Growth hormone receptor gene expression in sex-linked dwarf Leghorn chickens: evidence against a gene deletion

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ABSTRACT

GH receptor (GHR) mRNA has been identified in peripheral (liver and muscle) and central (brain and hypothalamus) tissues of sex-linked dwarf (SLD) Leghorn chickens. Total RNA was extracted from the tissues of immature (1 week, 4 week), pubertal (16 week) and adult (>24 weeks) SLD and K (the normally growing strain) Leghorn chickens. In both groups and all tissues, an mRNA moiety of 4·4 kb hybridized with cRNA probes derived from the rabbit hepatic GHR sequence. An additional low-abundance transcript of 2·8 kb was also identified in some tissues. An age-related increase in expression was observed in K and SLD hepatic GHR mRNA, suggesting normal regulation of SLD GHR gene transcription. Amplification of cDNA from K and SLD tissues in the presence of oligonucleotide primers coding for the intracellular or extracellular domains of the chicken GHR generated electrophoretically separable fragments of expected size. Restriction enzyme digestion of the products with EcoRI, BstNI, HaeIII, NcoI or BamHI produced smaller moieties of expected sizes in both strains. These results demonstrate that, in contrast to broiler SLDs, a GHR gene deletion is not responsible for the GHR dysfunction in Leghorn SLDs. Although the actual defect in GHR gene expression in SLD Leghorns remains to be identified, this study demonstrates that sex-linked dwarfism, like Laron dwarfism, is due to a heterogeneity of lesions.


INTRODUCTION

Sex-linked dwarfism in poultry is due to a recessive gene on the Z chromosome that phenotypically results in mature body weights 30–40% smaller than those of normal chickens (Guillaume, 1976; Tixier-Boichard et al. 1989; Decuypere et al. 1991). However, as sex-linked dwarfism is thought to have arisen independently in different strains of domestic fowl (Guillaume, 1976), its aetiology may be due to a variety of gene mutations.

In sex-linked dwarf (SLD) Leghorn chickens, genetically selected for egg laying, dwarfism is likely to be due to tissue resistance to growth hormone (GH) action. Although the pituitary GH gene is normally transcribed and the circulating GH concentrations are normal or supranormal in these birds (Scanes et al. 1983; Harvey et al. 1984), they are abnormally fat (Guillaume, 1976) and are deficient in plasma triiodothyronine (T₃) and insulin-like growth factor I (IGF-I) (Huybrechts et al. 1987, 1989). Since lipo-lysis (Scanes, 1991) and the production of IGF-I (Goddard & Boswell, 1991) and T₃ (Kühn et al. 1990) is GH dependent, the GH-receptor (GHR) gene may be deficient or defective in Leghorn SLDs. This possibility is supported by the inability of exogenous GH to promote growth or restore T₃ or IGF-I production in these birds (Bowen et al. 1987; Huybrechts et al. 1987; Leung et al. 1987b; Kühn et al. 1990) and by the greatly diminished binding of labelled GH to the plasma membranes of SLD tissues (Leung et al. 1987b; Vanderpooten et al. 1991b). The GHR gene in broiler SLDs genetically selected for meat production is, moreover, aberrantly transcribed or deficient in coding sequences in comparison with normal growing birds (Burnside et al. 1991). The possibility that the GHR gene in Leghorn SLDs may also be anomalous was therefore investigated in the present study.

MATERIALS AND METHODS

Tissues

Peripheral (liver, muscle and adipose) and central (hypothalamic and extrahypothalamic regions of the brain) tissues known to express the GHR gene (Mendelsohn, 1988; Attardo & Harvey, 1990) were rapidly dissected from freshly killed Leghorn SLDs (Cornell University flocks) and from a genetically related normally growing (K) strain. The tissues were immediately frozen in liquid nitrogen and stored at -80 °C. For comparative purposes, tissues were collected from immature (1 and 8 weeks of age), pubertal (16 weeks of age) and adult (> 24 weeks of age) birds, since the binding of labelled GH to the hepatic membranes of normally growing birds increases during growth (Leung et al. 1987b; Vanderpooten et al. 1991b).

