo Alto, CA). Primers for PurA and PurB were designed on the basis of the conserved region of zebrafish PurB (GenBank accession no. BC056517). On the basis of the resultant partial sequence from the PCR using zebrafish primers, RACE PCR primers used for both 3’ and 5’ ends of PurA and PurB cDNAs were then designed. All primers were designed with 60°C melting temperature. Touchdown PCR was used for cloning experiments in this study, 3 min of 95°C initial denaturing followed by 16 touchdown cycles from 68 to 60°C (annealing temperature, decrease of 0.5°C every cycle), and continued for another 25 cycles with 60°C annealing temperature. The resulting sequences for A. burtoni PurA and PurB cDNA containing the complete CDSs were verified by sequencing in both directions (Sequetech, Mountain View, CA).

Multiple alignment analysis of PurA and PurB from A. burtoni compared with 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, Valencia, CA). 3’-RACE cDNA for each tissue was synthesized (SMART cDNA synthesis kit; Clontech Laboratories). Touchdown PCR was then conducted using specific primers of A. burtoni PurA and PurB.

In situ hybridization. To colocalize PurAβ and GnRH1 mRNA expression, double in situ hybridization was used. Methods developed in our laboratory (88) were used, with minor modifications. Animals were euthanized 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 Scientific, Santa Clara, CA). Templates for radioactively labeled RNA probes specific for PurA and PurB 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 transcription (Ambion, Austin, TX) in the presence of 35S-labeled UTP (Amersham Biosciences, Piscataway, NJ). Brain slices were hybridized with the 35S-labeled sense or antisense probes and dipped in nuclear emulsion (NBT-2; Kodak, Rochester, NY) and exposed for ~1 mo at ~20°C. To identify GnRH1, probe was labeled with digoxigenin-coupled nucleotide triphosphates (Roche Applied Science, Indianapolis, IN) and visualized with 3,3’-diaminobenzidine staining using anti-digoxigenin-peroxidase primary antibody (Roche Applied Science) 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 Drs. P. L. Mellon and R. I. Weiner (60). All cell culture reagents were supplied by Invitrogen. GT1–7 cells were cultured in 100-mm master plates in Dulbecco’s modified Eagle’s medium (cat. no. 11995-065) and supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO2 at 37°C. Medium was replaced every 2–3 days until confluence was reached. For experiments, cells were seeded into 24-well plates and cultured under the same conditions. Cells utilized for chromatin isolation and transient transfection were of similar passage (passages 6–15) and confluence (~90%).

**Chromatin immunoprecipitation.** To isolate specific nucleoprotein complexes from tissues or 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 min by adding glycine to a final concentration of 0.125 M. After lysis, chromatin was fragmented by sonicating the lysate five times for 10 s (power level 3, Branson Sonifier 250; VWR International, West Chester, PA). Pre-cleared chromatin solution was then incubated with or without anti-
body (1 µg/ml) for 1 h 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 the immune complexes were eluted. Eluted immune complexes were incubated at 67°C for 4 h 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 S1).

Overexpression and transcriptional regulation in GT1–7 cells. Cloned full-length A. burtoni Purα and Purβ were subcloned into pcDNA3.1(+)–Myc-His-B mammalian expression plasmids (Invitrogen) between EcoRI and BsrBI. Expression plasmids encoding His-tagged mouse Purα and Purβ have been described previously (6). Mouse GT1–7 cells were transfected with 1 µg of plasmid and 3 µl of TransFast reagent (Promega, Madison, WI) in a 24-well plate according to the manufacturer’s protocol. Total RNA from these cells was purified (RNeasy Mini-plus Kit; Qiagen) after 48 h 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 Purα and Purβ to confirm the overexpression of the transfected genes (40). Mouse β-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 MS-assisted peptide mapping with classic homology on the basis of molecular cloning and functional tests.

