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Extrapituitary Pituitary Hormones:
GH and TSH in tissues of the domestic fowl (*Gallus domesticus*)

by

Amy Elizabeth Murphy

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of Master of Science.

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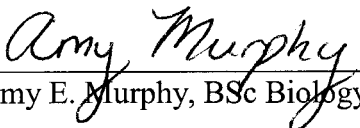**Name of Author:** Amy Elizabeth Murphy

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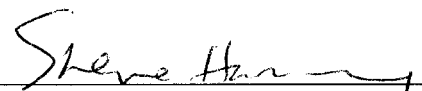
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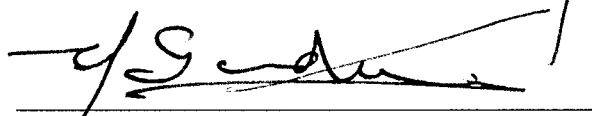
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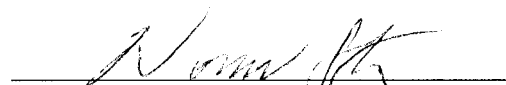
The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Extrapituitary Pituitary Hormones: GH and TSH in tissues of the domestic fowl (*Gallus domesticus*)** submitted by **Amy Elizabeth Murphy** in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

It is generally thought that pituitary hormones are not involved in early embryonic growth and development, since the pituitary gland does not develop until mid-late gestation. However, pituitary hormones have been localized in many extrapituitary tissues and in some early embryonic tissues. Therefore, extrapituitary "pituitary" hormones may have essential autocrine/paracrine roles in extrapituitary tissues and in the early embryo prior to pituitary differentiation.

Immunoreactive GH- and βTSH-like proteins were detected in numerous extrapituitary tissues of the ED7 (prior to the ontogeny of functional GH-secreting somatotrophs) chick embryo. The distribution of immunoreactive GH was different than that of immunoreactive βTSH. The transcription of both pituitary GH and βTSH is dependent on the transcription factor Pit-1. Immunoreactive Pit-1 like proteins of appropriate size (33kDa) and the Pit-1 transcript were also detected in the ED7 chick embryo. The distribution of Pit-1 immunoreactivity differed from that of GH and βTSH in some tissues. These results suggest GH and βTSH may have autocrine or paracrine roles during embryonic development and their transcription may be Pit-1 dependent in some ED7 chick embryo tissues and Pit-1 independent in others.

GH, GH-receptor and Pit-1 genes were also detected in the adult male chicken reproductive tract and the chicken testicular GH gene was cloned and sequenced revealing a novel chicken testicular GH transcript. These results provide further evidence for a role for growth hormone in reproductive physiology.

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LIST OF ABBREVIATIONS

ABC	avidin-biotin complex
ACTH	adrenocorticotropin
bp	base pairs
cDNA	complmentary dioxyribonucleic acid
cGH	chicken growth hormone
Cys	cysteine
DA	dopamine
DAB	diaminobenzidine
DIG	digoxigenin
ED	embryonic day
FSH	follicle stimulating hormone
Gap DH	glyceraldehyde-3-phosphate dehydrogenase
GH	growth hormone
GHR	growth hormone receptor
GHRH	growth hormone releasing hormone
hCS	human chorionic somatomammotropin
hGH-N	human growth hormone-normal
hGH-V	human growth hormone-variant
ICC	immunocytochemistry
IGF	insulin-like growth factor

IHC	immunohistochemistry
IR	immunoreactivity
kDa	kilodalton
LH	luteinizing hormone
mRNA	messenger ribonucleic acid
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pit-1/GHF-1	pituitary-specific transcription factor-1/ growth hormone factor-1
PRL	prolactin
RNA	ribonucleic acid
RT	reverse transcription
SDS	sodium dodecyl sulphate
SRIF	somatostatin
T3	L-3,5,3-triiodothyronine
T4	thyroxine
TRH	thyrotropin releasing hormone
TSH	thyroid stimulating hormone

Chapter 1

Literature Review:

Pituitary and Extrapituitary Hormones

I. GENERAL INTRODUCTION: The Expanding Endocrine System

Until recently, the endocrine system (as taught in the medical school curriculum) was thought to comprise a number of scattered glands throughout the body (the hypothalamus, pituitary, thyroid, parathyroid, pancreas, adrenal and gonads). By definition, they were characterized by being ductless glands that secreted their products (hormones) into the bloodstream that connected them. Other than this, it was difficult to characterize the endocrine system, as it was evidently comprised of neural and non-neural tissues, discrete glands or scattered cells, and individual, paired or multiple glands that contained one or more secretory cell-type, each of which might secrete one or more hormones. The distinctiveness of the endocrine system became even more problematic when it was realized that the brain, heart, lungs, kidneys, thymus, spleen and liver also had endocrine function and when the skin and cardiovascular system were thought to be the largest endocrine tissues of the body (Peart, 1977; Beh-Harari and Youdim, 1983; Henderson, 1987; Cantin and Genest, 1988; Anggard, 1990; Zouboulis, 2000).

Whilst it is now difficult to describe unique features of the endocrine system, it is equally difficult to define hormones, the classical messages of the endocrine system. Hormones were described as homeostatic regulators that acted at distant target sites, with a latency of response, after transportation through the blood system from their sites of production. This definition does not, however, differentiate "hormones" from "parahormones", like carbon dioxide and glucose (Kolata, 1982; Martin, 1985). Moreover, while a chemical messenger may have endocrine action following its release into the bloodstream, it is now realized that the same chemical may exert local (paracrine) actions on neighboring cells or direct (autocrine) actions on the cell of origin.

It may even be retained within the cell to exert "intracrine" action or be transported through a cellular matrix (juxtacrine) or body cavity (luminocrine) rather than through the bloodstream. These messengers may also act, dogmatically, through signal-transduction system, but any one may act through a variety of plasma membrane, cytoplasmic or nuclear receptors. Some may also act without receptor occupancy, through changes in the conformation of the plasma membrane or via intercellular communications via gap junctions. Many are also now known to act rapidly, with very little latency in the response. Many of these chemical messengers are also now known to be produced in more than one tissue or site within the body.

The ectopic (abnormal) production of hormones in "unusual" tissues is well established, although it was thought to be an epiphenomenon associated with malignancy and other pathologies (Barkan *et al.*, 1986; Morgello *et al.*, 1988; Coleman *et al.*, 2000; Ohta *et al.*, 2000; Indinnimeo *et al.*, 2001). It is, however, now known that many hormones are physiologically expressed in many sites in which they may have systemic or local actions. Thus, while growth hormone (GH), for instance, is generally considered as an endocrine factor produced by the pituitary gland, it is also thought to be a neurocrine, produced within the brain, a cytokine within the immune system and a growth factor in extrapituitary tissues (Render *et al.*, 1995a; Waters *et al.*, 1999; Harvey *et al.*, 2000a; Ramesh *et al.*, 2000). The presence of GH and TSH (thyroid stimulating hormone) (another hormone primarily produced by the pituitary gland) in extrapituitary tissues is the focus of the review and the research described.

II. GROWTH HORMONE (GH)

A. Pituitary GH

1. SOMATOTROPHS

Growth hormone (GH) is primarily produced in pituitary somatotrophs, which are the most abundant adenohypophyseal cell type (Porter, 1997). These cells have geographical and spatial distributions within the pituitary gland that are species-related (Harvey, 1995). In the chicken, most somatotrophs (>90%) are found in the caudal lobe of the adenohypophysis and account for more than 40% of the secretory cell types in the lobe (Hull *et al.*, 2000). The distinct cellular location of these cells therefore promotes paracrine relationships between somatotrophs and folliculostellate cells and other secretory cells types (Schwartz *et al.*, 1998). Corticotrophs are, for instance, closely associated with somatotrophs in the rat pituitary (DeNef, 1986), and adrenocorticotropin (ACTH) and GH reciprocally regulate somatotroph and corticotroph function (Schwartz and Perez, 1994).

