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Expression of the growth hormone gene in immune tissues

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It is well established that growth hormone (GH)-like proteins and mRNA are present in immune tissues, but it is not known whether this reflects ectopic transcription of the GH gene or the expression of a closely related gene. This possibility was, therefore, investigated. Immunoreactive (IR) GH-like proteins were readily measured by radioimmunoassay and immunoblotting in the spleen, bursa of Fabricius and thymus of immature White Leghorn chickens, in which IR-GH was similar in size and antigenicity to the major GH moieties present in the pituitary gland. RT-PCR of mRNA from these immune tissues, with oligonucleotide primers spanning the coding region of pituitary GH cDNA, also generated cDNA fragments identical in size (689 bp) to pituitary GH cDNA. *Bam*HI and *Rsa*I cleavage sites were located in these cDNA sequences in the same position as those in pituitary GH cDNA. These amplified cDNA sequences also contained sequences that hybridized, by Southern blotting, with a chicken pituitary GH cDNA probe, thus suggesting a high degree of homology between pituitary and immune GH transcripts. The nucleotide sequence of the PCR products generated from these immune tissues, determined by a modified cycle dideoxy chain termination method, were also identical to pituitary GH cDNA. This homology extended over 593 bp of the spleen cDNA (spanning nucleotides 70–663 of the pituitary GH cDNA and its coding region for amino acids 5–201), 613 bp of the bursa cDNA fragment (spanning nucleotides 63–676 of the pituitary GH cDNA and its coding region for amino acids 3–207) and 607 bp of the thymic cDNA fragment (spanning nucleotides 61–665 of pituitary GH cDNA and its coding region for amino acids 4–203). These results clearly establish that the GH mRNA is present in immune tissues, in which GH-IR proteins are present. The local production of GH within the immune system of the domestic fowl, therefore, suggests it has paracrine or autocrine roles in modulating immune function.

Keywords: growth hormone; gene; protein; immune tissues; pituitary glands; chickens

Introduction

It is now well-established that growth hormone (GH)-secreting cells are not confined to the adenohypophysis. Indeed, the widespread tissue localization of GH immunoreactivity (IR) suggests an almost ubiquitous transcription of the GH gene. Immunoreactive GH (GH-IR) and GH-like mRNA has, therefore, been located in the brain (see Harvey *et al.*, 1993, for review) and GH-like peptides have been detected in the pineal glands, mammary glands, liver, kidney, lung, muscle, colon, stomach, ovary and placenta (Kaganowicz *et al.*, 1979; Kyle *et al.*, 1981; Noteborn *et al.*, 1993; Selman *et al.*, 1994; Cook & Liebhaber, 1995). The GH-IR in the placenta reflects the tissue-specific expression of the GH-V gene (Cook & Liebhaber, 1995), a member of the GH-chorionic somatomammotropin gene family (Harvey, 1995), which may also be transcribed in pituitary cells (Nickel & Cattini, 1992). It is, however, uncertain if the GH-IR in other tissues reflects transcription of the GH gene or the expression of closely related genes.

In the immune system, GH-like proteins are secreted by rat and human lymphocytes *in vitro* (Weigent *et al.*, 1987, 1988; Weigent & Blalock, 1989; Hattori *et al.*, 1990, 1994; Kao *et al.*, 1992; Varma *et al.*, 1993) and by leukocytes *in vivo* (Baxter *et al.*, 1991). The incorporation of radioactive amino acids into these GH moieties (Weigent *et al.*, 1988) provides evidence for the local synthesis in immune cells. This is supported by the suppression of GH release from lymphocytes transfected with antisense oligonucleotides for GH mRNA (Weigent *et al.*, 1991a). Expression of GH-like genes in the immune system is also indicated by the localization of GH-like mRNA in circulating mononuclear cells and in thymic tissues and bone marrow (Weigent *et al.*, 1988, 1991b; Weigent & Blalock, 1989; Baxter *et al.*, 1991; Binder *et al.*, 1994; Maggiano *et al.*, 1994). Southern blotting of the cDNA fragments generated by reverse transcription and the polymerase chain reaction (RT-PCR) has also established some sequence homology between immune and pituitary GH mRNA (Weigent *et al.*, 1991b; Binder *et al.*, 1994). This homology is also supported by the digestion of immune and pituitary cDNA fragments with restriction endonucleases that cleave, in a similar manner, the cDNA sequences generated by RT-PCR (Weigent *et al.*, 1991b). It is, however, still uncertain if GH mRNA in immune tissues is identical to pituitary GH mRNA, especially as some authors suggest the GH-like and GH mRNA-like moieties in immune tissues are structurally different from those in the pituitary gland (Hiestand *et al.*, 1986; Baglia *et al.*, 1992). The sequence homology of GH mRNA in immune tissues and pituitary glands has, therefore, been determined in the present study, in which the immune tissues of chickens have been shown, for the first time, to be extrapituitary sites of GH gene expression.

Results

Immunoreactive cGH in immune tissues

The presence of immunoreactive GH-like material in immune tissues was investigated using radioimmunoassay. Serial dilutions of boiled crude saline extracts of the spleen, thymus, bursa and pituitary gland of the chicken displaced the binding of [^{125}I]GH tracer to GH antisera in a manner parallel to that of the standard (Figure 1). The amount of immunoreactive GH material in these immune tissues was, however, considerably less than that found in the pituitary gland (Table 1), but greater than that dectected in muscle, liver or duodenum (<0.01 ng/mg protein).