Mapping the DNA/protein-binding sites upstream of the GnRH1 gene. Double-stranded biotinylated DNA fragments (~1,150 bps) of the GnRH1 upstream region were generated by PCR. These fragments covered 3,489 bps with a 500- to 600-bps overlap (designated G1 to G5; Fig. 1B). 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 MS-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 mass of ~41 and 38 kDa were analyzed further (Fig. 1A). On the basis of their apparent molecular weight 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 S2 and Fig. 1). For example, a complete binding site for G3–41 and G3–38 in the −996 to +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, that 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 server using NCBIEntrez 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, Purβ (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 nine MS matches for the G3–41 band, of which four were confirmed by MS/MS peptide sequencing (Supplemental Table S3). The G3–38 band matched best to frog Purα protein (Supplemental Table S4), with a highly significant protein score of 319. Eight MS matches were found for the G3–38 band, of which four were confirmed by MS/MS peptide sequencing (Supplemental Table S4). 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 Purα and Purβ. From A. burtoni, we cloned the full-length 858-bp CDS of Purα (GenBank accession no. DQ630740), which predicted a protein of 285 amino acids. The full-length 891-bp CDS of Purβ (GenBank accession no. DQ630224), which predicted a protein of 296 amino acids, was also cloned. Multiple alignments (Fig. 2, A and B) and phylogenetic tests (Fig. 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 (Fig. 2, A and B): identities = 224/237 (94%) and positives = 229/237 (96%) for Purα; identities = 227/280 (81%) and positives = 242/280...
Purα and Purβ are highly conserved in their central ssDNA/RNA-binding domain but are more variable in NH2- and COOH-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 Purα and Purβ. Following submission of the cloned sequence of Purα and Purβ to GenBank, we conducted a new Mascot search with the original mass mapping data against the updated NCBIInt database. As expected, the A. burtoni sequence cosegregates with sequences from other fish species.
A. burtoni Purα and Purβ sequences also resulted in high protein scores, and the major MS peaks were found to be matched (Supplemental Tables S5 and S6). These results confirmed that bands G3–41 and G3–38 are indeed A. burtoni Purβ and Purα, respectively.

Tissue distribution of Purα and Purβ mRNA. To discover where Purα and Purβ are expressed in A. burtoni, we used reverse transcription PCR in a variety of tissues. These data showed that both Purα and Purβ are widely expressed in most tissues sampled (Fig. 3A). Purα 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. Purβ is expressed abundantly in most tested tissues but is absent in stomach.

To test whether Purα and Purβ are coexpressed in A. burtoni brain, we performed in situ hybridization. We detected Purα and Purβ mRNA in the preoptic area (Fig. 3B) where GnRH1 neurons reside. Double in situ hybridization results clearly demonstrated colocalization of Purα and Purβ mRNA in GnRH1 neurons and nearby cells. In addition, using PCR, we found that mouse (m)Purα and mPurβ are also expressed in mouse GT1–7 neuronal cells (data not shown).

To confirm the molecular size of A. burtoni (a)Purα and aPurβ, fish retina and brain lysate were probed via Western blotting using specific antibodies against Purα (A149) and Purβ (B302) (40). In both tissues, the endogenous aPurα and aPurβ proteins exhibited apparent molecular masses of ~38 and 41 kDa, respectively (Fig. 3C, left), which were consistent with the molecular weights of the GnRH1 promoter binding proteins found in DNA/protein binding assay (G3–38 for aPurα and G3–41 for aPurβ). However, endogenous aPurα is smaller than the endogenous mPurα (~42 kDa) detected GT1–7 cells, whereas aPurβ is larger than mPurβ (~39 kDa) (Fig. 3C, left). When expressed in GT1–7 cells, the apparent molecular masses of Myc-His-tagged aPurα and Myc-His-tagged aPurβ increased to 42 and 40 kDa, respectively (Fig. 3C, right). Although 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 aPurα compared with Myc-His-tagged aPurβ in GT1–7 cells was observed (from 38 to 42 kDa for aPurα vs. from 39 to 40 kDa for aPurβ). This finding implied that different posttranslational modifications, such as posttranslational processing (37), N-glycosylation, and/or phosphorylation (38), might distinguish aPurα from aPurβ in A. burtoni and mouse cells.

In vivo binding of Pur proteins to the upstream region of the GnRH1 gene. Although we successfully identified Purα and Purβ 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 Purα and Purβ to interact with the upstream region of the GnRH1 gene in vivo, we performed chromatin immunoprecipitation. We utilized two antibodies specifically against Purα (A149) and Purβ (B302) (40) to immunoprecipitate genomic DNA/Pur protein complexes from A. burtoni brain (Fig. 4A) and GT1–7 cells (Fig. 4B). Expected PCR products were observed only...
from the brain samples treated with Purα and Purβ antibodies. In GT1–7 cells, we also found strong binding between mPurβ and the mouse GnRH1 (mGnRH1) upstream region, whereas mPurα showed only very weak binding to the −909 to −387 segment of the mGnRH1 gene (Fig. 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 Purβ) are able to bind to the upstream region of GnRH1 gene in vivo.