Somatotrophs are characteristically large, ovoid shaped cells with large nuclei, although morphological heterogeneity is present in many species, including the domestic fowl (Hull *et al.*, 2000). This morphological heterogeneity may be related to differences in the composition of the intracellular organelles and to the production of heterogeneous GH moieties. This heterogeneity may also reflect the presence of cells that secrete more than one pituitary hormone: prolactin (PRL) – (mammosomatotrophs), thyrotropin (TSH) – (thyrosomatotrophs), ACTH – (corticosomatotrophs) and luteinizing hormone (LH) – (gonadosomatotrophs) having been identified in mammalian pituitary glands (Frawley

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and Boockfor, 1991; Childs, 2000; Vidal *et al.*, 2000). The presence of GH-secreting and non-GH-secreting somatotrophs (silent somatotrophs, Frawley and Boockfor, 1991) and of GH depleted and GH replete cells (Hull *et al.*, 2000) also contribute to somatotroph heterogeneity because of differences in GH content and immunocytochemical staining.

2. CHEMISTRY

The most abundant form of GH in the pituitary gland is a single chain 22 kilodalton (kDa) protein of approximately 191 amino acids. The basic GH structure is highly conserved amongst species and is characterized by 4 cysteine residues that form two disulfide bridges (Cys^{53}-Cys^{165} and Cys^{182}-Cys^{189}) and create large and small intramolecular loops, respectively (Charrier and Martal, 1988). These loops are thought to be important for receptor binding and signal transduction, although specific biological activities are thought to reside within discrete polypeptide fragments of the intact molecule (Lewis, 1984; Campbell and Scanes, 1995), suggesting the presence of multiple GH receptor (GHR) moieties.

In addition to 22 kDa GH (monomer GH), numerous GH moieties are also present in the pituitary gland (Aramburo *et al.*, 2000; 2001). These are thought to reflect differences in gene transcription or post-translational modifications of the protein. In chickens, these include charge variants (Aramburo *et al.*, 1989), glycosylated GH (Berghman *et al.*, 1987), phosphorylated GH (Aramburo *et al.*, 1990; 1992), oligomeric forms (dimers, trimers, quatramers and pentamers; Lewis, 1984; Stolar, 1984), and proteolytic cleavage forms (Aramburo *et al.*, 1989; Render *et al.*, 1995b). They are also known to be differentially produced in response to different GH-secretagogues

(Martinez-Coria *et al.*, 2002) and their relative abundance changes during ontogeny

(Aramburo *et al.*, 2000).

3. GENE

In humans, the pituitary GH gene (hGH-N) is a member of a family of five highly

homologous genes that are encoded within 55 kilobases (kb) on band q22-q24 on the long

arm of chromosome 17 (Parks, 1989). In addition to hGH-N these genes include human

chorionic somatomammotropin (hCS-A and hCS-B) (also called placental lactogens), a

hCS-pseudogene (hCS-L, that is not transcribed) and a placental hGH variant (hGH-V,

which is present as hGH-V$_1$, hGH-V$_2$, and hGH-V$_3$ proteins) (MacLeod *et al.*, 1992;

Lytras *et al.*, 1994; Misra-Press *et al.*, 1994; Boguszewski *et al.*, 1998).

These genes are differentially expressed in tissue- and time-dependent ways

(Nickel *et al.*, 1990; Eberhardt *et al.*, 1996). The hGH-N and hGH-V genes are both

expressed in the pituitary gland and placenta, although the hGH-N gene is preferentially

expressed in the pituitary while the hGH-V gene is preferentially expressed in the

placenta. Placental GH-like genes have also been found in sheep (Lacroix *et al.*, 1996)

and rats (Ogilvie *et al.*, 1990). Two GH genes have also been identified in the pituitary

glands of some fish (Agellon *et al.*, 1988; Lorens *et al.*, 1993; Law *et al.*, 1996), in which

a somatolactin gene (resembling both GH and prolactin) may additionally be expressed

(Rand-Weaver *et al.*, 1991; Astola *et al.*, 1996).

In addition to heterogeneous GH genes, the presence of two promotors for the

hGH-N gene (Courtois *et al.*, 1992; Giordano *et al.*, 1997; Rodrigues *et al.*, 1998)

indicates the existence of transcriptional heterogeneity. Alternate splicing of the hGH-N

pre-mRNA (in which exon 2 of the five exons is spliced to an alternative acceptor site located 45 bases within exon 3) results in the synthesis of a 20 kDa protein lacking 15 internal amino acid residues (32-46, inclusive) (Lewis *et al.*, 1980), indicating the presence of post-translational heterogeneity.

In birds, the pituitary GH gene has been cloned from the chicken (Zhvirblis *et al.*, 1987; Baum *et al.*, 1990; Tanaka *et al.*, 1992), turkey (Foster *et al.*, 1991), and duck (Chen *et al.*, 1988). These have close homology (>90% similarity) with each other and with mammalian GH genes (>60% homology) (Tanaka *et al.*, 1992). A unique transcript, coding for a truncated 16 kDa GH moiety (lacking the amino terminus of monomer GH), has also been identified in the retina of embryonic chicks (Takeuchi *et al.*, 2001). Numerous polymorphisms of the GH gene, as a result of differences in intron sequences (Kuhnlein *et al.*, 1997; Ip *et al.*, 2001), have also been identified in the chicken pituitary. Some of these polymorphisms are strain related and correlate with differences in egg production (Kuhnlein *et al.*, 1997; Ip *et al.*, 2001).

4. ONTOGENY

Embryonic or fetal growth is thought to be a "*growth-without-GH*" syndrome (Geffner, 1996), since much of it occurs prior to pituitary differentiation and the onset of GH secretion.

Although somatotrophs account for >40% of the cells in the caudal lobe of 4-6 week old chickens (Malamed *et al.*, 1988) immunoreactive somatotrophs are generally thought to be absent until mid-development (between embryonic day (ED) 10 or ED 12 of the 21 day incubation period), and even then they are sparse (0.2% of parenchymal

cells), small and poorly developed, with pleiomorphic secretory granules (Porter, 1997; Jozsa et al., 1979). The number, size and content of the secretory granules increases towards hatch, but mature, spherical secretory granules are not present until ED 15. The number of somatotrophs also increases towards hatch, when they account for almost 20% of the pituitary cells (Malamed et al., 1993). An earlier ontogeny of somatotrophs, in ED 7-ED 8 chick embryos, was reported by Gasc and Sar (1981) and Mikami and Takahashi (1987). Thommes et al. (1987) even suggested somatotrophs were present in Rathke's pouch in ED4.5 embryos, although others have concluded they are not present until ED 13 (Barabanov, 1991), ED 15 (Wingstrand, 1951), ED 18 (Tixier-Vidal, 1954) or ED 19 (Rahn, 1939). This controversy may reflect differences in methodology, but it is consistent with the ontogeny of somatotrophs in mammalian pituitary glands, which has been reported to occur mid-way through the first trimester of gestation in some studies (Baker and Jaffe, 1975; Asa et al., 1986), but mid-way through the last trimester in other studies (Chatelain et al., 1979; Wilson and Wyatt, 1993). It is, however, clear that the ontogeny of pituitary somatotrophs occurs after the onset of organogenesis, which must therefore occur in the absence of pituitary GH.

The ontogeny of pituitary somatotrophs occurs coincident with the ontogeny of GH synthesis. In the chicken, transcripts of the GH gene are present in the pituitaries of ED 12 embryos and increase in abundance towards hatch (Kansaku et al., 1994; McCann-Levorse et al., 1993). This induction of GH gene transcription is preceded by increased levels of the pituitary-specific transcription factor (Pit-1), which is thought to be essential for pituitary GH gene expression (Harvey et al., 2000b). The abundance of the pituitary GH transcript in ED 18 chick embryos is, however, <20% of that in hatched

chicks and <3% of that in 4-week-old birds. The GH content of the pituitary gland similarly doubles between ED 12 and ED 18, but even then it is <10% of that in newly hatched chickens and <1% of that in 3-week-old birds (McCann-Levorse *et al.*, 1993). The expression of the GH gene is therefore minimal during embryonic development.