Western analysis

The size of immunoreactive GH moieties detected in pituitary and immune tissues was subsequently investigated by Western blotting, using a polyclonal antibody raised against cGH (Figure 2). Several immunoreactive cGH-like bands were detected in the pituitary gland, with apparent molecular weights of 16, 22, 26 and 44 kDa, although most of the immunoreactivity was associated with the 26 kDa protein. In contrast, a single GH immunoreactive band was identified in bursa and thymus, which was identical in size to the 26 kDa

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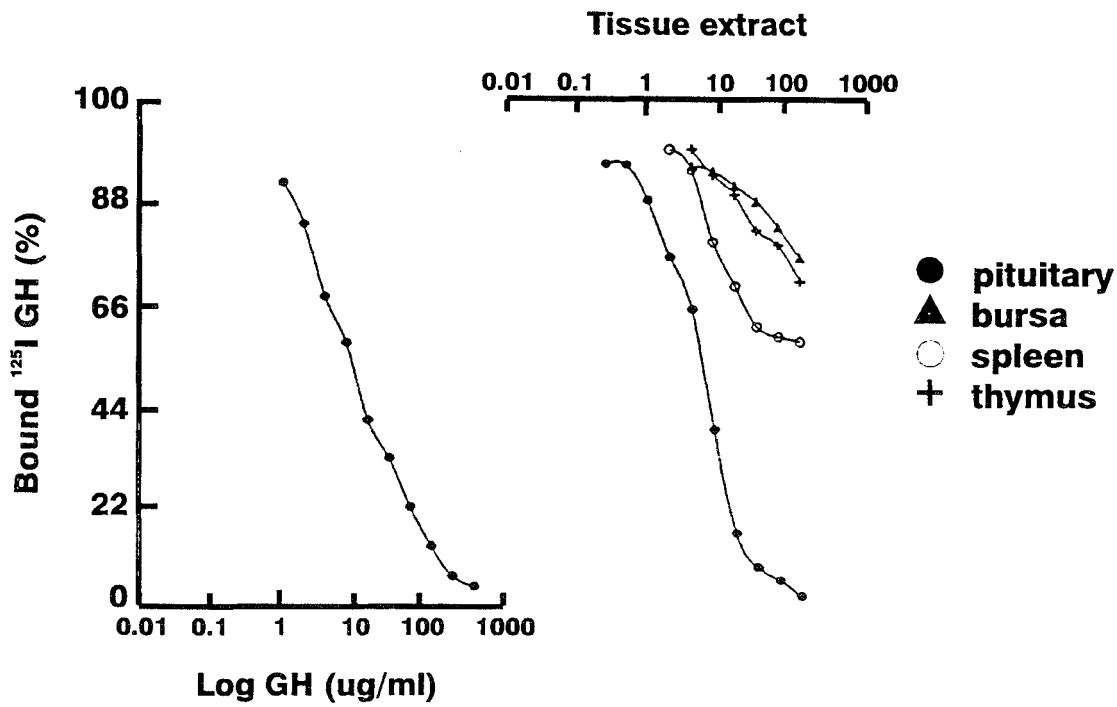

Figure 1 Cross-reactivity of crude saline homogenates of pituitary, spleen, thymus and bursa with a cGH antiserum. Dose response curves for the immune tissue extracts showed parallelism with the standard curve. The slopes of the lines lie within the 95% confidence limits for the GH standard (−0.57 to −0.64)

Table 1 Immunoreactive growth hormone levels in immune tissues of the chicken

Tissue extract	*GH-IR* (μg/mg protein)
Bursa	0.32
Spleen	3.61
Thymus	0.58
Pituitary	38.09
Muscle	ND
Duodenum	ND
Liver	ND

GH-IR levels as determined by cross-reactivity with GH antisera in a radioimmunoassay. ND, not detectable (<0.01 ng/mg protein)

protein in the chicken pituitary gland. This immunoreactive protein was also weakly present in spleen extracts; however, a 44 kDa GH-like protein was more abundant. Staining of all these bands was abolished by prior immunoadsorption of the primary antibody with excess rcGH or when the primary antibody was replaced with preimmune serum.

RT-PCR

To characterize the mRNA produced by chicken immune tissues, GH-specific sequences were amplified by reverse transcription and PCR using two oligonucleotide primers specific for chicken pituitary GH (Figure 6). cDNA moieties identical to that generated from the pituitary gland of appropriate size (689 bp) were amplified from each immune tissue (Figure 3). Under the same conditions, cDNA fragments were not generated from muscle or liver RNA (data not shown). The omission of RNA or Superscript from the initial RT reaction similarly failed to generate cDNA fragments (data not shown), indicating the specificity of the PCR reaction.

Restriction endonuclease digestion

Restriction endonuclease mapping of the pituitary GH gene has identified cleavage sites for the enzymes *Bam*HI and *Rsa*I. The possible occurrence of these sites in immune GH cDNA was, therefore, investigated. Digestion of the cDNA produced from bursa, spleen and thymus with *Bam*HI produced fragments of expected size (465 and 224 bp) (Figure 3). *Rsa*I digestion of these PCR products also produced bands of expected size (237 and 452 bp; data not shown).