Northern blot analysis

Total cellular RNA was extracted from pooled samples of chicken liver, muscle, fat, brain and hypothalamus (five birds per group). The tissues were extracted in guanidinium thiocyanate (5-5 mol/l), containing sodium citrate (25 mmol/l), 0·2% (w/v) β-mercaptoethanol and 0·5% (w/v) sodium lauryl sarcosine (pH 7·0) and collected after centrifugation (125 000 g, at 15 °C for 24 h) through a bed of caesium trifluoroacetic acid (Pharmacia Fine Chemicals, Uppsala, Sweden; density 2·01) containing EDTA (100 mmol/l), pH 7·0 (Okayama et al. 1987). The RNA was quantified spectrophotometrically and 5 μg aliquots were subjected to electrophoresis in 1% (w/v) agarose and 3·1% (w/v) formaldehyde gels and transferred to nylon membranes by capillary transfer. A rabbit GHR (rGHR) cRNA probe was constructed from a 638 bp BamHI/EcoRI fragment (donated by Dr W. I. Wood, Genetech, San Francisco, CA, U.S.A.) of the rabbit hepatic GHR cDNA sequence coding for portions of the extracellular and transmembrane domains (Leung et al. 1987a). The plasmid pGEM3Z (Promega Corporation, Madison, WI, U.S.A.) containing this sequence was linearized by BamHI digestion and transcribed by T7 polymerase using a riboprobe kit (Promega) in the presence of [α-32P]CTP (800 μCi/mm; New England Nuclear, Mississauga, Ontario, Canada). This probe specifically hybridizes with GHR RNA in chicken tissues (Fraser et al. 1990). Transcription of this plasmid with SP6 polymerase (Promega) generates a sense RNA probe that does not hybridize with chicken mRNA (Fraser et al. 1990). The cRNA probe was then hybridized with the immobilized RNA in 50% (w/v) formamide (containing NaCl (0·75 mol/l), Pipes (25 mmol/l), EDTA (25 mmol/l), 0·2% (w/v) sodium dodecyl sulphate (SDS), 1 × Denhardt’s reagent (0·1% (w/v) Ficoll, 0·1% (w/v) bovine serum albumin, 0·1% (w/v) polyvinylpyrrolidone), salmon sperm DNA (100 μg/ml) and yeast tRNA (100 μg/ml), pH 6·8) for 12 h at 68 °C, following a 3-h incubation in the absence of the probe. The nylon membranes were then washed twice in each of 2 ×, 0·5 × and 0·05 × SSC (1 × SSC = sodium citrate (0·015 mol/l) and NaCl (0·15 mol/l), pH 7·2) (containing 0·2% (w/v) SDS) at room temperature, 68 °C and 68 °C respectively. Membranes were then placed between intensifying screens and exposed to Kodak X-OMAT-AR film (Kodak, Rochester, NY, U.S.A.) for 1 day (liver and brain RNA) or 7 days (hypothalamus and adipose RNA). To correct for loading error, blots were stripped of the probe in Tris–HCl, pH 8·0 (1 mmol/l), EDTA (1 mmol/l) and 0·1 × Denhardt’s reagent and hybridized with a mouse γ-actin cRNA probe. The degree of specific hybridization of the GHR cRNA probe to hepatic RNA was quantified by laser densitometry and corrected in relation to hybridization of the γ-actin cRNA probe. The degree of hybridization and the age of the birds was correlated by Spearman’s rank correlation. The sizes of the RNA moieties hybridizing to the probe were determined by comparison with electrophoretically separable size markers (Boehringer Mannheim, Dorval, Quebec, Canada).