The ontogeny of pituitary somatotrophs also precedes the ontogeny of pituitary GH secretion. While immunoreactive somatotrophs have been identified in the pituitaries of ED 10 and ED 12 embryos (Porter, 1997), GH-secreting somatotrophs (detectable by the reverse hemolytic plaque assay) are not thought to differentiate until ED 14 and ED 16 (Porter *et al.*, 1995a,b). Somatotroph responsiveness to GH-releasing stimuli also only occurs during late embryogenesis, since the hypothalamus-pituitary axis does not become a functional unit until 1 week before hatching (Decuypere and Scanes, 1983; Thommes *et al.*, 1983; Porter *et al.*, 1995a). It is, therefore, not surprising that the secretion of GH into peripheral circulation does not occur until after ED 16 (Harvey *et al.*, 1998). Pituitary GH cannot, therefore, exert endocrine actions on tissue growth until late embryogenesis. It is, however, possible that GH produced in extrapituitary tissues prior to pituitary differentiation might be involved in early embryonic development (Harvey *et al.*, 1998).

B. Extrapituitary GH

1. DISTRIBUTION

It is now well established that the expression of the GH gene is not confined to the pituitary gland and occurs, in lesser amounts, in extrapituitary tissues (reviewed by Harvey and Hull, 1997; Hull and Harvey, 1998). For instance, in adult chickens, GH and

GH mRNA have been detected in the brain (Render *et al.*, 1995a), in which GH-immunoreactivity is widely distributed (Ramesh *et al.*, 2000). GH and GH mRNA are also present in the thymus, bursa and spleen of adult chickens (Render *et al.*, 1995b). Similarly, in mammals, GH and GH mRNA are present in the nervous system – Harvey *et al.*, 1993; Yoshizato *et al.,* 1998; immune system – Maggiano *et al.*, 1994; Recher *et al.*, 2001; integumentary system – Palmetshofer *et al.*, 1995; Slominski *et al.*, 2000; respiratory system – Allen *et al.*, 2000; alimentary system – Tresguerres *et al.*, 1999; Kyle *et al.*, 1981; cardiovascular system – Costa *et al.*, 1993; Wu *et al.*, 1996; Recher *et al.*, 2001; reproductive system – Hu *et al.*, 1999; Schwarzler *et al.*, 1997; Untergasser *et al.*, 1997; mammary glands – Mol *et al.*, 1995a;b; and teeth – Zhang *et al.*, 1997).

2. CHEMISTRY

Since extrapituitary GH has largely been detected immunologically, it must share epitopes with pituitary GH. It is also associated with proteins comparable in size to monomer GH in the pituitary gland (Render *et al.*, 1995a,b) and with numerous size variants (Render *et al.*, 1995a,b; Aramburo *et al.*, 2000; Luna *et al.*, 2002). However, the GH-immunoreactivity in the rat brain is more akin to human GH than to rat pituitary GH (Hojvat *et al.*, 1982), and this may indicate tissue-specific differences in GH structure (Harvey *et al.*, 1993).

3. GENE

The GH gene in the human (Martial *et al.*, 1979), rat (Seeburg *et al.*, 1977) and chicken (Tanaka *et al.*, 1992) has been cloned and sequenced. In all cases it consists of 5

exons and 4 introns. The GH cDNA in chicken (Render *et al.*, 1995b), rat (Weigent and Blalock, 1991) and human (Melen *et al.*, 1997) immune tissues is identical to that in the corresponding pituitary glands. The GH gene in the human placenta (Boguszewski *et al.*, 1998), testis (Berger *et al.*, 1999) and ovary (Schwarzle *et al.*, 1997; Untergasser *et al.*, 1997) does, however, include the placental variant (hGH-V) that codes for hGH-V$_1$, hGH-V$_2$ and hGH-V$_3$. A novel, truncated GH transcript has also been identified in the eyes of chick embryos (Takeuchi *et al.*, 2001). These authors cloned a novel GH cDNA (s-cGH) that consisted of two exons; the first corresponds to a part of intron 3 and exon 4 of the pituitary GH gene, the second exon corresponds to exon 5 of pituitary GH mRNA. The portion coded by this cDNA had a deduced molecular size of 16.5 kDa and 140 amino acid residues, lacking the signal peptide and the N-terminal 71 amino acids of 22 kDa pituitary GH. Its N-terminus would have a unique sequence of 20 amino acids, whereas the C-terminal 120 amino acids (positions 97-216 of the GH prohormone) would be identical to 22 kDa pituitary GH and contain helixes 3 and 4 that bind to the GH receptor (GHR). The conserved four cysteine residues in the C-terminus would permit the formation of two intramolecular disulfide bridges and a secondary (coil) structure as in the corresponding region of 22 kDa pituitary GH. These structural similarities of s-cGH with 22 kDa pituitary GH suggests it could bind to, and activate, tissue GHRs. Interestingly, as the leucine residues in the third helical domain of the s-cGH are arranged every 7 amino acids, it also has the characteristic structure of a leucine-zipper transcription factor and it may thus participate in gene regulation. However, as the N-terminus is hydrophilic and unlikely to serve as a secretory signal peptide, s-cGH

probably acts as an intracrine within the cells expressing this GH isoform. The identity of the cells expressing this isoform is, however, unknown.

4. ONTOGENY

Prior to the appearance of GH-immunoreactivity in pituitary somatotrophs (at approximately ED 12; Porter, 1997) and in Rathke's pouch of chick embryos (at ED 4.5; Thommes *et al.*, 1987; at ED 7; Harvey *et al.*, 2000a) GH-immunoreactivity is present in the endoderm and ectoderm layers of ED 1 and ED 2 embryos (Wang, 1989). Harvey *et al.* (2000a) similarly found that GH immunoreactivity was almost ubiquitously present in peripheral tissues of ED 3 chick embryos and was widespread in ectodermal, mesodermal and endodermal derivitives at ED 5, although not present in every cell of each tissue. Immunoreactive GH was particularly abundant in the neural tube, notochord, limb bud, somites, heart, stomach, liver, kidney, Wolffian duct and the amnion. By ED 8, GH-immunoreactivity was still widespread and now present in limb bud chondrocytes and osteocytes. The presence of GH-immunoreactivity in the Mullerian ducts of male and female chick embryos between ED 5 and ED 12 was also reported by Wang (1989). This immunoreactivity persists in most tissues until at least mid-late embryogenesis (Harvey *et al.*, 1998), and persists in neural (Render *et al.*, 1995a; Ramesh *et al.*, 2000; Harvey *et al.*, 2000a) and immune (Render *et al.*, 1995b) tissues neonatally and through sexual maturity. The GH-immunoreactivity in some tissues (eg. the heart, liver, muscle and mesonephros) is, however, lost by ED 12 -- ED 18 of embryonic development (Harvey *et al.*, 1998; 2000a), suggesting tissue-specific regulation of the extrapituitary GH gene.

In the embryonic chick, GH-immunoreactivity was identified by its cross-reactivity with four antisera raised against chicken GH and by a lack of staining following the preabsorption of the antisera with recombinant chicken GH. Western blotting also showed that this immunoreactivity was associated with proteins identical in size to monomer (26.5 kDa) and dimer (53 kDa) GH in the pituitary glands of adult chickens (Harvey et al., 1998) and was present in the trunks of ED 7 embryos (Harvey et al., 1998).

The detection of GH-immunoreactivity in early chick embryos occurred prior to the differentiation of pituitary somatotrophs. It also occurred before the appearance of GH in peripheral plasma (on ED 17; Harvey et al., 1979; Harvey et al., 1998) and, indeed, prior to the maturation of the cardiovascular system (before ED 4). This GH immunoreactivity is therefore likely to reflect an extrapituitary expression of a GH gene. This possibility is supported by the detection, by RT-PCR, of GH mRNA in the bodies of ED 2 – ED 5 chick embryos (Harvey et al., 2000b; 2001a) and in the headless bodies of ED 7 embryos (Harvey et al., 1998). As Pit-1 mRNA was also detected in the same tissues (Harvey et al., 2000b), this early expression of the GH gene may be dependent on an earlier or contemporaneous expression of the Pit-1 gene, since GH gene expression is thought to be Pit-1 dependent.