Southern analysis

Southern analysis was used to confirm that the cDNA generated from bursa, spleen and thymus shared sequence homology with pituitary GH cDNA. A radiolabelled cGH cDNA probe hybridized with the PCR fragments amplified from each of the tissues. In each case, hybridization occurred with a single cDNA moiety of approximately 689 bp (Figure 4) as predicted (Lamb *et al.*, 1988) and observed (Figure 4) for pituitary GH cDNA. This hybridization was specific, since it did not occur in samples from muscle and liver (data not shown). To exclude the possibility that the probe was hybridizing with the primers used to generate the cDNA in the PCR, the probe was digested with *Bst*XI and *Hae*II to remove these sequences prior to hybridization.

Nucleotide sequencing

Nucleotide sequences were determined for fragments (593 bp, 613 bp and 604 bp, respectively) of the cDNAs produced by RT-PCR of spleen, bursa and thymic RNA (Figure 5). These sequences were completely homologous with the published (Tanaka *et al.*, 1992) and observed (data not shown) pituitary GH cDNA sequences. This homology extended through all 593 bp of the spleen cDNA (spanning nucleotides 70–663 of the pituitary GH cDNA and its coding region for amino acids 5–201), through all 613 bp of the bursa cDNA fragment (spanning nucleotides 63–676 of the pituitary GH cDNA and its coding region for amino acids 3–207) and through all 604 bp of the thymic cDNA fragment (spanning nucleotides 61–665 of the pituitary GH cDNA and its coding region for amino acids 4–203).

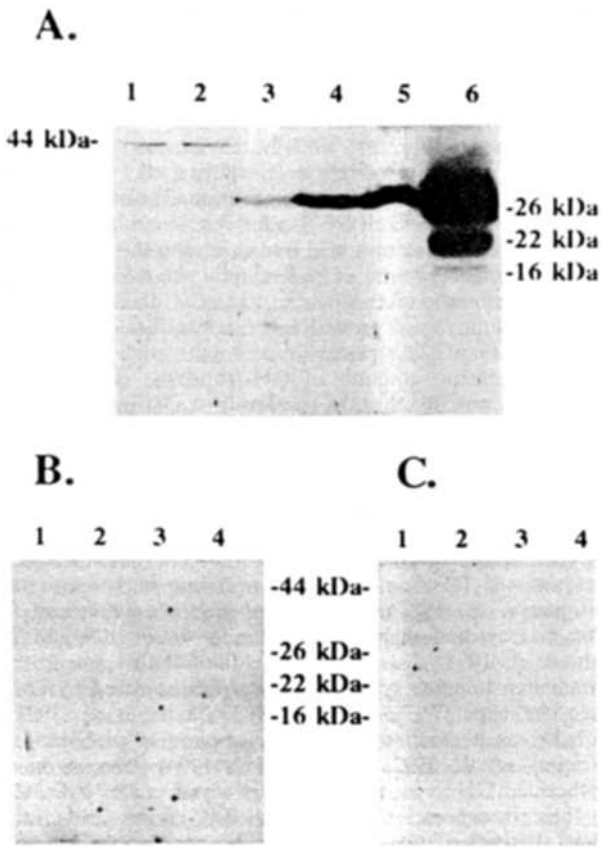

Figure 2 Immunoblot detection of GH in chicken immune tissues. Crude tissue homogenates (spleen, lanes 1 and 2; thymus, lanes 3 and 4; bursa, lane 5 and pituitary, lane 6) were subjected to reducing SDS–PAGE electrophoresis, transferred to PVDF membranes and incubated with a chicken GH antisera (αcGH) (A). Identical blots were also incubated with αcGH that had been pre-absorbed with excess recombinant cGH (B) or with preimmune rabbit serum (C). Immunoreactive proteins were visualized using HRP-labelled anti-rabbit IgG and enhanced chemiluminescence

Discussion

The demonstration of GH mRNA in immune tissues of the domestic fowl suggests they are extrapituitary sites of GH synthesis, as reflected by the coincident localization of GH-IR in the spleen, bursa and thymus.

This is the first demonstration of GH mRNA sequences in immune tissues that code for pituitary GH. The homology with pituitary GH mRNA was established by PCR, endonuclease digestion, Southern blotting and nucleotide sequencing. These results, therefore, indicate that the GH gene expressed in the immune system is homologous to the pituitary GH gene and unlikely to be a variant or closely related GH

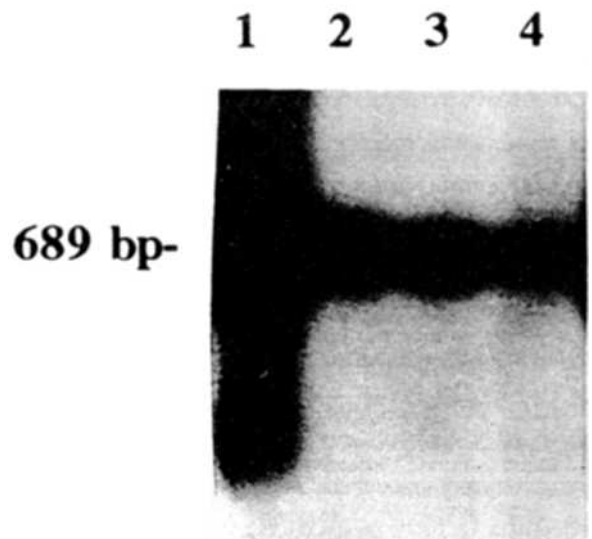

Figure 4 Southern analysis. RT–PCR products amplified from chicken pituitary (lane 1), bursa (lane 2), spleen (lane 3) and thymus (lane 4) RNA were transferred onto nylon membranes and analysed using a radiolabelled cGH cDNA fragment that did not contain the primer sequences

