Heterogeneous nuclear ribonucleoprotein A/B and G inhibits the transcription of gonadotropin-releasing-hormone 1

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Gonadotropin-releasing hormone 1 (GnRH1) causes the release of gonadotropins from the pituitary to control reproduction. Here we report that two heterogeneous nuclear ribonucleoproteins (hnRNP-A/B and hnRNP-G) bind to the GnRH-I upstream promoter region in a cichlid fish Astatotilapia burtoni. We identified these binding proteins using a newly developed homology based method of mass spectrometric peptide mapping. We show that both hnRNP-A/B and hnRNP-G co-localize with GnRH in the pre-optic area of the hypothalamus in the brain. We also demonstrated that these ribonucleoproteins exhibit similar binding capacity in vivo, using immortalized mouse GT1–7 cells where overexpression of either hnRNP-A/B or hnRNP-G significantly down-regulates GnRH1 mRNA levels in GT1–7 cells, suggesting that both act as repressors in GnRH1 transcriptional regulation.

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Keywords: Gonadotropin-releasing hormone 1 (GnRH1); Heterogeneous nuclear ribonucleoprotein A/B (hnRNP-A/B); Heterogeneous nuclear ribonucleoprotein G (hnRNP-G); Transcriptional regulation; Mass spectrometric peptide mapping; Hypothalamic–pituitary–gonadal (HPG) axis

Introduction

In all vertebrates, reproduction is controlled by the hypothalamic–pituitary–gonadal (HPG) axis (Cheng et al., 1998; Faulkes and Abbott, 1991; Gahr, 2004; McComb, 1987) via gonadotropin-releasing hormone (GnRH1) released from the brain into the pituitary to regulate reproductive capacity. In many species, social interactions provide important control of the HPG axis, as in adult males of an African cichlid fish, Astatotilapia burtoni. In this species, gaining or losing social dominance induces specific changes in the structure and function of GnRH-containing neurons in the hypothalamus allowing analysis of the molecular basis of changes in reproductive capacity (Davis and Fernald, 1990; Fernald, 2002; Greenwood and Fernald, 2004; White and Fernald, 1993).

At the cellular level, GnRH1 is known to be controlled by multiple signals including growth factors, secondary messengers, steroid hormones, neurotransmitters, and neuropeptides (Belsham and Lovejoy, 2005; Haggood et al., 2005; Nelson et al., 1998). Using immortalized GnRH1 releasing cell lines (GT1–7) (Liposits et al., 1991; Mellon et al., 1990; Weiner et al., 1992; Wetsel, 1995), a proximal promoter (Enaly and Mellon, 1995; Kepa et al., 1992) and distal enhancer regions (Kepa et al., 1996; Lawson et al., 1996; Whyte et al., 1995) critical for mammalian GnRH1 gene regulation have been identified. The proximal promoter is important for basal GnRH1 gene expression and the distal promoter for GnRH1 neuron-specific expression because of an enhancer region. Both regions could bind to numerous dsDNA-binding proteins.

Transcription factors, including the GATA-factor families (Lawson et al., 1998, 1996), octamer-binding transcription factor-1 (Oct-1) (Clark and Mellon, 1995; Enaly et al., 1998), Otx2 (Kelley et al., 2000), and three-amino acid loop extension (TALE) homeodomain proteins (Rave-Harel et al., 2004) have been shown to be important in the transactivation of the rat GnRH1 gene. In contrast, COUP-TFI or CCAAT/enhancer binding protein-β (C/EBP-β) may be involved in the melatonin mediated repression of GnRH1 gene expression (Gillespie et al., 2004). Interactions between the proximal promoter and enhancer are required for optimal expression of the GnRH1 gene in GT1 cells (Nelson et al., 2000).

The regulatory mechanisms controlling GnRH1 expression in cold-blooded vertebrates remain unknown, in part because there is no well-characterized GnRH releasing cell line derived from those species. Nevertheless, bioinformatic analysis suggests that there are multiple upstream putative consensus protein binding sites in GnRH1 for adequate regulation (Kitahashi et al., 2005).

To understand how GnRH1 transcription is regulated, we analyzed GnRH1 upstream binding proteins in the teleost A. burtoni. Here we describe two new GnRH1 upstream binding proteins that may repress GnRH1 transcription: hnRNP-A/B and hnRNP-G. Both proteins belong to a previously reported ssRNA binding protein super family of heterogeneous nuclear ribonucleoproteins. Our results suggest that this newly described function of hnRNP-A/B and hnRNP-G may be conserved across vertebrates.

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Results

To identify the DNA-binding proteins that interact with the upstream region of the *A. burtoni* GnRH1 gene, we combined mass mapping with classic homology based molecular cloning followed by functional tests (Fig. 1).

