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COMPARISON OF GENE EXPRESSION IN NORMAL AND GROWTH HORMONE RECEPTOR-DEFICIENT DWARF CHICKENS REVEALS A NOVEL GROWTH HORMONE REGULATED GENE

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Because of a dysfunctional growth hormone (GH) receptor there is an absence of GH-dependent gene expression in the sex-linked dwarf chicken. Therefore, a comparison of mRNAs expressed in normal and dwarf chickens should lead to the identification of mRNAs that are regulated by GH action. We have compared gene expression in normal and dwarf chickens using the mRNA differential display technique. A combination of three anchored oligo dT primers and 15 random decamers were used to detect at least 75 differentially expressed mRNAs. One of these, designated GHRG-1, hybridizes to a 0.9 kb transcript found only in liver and in normal chickens shows a pattern of developmental expression which parallels the plasma GH profile. A GHRG-1 cDNA clone was isolated that encodes a 120 amino acid peptide with no homology to any known gene. Sequence of the promoter from a genomic clone shows a region with strong similarity to the GH response element identified in the serine protease inhibitor gene, Spi 2.1. These results suggest that GHRG-1 is a novel GH regulated gene.

Growth hormone (GH) plays a major role in growth, development and metabolism, yet there is only limited information on genes whose expression is controlled by GH. GH administration alters specific mRNA levels of hepatic IGF-I (1), c-*fos* and c-*jun* (2), members of the cytochrome P_{450}IIC family (3), lipoprotein lipase (4), and genes of the serine protease inhibitor (Spi) family (5-8). Hypophysectomized (hypox) rats have been used in the past to identify GH-regulated genes by comparing expression with and without GH replacement; this model has been used to identify two homologous GH-regulated genes, Spi 2.1 and Spi 2.3 [(5,9); nomenclature based on (6)]. Their physiological role in GH action is not yet deciphered (10), but a GH-responsive element (GHRE) in the promoter of Spi 2.1 has been identified (6,11). A GH-dependent nuclear factor that interacts with this sequence has been partially characterized (9). However, comparison of the putative GHRE with the sequence of the human IGF-1 promoter (12) or the GH-responsive c-*fos* promoter (13) does not indicate a consensus GHRE sequence. The identification of additional target genes for GH is necessary for mapping of the *cis*-regulatory elements.

The GH receptor (GHR) deficient sex-linked dwarf chicken (14) is a valuable model for understanding GH-action at the molecular level. We have identified mutations in the GHR gene

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that account for the absence of GH action in these birds (15,16). We have used the mRNA differential display technique (17) to compare gene expression in liver of normal (*DwDw*) and dwarf chickens (*dwdw*) for identification of novel GH-regulated genes and the promoter sequences required for transcriptional activation by GH. We report the initial characterization of one differentially expressed gene, designated GHRG-1 (GH Regulated Gene-1).

MATERIAL AND METHODS

Experimental animals. The *DwDw* and *dwdw* chickens used in this study are maintained at the University of Georgia and are of the same genetic background (18). Fertile eggs were shipped by air freight to the University of Delaware for hatching. Animals were handled in accordance with the principles and procedures outlined by the University's Animal Care and Use Committee.

Materials. RNAmap mRNA Differential Display system was purchased from GenHunter Corporation (Brookline, MA). Radiolabeled nucleotides were purchased from New England Nuclear (Boston, MA). Oligonucleotides were prepared by Biosynthesis, Inc. (Lewisville, TX). λZap chicken liver cDNA library was purchased from Stratagene (La Jolla, CA). PCR products were cloned using the pCR-Script SK(+) cloning kit obtained from Stratagene (La Jolla, CA). Poly(A)Tract mRNA isolation system, Magic PCR Preps, Magic Lambda Preps, Magic Minipreps DNA purification systems and enzymes were purchased from Promega (Madison, WI). RediPrime random primer labeling kits were obtained from Amersham (Arlington Heights, IL) and sequencing reagents were from US Biochemicals (USB, Cleveland, OH).

Differential display. Poly(A) RNA (0.5 μg), prepared from 5 week-old *DwDw* and *dwdw* chickens, was reverse transcribed with each of the four anchored oligo dT primers, 5'GMT_{12}3', 5'AMT_{12}3', 5'TMT_{12}3' and 5'CMT_{12}3' where 'M' is a degenerate mixture of dA, dC and dG. Amplification and isolation of cDNA fragments were performed in accordance with the manufacturer's instructions. The resulting PCR products were treated with Klenow DNA polymerase (19), cloned into pCR-Script SK(+) vector and sequenced with the Sequenase 2.0.