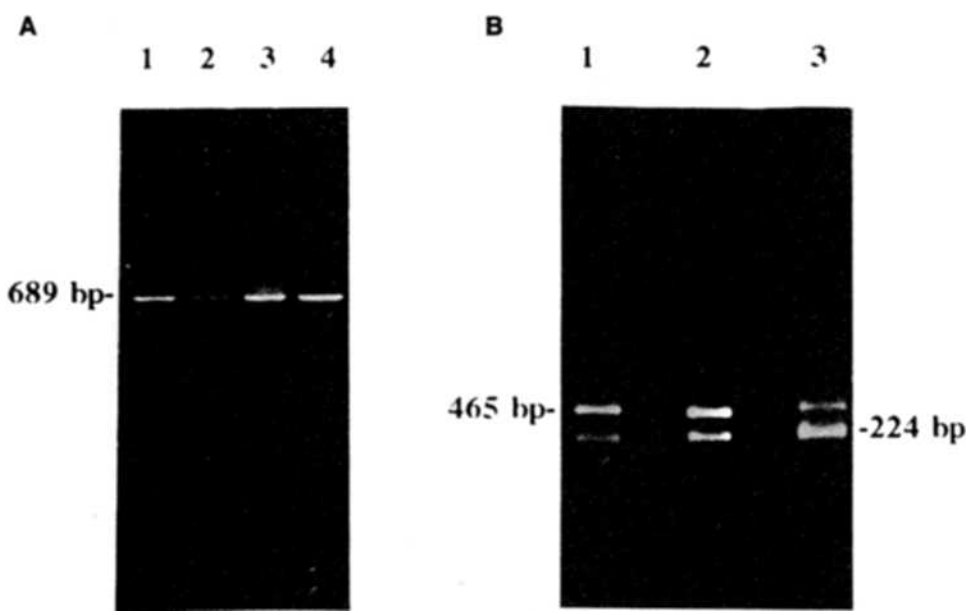

Figure 3 Analysis of immune GH transcripts by the polymerase chain reaction (PCR). RNA from chicken bursa (lane 1), spleen (lane 2), thymus (lane 3) and pituitary gland (lane 4) was reverse transcribed and amplified in the presence of oligonucleotide primers (CRE1 (exon 1) and CRE2 (exon 5) (A). The amplified cDNA was visualized by electrophoresis in ethidium bromide-stained minigels, and size of the fragments was determined by the comparative migration of ΦX174RF DNA/*Hae*III size markers. (B) *Bam*HI digests of bursa (lane 1) spleen (lane 2) and thymus (lane 3) cDNA are also shown

gene (Harvey, 1995), especially as chickens are non-placental animals and thus unlikely to have placental lactogens or a GH-V gene. Although GH-like mRNA has previously been detected in rat and human tissues and characterized by PCR and Southern blotting, these moieties have never been sequenced (Weigent *et al.*, 1988, 1991b; Baxter *et al.*, 1991; Binder *et al.*, 1994; Maggiano *et al.*, 1994). Indeed, the GH-like mRNA in rat lymphocytes has also been suggested to be larger than pituitary GH mRNA (Hiestand *et al.*, 1986).

The presence of GH mRNA in chicken immune tissues was not due to contamination with genomic DNA. The oligonucleotide primers used for RT–PCR were designed from two different exons and larger fragments containing intron sequences would have been amplified from genomic DNA. The possible contamination of PCR reactions by cDNA was also controlled by performing reverse transcription in the absence of RNA or reverse transcriptase. The specificity of the findings was also demonstrated by the use of pituitary and muscle RNAs as positive and negative controls, respectively. The occurrence of GH mRNA in chicken immune tissues is also unlikely to be an artefact of 'illegitimate' gene transcription (Chelley *et al.*, 1988, 1989; Ruano *et al.*, 1989). Although the abundance of GH mRNA in these tissues is likely to be far less than that in the pituitary gland, it is not a rare transcript and did not require a second (booster) round of PCR amplification for detection. The GH gene in chicken immune tissues is, therefore, likely to be constitutively expressed in amounts that are physiologically relevant. This possibility is supported by the detection of significant amounts of GH-IR in the same tissues. Indeed, it is possible that the low levels of GH in the plasma of hypophysectomized chickens (Harvey *et al.*, 1987; Lazarus & Scanes., 1988) may reflect the extrapituitary secretion of GH from immune tissues, especially as hypophysectomy does not impair immune GH production in rats (Weigent *et al.*, 1992).