**GnRH1 gene upstream sequence**

Using inverse PCR, we cloned a total of 7235-bp of *A. burtoni* GnRH1 gene sequence. Double stranded biotinylated DNA fragments (~1150 bp) of the upstream region were generated by PCR (Fig. 2A). These fragments covered 3489 bp upstream region with 500–600 bp overlapping with each other (Fig. 2C). Since multiple transcription initiation sites for GnRH1 have been found in other species (Dong et al., 1996, 1993; Kepa et al., 1992; Radovick et al., 1990; Von Schalburg and Sherwood, 1999), we predicted that there might be more transcription initiation sites than previously predicted (White and Fernald, 1998). To avoid missing any upstream sequence, we included the sequence corresponding to the GnRH1 mRNA 5′ untranslated region to the translation start codon (+165).

**Mapping the DNA/protein binding sites upstream of the GnRH1 gene**

Using mass spectrometry compatible silver staining, GnRH1 upstream binding proteins were visualized in an SDS–PAGE gel. At least seven novel bands could be unambiguously identified. In this study, three bands with molecular weight (MW) around 37 kDa, 42 kDa, and 48 kDa (Fig. 2B) were analyzed further. Based on their

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Fig. 1. Scheme for identifying novel GnRH1 upstream binding protein. This whole procedure consisted of four steps: in vitro DNA/protein binding assay and purification of the sequence-specific DNA-binding proteins; mass spectrometric peptide mapping to identify the novel binding protein based on homology database searching; verification of the mass mapping results by molecular cloning; functional tests including co-localization of the identified protein in GnRH releasing neurons, in vivo binding ability of the identified protein to GnRH1 upstream and functional tests of transcriptional regulation.

Fig. 2. Mapping DNA/protein binding sites in the GnRH1 upstream by immobilizing upstream fragments using magnetic beads. (A) DNA electrophoresis of the biotinylated double stranded fragments generated by PCR using 5′ biotin labeled sense primers. G1 to G5, upstream fragments of GnRH1 gene sequence; Ctr, a control fragment from the cDNA coding sequence of PCNA; MK, 1 kb DNA ladder (Invitrogen); (B) mass spectrometry compatible silver stain of the captured binding proteins in SDS–PAGE gel. Compared with the control, 37 kDa (gray arrows), 42 kDa (black arrows), and 48 kDa (white arrows) bands were observed in SDS–PAGE gel. SB, SeeBlue pre-stained protein ladder; SB+, SeeBlue-plus pre-stained protein ladder (Invitrogen). (C) Schematic representation of the GnRH1 upstream fragments (gray lines) and the deduced binding sites of the 37 kDa, 42 kDa, and 48 kDa binding proteins.
molecular weights and high band intensity, we labeled these binding proteins as G1–37, G1–42, and G5–48. The DNA-binding sites in GnRH1 upstream sequence could then be deduced by comparing the covered regions of the fragments (Table 1 and Fig. 2C). For example, there may not be a 42 kDa protein binding site in −2692 to −383 region due to the absence of the 42 kDa band in lane G2, G3, and G4. Similarly, G1–37 binding sites may be located in −3325 to −2693, around −1536, and −382 to +164. For binding protein G5–48, there might be no binding site in −2692 to −1537 region due to the absence of the 48 kDa band. The fact that lane G1 (for 37 kDa and 42 kDa) and lane G5 (for 48 kDa) had much stronger signals than other lanes indicate that either there are multiple binding sites or the binding sites there have higher binding affinity to these proteins.

**Mass spectrometric peptide mapping of the binding proteins**

To characterize the putative binding proteins, we performed mass spectrometric (MS) peptide mapping for 37 kDa, 42 kDa, and 48 kDa bands. Both MS and MS/MS data were analyzed (Mascot sever and NCBIInt database). Band G1–42 matched an unnamed protein (gi|47221330) from puffer fish (Tetraodon nigroviridis) with the highest protein score, 80, higher than the threshold 78 (P < 0.05). There are 4 MS matches for G1–42 band, in which one MS/MS peptide sequence (IFVGG-LNPEA-TEETI-R) was also found. Total coverage of the whole protein sequence is about 12%. BLAST showed this unnamed puffer fish protein to be homologous to heterogeneous nuclear ribonucleoproteins A/B (hnRNP-A/B). In a smaller molecular weight than the band observed on SDS–PAGE (48 kDa) which could be explained by glycosylation of the protein.