In addition to embryonic chicks, GH and GH mRNA have been detected in preimplantation mouse embryos (Pantaleon et al., 1997). Yang et al. (1999) similarly found GH-immunoreactivity and GH mRNA in trout embryos during gastrulation and organogenesis, although they did not determine its tissue distribution during ontogeny. Izadyar et al. (2000) have also found GH and GH mRNA in bovine embryos prior to

blastocyst formation and blastocyst hatching. Hojvat *et al.* (1982a) had earlier found that the appearance of GH-immunoreactivity in the brains of fetal rats occurs before its ontogenic appearance in the pituitary gland. A widespread expression of the GH gene in peripheral tissues of the rat fetus (eg. in gut, liver, muscle and the cardiovascular system) has also been observed (Costa *et al.*, 1993; Recher *et al.*, 2001), even though GH gene expression in these tissues is not seen neonatally. GH gene expression therefore appears to be widespread during early development and before the ontogeny of pituitary GH expression. It is therefore likely that GH acts as a local growth factor during early development rather than as a pituitary endocrine.

5. PARACRINE/AUTOCRINE ROLES?

In addition to GH, GHRs have been identified in many extrapituitary sites of GH production (Harvey and Hull, 1997; Hull and Harvey, 1998). It is therefore likely that GH may have local autocrine/paracrine actions in those sites. For instance, the presence of GH and GHR in the skin (Lobie *et al.*, 1990a; Garcia-Aragon *et al.*, 1992; Costa *et al.*, 1993; Palmetshofer *et al.*, 1995; Slomiski *et al.*, 2000) suggests the presence of an autocrine/paracrine system of GH action in the integument. The presence of GH and GHR in the nervous system (Harvey *et al.*, 2001a), respiratory system (Garcia-Aragon *et al.*, 1992; Costa *et al.*, 1993; Batchelor *et al.*, 1998; Allen *et al.*, 2000), digestive system (Kyle *et al.*, 1981; Lobie *et al.*, 1990b; Garcia-Aragon *et al.*, 1992; Costa *et al.*, 1993; Mori and Devlin, 1999), immune system (Kyle *et al.*, 1981; Lobie *et al.*, 1990a; Costa *et al.*, 1993; Wu *et al.*, 1996), and the musculoskeletal system (Kyle *et al.*, 1981; Garcia-Aragon *et al.*, 1992; Werther *et al.*, 1993; Zhang *et al.*, 1997; Harvey *et al.*, 1998; 2000a)

similarly suggests autocrine/paracrine roles for GH in these tissues. This possibility is supported by the presence of inducible GH-response genes (eg. GH-response gene-1 and insulin-like growth factor-1) in many of these tissues (Harvey *et al.*, 2001a,b). Moreover, in many tissues, local actions of GH have been demonstrated that are blocked by GH-immunoneutralization. For instance, the blockade of endogenous GH in fetal rats prevents the differentiation of the Wolffian duct (Nguyen *et al.*, 1996). GH-immunoneutralization similarly blocks the GH-induced glucose transport in preimplantation mouse embryos (Pantaleon *et al.*, 1997), the hatching of murine (Drakakis *et al.*, 1995; Fukaya *et al.*, 1998) and bovine (Izadyar *et al.*, 2000) blastocysts, haemopoietic function in human lymphocytes (Sabharwal and Varma, 1996) and mammary development in dogs (Mol *et al.*, 1996). Antisense oligonucleotides for the GH gene similarly block the actions of endogenous GH in the rat immune system (Weigent *et al.*, 1991). The local production of GH in cultured *BRL* cells is similarly unable to prevent apoptosis following transfection with a mutant GHR gene (Liu *et al.*, 1997).

III. THYROID STIMULATING HORMONE (TSH)

A. Pituitary TSH

1. THYROTROPHS

Pituitary TSH is synthesized in, and secreted from, basophilic cells called thyrotrophs (Nathanielsz, 1976). Thyrotrophs are stellate shaped and contain TSH secretory granules up to 200 nm in diameter (Harrisson *et al.*, 1982). The Golgi bodies and the endoplasmic reticulum are well developed and extensive, suggesting high TSH

synthetic activity. Thyrotrophs occur in low abundance in the pituitary gland, accounting for only 1-2% of cells in the rat adenohypophysis (Manger, 1989) and about 2% of the cells in the late chick embryo (Porter *et al.*, 2000). In Aves, a restricted distribution of thyrotrophs has been seen in the cephalic (or rostral) lobe of the anterior pituitary gland, although other investigators have localized TSH in both lobes (Table 1.1). Thyrotrophs are not a fixed population and can increase by both hyperplasia and hypertrophy in response to increased stimulation (Torres *et al.*, 1995).

2. CHEMISTRY

Thyroid stimulating hormone (TSH), or thyrotropin, is the primary regulator of the thyroid gland. TSH stimulates the release of the thyroid hormones, triiodothyronine (T_3) and thyroxine (T_4), (Radke and Chiasson, 1977). TSH, like luteinizing hormone (LH), follicle stimulating hormone (FSH) and chorionic gonadotropin (CG) is a heterodimeric glycoprotein consisting of noncovalently bound α and β subunits (Holdstock and Burrin, 1994). The α subunit is common to all glycoprotein hormones and conveys species specificity, while the β subunit is specific to each hormone and determines hormonal activity (Pierce and Parsons, 1981; Querat, 1994). The α and β subunits are synthesized as separate moieties from different genes (Hsieh *et al.*, 2000). The TSH holoprotein molecular weight is estimated at 25 – 33 kDa for mammals (Pierce, 1971; Jacobson *et al.*, 1977) and 28.3 – 38 kDa for chickens (Prada *et al.*, 2000; Porter *et al.*, 2000). Subunit molecular weights are estimated at 18 – 22 kDa in mammals (Pierce and Parsons, 1981) and 17 – 18 kDa in chickens (Krishnan *et al.*, 1994; Porter *et al.*, 2000). Different isoforms of TSH exist and differ in biological activity. However, the

differences in the isoforms appear to be in oligosaccharide structure and not in the

peptide backbone (Beck-Reccoz and Persani, 1994; Szkudlinski *et al.*, 1995).

3. GENE

The chicken βTSH gene has been cloned and the cDNA sequence determined

(Gregory and Porter, 1997: Kato *et al.*, 1998). It has 93-94% homology with TSH from

other avian species (e.g. the duck, Hsieh *et al.*, 2000; quail, Kato *et al.*, 1997), 67-69%

homology with mammalian TSH (e.g. human TSH, Guidon *et al.*, 1988; bovine TSH,

Maurer *et al.*, 1984; rat TSH, Croyle and Maurer, 1984), 61% homology with frog TSH

(Buckbinder and Brown, 1993); and lesser homology (44-52%) with TSH from teleosts

(e.g. trout, Ito *et al.*, 1993; salmon, Martin *et al.*, 1999; eel, Salmon *et al.*, 1993). There

are three areas within the βTSH cDNA sequences that are highly conserved among

vertebrates, and Area I is thought to have an important function concerning the

mechanism of βTSH action (Gregory and Porter, 1997).

4. CONTROL OF PITUITARY TSH

TSH synthesis and secretion are controlled by hypothalamic and thyroid

hormones. Thyrotropin releasing hormone (TRH) increases TSH secretion from the

pituitary of chickens *in vitro* (Scanes, 1974; Breneman and Rathcamp, 1973) and

increases βTSH mRNA in ducks (Hsieh *et al.*, 2000). T_4 and T_3 downregulate βTSH

mRNA formation in chickens and ducks via negative feedback (Gregory and Porter,

1997; Hsieh *et al.*, 2000). Somatostatin (SRIF) and dopamine (DA) also have inhibitory

effects on TSH synthesis and secretion (Chin *et al.*, 1993), which is also suppressed by gonadal steroids (Tixier-Vidal and Assenmacher, 1961, Tixier-Vidal *et al.*, 1972).

5. ONTOGENY

Thyrotrophs appear ontogenically prior to somatotroph, lactotroph and gonadotroph differentiation in mammals (Burrows *et al.*, 1999) and birds (Porter, 1997). A large number of transcription factors are located in Rathke's pouch and required for the formation of thyrotrophs, as well as other hormone secreting cells, and include *Hesx1, Pitx1, Msx1, Lbd1,3, Pit1, Lhx2, Lhx3, Pitx2, Gata2* and *Egr1* (reviewed by Burrows *et al.*, 1999). Thyrotroph embryonic factor (TEF) also activates the TSHβ gene promotor (Drolet *et al.*, 1991).