Northern blot analysis. Preparation and analyses of RNA were performed essentially as described (15). cDNAs were random primer labeled and blots were washed with a final stringency of 2X SSC, 0.1% SDS at 60°C. Probes were removed from the blot, by shaking at room temp for 15 min, in 0.2X SSC and 0.1% SDS, at 100°C.

cDNA and genomic library screening. A λZap cDNA library (Stratagene) prepared from 7-week-old broiler chicken liver was screened by hybridization of duplicate filter lifts (6 X 10^5 pfu) with the random primer labeled fragment of GHRG-1. Positive clones were plaque purified and plasmid excised using the *in vivo* excision protocol as per the manufacturer's instructions. cDNA clones were sequenced with primers flanking the polylinker and primer walking. Comparison of DNA homology with NCBI DNA databases was performed using BLAST (20,21).

A GHRG-1 cDNA fragment was used to screen a chicken genomic library (10^6 plaques) in λGEM-11. Positive clones were plaque purified and analyzed by Southern blot analysis. The fragments of the genomic clones containing the 5' end of the gene were identified by hybridization to a fragment of the cDNA containing the 5' end.

Primer extension analysis of transcriptional start site. An oligonucleotide complementary to nucleotides (nt) +292 to +319 of GHRG-1 cDNA (Fig. 3) was labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP and used to prime a reverse transcriptase reaction (19) with poly(A) RNA from a liver of a 5 week old broiler chicken . A comparison of the migration of the primed readout product with the sequence of pBluescript with T3 primer on a 6% sequencing gel indicated the size of the product and the location of the start site.

RESULTS AND DISCUSSION

Expression of GHRG-1 in normal and dwarf chickens

A comparison of gene expression by mRNA differential display in the livers of age-matched normal and dwarf chickens revealed a large number of differentially expressed genes.

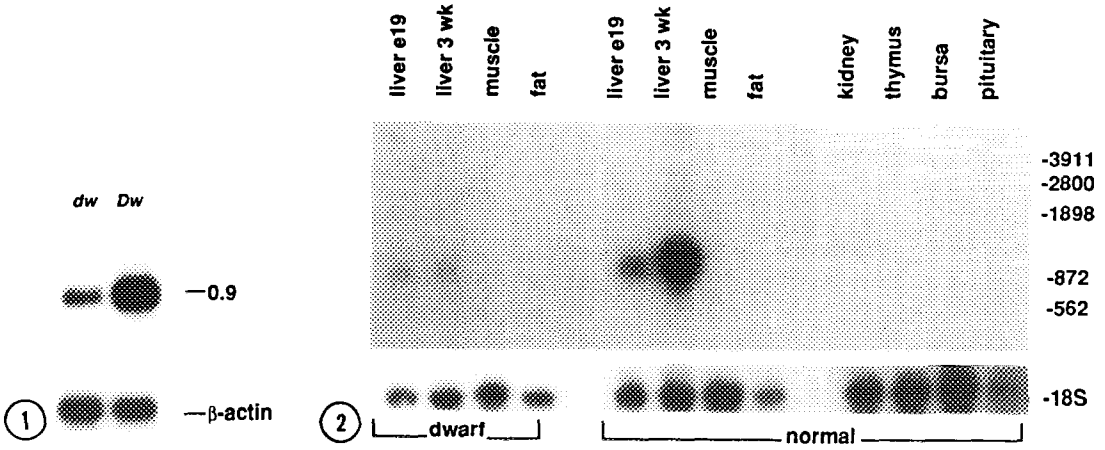

Figure 1. Northern blot analysis of GHRG-1 in normal (*Dw*) and dwarf (*dw*) RNA. Poly(A) RNA (2 μg) from liver was electrophoresed, transferred to a nylon membrane and UV crosslinked, and hybridized to a GHRG-1 cDNA probe. The blots were exposed for 6 h with intensifying screens at -70°C. Hybridization of the same blot to a probe for chicken β-actin indicates an equal amount of RNA in both lanes.

Figure 2. Total RNA (15 μg) prepared from different tissues of normal and dwarf chickens was hybridized to a GHRG-1 cDNA probe. Liver samples were obtained from embryos at day 19 of incubation (e19) and three-week-old (3 wk) normal and dwarf chickens. Hybridization of the same blot to a 18S ribosomal RNA probe indicates intact RNA in all lanes. Migration of an RNA ladder is shown on the right.

Using 27 different combinations of primer pairs (3 anchored oligo dTs and 15 random decamers), a total of about 700 bands were revealed, of these, 42 were expressed at higher levels in *DwDw* chickens and 33 were expressed at higher levels in the *dwdw* chicken (not shown).