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                                                    100 (815)
CCAGGCTCGTGGTTTTCTCCTCTCCTCATCGCTGTGGTCACGCTGGGACT
                                                    150 (865)
GCCGCAGGAAGCTGCTGCCACCTTCCCTGCCATGCCCCTCTCCAACCTGT
                                                    200 (915)
TTGCCAACGCTGTGCTGAGGGCTCAGCACCTCCACCTCCTGGCTGCTGAG
                                                    250 (1407)
ACATACAAAGAGATTGAACGCACCTATATTCCGGAGGACCAGAGGTACAC
                                                    300 (1457)
CAACAAAAACTCCCAGGCTGCGTTTTGTTACTCAGAAACCATCCCAGCTC
                                                    350 (1808)
CCACGGGGAAGGATGACGCCCAGCAGAAGTCAGACATGGAGCTGCCTCGG
                                                    400 (1858)
TTTTCACTGGTTCTCATCCAGTCCTGGCTCACCCCCGTGCAATACCTAAG
                                                    450 (1908)
CAAGGTGTTCACGAACAACTTGGTTTTTGGCACCTCAGACAGAGTGTTTG
                                                    500 (1958)
AGAAACTAAAGGACCTGGAAGAAGGGATCCAAGCCCTGATGAGGGAGCTG
                                                    550 (3074)
GAGGACCGCAGCCCGCGGGGCCCGCAGCTCCTCAGACCCACCTACGACAA
                                                    600 (3124)
GTTCGACATCCACCTGCGCAACGAGGACGCCCTGCTGAAGAACTACGGCCT
                                                    650 (3174)
GCTGTCCTGCTTCAAGAAGCATC1GCACAAGGTGGAGACCTACCTGAAGGT
                GATGAAGTGCCG
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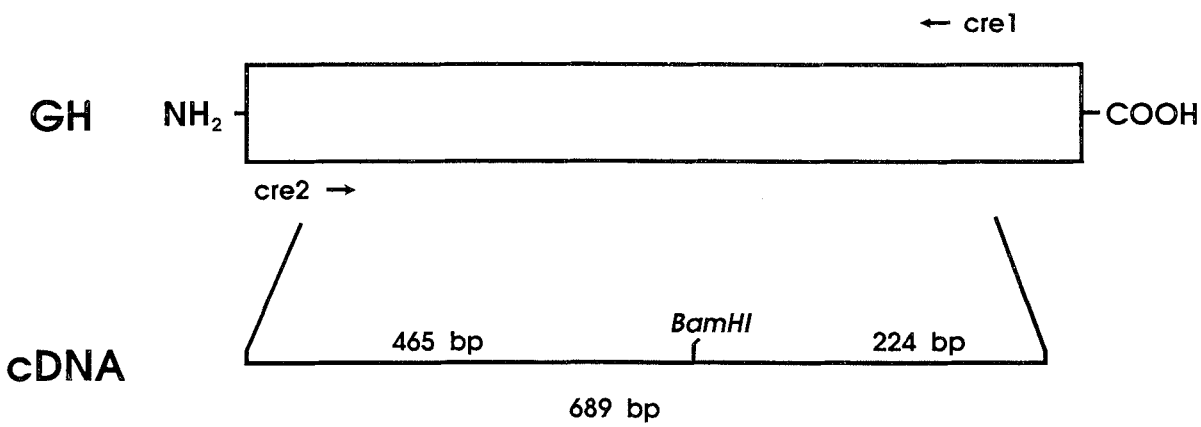
Figure 5 The nucleotide sequence of the cDNA resulting from RT–PCR of bursa RNA with CLR1 and CLR2. Bases are numbered from the 5' end of the clone. The last digit of the nucleotide number is aligned with the corresponding cDNA base. For comparative purposes, corresponding nucleotide numbers of the chicken growth hormone gene are indicated in brackets (Tanaka *et al.*, 1992)

Constitutive expression of a GH-like gene has also been demonstrated in unstimulated rat and human lymphocytes (Weigent *et al.*, 1988; 1991a,b; Baxter *et al.*, 1991) and in bone marrow and thymic tissue (Binder *et al.*, 1994; Maggiano *et al.*, 1994). The expression of the GH-like gene in the mammalian immune system is, however, stimulated by antigenic challenge (Weigent *et al.*, 1988; Baxter *et al.*, 1991), probably as a result of increased lymphocyte proliferation (Weigent *et al.*, 1992; Binder *et al.*, 1994). The *de novo* synthesis of GH in mammalian lymphocytes is also indicated by their incorporation of radiolabelled amino acids into newly synthesized GH-IR proteins, by cyclohexamide- and actinomycin D-sensitive pathways (Weigent *et al.*, 1988; Hattori *et al.*, 1994). Indeed, although some immune proteins differ from pituitary GH in molecular size (Weigent *et al.*, 1988; Baglia *et al.*, 1992), lymphoid cells in the rat (Weigent & Blalock, 1989; Baxter *et al.*, 1991; Weigent *et al.*, 1991a,b) and human (Weigent & Blalock, 1989; Hattori *et al.*, 1990, 1994; Varma *et al.*, 1993), and lymphocyte cell lines (Kao *et al.*, 1992; Yang *et al.*, 1993) appear to produce proteins that closely resemble pituitary GH in molecular size, antigenicity and bioactivity.