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<th>G4</th>
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<td>No band</td>
<td>Very weak</td>
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<tr>
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<td>Very weak</td>
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**Table 1** Summary of the GnRH1 upstream binding proteins deduced by the in vitro DNA/protein binding assay

**Molecular cloning of hnRNP-A/B and hnRNP-G**

The full-length 951-bp coding sequence (CDS) of hnRNP-A/B (GenBank Accession No. DQ630738) predicted a protein of 316 amino acids (GenBank Accession No. ABG02277) which was cloned from A. burtoni. The full-length 1176-bp coding sequence (CDS) of A. burtoni hnRNP-G predicted a protein of 391 amino acids and had a calculated molecular weight of ~43.085 kDa. This is a smaller molecular weight than the band observed on SDS–PAGE (48 kDa) which could be explained by glycosylation of hnRNP-G (Soulard et al., 1993). Multiple alignment (Figs. 3A and C) and a phylogenetic test (Figs. 3B and D) were performed for both hnRNP-A/B and hnRNP-G (or RBMX) with their homologs from other species. Identical branching patterns in the phylogenetic trees were generated regardless of method used to produce the tree (data not shown). Analysis across species proved that these proteins are conserved from fish to humans (Fig. 3): Identities = 147/182 (80%), Positives = 164/182 (90%) for hnRNP-A/B and 154/182 (85%), Positives = 164/182 (90%) for hnRNP-G.

**Internal sequencing of hnRNP-A/B to confirm the mass mapping result**

To verify the reliability of the results produced by mass mapping and database searching based on homology among species, we performed internal de novo sequence for the 42 kDa band with large scale purification of pooled G1–42 bands stained by Coomassie Blue. Two peptides were sequenced (Peptide1: DLKDY-FSK and Peptide2: IFVGG-LNPEA-TEETI-R). We then combined both peptide sequences and looked for short, nearly exact matches using BLAST program and GenBank protein database. BLAST results found both peptides in hnRNP-A/B from puffer fish, chick, mice, rat, and human with no or only one mismatched amino acid (data not shown), which confirmed the result from mass mapping. Furthermore, Peptide2 was also found in the original MS and MS/MS data.
and Identities = 210/401 (52%), Positives = 231/401 (57%) for hnRNP-G. hnRNP-A/B is highly conserved in its RNA recognition domain (RRD, at 57 to 129 and 141 to 213 amino acid) and more variable at the C-terminals. hnRNP-G is highly conserved in its RNA recognition domain (RRD, between 9 and 82 amino acid) and more variable at the C-terminals. Phylogenetic analysis reveals that the cichlid sequences are closer to *T. nigroviridis* than *Danio rerio*.

**Confirmation of the mass mapping results by analysis of cloned A. burtoni sequences of hnRNP-A/B and hnRNP-G**

Following submission of the cloned sequences of HnRNP-A/B and hnRNP-G to GenBank, we conducted a new search (Mascot) using the original mass mapping data to probe the updated NCBI database. As expected, the new search found the *A. burtoni* hnRNP-A/B

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**Fig. 3.** Molecular cloning of HnRNP-A/B and HnRNP-G from *A. burtoni*. Vector-NT software (Invitrogen) was used to generate multiple alignment of predicted protein sequences for *A. burtoni* hnRNP-A/B (A) or hnRNP-G (RBMX) (C) and sequences from other fish species, mouse, and human. Two conserved RRD and RRM domains were indicated by an open box and peptides matched in mass mapping (black) and internal sequencing (red) data were underlined. The phylogenetic tree for hnRNP-A/B (B) or hnRNP-G (RBMX) (D) was generated by Mega 3.1 using neighbor-joining and bootstrap test. Note that the *A. burtoni* sequence co-segregates with sequences from the other fish species.
sequence with a much higher protein score of 102 and better sequence coverage of 25% (Table 2). In addition, three more observed MS numbers were found in *A. burtoni* hnRNP-A/B sequence (Table 2). Furthermore, the peptide sequences obtained by internal sequencing (DLKDY-FSK and IFVGG-LNPEA-TEETI-R) were found in *A. burtoni* hnRNP-A/B (Table 2 and Fig. 3A). Similarly, the new search for G5–48 band found *A. burtoni* hnRNP-A/B sequence with a very high protein score of 381 and sequence coverage of 55%. In addition, 23 observed MS numbers and six MS/MS peptide sequences were found in *A. burtoni* hnRNP-G sequence (Table 3). Peptide sequence GPAPP-VERGY-PPR found in the original search with a small ion score (only 5) was not found in the new search (Table 3), indicating a false positive match. Nevertheless, five new MS/MS peptide sequences were discovered in the subsequent search (DGYGG-GREPR, DRDPY-GPPPP-R, SYMDR-PSGGS-YR, SAPSG-PSMR-PMSR, and SLDGK-PIKVE-QATKP-QFESA-GR, Table 3 and Fig. 3C). These results strongly confirmed that bands G1–42 and G5–48 are indeed *A. burtoni* hnRNP-A/B and hnRNP-G respectively and clearly demonstrated the feasibility of this new homology based strategy designed to identify novel binding proteins.

Fig. 3 (continued).
Table 2
Confirmation of mass mapping result after cloning *A. burtoni* hnRNP-A/B

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Peptides in bold were confirmed by MS/MS peptide sequencing and internal sequencing data.