Polymerase chain reaction (PCR)

Total RNA extracted from the liver, muscle, hypothalamus and brain was also reverse transcribed by Superscript (100 U; BRL, Burlington, Ontario, Canada) in the presence of 100 pmol of oligodeoxythymine primer (Boehringer Mannheim), excess nucleotides (10 mmol each of dCTP, dTTP, dATP and dGTP/l, Boehringer Mannheim) and 5 × H-RT buffer (BRL). Reactions were also prepared in the absence of RNA or Superscript, to serve as negative controls. The reaction mixtures were diluted with double-distilled water (500:1, v/v) and aliquots of liver, muscle and brain cDNA (5% of total volume) were added to a PCR cocktail containing 5'-oligomer chicken (c) GHR sense (5'-CCTCGATTTGGATA CCATATTGTGTTAAGC-3') and 3'-oligomer cGHR antisense primers (5'-CTGTTACGGCCA GCCCACACACTCCGAAG-3') (15 pmol of each), deoxynucleotides (1-25 mmol of each/l), 1 × PCR reaction buffer (KCl (50 mmol/l), Tris–HCl (10 mmol/l), pH 8·4, MgCl2 (1·5 mmol/l)), gelatin (20 μg/ml) and Thermus aquaticus (Taq) DNA polymerase (5 U, BRL). The primers were based on the known sequence of a portion of the cGHR transcript coding for the extracellular domain between nucleotides 303 and 798 (Burnside et al. 1991). Reverse-transcribed RNA from liver, muscle, hypothalamus...
and brain was also amplified in the presence of 5' sense (5'-CTGCGGCGCACGAGCACATTCACAGGTTAA-3') and 3' antisense (5'-AAGCGGCCGCGCAAGGATGTCGATTTC-3') oligonucleotides coding for a portion of the intracellular domain of the receptor between nucleotides 844 and 1667 (Burnside et al. 1991). The mixtures were overlayered with mineral oil (v/v), and heat-denatured at 94 °C for 2 min before 30 cycles of annealing (at 45 °C for 1 min), extension (at 72 °C for 3 min) and denaturing (at 94 ºC for 1 min) in a genetic thermal cycler (Precision Instruments, Chicago, IL, U.S.A.). The hepatic PCR products were also digested for 2 h with restriction endonucleases (BamHI, NcoI, HaeIII or EcoRI) for known cleavage sites in the regions coding for the extracellular (at nucleotides 445 and 473 for BamHI and nucleotide 449 for NcoI) and intracellular (at nucleotide 1330 for HaeIII and nucleotide 1394 for EcoRI) domains of the receptor respectively (Burnside et al. 1991). The PCR and digestion products were electrophoresed in 1.4% (w/v) agarose ethidium bromide-stained minigels and compared with HaeIII-digested pUC18 or EcoRI/BamHI-digested size markers. PCR products were purified directly from the amplification mixture using the Promega Magicprep Kit (Promega) and partially sequenced (Department of Biochemistry, University of Alberta).

**Radioligand binding**

The binding of GH to liver plasma membranes from adult SLD and K birds was determined as previously described (Fraser et al. 1990). Briefly, hepatic plasma membranes isolated from eight chickens were prepared separately by centrifugation and ultracentrifugation after homogenization in 25 mmol Tris–HCl buffer/1, pH 7.4. The membranes were then incubated at room temperature for 4 h with 125I-labelled ovine GH (60 μCi/μg; NIADDK-oGH-14; Bethesda, MD, U.S.A.) in the presence or absence of unlabelled GH (2 μg/ml) to determine non-specific binding. Bound and free radioactivity were separated by centrifugation.

**RESULTS**

**Radioligand binding**

Specific binding of the GH tracer to hepatic membranes derived from the livers of eight adult SLDs (0.5 ± (s.e.m.) 0.27% of total counts incubated) was significantly less (P < 0.001, Student's t-test) than that to membranes derived from eight controls (K strain) (17.6 ± 0.8% of total counts). In both cases the non-specific binding was < 10% of the total counts.

**Northern blot analysis**

The rGHR cRNA probe specifically hybridized with a major transcript of 4.4 kb found in total RNA isolated from the liver of K and SLD birds (Figs 1 and 2). In both strains, an age-related increase in the amount of this hybridizing moiety was apparent in two separate blots (K: r = 0.952 (n = 8) P < 0.01; SLD: r = 0.976 (n = 7) P < 0.01, Spearman's rank correlation) (Fig. 2). At each age the abundance of this message in the liver of the dwarf birds was comparable with that in the controls. A smaller transcript of 2.7 kb hybridizing with the rGHR cRNA probe was also evident in hepatic RNA from both strains when the same blots were exposed for 72 h (data not shown). In each strain this transcript accounted for approximately 45% of the total hybridization. The larger transcript was ubiquitously present in other tissues of both strains, although the smaller transcript was only detected in the brain of the K strain and was undetectable in hypothalamic and fat RNA from SLD and K birds when the blots were exposed for 7 days (Fig. 3). Hybridization with an intermediate transcript of 3.1 kb was also evident in RNA extracted from the adipose tissues of both strains.