The most abundant GH moiety detected in the chicken bursa and thymus by Western analysis had a molecular size



Primers

cre1: 5' gcctcagatggtgcagttgctctctccgaa3'

cre2: 5' cgttcaagcaacacctgagcaactctcccg3'

Figure 6 (A) Olignucleotide primers (CRE 1 and CRE 2) used in the polymerase chain reaction (PCR) to amplify the coding region of the GH gene. (B) Sites of primer annealing and restriction endonuclease digestion site on the GH cDNA

of 26 kDa, similar to that in the pituitary gland. Although this is slightly larger than the predicted size of the 216 amino acid monomer protein, this is likely to reflect its post-translational processing and is consistent with the size of pituitary GH previously determined by SDS–PAGE (Lamb *et al.*, 1988; Scanes *et al.*, 1993). A larger immunoreactive protein of 44 kDa was, however, the major GH moiety in the chicken spleen. The post-translational processing of the GH protein in the chicken spleen may, therefore, be tissue-specific, since this variant was not detected in the bursa or thymus, although it was present in pituitary extracts. Multiple forms of GH-IR have also been described in rat and human lymphocytes, and these large forms may represent oligomers or protein-bound variants (Weigent *et al.*, 1987; Hattori *et al.*, 1990).

The expression of the GH gene in the pituitary gland is thought to be dependent upon a transcription factor, Pit-1, that was originally thought to be pituitary-specific (Harvey, 1995). However, this factor is also likely to regulate the transcription of the GH-like gene in mammalian immune tissues, since it has been located in lymphoid tissues of the rat (Delhase *et al.*, 1993). A similar regulatory mechanism of gene expression may also be responsible for GH synthesis in the chicken, since an upstream region of the chicken pituitary GH gene contains a nucleotide sequence that is highly homologous to the Pit-1 proximal binding site detected in the rat GH gene promoter (Tanaka *et al.*, 1992).

The expression and presumed translation of the GH gene in the chicken immune system axiomatically implies paracrine or autocrine roles for GH in immune regulation. Indeed, it is well established that GH stimulates immune function in mammalian species and exogenous GH can prevent thymic involution in aging rats (see Kelley, 1991; Gelato, 1993; Weigent & Blalock, 1995, for reviews). In chickens, GH similarly promotes the hyperplasia of lymphoid tissues and the proliferation of lymphocyte cells (Scanes *et al.*, 1990; Haddad & Marshaly, 1991). The treatment of hypophysectomized birds with exogenous GH restores the growth of their primary lymphoid organs and protects against age-associated bursal atrophy (Scanes *et al.*, 1990). The depressed humoral immune response of GH-deficient dwarf chickens can also be improved by GH infusion (Marsh *et al.*, 1984). More recently, Haddad & Marshaly (1991) demonstrated increased natural killer cell activity in GH-treated chickens and Marsh *et al.* (1992) found exogenous GH increased the numbers of the $CD4^+$ and $CD8^+$ cells.

The occurrence of GH cDNA in the immune system of the domestic fowl is, therefore, also of evolutionary significance. Although GH-secreting cells in higher vertebrates are largely confined to the pituitary gland, GH-like proteins are present in primitive vertebrates and invertebrates lacking pituitary somatotrophs or pituitary glands, respectively (Harvey *et al.*, 1993). Growth hormone-like moieties are, nevertheless, located in the hemocytes of the primitive vertebrate *Viviparus ater* (Ottaviani *et al.*, 1992), which are responsible for immune function in this gastropod (Ottaviani, 1989). Immune regulation may, moreover, have been the ancestral function of this hormone, prior to its roles in growth and development and prior to the localization of GH in pituitary somatotrophs.

In summary, these results demonstrate, for the first time, that GH mRNA coding for pituitary GH is present in immune tissues of the domestic fowl, in which GH is likely to have local roles in immune regulation.

Materials and methods

Animals and tissues

Immune tissues (spleen, thymus and bursa of Fabricius) were rapidly dissected from immature male White Leghorn chickens, in which pituitary GH (Scanes *et al.*, 1993) and the pituitary GH gene (Lamb *et al.*, 1988; Tanaka *et al.*, 1992) are well characterized. The tissues were immediately frozen in liquid nitrogen and stored at −70°C prior to analysis. For comparative purposes, liver and pituitary glands were also similarly collected.

Radioimmunoassay (RIA)

The presence of GH-IR in immune tissues was first investigated by radioimmunoassay. Tissues were homogenized (1 g/10 ml) in RIA buffer (0.04 M $NaPO_4$, 0.15 M NaCl, 0.1% sodium azide, 0.01 M EDTA, 0.5% ovalbumin; pH 7.0) and centrifuged at 2000 *g* at 4°C for 30 min. Supernatants were boiled for 10 min to remove endogenous enzyme activity and cGH was measured in duplicate using a double antibody RIA (Harvey & Scanes, 1977). The IC_{50} for the assay was 3.67 ± 0.37 ng/tube and 10% inhibition of the tracer binding was 1.23 ± 0.19 ng/tube. Intra-assay and inter-assay coefficients of variation were 6.4 and 17.75%, respectively (Harvey & Scanes, 1977).