One differentially expressed gene, designated GHRG-1, was chosen for further analysis. The partial cDNA recovered from the display gel was reamplified, random primer labeled in the presence of [^{32}P]dCTP and used as a probe on Northern blots of liver mRNA from *DwDw* and *dwdw* chickens. GHRG-1 hybridizes to a band of 0.9 kb, which is expressed at high levels in *DwDw* chicken and under-expressed in the livers of *dwdw* chickens (Fig. 1). In addition, the expression of GHRG-1 appears to be limited to liver (Fig. 2), which is a primary target for GH action.

Developmental regulation of GHRG-1

The expression of a GH-regulated gene should follow the plasma GH profile during development (Fig. 3A). Northern blots of total RNA from the liver of *DwDw* and *dwdw* chickens at different ages are shown in Fig. 3. The pattern of GHRG-1 expression in *DwDw* chickens (Fig. 3B) parallels the plasma profile of GH with peak expression at 3-4 weeks of age. In contrast, no developmental changes in GHRG-1 expression were noted in liver RNA of *dwdw* chickens (Fig. 3C). The pattern of GHRG-1 expression is similar to that of chicken IGF-I, a well known GH-regulated gene (22). In rats, the developmental expression of two GH-regulated genes, Spi 2.1 and 2.3, also parallels serum GH levels (23,24). And similar to what has been described in rats (23), expression of GH regulated genes does not correlate with GHR gene expression, which in chickens increases from hatching until reaching a plateau in mature animals (22).

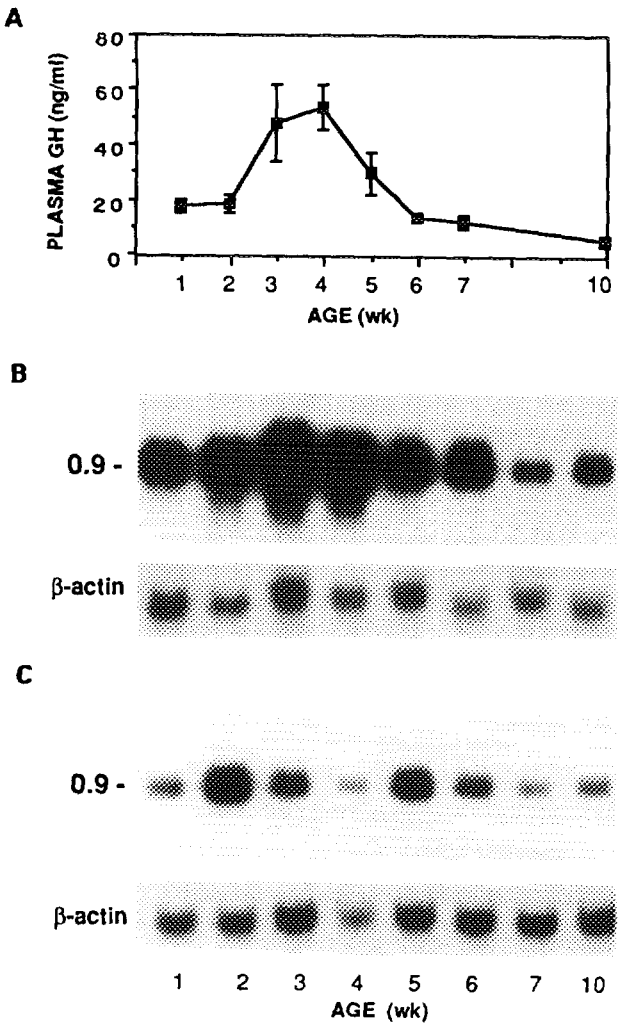

Figure 3. Developmental profile of plasma GH levels (**A**) (22) and GHRG-1 mRNA (**B**) in normal chickens. GHRG-1 expression in the liver of *dwdw* chickens (**C**). Hybridization of the blots to a probe for chicken β-actin indicates equal amounts of RNA in all lanes.