Embryonic chick thyroids cultivated *in vitro* first respond to TSH stimulation on ED 6 (Tixier-Vidal, 1959) or ED 6.5 (Thommes and Hylka, 1978), and embryonic chick pituitary glands are responsive to TRH stimulation (as indicated by increased circulating T_4 levels) by ED 6.5 (Thommes and Hylka, 1978). However, normal thyroid activity does not become pituitary dependant until between ED 10 and 12, when TSH becomes abundant in the pituitary gland (Thommes and Hylka, 1978). Pituitary βTSH mRNA is not present until ED 11 and increases toward hatch with a high peak on ED 19 (Gregory *et al.*, 1998). In contrast, Geris *et al.* (1998) found that plasma TSH indices dropped toward hatch. Thommes *et al.* (1983) found immunoreactive βTSH in Rathke's pouch at ED 6.5, but it remained in low abundance until ED 11.5, when both lobes of the anterior pituitary gland were distinct.

6. ACTIONS

The major function of pituitary TSH in the embryo is thought to be the regulation of thyroid function. Pituitary TSH begins to regulate thyroid activity in the chick embryo between ED 10.0 and ED 12.0 (Thommes and Hylka, 1977). Circulating T_4 levels fail to increase after ED 10 – 11.5 in hypophysectomized chicks (Thommes et al., 1977), indicating pituitary control of T_4 release at this stage. Iqbal et al. (1987) found that chronic TSH exposure in ED 15 chicks stimulates increased thyroid peroxidase activity, and Hamori et al. (1959) demonstrated increased thyroid iodine uptake after ED 11 after surgical decapitation reduced thyroid ^{131}I uptake rates after ED 11. Experiments by Kuhn et al. (1988) showed that TSH stimulation in ED 17 and 18 chicks increased T_3, T_4 and reverse (r) T_3 levels, but did not increase hepatic 5'-monodeiodination (5'-D) activity.

TSH is known to have other actions in the embryo unrelated to thyroid stimulating activities. TSH is the dominating lipolytic hormone *in vitro* during the neonatal period (Marcus et al., 1988) and it regulates lipolysis in neonatal human adipocytes to help with the metabolic adaptation after birth (Janson et al., 1998). Haraguchi et al. (1999) have also found that TSH induces proliferation and inhibits differentiation of cultured rat preadipocytes, and Bell et al. (2000) speculate a role for TSH in adipose development. In addition, TSH is known to have roles in embryonic gonadal tissues. TSH stimulates growth and differentiation of the gonad (Csaba et al., 1980; Shanin et al., 1982a,b), migration of germ cells to the gonads (Shanin et al., 1982b) and mitosis of the oocyte (Csaba et al., 1980).

B. Extra-Pituitary TSH

TSH and TSHRs are expressed in a variety of tissues in different species (Table1.2 and 1.3). Extra-pituitary TSH may be produced locally within these tissues and act as an autocrine or paracrine in these sites.

1. TSH: A NEURAL AUTOCRINE/PARACRINE?

The brain is thought to be an extra-pituitary site of TSH synthesis since brain TSH is present prior to pituitary TSH in the rat (Hojvat et al., 1982a,b), so brain TSH is unlikely to be from peripheral blood. This possibility is also supported by the pituitary independent changes in brain TSH concentration that occur diurnally in rats (Ottenweller and Hedge, 1982), during growth (Emanuele et al., 1985), and with endocrine manipulations (Hojvat et al., 1985; De Vito et al., 1985).

Neural TSH may act in an autocrine or paracrine manner since TSH receptor (TSHR)-like protein and TSHR mRNA are also found in the brains of mammals (Labudova et al., 1999; Crisanti et al., 2001; Bockmann et al., 1997a,b) and fish (Kumar et al., 2000). Indeed, TSH has been shown to have actions in neural tissues. In astroglial cells TSH stimulates the release of arachidonic acid and the activity of type II 5'-iodothyronine-deiodinase (Saunier et al., 1993) and stimulates mitogen-activated protein kinase isozymes (Tournier et al., 1995). TSH may also regulate TSHR expression in the brain (Crisanti et al., 2000). It has, furthermore, been speculated that TSH may act as a neuromodulator in the human retina, and to regulate retinal blood flow (Fernandez-Trujillo et al., 1996). It may also act as a modulator of synaptic transmission and a regulator of trophic function in the chicken retina (Prada et al., 2000).

2. TSH: AN IMMUNE AUTOCRINE/PARACRINE?

Immune tissues have been found to produce TSH. Human lymphocytes produce a TSH-like peptide (Smith *et al.,* 1983) and express a βTSH gene (Peele *et al.,* 1993). Harbour *et al.* (1989) have demonstrated TSH mRNA in, and *in vitro* TSH secretion from, T cell lines. In addition, Bodey *et al.* (2000) have shown TSH secretion from human thymic reticulo-epithelial cells. Extra-pituitary immune TSH likely acts in an autocrine or paracrine manner, since TSH receptor mRNA and protein have been found extensively in immune cells and TSH is known to have effects in immune function.

TSHRs have also been located in the immune systems of mammals (Table 1.3) suggesting that immune TSH may act in an autocrine or paracrine manner. TSH has many stimulatory effects on the immune system and it has been speculated that the primary source of immune TSH is not the pituitary gland, but the immune system itself (Bagriacik and Klein, 2000). TSH has been shown to increase the cytotoxic activity of natural killer (NK) cells and to act as a costimulatory factor for T-cell proliferation (Provinciali *et al.,* 1992). It also enhances phagocytic activity and cytokine responses of TSH stimulated murine dendritic cells (Bagriacik and Klein, 2000), as well as increasing cytokine responses of hematopoietic cells (Whetsell *et al.,* 1999; Delgado *et al.,* 1998). TSH can alter the immunological composition of lymphoid cells in the intestinal epithelium (Wang *et al.,* 1997). In addition, TSH modulates human lymphocyte activation, as demonstrated by blocking TRH stimulation with administration of an anti-ratTSH antibody (Raiden *et al.,* 1995).

3. TSH: A PITUITARY AUTOCRINE/PARACRINE?

The pituitary gland is the major site of TSH synthesis and secretion. Recently TSHRs have been localized in the pituitary gland suggesting autocrine/paracrine roles for TSH in pituitary function. Prummel *et al.* (2000) and Theodoropoulou *et al.* (2000) found TSHR mRNA and protein in folliculostellate cells and thyrotrophs of the human pituitary. Together, these results indicate the presence of a short-loop feedback mechanism through which pituitary thyrotrophs autoregulate their synthesis and secretion of TSH (Theodoropoulou *et al.*, 2000), and paracrine actions of TSH on the function of folliculostellate cells (Prummel *et al.*, 2000). The concept of pituitary hormone autoregulation is supported by other studies identifying prolactin (Jin *et al.*, 1997) and GH (Mertani *et al.*, 1995) receptors in the pituitary gland.

4. EMBRYONIC EXTRAPITUITARY TSH

Extrapituitary TSH in the embryo or fetus has, thus far, only been localized in the extahypothalamic rat brain (Hojvat *et al.*, 1982a). Brain TSH is not considered to be of pituitary origin since circulating radiolabeled TSH does not cross the blood-brain barrier in the fetus and radiolabeled TSH in the mother does not cross into fetal circulation (Hojvat *et al.*, 1982a). βTSH protein was detected in the fetal rat brain at ten days of fetal life, five days prior to its detection in the anterior pituitary at fifteen days of fetal life, and persists throughout development (fetal, neonatal, prepubescent and adult) of the rat brain (Hojvat *et al.*, 1982a). Roles for brain TSH in the fetal rat have yet to be determined; however, actions for neural TSH in adults have been suggested (Theodoropoulou *et al.*, 2000).