Western analysis

The molecular size of immune proteins with GH-IR was investigated by Western analysis. Frozen tissue (1 g/10 ml) was homogenized in homogenization buffer (1% sodium dodecyl sulphate (SDS); 1 mM phenylmethylsulphonylfluoride (PMSF); 10 μg/ml aprotinin; Sigma Chemical Company, St. Louis, MO) using a Brinkman Polytron homogenizer (Brinkman Instruments, IL, USA). The homogenates were centrifuged at 2000 *g* for 5 min at 4°C and the supernatants collected. Protein content was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as standard and coomassie blue as indicator. Samples containing 30 μg protein were diluted 1:1 with loading buffer (0.06 M Tris.Cl, pH 6.8; 10% glycerol; 2% SDS; 8% 2-β-mercaptoethanol; 0.01% bromophenol blue) and heated to 55°C for 15 min prior to electrophoretic separation on 15% SDA-polyacrylamide gels. After separation, the gels were equilibrated in transfer buffer (25 mM Tris; 192 mM glycine; 20% methanol) for 30 to 60 min and electrotransferred to Immobilon-P PVDF membranes (Millipore, Bedford, MA, USA) at 30 V for 4 h at 4°C. The membranes were blocked in 5% non-fat dry milk dissolved in tris buffered saline (TBS: 25 mM Tris.HCl, pH 7.5; 0.5 M NaCl) for 1 h at room temperature. GH-IR was detected by incubation of membranes with a rabbit polyclonal antibody raised against cGH (Harvey & Scanes, 1977; Tai & Chadwick, 1977) (1:2000) for 4 h at room temperature. Antibody binding for cGH was visualised using an anti-rabbit IgG horseradish peroxidase conjugate (Amersham, Mississauga, ON, Canda) diluted 1:2000 in TBS/5% non-fat dry milk or with a concanavalin A hoseradish peroxidase conjugate (Con-A-HRP: 4 μg/ml; Sigma) for 4 h. Immunoreactive bands were visualized by incubating membranes in ECL reagent (Amersham) for 1 min and exposing membranes to Kodak X-AR film (Kodak, Rochester, NY, USA).

Ribonucleic acid (RNA) extraction

The possible expression of the GH gene in immune tissues of the chicken was investigated using the PCR. Total cellular RNA was extracted from immune tissues, livers and pituitaries by Polytron homogenization (Brinkman Instruments) in 5.5 M guanidium thiocyanate (containing 25 mM sodium citrate, 0.5% (w/v) sodium lauryl sarcosine, pH 7.0 and 0.2% 2-β-mercaptoethanol) and collected after isopycnic ultracentrifugation (125,000 *g* at 22°C for 24 h) through a gradient of caesium trifluoracetic acid (Pharmacia Fine Chemicals, Uppsala, Sweden; Density 1.51 ± 0.01 g/ml) containing 0.1 M EDTA (pH 7.0). The RNA was resuspended in diethylpyrocarbonate (DEPC) treated water and quantified spectrophotometrically at 260 nm. In all samples, optical

density ratios (measured at 260 and 280 nm) were between 1.8 and 2.1. Purity of RNA was also determined by electrophoresis in 1% (w/v) agarose gels stained with ethidium bromide (Maniatis *et al.*, 1982).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was reverse transcribed into complementary deoxyribonucleic acid (cDNA) by reverse transcriptase (100 U superscript; BRL, Burlington, ON, Canada) in the presence of 50 pmol of a specific antisense oligonucleotide primer (5′-GCCTCAGATGGTGCAGTTGCTCTCTCCGAA-3′) for pituitary GH cDNA (Lamb *et al.*, 1988) or 100 pmol oligodeoxythymidine primer (Boehringer Mannheim, Laval, PQ, Canada), 5X-HRT buffer (BRL) and excess (10 mM of each) deoxynucleotides. The reaction mixture was incubated for 1 h at 37°C and 30 min at 42°C. Negative controls were also performed, in which superscript or RNA was omitted from the reverse transcription mixture. An aliquot (0.05%) of the cDNA was then amplified for 30 cycles in a mixture containing 1 × PCR buffer (80 mM KCl; 16 mM Tris.HCl, pH 8.4; 1.5 mM $MgCl_2$; 0.1% Triton X-100), 200 μM of each deoxynucleotide, 15 pmol of both 5′ oligomer sense and 3′oligomer antisense primers and *Thermus aquaticus* (*Taq*) DNA polymerase (5 U; Promega, Madison, WI, USA). Amplifications of the cDNA were performed using primers CRE1 (5′-CGTTCAAGCAACACCTGAGCAACTCTCCCG-3′) and CRE2 (5′-GCCTCAGATGGTGCAGTTGCTCTCTCCGAA-3′). These primers were based on the known sequence of the cGH cDNA (Lamb *et al.*, 1988) and were designed to generate a 689 bp fragment of chicken GH cDNA, including the entire 216 amino acid coding sequence (Lamb *et al.*, 1988; Figure 6). The PCR mixtures were overlaid with mineral oil (v/v) and amplifications were carried out for 30 cycles at 94°C for 1 min (denaturing), 50°C for 1 min (annealing) and 72°C for 2 min (extension), with a final extension (72°C for 10 min) in a genetic thermal cycler (MJ Research, Watertown, MA, USA). The PCR products were resolved by 1.5% (w/v) agarose gel electrophoresis and visualized by ethidium bromide staining.