GHRG-1 cDNA and genomic sequence analysis

The partial GHRG-1 cDNA cloned from the differential display gel was used to obtain a clone from a chicken liver cDNA library (Fig. 4). Based on the results of transcriptional start site analysis (Fig. 5), the clone from the library lacks sequences in the 5' untranslated region (UTR). Thus, the sequence of the full length cDNA was generated by combining 110 bp sequence from a genomic clone with the sequence of the cDNA clone. Using the ATG at nt 229 as the initiator methionine, a 120 amino acid open reading frame was found. The full length cDNA of GHRG-1 contains 227 bp of 5' UTR and 246 bp of 3' UTR. There is a consensus poly(A) signal (ATTAAA) in the 3' UTR 27 bp upstream of the poly(A) tail. The predicted amino acid sequence does not show any striking similarity with any known sequence. This small protein is predominantly hydrophilic and lacks a signal peptide, indicating it is likely to be an intracellular protein. There are four cysteine

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ACATGTTCAGGTAAGATGTTCAGAATTTTTCAGTCATTTTTCATATGGCTTAGCTAAGAAAAACACTGAACTCTAATGG  79
AACTGTGTGTCTGCCTCAGGATTTCCAGTGTACGATTATGAACTCCCTGTCACAGAAGAGGCTCTCAATGCTTCTATTG 158
CAAGGATCAATTCTCAGACTTGGGGCCCAAACCTGTATGGAGTTGTCAGGAGCCACGTTAGACACGTTGAC ATG TGG 235
                                                                      M   W   2

AAC AGC AAT GAT TAT AGA CTA GAG CTG CAG CTC AGT ATT CGT GAA ACC GAA TGC ACA AAA 295
 N   S   N   D   Y   R   L   E   L   Q   L   S   I   R   E   T   E   C   T   K  22

GCT TCA GGA AGA GAC CCA TTT ACA TGT GGC TTC AAA GTA GGG CCT TTT GTG CCA ACT GCT 355
 A   S   G   R   D   P   F   T   C   G   F   K   V   G   P   F   V   P   T   A  42

GTC TGC AAA AGT GTT GTA GAA GTC TCC AGT GAG CAG ATT GTG AAT GTT ATT GTG CGA TGC 415
 V   C   K   S   V   V   E   V   S   S   E   Q   I   V   N   V   I   V   R   C  62

CAT CAG AGC ACA TTC AGC TCT GAA TCG ATG AGC AGT GAG GAG ATG ACG TAT ATG CTG ATG 475
 H   Q   S   T   F   S   S   E   S   M   S   S   E   E   M   T   Y   M   L   M  82

ACG GAC CCA AGG AAG CGA GGC AGC AGT CGC TCC GAA GCC TTC TCA TCA AGG GGA AGA GGC 535
 T   D   P   R   K   R   G   S   S   R   S   E   A   F   S   S   R   G   R   G  102

CAC AGC AAT GGT GAC TGG CGT AAA CCT GAT TAT ACT AGC CCT GGC AAG GTT GAA TAA TGC 595
 H   S   N   G   D   W   R   K   P   D   Y   T   S   P   G   K   V   E  end

AATTTAGGAAAAACTATTCTGTGATGAAGTGAGTCTTTCCTTAAAATCACCTTCTGCTTTACAGCCAAGTGGCCATTGG 674
ATGAGTTCATCGGGTGCTGAATGGATGCACTGCTCAGTCAATAGTGTCCTGACATATTACAGCTCATCGGAAGGACTGT 753
CTCAGACACCTAATGTAACTGTCTAGTATGCATTGTACCATCTCATAGCAATGATATTAAAGGATCAAAGGATTGCTCT 832
TGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Figure 4. Nucleotide and predicted amino acid sequence of GHRG-1 cDNA. The nucleotides are numbered from the 5' end with the transcriptional start site as +1. Introns, indicated by **I** were located by comparison to the sequence of GHRG-1 genomic clones. The underlined region in the sequence corresponds to the primer used for start-site analysis and the poly(A) signal is in bold type. The potential SH2 domain binding motif is boxed.

residues which could be involved in protein folding. There is a high percentage of serine residues, which suggests this is a short-lived protein, because proline, glutamine, serine and threonine-rich proteins tend to have short half lives (25). A motif, YMLM, that could be involved in binding to SH2 domains (26) is present. This sequence is found in proteins which interact with receptor signalling complexes. No other consensus amino acid motifs were detected.

A genomic clone was obtained and the promoter was partially sequenced (Fig. 6A). A comparison of the genomic clone to the cDNA shows that the first exon contains untranslated sequence and the first in-frame methionine is located in exon-2. The region upstream of the start site does not show a consensus TATA box, although there is an (A+T)-rich sequence 27 bp upstream of the start site.

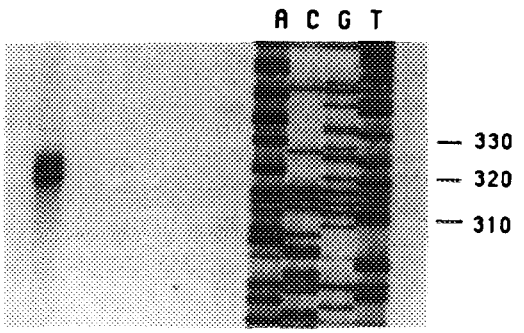

Figure 5. Primer extension mapping of the transcriptional start site. The *left panel* shows the extended product using the primer underlined in Fig. 4 and poly A-purified chicken liver RNA as template. The *right panel* shows sequence of pBluescript generated with T3 primer which was used to size the primed readout product.