**Polymerase chain reaction**

A single moiety of 804 bp was generated when cDNA reverse-transcribed from hepatic, muscle and brain RNA from SLD and K birds was amplified by PCR in the presence of 3'- and 5'-oligonucleotide primers.
coding for the intracellular domain of the GHR (Fig. 4). Digestion of both hepatic PCR products with EcoRI (Fig. 4) and HaeIII (data not shown) produced fragments of the expected sizes (533 bp and 271 bp, 469 bp and 337 bp respectively). In the presence of 3’- and 5’-primers coding for portions of the extracellular and transmembrane regions of the GHR, a single moiety of the appropriate size (496 bp) was amplified from hepatic, muscle, brain and hypothalamic cDNA (Fig. 5). Digestion of both hepatic PCR products with BamHI generated identical fragments (325 bp and 171 bp) of predicted sizes (Fig. 5). Digestion of the cDNA with NcoI also produced fragments of the expected sizes (350 bp and 150 bp) (data not shown). In both PCR reactions, these fragments were not generated when the RNA was not transcribed by Superscript or was absent (results not shown). The sequence of the extracellular PCR fragment between nucleotides 393 and 621 and the intracellular PCR fragment between nucleotides 903 and 1232 was more than 99% identical in K and SLD samples (Fig. 6).

**DISCUSSION**

These results clearly demonstrate that the GHR dysfunction in Leghorn SLDs is not due to a GHR gene deficiency or gene deletion. The GHR gene transcripts in SLD tissues were of equal abundance and identical size to those in the K strain, as were the PCR products generated with oligonucleotide primers for the extracellular and intracellular sequences of the GHR. The coding sequences for portions of the GHR cDNA in Leghorn SLDs were very similar to those in the K strain, and in accordance with the sequence published by Burnside et al. (1991). Cleavage sites for the restriction endonucleases BamHI, NcoI, HaeIII and EcoRI must also be similarly located on the GHR genes. These results are, however, in contrast with those of Burnside et al. (1991), who found a 1-0-1-7 kb deletion in the GHR transcript of SLD broilers by Northern blot analysis and a GHR cDNA polymorphism by Southern blot analysis. It is, however, possible that undetected base pair point mutations occur along the length of the GHR in SLD Leghorn.
chickens, small deletions occur in areas outside those covered by the oligonucleotide primers or the untranslated regions of the gene are structurally defective. A dysfunction or deficiency in the translation of the GHR message may also account for the failure of labelled GH to bind to tissue membranes from Leghorn SLDs and the inability of exogenous GH to stimulate IGF-I or T3 production in these birds.

**Figure 4.** (a) Ethidium bromide-stained 1-4% agarose gel of cDNA reverse-transcribed from the total RNA extracted from control Cornell (K) (lane 1) and sex-linked dwarf (SLD) Leghorn chicken (lane 2). Brain cDNA was amplified by the polymerase chain reaction (PCR) with 3'- and 5'-oligonucleotide primers for the intracellular domain of the chicken liver GH receptor cDNA prior to electrophoresis. (b) Total RNA extracted from K (lane 1) and SLD (lane 2) muscle, and K (lane 3) and SLD (lane 4) liver was similarly reverse-transcribed and amplified. EcoRI digests of the PCR products derived from adult K (lane 5) and SLD (lane 6) hepatic cDNA samples are also shown. The size of the various fragments was determined by comparison with the migration of HaeIII-digested pUC18 and EcoRI/BamHI-digested λ size markers.