Restriction endonuclease digestion

GH cDNA fragments produced by RT-PCR were further characterized by restriction endonuclease digestion with *Bam*HI or *Rsa*I (5 U/mg DNA; BRL) for 2 h at 37°C, to determine if the cDNA contained the restriction sites present in pituitary GH cDNA. Products were identified by electrophoresis of the reaction mixtures in ethidium bromide-stained 1.5% (w/v) agarose gels. The sizes of the digested fragments were determined by comparison with the migration of PCR size markers (Promega).

cDNA probe synthesis

The identity of the GH-like PCR products was also investigated by Southern analysis, using a cDNA probe constructed from an 803 bp fragment of the cGH cDNA sequence (kindly provided by Dr Douglas Foster, Dept. Animal Science, University of Minnesota). The plasmid pUC119 was linearized by restriction enzyme digestion with *Eco*RI (5 U/μg DNA; BRL) and the cDNA fragment was isolated from the vector DNA by electrophoretic separation on 1.5% agarose gels. The cDNA was then purified using Geneclean II (BioCan Scientific, Mississauga, ON, Canada) and restriction endonuclease-digested with *Bst*XI and *Hae*II (5 U/μg DNA; BRL) to produce a 613 bp fragment which did not contain the oligonucleotide primer sequences used in RT-PCR. The cDNA probe was then radiolabelled by random priming (BRL). Template cDNA was incubated with nucleotide mix (dATP, dGTP and dTTP; 0.5 mM each), ^{32}P-labelled dCTP (Dupont, Mississauga, ON, Canada), and random hexamer primers and the synthesis of radiolabelled template was carried out by Klenow fragment (DNA polymerase 1; 3 U). Radiolabelled template was purified from excess nucleotides and enzyme by Nuctrap push columns (Stratagene, La Jolla, CA, USA) and resuspended in double-distilled water prior to incubation with membranes.

Southern blot analysis

Southern blotting was utilized to determine if the PCR products generated from the mRNA in immune tissues were homologous to pituitary GH cDNA. The PCR reaction mixtures were electrophoresed through a 1.5% (w/v) agarose gel and the cDNAs were transferred to Hybond N nylon membrane (Amersham) by capillary transfer, prior to the baking of the membranes at 80°C for 2 h under vacuum. Membranes were prehybridized for 3 h at 42°C in 50% formamide containing 5 × SSC (1 × SSC: 0.15 M NaCl; 0.015 M sodium citrate, pH 7.2), 5 × Denhardts, 1% SDS, and 50 μg/ml salmon sperm DNA (Sigma) and hybridized under the same conditions for 8 h in the presence of 10% dextran sulphate and newly synthesized probe. Following hybridization, the membranes were washed sequentially at 60°C in decreasing concentrations of SSC (2 ×, 1 ×, 0.5 × and 0.1 × SSC; 15 min each). The membranes were exposed to Kodak X-AR film (Kodak) between intensifying screens for 1 to 24 h at −70°C.

Nucleotide sequencing

The similarity of the GH-like mRNA in immune and pituitary tissues was further investigated by nucleotide sequencing of the cDNA fragments generated by RT-PCR. The PCR reaction products were electrophoresed in 1.5% (w/v) agarose ethidium bromide-stained minigels (Maniatis *et al.*, 1982). The appropriately sized ethidium bromide-stained DNA band (689 bp) was excised from the gel and purified from excess nucleotides and agarose using Geneclean II (BioCan Scientific). The fragment was then resuspended in double distilled water prior to sequencing. PCR fragments were cycle sequenced (both strands) using the silver sequence method (Promega) which is a modification of the cycle dideoxy chain termination method (Sanger *et al.*, 1977). Briefly, 2 pmol template DNA was added to a mixture of 5 × sequencing buffer (250 mM Tris.HCl pH 9; 10 mM $MgCl_2$: Promega), 5′-oligomer sense (CGTTCAAGCAACACCTGAGCAACTCTCCCG) or 3′-oligomer antisense (GCCTCAGATGGTGCAGTTGCTCTCTCCGAA) primer (4.5 pmol), sequencing grade *Taq* DNA polymerase (Promega) and one of the four silver sequence deoxy/dideoxy nucleotides (d/ddATP, d/ddCTP, d/ddGTP or d/ddTTP). The primers were based on the published sequence of GH cDNA (Lamb *et al.*, 1988). The mixture was overlaid with mineral oil (v/v) and heat denatured at 95°C for 2 min before 55 cycles of 95°C for 30 s (denaturing step) and 70°C for 30 s (annealing/extension step) in a genetic thermal cycler (MJ Research). The reaction was terminated by addition of DNA sequencing stop solution (10 mM NaOH; 95% formamide; 0.05% bromophenol blue; 0.05% xylene cyanol). cDNA from each tissue was sequenced five to six times to increase fidelity of results. Sequenced products were heated at 70°C for 2 min prior to loading on 6% (v/v) acrylamide/bis-acrylamide gels (19:1), containing 1X TBE (0.089 M Tris; 0.089 M boric acid; 20 mM EDTA, pH 8) and electrophoresed at 1800 V for 1.5 to 2.5 h. After electrophoresis, the gel plates were separated and the gel fixed in 10% glacial acetic acid, stained with silver nitrate (1 g/L) solution containing 0.05% formaldehyde and developed with chilled (10 to 12°C) Na_2CO_3 solution (30 g/L), containing sodium thiosulphate (2 mg/L) and formaldehyde (0.05%). The developing reaction was terminated by addition of 10% glacial acetic acid (v/v), and gels were air dried, prior to exposure to EDF film (Kodak) for 10 to 15 s.

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