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IV. PITUITARY-SPECIFIC TRANSCRIPTION FACTOR-1 (PIT-1)

A. General Introduction

Pituitary-specific transcription factor-1 (Pit-1), or growth hormone factor-1 (GHF-1), is a member of the POU-homeodomain family of proteins. It binds to and transactivates promoters of growth hormone, prolactin and β-thyroid stimulating hormone genes (Bodner *et al.*, 1988). Pit-1 also autoregulates its own expression, while the initial expression of Pit-1 is induced by the Prophet of Pit-1 (PROP-1) gene (Sornson *et al.*, 1996). In mammals, Pit-1 is a 33 kDa, 291 amino acid protein that binds to two adjacent sites in the GH, PRL and βTSH promoter regions and binding to both sites is necessary for transcription (Harvey *et al.*, 2000b).

The Pit-1 gene has been cloned and sequenced in mammals (bovine, Bodner *et al.*, 1988; rat, Bodner *et al.*, 1988, Ingraham *et al.*, 1988; mouse, Li *et al.*, 1990; swine, Tuggle *et al.*, 1993; human, Tatsumi *et al.*, 1992) and several transcripts have been identified. These include, in addition to the major Pit-1, moiety Pit-1α, the variants Pit-1β, Pit-1T, Pit-1γ, Pit-1Δ3 and Pit-1Δ4, which arise by alternative transcription, initiation and alternative splicing (Theill *et al.*, 1992; Konzak and Moore, 1992; Haugen *et al.*, 1993), and act differently on the promoters of target genes. In Aves, Pit-1 has been cloned and sequenced from chicken (Tanaka *et al.*, 1999; Van As *et al.*, 2000) and turkey (Wong *et al.*, 1992; Kurima *et al.*, 1998) pituitary glands. Three different transcripts have been identified in the turkey: tPit-1, tPit-1β and tPit-1W (Kurima *et al.*, 1998) and in the broiler chicken: ggPit-1, ggPit-1β and ggPit-1W (Van As *et al.*, 2000), although Tanaka *et al.* (1999) only identified two Pit-1 transcripts (cPit-1α, homologous to ggPit-

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1, and cPit-1γ, homologous to ggPit-1W) in a layer strain of chicken. The three chicken

Pit-1 transcripts have a high degree of homology with the three turkey Pit-1 transcripts

(Van As *et al.*, 2000). The mammalian Pit-1 mRNA consists of six exons and five

introns, while turkey Pit-1 mRNA consists of seven exons and six introns (Kurima *et al.*,

1998).

Pit-1 expression is present in the pituitary gland of mammals prior to GH, PRL

and βTSH expression (Burrows *et al.*, 2000), and is in fact necessary for the ontogeny of

somatotrophs, lactotrophs and thyrotrophs (Camper *et al.*, 1990; Li *et al.*, 1900; Castrillo

et al., 1991). This is demonstrated in Pit-1 deficient dwarf Snell and Jackson mice,

which have dysfunctional mutations of the Pit-1 gene (Cohen *et al.*, 1996), and Ames

mice, which have a mutation in the PROP-1 gene that results in Pit-1 deficiencies

(Anderson and Rosenfeld, 1994). Both strains of mice are deficient in somatotrophs,

lactotrophs and thyrotrophs (Chen *et al.*, 1990). In chickens, Pit-1 mRNA has been

localized in the pituitary as early as embryonic day (ED) 5, which was thought to precede

the gene expression and secretion of GH, PRL and βTSH (Van As *et al.*, 2000).

B. Pituitary Specificity?

Pit-1 mRNA is present in all five anterior pituitary hormone secreting cell types

of the human, porcine and rat pituitary (Malagon *et al.*, 1996), although the Pit-1 protein

was thought to be localized only in somatotrophs, lactotrophs and thyrotrophs (Simmons

et al., 1990; Asa *et al.*, 1993; Puy and Asa, 1996), indicating cell specific regulation at a

transcriptional level. However, other studies have found Pit-1 protein co-localized in rat

gonadotrophs (Vidal *et al.*, 1998) and in all five cell types of the porcine anterior pituitary

gland (Malagon *et al.*, 1996). Moreover, Lin *et al.* (1994) have identified a Pit-1 independent line of pituitary thyrotrophs that are present in the fetal rat but disappear in the adult. This suggests that some fetal pituitary cells expressing GH, PRL and βTSH may do so independently of Pit-1.

Pit-1 was thought to be specific to the pituitary because early studies indicated that it was absent from intestine, liver, lung, spleen, thyroid, thymus, pancreas, kidney, muscle, heart, testis, prostate, uterus, brain, and neural retina, as well as absent from fibroblasts, hepatocytes, and placental cells (Bodner and Karin, 1987; Ingraham *et al.*, 1988; Simmons *et al.*, 1990; Lew *et al.*, 1993). However, more recent studies have located Pit-1 protein and mRNA in a variety of extrapituitary sites (Table 1.4). Pit-1 is present in the developing nervous system of rats in the brain and neural retina, but is thought to be absent in the adult rat brain (He *et al.*, 1989), although Torner *et al.* (1999) demonstrated the presence of Pit-1 in the brain of female rats at estrous. Pit-1 has also been localized in immune tissues. Pit-1 mRNA has been shown, by RT-PCR and Southern blotting, to be present in human and rat bone marrow, and in human, rat and chicken spleen and thymus (Table 1.4). Similarly, a Pit-1 mRNA isoform has been found in fetal bovine thymocytes and splenocytes (Chen *et al.*, 1997), and Kooijman *et al.* (1997b) have found a Pit-1 splice variant in circulating human neutrophils. Since the transcription of pituitary GH, PRL and βTSH is Pit-1 dependent, extrapituitary expression of these hormones may be dependent on Pit-1 as well. The expression of GH, PRL and βTSH in extrapituitary tissues may, however, be Pit-1 independent.

This possibility is supported by several studies. GH gene expression occurs in the bone marrow of the Pit-1 deficient Snell dwarf mouse (Blalock and Weigent, 1994;

Kooijman *et al.*, 1997b). Also, GH production in the canine mammary gland (Lantinga-van Leeuwen *et al.*, 1999) and in the pars tuberalis (Bockmann *et al.*, 1997a; Sakai *et al.*, 1999b) occurs in the absence of Pit-1. Moreover, although Pit-1 is present in the placenta, Pit-1 has no effect on placental hGH-V expression (Schanke *et al.*, 1997) and is not required for hGH-V expression in peripheral mononuclear blood cells, since this expression occurs in Pit-1 deficient individuals that are unable to express the hGH-N gene (Melen *et al.*, 1997). Similarly, prolactin gene expression occurs in extrapituitary tissues that do not express Pit-1. PRL is expressed in uterine sarcoma cells in the absence of Pit-1 (Gellersen *et al.*, 1995), as well as in kidney and decidual cells (Sun *et al.*, 1994). The expression of prolactin in lymphocytes and endometrial stroma (Gellersen *et al.*, 1995) and the rat brain (Emanuele *et al.*, 1992) is also Pit-1 independent.

Pit-1 may control the expression of some extrapituitary GH, PRL and βTSH since it has been co-localized with GH and PRL in some tissues. For example, Chen *et al.* (1997) and Kooijman *et al.* (1997b) co-localized GH with Pit-1 in bovine lymphoid cells and human neutophils, respectively, while Sakai *et al.* (1999a) found PRL and Pit-1 in murine kidney cells. However, Pit-1 expression in extrapituitary sites might be unrelated to GH, PRL and βTSH expression since Pit-1 is known to have other roles which include regulating the activity of the renin gene (Gilbert *et al.*, 1994; Catanzaro *et al.*, 1994; Germain *et al.*, 1996) and the genes for the somatostatin (Baumeister and Meyerhof, 1998) and GHRH (Lin *et al.*, 1992; Iguchi *et al.*, 1999; Miller *et al.*, 1999; Gaylinn, 1999) receptors.

V. SUMMARY

The growth and development of the vertebrate embryo is a complex process involving many different spatially and temporally produced growth factors. Early embryonic growth is considered to be independent of pituitary hormones since the pituitary gland does not produce and secrete hormones until mid-late gestation in mammals (Wilson and Wyatt, 1993) and until mid-incubation in birds (Thommes and Hylka, 1978; Porter *et al.*, 1995a,b; Gregory *et al.*, 1998). Therefore, early embryonic development must be under the control of locally produced factors exerting their effects in autocrine or paracrine manners.