Figure 6. **A**. Partial sequence of the promoter region of GHRG-1. The nucleotide sequence showing identity of 11 out of 13 base pairs of the Spi 2.1 GHRE is boxed. The bent arrow indicates the transcriptional start site. **B**. Sequence similarity between rat Spi 2.1 GHRE and similar element in GHRG-1. The two GAS-like elements in the Spi 2.1 promoter are boxed (27,28), and mismatched bases are shown in lowercase letters.

A comparison of the 5' flanking region of GHRG-1 to the Spi 2.1 GHRE [-147 to -103 (9)] reveals the presence of a region with striking sequence similarity (11 of 13 bp identical) which is 7 bp upstream of the start site (Fig. 6B). In the non-GH-responsive Spi 2.2 gene [nomenclature based on (6)], this region is identical to Spi 2.1, with the exception of 2 bases, which are included in the 13 bases of sequence similarity between Spi 2.1 and GHRG-1 ($^{-126}$TG -> CC). This region in the GHRG-1 gene is found within the γ-interferon activated sequence (GAS)-like element of the Spi 2.1 gene (-129 to -121) that forms a GH-dependent complex with nuclear proteins (27); however, the palindrome-like sequence in GHRG-1 is not the same as the GAS-like palindrome. There is no region in the GHRG-1 promoter that resembles the second GAS-like element of the Spi 2.1 gene (-144 to -136) (27,28); this element does not appear to be involved in GH regulation, since deletion of these sequences does not abolish GH responsivity of the GH promoter (7) and no GH-dependent nuclear complexes are formed with this sequence (27). Another Spi 2.1 promoter element involved in GH regulation, the GAGA motif, has also been described (7). However, sequences similar to this GH responsive element are not found in GHRG-1. Preliminary transient expression studies in chicken embryonic hepatocytes with 1800 bp of the GHRG-1 promoter indicates it is indeed GH-responsive (data not shown). While it is tempting to speculate that the region homologous to the Spi 2.1 GHRE is involved in GH-regulated expression of GHRG-1, comprehensive functional studies of the GHRG-1 promoter are needed to confirm the location and activity of the GHRE.

Several other consensus binding sites for transcription factors could also be present in the promoter, including liver-specific HNF-3 (82% identity, -32 to -22) (29) and HNF-5 (100% identity, -170 to -164) (30). Other potential consensus motifs of interest are a half site

glucocorticoid response element (GRE, 100%, -475 to -470) (31), interleukin-6 response element (IL6-RE, 100%, -427 to -422) (32) and C/EBP binding site (89% -541 to -533) (33).

We have demonstrated that differential display PCR can be used to isolate genes that are expressed as a consequence of GH action, using *DwDw* and GHR-deficient *dwdw* chickens. Because of the absence of GH action in the *dwdw* chickens, it is likely that expression of many of these differentially expressed genes are controlled directly or indirectly by GH. It is also likely that most of these are unique, since the probability of the same random decamer amplifying the same mRNA at two different locations is low. Previous studies designed to identify GH-regulated genes have relied on GH-replacement in hypox or hypothyroid animals (5,23,34). The use of *DwDw* and *dwdw* chickens for identification of GH-regulated genes has advantages over the ablation models, since the pituitary gland of the *DwDw* chicken is functional and capable of providing pulsatile secretion of endogenous GH. Several studies have pointed to the importance of intermittent secretion of GH on the biological responses (35-37). The PCR-based cloning approach for detecting differentially regulated genes is far more sensitive than previously used techniques, because large numbers of genes can be simultaneously visualized. In an analysis of translational products of RNA from GH-treated hypothyroid rats, Liaw *et al.*, (34) identified 8 proteins which were consistently altered by GH treatment, from 150-250 different products that could be resolved by 2-dimensional gel electrophoresis (34). Differential screening of a rat hepatic cDNA library with cDNA derived from hypox and GH treated-hypox rats also only yielded a few clones of interest (5). These procedures favor identification of high abundance genes, which would allow genes with low copy number to escape detection. Thus, the differential display technique in our model system, *DwDw vs. dwdw*, is likely to generate considerably more information on GH-regulated genes. And, even in the absence of information on gene function, novel GH regulated genes can be used for the identification of *trans*-acting factors involved in GH signal transduction.

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