**Location of hnRNP-A/B and hnRNP-G mRNA**

To discover where hnRNP-A/B and hnRNP-G are located in *A. burtoni*, we used reverse transcription PCR in a variety of tissues (Fig. 4A). These data showed that hnRNP-A/B is highly expressed in most tissues we sampled, including brain, retina, pituitary, muscle, gill, gut, liver, kidney, ovary, testicle, and heart; with reduced expression in spinal cord and no expression in stomach. hnRNP-G is highly expressed in retina, pituitary, muscle, liver, testicle, and heart, with reduced expression in brain, gill, spleen, gut, kidney, and ovary and no expression in spinal cord and stomach.

To locate expression in the brain, we performed *in situ* hybridization for hnRNP-A/B and hnRNP-G. We found the mRNA of both binding proteins in many brain regions (data not shown) including the pre-optic area (Fig. 4B) where GnRH1 neurons are located. Double *in situ* hybridization showed that hnRNP-A/B or hnRNP-G mRNA and GnRH1 mRNA were co-localized and that some nearby cells also express hnRNP-A/B and hnRNP-G (Fig. 4C). The successful cloning of mouse hnRNP-A/B (mhnRNP-A/B) and hnRNP-G (mhnRNP-A/B) from GT1–7 cells suggests that they are also present in this cultured mouse GnRH1 releasing neuron (data not shown).

**Table 3**
Confirmation of mass mapping result after cloned *A. burtoni* hnRNP-G

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Peptides in bold were confirmed by MS/MS peptide sequencing data.
this is a false positive due to the buffering conditions in vitro. To confirm the in vivo binding capacity of hnRNP-A/B and hnRNP-G to the upstream region of mouse GnRH1 gene, we performed chromatin immunoprecipitation. Since there is no available antibody against A. burtoni hnRNP-A/B or hnRNP-G, we utilized an antibody against mouse hnRNP-A2/B1 or mammalian hnRNP-G (C-17) to immunoprecipitate the in vivo DNA/protein complex from GT1–7 cells. Only expected PCR products were observed from the samples treated with hnRNP-A2/B1 or C-17 antibody. All negative controls (bacteria genomic DNA, sample without antibody or with an antibody against hnRNP-M1-4) did not identify any immunoprecipitant relevant sequence. Thus, the ChIP result did confirm that

![Figure 4. Localization of hnRNP-A/B and hnRNP-G mRNA in A. burtoni.](image)

(A) mRNA of hnRNP-A/B and hnRNP-G in various A. burtoni tissues. 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. (B (20× magnification) and C (40× magnification)) In situ hybridization of hnRNP-A/B or hnRNP-G mRNA and GnRH1 mRNA in the pre-optic area of the A. burtoni brain. GnRH1 releasing neurons were stained by DAB (brown in upper panel, bright field) and hnRNP-A/B or hnRNP-G mRNA were visualized by silver grains developed in 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.

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mhnRNP-A/B and mhnRNP-G proteins are able to bind to the upstream region of mouse GnRH1 gene (mGnRH1) (Fig. 5).

Overexpression of hnRNP-A/B and hnRNP-G down-regulates mouse GnRH1 in GT1–7 cells

We conducted a functional test of hnRNP-A/B and hnRNP-G on the transcriptional regulation of mGnRH1 in the GT1–7 cell line. Overexpression of A. burtoni hnRNP-A/B (aA/B), ahnRNP-G (aG), mouse hnRNP-A/B (mA/B), or mhnRNP-G (mG) reduced the transcripts of mGnRH1 to 72.60%, 78.33%, 66.72%, and 77.11% of the control cells transfected with empty plasmid (Fig. 6A). Overexpression of hnRNP-A/B and hnRNP-G were further confirmed by western blot analysis (Fig. 6B). In each case, despite the overexpression of the hnRNP-A/B or hnRNP-G, the inhibition of the high level of mGnRH1 expression in GT1–7 cells is moderate, indicating that the overexpression of hnRNP-A/B or hnRNP-G alone is insufficient to totally repress transcription. This phenomenon has also observed in other hnRNP-A/B targeted genes such as the apo very low density lipoprotein II (apoVLDL II) gene (Smidt et al., 1995).