The presence of "pituitary" hormones in extrapituitary tissues is largely based on protein immunoreactivity. This does not, however, provide much information on their structural characteristics nor on the genes that express these proteins. It could also result from artefactual cross-reactivity in the immunological techniques used. Further characterization of extra-pituitary phenomena is therefore warranted and the focus of the research described in this thesis.

Table 1.1 betaTSH in Avian Anterior Pituitary

Cephalic Lobe	Species	Reference
	chicken	Porter *et al.* , 2000
		Bergman *et al.* , 1993
	drake	Sharpe *et al.* , 1979
	duck	Marchand and Bugnon, 1972, 1973
	Japanese Quail	Mikami *et al.* , 1975

Both Lobes	Species	Reference
	Chinese quail	Harrisson *et al.* , 1982
	duck	Hassan, 1975
		Tixier-Vidal *et al.* , 1961
	goose	Hassan, 1975
	chicken	Radke and Chiasson, 1974
	pigeon	Matsuo *et al.* , 1969
	White-crowned sparrow	Matsuo *et al.* , 1969
	Orange weaver	Gourdji, 1965

Table 1.2 betaTSH in Extrapituitary Tissues

Tissue	Species	mRNA	Protein	Reference
Brain	Monkey		x	Hojvat *et al.*, 1982b
	Rat		x	Hojvat *et al.*, 1982a
			x	Ottenweller and Hedge, 1982
			x	De Vito *et al.*, 1985
			x	De Vito *et al.*, 1989
Retina	Human		x	Fernandez-Trujillo *et al.*, 1996
	Chicken		x	Prada *et al.*, 2000
Immune	Human		x	Smith *et al.*, 1983
		x	x	Harbour *et al.*, 1989
		x		Peele *et al.*, 1993
			x	Bodey *et al.*, 2000
Gut	Rat		x	Wang *et al.*, 1997
CSF	Human		x	Schaub *et al.*, 1977
Amniotic Fluid	Rat		x	Tang *et al.*, 1984
Placenta	Human		x	Harada and Hershman, 1978

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Table 1.3 betaTSH receptor in Extrapituitary Tissues

Tissue	Species	mRNA	Protein	Reference
Brain	Human		x	Lubudova *et al.* , 1999
		x		Crisanti *et al.* , 2001
	Sheep	x		Bockmann *et al.* , 1997b
	Rat	x		Crisanti *et al.* , 2001
	Bony Fish	x		Kumar *et al.* , 2000
Immune	Human		x	Coutelier *et al.* , 1990
		x		Francis *et al.* , 1991
		x	x	Dutton *et al.* , 1997
		x		Spitzweg *et al.* , 1999
	Rat	x		Murakami *et al.* , 2001
	Mouse		x	Bagriacik and Klein, 2000
Heart	Human	x		Drvota *et al.* , 1995
	Pig	x		Sellitti *et al.* , 1997
	Mouse	x		Drvota *et al.* , 1995
	Bony Fish	x		Kumar *et al.* , 2000
Muscle	Human		x	Hiromatsu *et al.* , 1996
		x		Busuttil and Frauman, 2002
	Bony Fish	x		Kumar *et al.* , 2000
Fat	Human	x		Hiromatsu *et al.* , 1996
		x		Crisp *et al.* , 1997
		x	x	Bell *et al.* , 2000
	Rat	x		Endo *et al.* , 1993, 1995
	Guinea Pig		x	Gennick *et al.* , 1986
		x		Roselli-Rehfus *et al.* , 1992
Reproductive	Chicken		x	Csaba *et al.* , 1980
			x	Shanin *et al.* , 1982a,b
			x	Woods *et al.* , 1991
	Bony Fish	x		Kumar *et al.* , 2000
Kidney	Human	x	x	Dutton *et al.* , 1997
		x		Sellitti *et al.* , 2000
	Mouse	x		Sellitti *et al.* , 2000

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Table 1.3 (cont)

Tissue	Species	mRNA	Protein	Reference
Pituitary	Human	x	x	Prummel *et al.*, 2000
		x	x	Theodoropoulou *et al.*, 2000
Adrenal	Human	x	x	Dutton *et al.*, 1997
Skin	Human		x	Daumerie *et al.*, 2002

Table 1.4 Pit-1 mRNA/protein in Extrapituitary Tissues

Tissue	Species	Reference
Brain	Rat	He *et al.*, 1989
	Mouse	Dolle *et al.*, 1990
	Chicken	Yoshizato *et al.*, 1998
Placenta	Human	Bamberger *et al.*, 1995
	Monkey	Schanke *et al.*, 1997
	Rat	Bamberger *et al.*, 1995
		Lee *et al.*, 1996
Spleen	Human	Delhase *et al.*, 1993
	Bovine	Chen *et al.*, 1997
	Rat	Delhase *et al.*, 1993
	Chicken	Harvey *et al.*, 2000b
Bone Marrow	Human	Delhase *et al.*, 1993
	Rat	Delhase *et al.*, 1993
Thymus	Human	Delhase *et al.*, 1993
	Bovine	Chen *et al.*, 1997
	Rat	Delhase *et al.*, 1993
	Chicken	Harvey *et al.*, 2000b
Lymphocytes	Human	Delhase *et al.*, 1993
Neurophils	Human	Kooijman *et al.*, 1997b
Kidney	Murine	Sakai *et al.*, 1999a

VI. REFERENCES

Agellon LB, Davies SL, Lin CM, Chen TT, Powers DA. (1988) Rainbow trout has two genes for growth hormone. *Molecular Reproduction and Development.* 1:11-7.

Allen JT, Bloor CA, Kedia RK, Knight RA, Spiteri MA. (2000) Expression of growth hormone-releasing factor, growth hormone, insulin-like growth factor-1 and its binding proteins in human lung. *Neuropeptides.* 34:98-107.

Andersen B, Rosenfeld MG. (1994) Pit-1 determines cell types during development of the anterior pituitary gland. A model for transcriptional regulation of cell phenotypes in mammalian organogenesis. *Journal of Biological Chemistry.* 269:29335-8.

Anggard EE. (1990) The endothelium--the body's largest endocrine gland? *Journal of Endocrinology.* 127:371-5.

Aramburo C, Carranza M, Sanchez R, Perera G. (1989) Partial biochemical and biological characterization of purified chicken growth hormone (cGH). Isolation of cGH charge variants and evidence that cGH is phosphorylated. *General and Comparative Endocrinology.* 76:330-9.

Aramburo C, Donoghue D, Montiel JL, Berghman LR, Scanes CG. (1990) Phosphorylation of chicken growth hormone. *Life Science.* 47:945-52.

Aramburo C, Montiel JL, Proudman JA, Berghman LR, Scanes CG. (1992) Phosphorylation of prolactin and growth hormone. *Journal of Molecular Endocrinology.* 8:183-91.

Aramburo C, Luna M, Carranza M, Reyes M, Martinez-Coria H, Scanes CG. (2000) Growth hormone size variants: changes in the pituitary during development of the chicken. *Proceedings of the Society for Experimental Biology and Medicine.* 223:67-74.

Aramburo C, Carranza M, Reyes M, Luna M, Martinez-Coria H, Berumen L, Scanes CG. (2001) Characterization of a bioactive 15 kDa fragment produced by proteolytic cleavage of chicken growth hormone. *Endocrine.* 15:231-40.

Asa SL, Kovacs K, Laszlo FA, Domokos I, Ezrin C. (1986) Human fetal adenohypophysis. Histologic and immunocytochemical analysis. *Neuroendocrinology.* 43:308-16.

Asa SL, Puy LA, Lew AM, Sundmark VC, Elsholtz HP. (1993) Cell type-specific expression of the pituitary transcription activator pit-1 in the human pituitary and pituitary adenomas. *Journal of Clinical Endocrinology and Metabolism.* 77:1275-80.

Astola A, Pendon C, Ortiz M, Valdivia MM. (1996) Cloning and expression of somatolactin, a pituitary hormone related to growth hormone and prolactin from gilthead seabream, Sparus aurata. *General and Comparative Endocrinology*. 104:330-6.

Bagriacik EU, Klien JR. (2000) The thyrotropin (thyroid-stimulating hormone) receptor is expressed on murine dendritic cells and on a subset of CD45Rbhigh lymph node T cells: functional role for thyroid-stimulating hormone during immune activation. *Journal of Immunology*. 164:6158-65.