**Figure 5.** (a) Ethidium bromide-stained 1-4% agarose gel of cDNA reverse-transcribed from total RNA extracted from control Cornell (K) (lane 1) and sex-linked dwarf (SLD) Leghorn (lane 2) hypothalami, K (lane 3) and SLD (lane 4) liver, K (lane 5) and SLD (lane 6) brain, and K (lane 7) and SLD (lane 8) muscle. The cDNA was amplified by the polymerase chain reaction with 3'-and 5'-oligonucleotide primers for the extracellular and transmembrane domains of chicken liver GH receptor cDNA. (b) BamHI digests of K (lane 1) and SLD (lane 2) amplified hepatic cDNA. The sizes of the various fragments were determined by comparison with the migration of HaeIII-digested pUC18 and EcoRI/BamHI-digested λ size markers.
The aetiology of dwarfism in Leghorn and broiler SLDs is therefore likely to be due to different lesions in the GHR gene. Sex-linked dwarfism in poultry would thus appear to resemble closely Laron dwarfism in humans, in which subpopulations with or without discontinuous deletions or point mutations of the GHR gene have been identified (Amselem et al. 1989; Godowski et al. 1989). The SLD phenotype in poultry may thus result from a plurality of GHR gene anomalies, and provide a valuable model for the aetiology and treatment of this human disease. Elucidation of the different lesions of the GHR gene in SLD poultry may also provide an insight into the signal transduction pathways that mediate GH action.

The major GHR gene transcript in both Leghorn strains was approximately 4.4 kb, as previously observed in broiler fowl (Burnside et al. 1991). The production of this transcript increased with age, possibly reflecting the maturational decline in circulating GH levels (Leung et al. 1987b; Tixier-Boichard et al. 1992). The hepatic GHR is down-regulated by exogenous and endogenous GH (Leung et al. 1986; Vasilatos-Younken et al. 1990; Vanderpooten et al. 1991a) and the maturational decline in GH secretion

**Figure 6.** Partial sequences of the polymerase chain reaction products amplified from cDNA reverse-transcribed from RNA isolated from control Cornell (K) and sex-linked dwarf (S) birds. Sequences coding for the (a) intracellular and (b) extracellular domains of the receptor were amplified using primers khu11 and khu12 or khu9 and khu10 respectively. N represents sites in which the identity of nucleotide was unclear.
may therefore up-regulate GHR gene expression. However, while the binding of GHR to hepatic membranes also increases with age in normal growing birds (Leung et al. 1987b; Vasilatos-Youen et al. 1990; Vanderpooten et al. 1991a), hepatic binding is not age-related in Leghorn SLDs (Vanderpooten et al. 1991a). Translation of the GHR mRNA in Leghorn SLDs may therefore be defective.

In addition to the major GHR transcript, a smaller 2-7 kb transcript was also observed in liver and brain mRNA from normal growing Leghorns and in SLD hepatic mRNA. This moiety may result from the tissue degradation of the 4-4 kb transcript, or indicate alternative transcription of the GHR gene in a tissue- and/or strain-specific way. The heterogeneity of the GHR is well established; GHR receptors having a variety of affinities (Hughes, 1979), molecular weights (Wallis et al. 1987) and subcellular locations (Herington et al. 1988) have been identified. Serum binding proteins of high (Fraser et al. 1990; Jones et al. 1990) and low affinity (Leung 1987a; Baumann et al. 1988; Spencer et al. 1988; Huybrechts et al. 1989; Smith et al. 1989; Baumann, 1991) also exist with varying degrees of homology to the membrane-bound hepatic receptor. Multiple mRNAs coding for the GHR/GH-binding protein have been isolated in rat liver (Donner, 1983; Tiong et al. 1989) and mouse liver (Smith et al. 1989). In brain and liver samples, the various GHR transcripts are present in similar ratios in SLD and K birds. In both strains only the large GHR transcript was detected in the hypothalamus and the 3-1 kb GHR transcript was confined to adipose mRNA. Transcription of the GHR gene or the intracellular processing of the transcripts may therefore be quantitatively or qualitatively tissue-specific.

In conclusion, sex-linked dwarfism in poultry is due to a plurality of causes. In contrast to broiler SLDs, transcription of the GHR gene of SLD Leghorn chickens is not anomalous; regions of the gene coding for the extracellular and intracellular regions of the receptor are present and largely intact.

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