Overexpression of Purβ downregulates mouse GnRH1 in GT1–7 cells. We also investigated whether Pur proteins transcriptionally regulate mGnRH1 expression in the GT1–7 cell line. Overexpression of either aPurα or mPurβ resulted in a significant reduction in mGnRH1 transcripts (Fig. 5A). The level of mGnRH1 mRNA in aPurβ-transfected cells was reduced to 79.2% of the control cells transfected with empty plasmid. Overexpression of mPurβ 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 Purβ alone was insufficient to silence the transcription of the mGnRH1 gene completely. We did not observe any change of GnRH1 expression in Purα-transfected cells, which is consistent with the lower apparent affinity and/or stoichiometry of Purα for the GnRH1 upstream regulatory region observed by ChIP assay (Fig. 4). Overexpression of Pur proteins in GT1–7 cells was confirmed by Western blot analysis (Fig. 5, B and C).

Putative Pur protein binding sites in GnRH1 gene. Finally, we compared the upstream sequence of A. burtoni and the mouse GnRH1 genes for putative Pur protein binding sites. Besides the Pur binding consensus sequence GGGAGA (83), Purα and Purβ have also been found to interact with purine-rich ssDNA sequences of the general form, (GG)n (3, 4, 28, 53), where N is A/T/C (H) and with at least two repeats minimally required for ssDNA binding (50). Therefore, we searched the A. burtoni GnRH1 gene (GenBank accession no. AF076961) and mouse GnRH1 gene (GenBank accession no. NT_039606) with these putative Purα and Purβ binding sequences (Fig. 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 (−1,600 to −1,100 bp) of the A. burtoni GnRH1 gene. There is one GGGAGA site on each DNA strand in the proximal promoter region (−800 to −200 bp) of the mouse GnRH1 gene. The GGGAGA sites are located in −1,600 to −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 (Figs. 1 and 4 and Supplemental Table S2).

**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 octamer-binding transcription factor-1 (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 downregulation) 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 Purα and Purβ as novel GnRH1 upstream binding proteins in both A. burtoni brain and mouse GT1–7 neuronal cells and subsequently confirmed that Purβ 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), Oct-1 (14, 22), orthodenticle homeobox 2 (Otx2) (39), and three-amino acid loop extension 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 to −249 bp), where two Otx2 binding sites were found (47). The MAP kinase pathway (46) and another proximal promoter region (−75 to −67 bp) binding protein, early growth response gene (17), were found to be responsible for insulin-induced mouse GnRH1 expression. In contrast, chicken ovalbumin upstream promoter-TFI or C/EBPβ may be involved in the melanominimizediated repression of rat GnRH1 gene expression (29). The binding of Oct-1 to the distal negative glucocorticoid response element 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 Purβ in the upstream region of the mouse GnRH1 gene. Because Purβ functions as a transcriptional repressor in GT1–7 cells, it is reasonable to speculate that Purβ binding may disrupt the interaction between the proximal promoter and distal enhancer region of the GnRH1 gene. Although the Purα protein also showed an ability to bind to a region of the GnRH1 gene co-occupied by Purβ (Fig. 4), the overexpression of Purα protein did not have a significant effect on GnRH1 mRNA synthesis (Fig. 5).

The apparent functional differences observed between ectopically expressed Purα and Purβ raise several questions for future investigation. First, does the intrinsic binding affinity and stoichiometry of Purα for selected elements in this region differ quantitatively from that of Purβ? Second, is the inhibitory effect of Purβ on GnRH1 gene transcription mediated by interaction with multiple regulatory sites in the upstream region of GnRH1 gene? Third, do endogenous Purα and Purβ 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 Purβ, also involved in the regulation of GnRH1 gene expression?

It is important to note that functionally relevant interactions among Purα, Purβ, and other ssDNA/RNA-binding proteins have been observed in a number of different genes. For example, in the smooth muscle α-actin gene, Purα and Purβ appear to collaborate with the pyrimidine-rich strand-binding protein YB-1/MSY1 to repress muscle CAT 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 Purα and Purβ, evidence suggests that these proteins may help stabilize the single-stranded state during replication initiation (75). Curiously, coordinate downregulation of both hnRNP-A/Bs and Purα has

Fig. 6. Schematic representation of A. burtoni and mouse GnRH1 genes with putative Pur protein-binding sites. GnRH1 genes were analyzed, and putative Purα and Purβ binding sites (GGGAGA and GGHGGH) were plotted. Symbols for binding sites on the top side of the gene indicate that they are on the sense strand. Bottom 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 Purα and Purβ were deduced from the in vitro DNA/protein-binding assay (Supplemental Table S2 and Fig. 1).
been demonstrated in the anterior pituitary gland of ovariecto-
mized rats by addition of estrogen (5). Beside hnRNP-A/B, hnRNP-K has also been reported to act together with Purα 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 corepress GnRH gene expression in hypothalamic-pituitary-gonadal axis.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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