Putative transcription factor binding sites and hormone response elements in GnRH1 gene

Finally, the A. burtoni GnRH1 (GenBank Accession No.AF076961) and mouse GnRH1 (GenBank Accession No.NT_039606) gene sequences were analyzed by a transcription factor prediction program, MatInspector. A stringent searching condition was used to reduce the false positive predictions by choosing 0.85 as the matrix similarity threshold resulting in 721 putative protein binding sites as opposed to the default optimized matrix similarity threshold (<0.85, found 1142 binding sites). Binding sites of well-known transcriptional factors and hormone response elements were plotted (Fig. 7). In summary, there are 9 sites for AP1, 5 for SP1, 6 CAAT-boxes, 20 for CREB sites, 3 for estrogen receptor, 2 for androgen and...
Discussion

GnRH1 transcriptional regulation

Our results (Fig. 7) showed that upstream of A. burtoni GnRH1 genes contain predicted potential transcription factor binding motifs for various DNA-binding proteins, including nuclear receptors, consistent with reports from other cichlid species (Kitahashi et al., 2005). Although searching for transcription factor binding sites based on short consensus sequence may produces false positives, novel binding sites may remain undiscovered. In cold-blooded vertebrates, none of the identified putative binding sites in GnRH1 gene has been investigated for possible direct binding activity. However, some evidence of transcriptional regulation of GnRH1 gene (e.g. overexpression or down-regulation) has been reported for putative binding proteins such as the sex hormone receptors, ER (Parhar et al., 2000), PR (Parhar et al., 1998), TR (Dubois et al., 2001; Iela et al., 1994), and GR (Fox et al., 1997; White et al., 2002). In this study, we sought direct evidence for regulation of GnRH1 at the level of the transcription.

Discovery of hnRNP-A/B and hnRNP-G as transcriptional regulator of GnRH1 in A. burtoni

To understand the transcriptional regulation of GnRH1, we assessed very small quantities of proteins bound to the GnRH1 gene, analyzing the sequence and then testing its role experimentally in cultured cells (Fig. 1). We identified several novel GnRH1 upstream binding proteins including hnRNPA/B and hnRNPG, as transcriptional repressors.

The hnRNPA family is a large nucleoprotein family including more than 20 known proteins (34 kDa to 120 kDa) that were identified as splicing factors modulating RNA processing (Krecic and Swanson, 1999). The hnRNPA/B subfamily is typified by its characteristic primary structure containing two RNA recognition domains (RBDs) followed by a glycine-rich C-terminus (Mayeda et al., 1994). The DNA-binding sequence domain of hnRNPA/B is different from the transcriptional regulation domain (Gao et al., 2004a). In rodent, the larger hnRNPA/B isoform, p40, exhibits an inhibitory activity on the target gene (Saitoh et al., 2002). In A. burtoni, we also observed two hnRNPA proteins (42 kDa and 37 kDa bands) that bind to the GnRH1 upstream region with similar molecular weight as rodent’s hnRNPA/B isoforms (Table 1 and Fig. 2). The predicted binding sites for hnRNPA/B based on the known core sequence were also found in the fragments where both proteins bind (Fig. 7).

In the transcriptional regulation of osteopontin (OPN), hnRNPA/B and hnRNPU proteins function antagonistically (Gao et al., 2005): hnRNPA/B dissociates from promoter binding site with subsequent derepression of OPN promoter activity (Gao et al., 2004a,b). hnRNPU then binds to the same site to further activate OPN promoter activation (Gao et al., 2005), indicating that an interaction among hnRNPs might be important for their in vivo functions (Kim et al., 2000). Here we also found that two hnRNPs bind to the upstream of the same gene at similar regions (Fig. 7), implying that a possible interaction between them might exist. Chicken single-strand D-box binding factor (ssDBF), a homolog of mammalian hnRNPA/B, acts as transcriptional silencer of apoLDL II and heterologous viral promoter via binding to the D-box and CARG-box respectively. Rat hnRNPA/B homolog, termed AIIFC1, is involved in repressing rat aldolase B (AldB) gene expression and in DNA replication initiation (Saitoh et al., 2002; Yabuki et al., 2001). In addition to its repressive function, hnRNPA/Bs have also been shown to be necessary for the transcriptional activation of the rat serine protease inhibitor 2 (spi-2) gene (Leverrier et al., 2000).

hnRNPG belongs to another hnRNPs subfamily with two more members, RBMY and hnRNPGT, with similar structures: an N-terminal RNA recognition motif (RRM) followed by amino acids rich in serine, arginine, and glycine (Elliott, 2004). All three are nuclear proteins with a previously reported role in regulating pre-mRNA splicing (Elliott, 2004). Interestingly, members of this subfamily have been reported as potential factors modulating the...
reproductive system of males (Delbridge et al., 1999; Ma et al., 1993; Venables et al., 2000; Westerveld et al., 2004). Human hnRNP-G (also named RBMX) gene is coded on the X chromosome and is expressed in many tissues (Lingenfelter et al., 2001) with a differential expression pattern with a characteristic tissue specificity (Nasim et al., 2003) supported in our PCR data in various fish tissues (Fig. 4A).

The wide distribution of hnRNP-G protein suggests that it might play an important role in somatic cells other than the testis. The human hnRNP-G gene is located on the X chromosome at Xq26 where several X-linked mental retardation (XLMR) syndromes have also been mapped (Delbridge et al., 1999), suggesting a possible involvement of hnRNP-G in these mental disorders. In zebrafish, knockdown experiment of hnRNP-G by antisense morpholino indicated that hnRNP-G is required for normal embryonic development (Tsend-Ayush et al., 2005). Although current studies on the hnRNP-G protein subfamily have been focused on their function of pre-mRNA binding and splicing, other hnRNPs have already been identified as ssDNA/dsDNA-binding proteins.

Transcriptional activation or repression of RNA polymerase II dependent genes such as GnRH1 is often mediated by sequence-specific DNA-binding proteins or transcription factors which re-

Table 4

<table>
<thead>
<tr>
<th>Consensus sequence</th>
<th>Format</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoVLVD II D-box</td>
<td>ssDNA</td>
<td>Transcriptional repressor</td>
<td>Smidt et al. (1995)</td>
</tr>
<tr>
<td>RSV CArG-box</td>
<td>ssDNA</td>
<td>Transcriptional repressor</td>
<td>Smidt et al. (1995)</td>
</tr>
<tr>
<td>OPN promoter</td>
<td>dsDNA</td>
<td>Transcriptional repressor</td>
<td>Gao et al. (2004a)</td>
</tr>
<tr>
<td>5’ end of Feline Parvovirus</td>
<td>ssDNA</td>
<td>Modify virus replication</td>
<td>Wang and Parrish (1999)</td>
</tr>
</tbody>
</table>

Based on known binding sequences found in other genes, a consensus binding sequence is deduced (DRTWKGDAV).
spond to extracellular signals by interacting with key cis-acting regulatory elements. Transcription factors generally bind to double-stranded DNA recognition sites distal to the TATA-box and influence promoter activity (Johnson, 1995; Tjian and Maniatis, 1994). Although traditional transcription factors are double-stranded DNA (dsDNA) binding proteins, in recent years, many sequence-specific, single-stranded DNA (ssDNA)/RNA (ssRNA)-binding proteins have been discovered that either activate (Duncan et al., 1996; Haas et al., 1995b; Tomonaga and Levens, 1996) or repress (Aliki and Groner, 1993; Gupta et al., 2003; Haas et al., 1995a; Kelm et al., 1999; Lindzey et al., 1994; Pan et al., 1990; Tanuma et al., 1995; Wilkinson et al., 1990) transcriptional activity.

Many such single-strand DNA-binding proteins are important for gene transcriptional regulation. For example, the far upstream element binding proteins (FBPs) and heterogeneous nuclear ribonucleoproteins have been discovered that either activate (Duncan et al., 1996; Kelm et al., 1999; Lindzey et al., 1994; Pan et al., 1990; Tanuma et al., 1995; Wilkinson et al., 1990) transcriptional activity.

Animals and materials

We used tissue from an African cichlid fish, *A. (Haplochromis) burtoni*, bred from wild-caught stock (Fernald, 1977; Fernald and Hirata, 1977) 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) (Fernald, 1977). All fish used in this study were sexually mature males with body sizes ca. 7–9 cm and body weights ca. 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 obtained from Invitrogen (Carlsbad, CA) (Supplementary Table 1) and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO) if not otherwise specified.

**Genomic DNA extraction and inverse PCR**

Genomic DNA was extracted from fish (DNeasy, Qiagen, Valencia, CA) and inverse PCR, based on the known GnRH1 gene sequence (GenBank Accession No.AF0769961), was repeatedly performed until a 7235-bp sequence of *A. burtoni* GnRH1 gene had been cloned. Briefly, 1 μg genomic DNA was digested with 5–10 units restriction enzyme (New England Biolabs, Ipswich, MA) in 50 μl reaction for 2 h. Several enzymes were utilized in each digestion to guarantee production of a suitable fragment with the appropriate length for forming a loop. The enzyme was inactivated at 65 °C for 20 min. Genomic DNA fragments were diluted to 500 μl and then circularized by 10 units T4-DNA ligase (Invitrogen, Carlsbad, CA) at 16 °C overnight followed by ethanol precipitation at −20 °C overnight. Touchdown PCR was used for cloning experiments in this study: 3 min 95 °C initial denature process 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. All primers were designed with 60 °C melting temperature based on the known sequence of *A. burtoni*.

**DNA/Protein in vitro binding assay**

The protocol for identifying the GnRH1 upstream binding proteins was based on Nordhoff’s method (Nordhoff et al., 1999). Thirteen fish brains were homogenized in 12 ml buffer A (10 mM Tris pH 7.4, 1 mM EDTA, 0.5 mM EGTA, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 10 mM Na3PO4, 1 mM NaVO4, with 1× proteinase inhibitors: 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM PMSF). The crude lysate was mixed with 3 ml 5× large-volume low-salt (LVLs) binding buffer (100 mM HEPES, pH 7.9, 250 mM KCl, 5 mM MgCl2, 50% glycerol, 5 mM DTT, and 0.5 mg/ml poly-d(I.C)). The brain lysate was then pre-cleared with 1 mg Dynabeads (Invitrogen, Carlsbad, CA) without immobilized DNA probe for 2 h at 4 °C with slow rotation. Insoluble pellets were spun down at 14,000 rpm for 10 min at 4 °C and the supernatant was collected. 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 primer was 5′-biotin labeled. These double stranded DNA fragments were then immobilized by Dynal M-280 streptavidin-coated magnetic beads (Invitrogen) following the manufacturer’s instructions. The pre-cleared supernatant was then aliquoted and incubated with the immobilized dsDNA.
μ followed by 250 Edman sequencing (Applied Biosystems sequenator, Procise HT). From 6% acetonitrile/0.1% trifluoroacetic acid to 42% acetonitrile/0.09% C18, 1×150 mm, 5 micron particle size, 300 Å pore size) with a gradient internal sequencing matches with a significant protein score. Analyzed again by Mascot server with the updated NCBInt database to

burtoni was then performed to find more homologs to use in designing degenerate primers for hnRNP-A/B and hnRNP-G. The predicted protein sequences from the cDNA sequence were submitted to GenBank and the original MS/MS data were analyzed again by Mascot server with the updated NCBInt database to verify whether the cloned protein could also be found in the resulted matches with a significant protein score.

Internal sequencing

The pooled Coomassie Blue stained protein bands with the same molecular weight found in silver stained gel were used for internal sequencing. After partially digesting the protein, peptides were purified by HPLC (Michrom Bioreources, Magic 2002) using columns (Reliasil C18, 1 × 150 mm, 5 micron particle size, 300 Å pore size) with a gradient from 6% acetonitrile/0.1% trifluoroacetic acid to 42% acetonitrile/0.09% trifluoroacetic acid over 40 min. Separated peptides were sequenced by Edman sequencing (Applied Biosystems sequenator, Procise HT).

Molecular cloning of A. burtoni hnRNP-A/B and hnRNP-G

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 brain total RNA was performed (SMART RACE cDNA Amplification Kit, Clontech, Palo Alto, CA). Based on published sequences (D. rerio—GenBank Accession No.BG66454; T. nigroviridis—GenBank Accession No.CNS0EWP5 and CNS0GP1), degenerate primers for hnRNP-A/B were designed. Degenerate primers for hnRNP-G were designed based on published sequences (D. rerio—GenBank Accession No. AJ717349; Macropus eugenii—GenBank Accession No.AF034741; T. nigroviridis—GenBank Accession No.CNS0G9EN). Based on the partial sequence from the degenerate PCR, RACE PCR primers used for both 3′ and 5′ ends of hnRNP-A/B and hnRNP-G cDNA were then designed. All primers were designed with 60 °C melting temperature. Touchdown PCR was used for all cloning experiments. The resulting sequences for A. burtoni hnRNP-A/B (GenBank Accession No.DQ630738) and hnRNP-G (GenBank Accession No.DQ630739) cDNA containing the complete coding sequence were verified by sequencing in both directions (Sequenew, Mountain View, CA). Multiple sequence alignment analysis of A. burtoni compared with other species’ hnRNP-A/B or hnRNP-G was performed (Vector-N software, Invitrogen). The phylogenetic tree for hnRNP-A/B across various species was generated (Mega 3.1) using neighbor-joining and bootstrap tests (Kumar et al., 2004).

RNA localizations in organs

PCR was performed on selected 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 their 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). Touch-down PCR was then conducted using specific primers of A. burtoni hnRNP-A/B and hnRNP-G.

In situ hybridization

To co-localize simultaneously hnRNP-A/B, hnRNP-G with GnRH expression, double in situ hybridization was used. Methods developed in our laboratory (Chen and Fernald, 2006) were used with minor modifications. Animals (1 year old reproductive male fish, ~8 cm in length and ~18 g in weight) were killed by rapid cervical transection, brains were immediately embedded in OCT Compound (Tissue-Tek, Torrence, CA) and flash frozen. Coronal tissue sections for were cut using a cryostat (Microm, Zeiss, Thornwood, NY) at 14 µm and thaw mounted onto slides (Superfrost, Fisher, Santa Clara, CA). Templates for radioactively labeled RNA probes specific for hnRNP-A/B and hnRNP-G 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 RNA probe from the end with T7 promoter. PCR products were obtained by gel purification kit (Qiagen). RNA probes were then synthesized by T7 transcriptionase (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, dipped in nuclear emulsion (NBT-2; Kodak, Rochester, NY), and exposed for approximately 1 month at ~20 °C. To identify GnRH1, the probe was labeled with digoxigenin (DIG) labeled 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. Digital photomicrographs were acquired under both bright field and dark field illumination (Spot camera, Diagnostic Instruments, Sterling Heights, MI, Axioscope, Zeiss).

Cell culture

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 were 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 were of similar passage (passage 6–15) and confluence (~90%).

Chromatin immunoprecipitation

To isolate chromatin, an immunoprecipitation (ChIP) protocol modified from Braunstein and co-workers was used (Alberts et al., 1998; Braunstein et al., 1993; Meluh and Koshland, 1997). Chromatin/Protein complexes in cultured GT1–7 cells were cross-linked by addition of formaldehyde to a final concentration of 1% in phosphate buffered saline (PBS). Cross-linking reaction was stopped after 10 min by adding glycine to a final concentration of 0.125 M. Cells were then spun down at 5000 rpm for 5 min and resuspended in buffer A-NP40 (85 mM KCl, 5 mM Pipes (pH 8.5), 50 mM NaF, 10 mM Na2HPO4-10H2O, 1 mM Na4P2O7, 0.5% NP-40, 1% proteinase inhibitors: 5 µg/ml aprotinin, 5 µg/ml leupeptin, and 1 mM PMSF) and placed on ice for 10 min followed by centrifugation at 7000 rpm for 10 min. Lysis buffer (10 mM EDTA, 50 mM Tris–HCl (pH 8.0), 1% SDS, 50 mM NaF,
Overexpression and transcriptional regulation in GT1–7 cells

Cloned full-length A. burtoni hnRNP-A/B (a/B), hnRNP-G (aG), mouse hnRNP-A/B (m/A/B), and hnRNP-G (m/G, from GT1–7 cells) were subcloned into pXFLAG-CMV-7.1 Expression Vector (Sigma) between HindIII and EcoRI. Mouse GT1–7 cells were transfected by 1 μg empty plasmid or plasmid containing aA/B, mA/B, aG, or mG with 3 μl TransFast (Promega, Madison, WI) in 24-well plate according to 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 (Zhao and Fernald, 2005). Transfected cells were also analyzed using western blot to confirm the overexpression of the transfected genes. Cells were treated with 0.5 μl of ice-cold total lysis buffer (10 μl Tris pH 7.4, 1 μl EDTA, 0.5 mM EGTA, 150 mM NaCl, 1% Triton X–100, 50 mM NaF, 10 mM Na₄P₂O₇, 1 mM Na₃VO₄, 1× proteinase inhibitors: 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM PMSF) was then added to pellet (Nuclear) and sat on ice for 10 min. The cells were then sonicated five times for 10 s (Power level 3, Branson Sonifier 250, VWR International, West Chester, PA) and spun down at 14000 rpm for 10 min. The supernatant was then diluted tenfold in IP buffer (1.2 mM EDTA, 16.7 mM Tris–HCl (pH 8.0), 0.01% SDS, 1.1% Triton X–100, 167 mM NaCl, 50 mM NaF, 10 mM Na₄P₂O₇, 10 mM H₂O, and 1 mM Na₃VO₄, 1× proteinase inhibitors: 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM PMSF) with 20 mg/ml sonicated bacterial genomic DNA and 1 mg/ml bovine serum albumin (BSA). The chromatin solution was then pre-cleared for 1 h at 4 °C on a rotating wheel by protein A-Magnetic beads (Invitrogen, preadsorbed in IP buffer with bacterial genomic DNA and BSA for 1 hour). Next the supernatant was incubated with/without antibody for 1 h at 4 °C. Antibody against hnRNPA2/B1 was purchased from Abcam (Cambridge, MA). Antibody against hnRNPG (C-17) or hnRNPM1–4 was purchased from Santa Cruz Biotech (Santa Cruz, CA). The preadsorbed protein A-Magnetic beads were added to precipitate the immunocomplex overnight at 4 °C. The beads were washed twice by W1 buffer (2 mM EDTA, 20 mM Tris–HCl (pH 8.0), 0.1% SDS, 1.1% Triton X–100, and 150 mM NaCl), once by W2 buffer (1 mM EDTA, 10 mM Tris–HCl (pH 8.0), 1% NP-40, 0.25 M LiCl, and 1% deoxycholate), and twice by TE buffer (10 mM Tris–Cl (pH 7.5) and 1 mM EDTA) for 5 min each time. The immune complexes were eluted by adding Elute buffer (0.25 ml 1% SDS, 0.1 M NaHCO₃, and 0.3 M NaCl) and rotating for 15 min at room temperature. The supernatant was then incubated in a 67 °C for 4 to 5 h to reverse the formaldehyde cross-link followed by phenol/chloroform extraction and then ethanol precipitated at −20 °C overnight. PCR was performed to detect the presence of the upstream promoter region of mouse GnRH1 followed by electrophoresis analysis.

Prediction of transcription factor binding sites

Both A. burtoni GnRH1 gene (GenBank Accession No. AF076961) and mouse GnRH1 gene were analyzed by MatInspector (www.genomatix.de) (Cartharius et al., 2005; Quandt et al., 1995) to predict the putative protein binding sites.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mcn.2007.08.015.

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