Baker BL, Jaffe RB. (1975) The genesis of cell types in the adenohypophysis of the human fetus as observed with immunocytochemistry. *American Journal of Anatomy*. 143:137-61.

Bamberger AM, Bamberger CM, Pu LP, Puy LA, Loh YP, Asa SL. (1995) Expression of pit-1 messenger ribonucleic acid and protein in the human placenta. *Journal of Clinical Endocrinology and Metabolism*. 80:2021-6.

Barabanov VM. (1991) The determination of the cytodifferentiation of the adenohypophysis in embryonic development. *Ontogenez*. 22:175-81.

Barkan AL, Shenker Y, Grekin RJ, Vale WW, Lloyd RV, Beals TF. (1986) Acromegaly due to ectopic growth hormone (GH)-releasing hormone (GHRH) production: dynamic studies of GH and ectopic GHRH secretion. *Journal of Clinical Endocrinology and Metabolism*. 63:1057-64.

Batchelor DC, Lewis RM, Breier BH, Gluckman PD, Skinner SJ. (1998) Fetal rat lung epithelium has a functional growth hormone receptor coupled to tyrosine kinase activity and insulin-like growth factor binding protein-2 production. *Journal of Molecular Endocrinology*. 21:73-84.

Baum D, Graser G, Heib M, Krampitz G. (1990) Chicken growth hormone: cDNA-synthesis and base sequence. *Comparative Biochemistry and Physiology B*. 96:491-5.

Baumeister H, Meyerhof W. (1998) Involvement of a Pit-1 binding site in the regulation of the rat somatostatin receptor 1 gene expression. *Annals of the New York Academy of Science*. 865:390-2.

Beck-Reccoz P and Persani L. (1994) Variable biological activity of thyroid-stimulating hormone. *European Journal of Endocrinology*. 131:331-40.

Bell A, Gagnon A, Grunder L, Parikh SJ, Smith TJ, Sorisky A. (2000) Functional TSH receptor in human abdominal preadipocytes and orbital fibroblasts. *American Journal of Physiology: Cell Physiology*. 279C:335-40.

Ben-Harari RR, Youdim MB. (1983) The lung as an endocrine organ. *Biochemical Pharmacology*. 32:189-97.

Berger P, Untergasser G, Hermann M, Hittmair A, Madersbacher S, Dirnhofer S. (1999) The testis-specific expression pattern of the growth hormone/placental lactogen (GH/PL) gene cluster changes with malignancy. *Human Pathology.* 30:1201-6.

Berghman LR, Lens P, Decuypere E, Kuhn ER, Vandesande F. (1987) Glycosylated chicken growth hormone. *General and Comparative Endocrinology.* 68:408-14.

Berghman LR, Darras VM, Chiasson RB, Decuypere E, Kuhn ER, Buyse J, Vandesande F. (1993) Immunohistochemical demonstration of chicken hypophyseal thyrotropes and development of a radioimmunological indicator for chicken TSH using monoclonal and polyclonal homologous antibodies in a subtractive strategy. *General and Comparative Endocrinology.* 92:189-200.

Blalock JE, Weigent DA. (1994) Pituitary control of immune cells. *Immunology Today.* 15:39.

Bockmann J, Bockers TM, Winter C, Wittkowski W, Winterhoff H, Deufel T, Kreutz MR. (1997a) Thyrotropin expression in hypophyseal pars tuberalis-specific cells is 3,5,3'-triiodothyronine, thyrotropin-releasing hormone, and pit-1 independent. *Endocrinology.* 138:1019-28.

Bockmann J, Winter C, Wittowski W, Kreutz MR, Bockers TM. (1997b) Cloning and expression of a brain-derived TSH receptor. *Biochemical and Biophysical Research Communications.* 238:173-78.

Bodey B, Bodey Jr. B, Siegel SE, Kaiser HE. (2000) The role of the reticulo-epithelial (RE) cell network in the immuno-neuroendocrine regulation of intrathymic lymphopoiesis. *Anticancer Research.* 20:1871-1888.

Bodner M, Karin M. (1987) A pituitary-specific trans-acting factor can stimulate transcription from the growth hormone promoter in extracts of nonexpressing cells. *Cell.* 50:267-75.

Bodner M, Castrillo JL, Theill LE, Deerinck T, Ellisman M, Karin M. (1988) The pituitary-specific transcription factor GHF-1 is a homeobox-containing protein. *Cell.* 55:505-18.

Boguszewski CL, Svensson PA, Jansson T, Clark R, Carlsson LM, Carlsson B. (1998) Cloning of two novel growth hormone transcripts expressed in human placenta. *Journal of Clinical Endocrinology and Metabolism.* 83:2878-85.

Breneman WR and Rathkamp W. (1973) Release of thyroid stimulating hormone from chick anterior pituitary glands by thyrotropin releasing hormone (TRH). *Biochemical and Biophysical Research Communications.* 52: 189-94.

Buckbinder L and Brown DD. (1993) Cloning and developmental expression of *Xenopus laevis* prolactin and thyrotropin genes. *Proceedings of the National Academy of Science USA.* 90:3820-4.

Burrows HL, Douglas KR, Camper SA. (1999) Genealogy of the anterior pituitary gland: tracing a family tree. *Trends in Endocrinology and Metabolism.* 10:343-52.

Busuttil BE, Frauman AG. (2002) TSH receptor expression in cardiac muscle tissue. *Journal of Clinical Endocrinology and Metabolism.* 87:2994-

Campbell RM, Scanes CG. (1995) Endocrine peptides 'moonlighting' as immune modulators: roles for somatostatin and GH-releasing factor. *Journal of Endocrinology.* 147:383-96.

Camper SA, Saunders TL, Katz RW, Reeves RH. (1990) The Pit-1 transcription factor gene is a candidate for the murine Snell dwarf mutation. *Genomics.* 8:586-90.

Cantin M, Genest J. (1988) The heart as an endocrine gland. *Pharmacology Research Communications.* 20 Suppl 3:1-22.

Castrillo JL, Theill LE, Karin M. (1991) Function of the homeodomain protein GHF1 in pituitary cell proliferation. *Science.* 253:197-9.

Catanzaro DF, Sun J, Gilbert MT, Yan Y, Black T, Sigmund C, Gross KW. (1994) A Pit-1 binding site in the human renin gene promoter stimulates activity in pituitary, placental and juxtaglomerular cells. *Kidney International.* 46:1513-5.

Charrier J, Martal J. (1988) Growth hormones. 1. Polymorphism (minireview). *Reproduction, Nutrition and Development.* 28(4A):857-87.

Chatelain A, Dupouy JP, Dubois MP. (1979) Ontogenesis of cells producing polypeptide hormones (ACTH, MSH, LPH, GH, prolactin) in the fetal hypophysis of the rat: influence of the hypothalamus. *Cell and Tissue Research.* 196:409-27.

Chen HT, Pan FM, Chang WC. (1988) Purification of duck growth hormone and cloning of the complementary DNA. *Biochimica et Biophysica Acta.* 949:247-51.

Chen HT, Schuler LA, Schultz RD. (1997) Growth hormone and Pit-1 expression in bovine fetal lymphoid cells. *Domestic Animal Endocrinology.* 14:399-407.

Chen RP, Ingraham HA, Treacy MN, Albert VR, Wilson L, Rosenfeld MG. (1990) Autoregulation of pit-1 gene expression mediated by two cis-active promoter elements. *Nature.* 346:583-6.

Childs GV. (2000) Growth hormone cells as co-gonadotropes: partners in the regulation of the reproductive system. *Trends in Endocrinology and Metabolism.* 11:168-75.

Chin WW, Carr FE, Burnside J, Darling DS. (1993) Thyroid hormone regulation of thyrotropin gene expression. *Recent Progress in Hormone Research.* 48:393-414.

Cohen LE, Wondisford FE, Radovick S. (1996) Role of Pit-1 in the gene expression of growth hormone, prolactin, and thyrotropin. *Endocrinology and Metabolism Clinics of North America.* 25:523-40.

Coleman RL, Lindberg G, Muller CY, Miller DS, Hameed A. (2000) Ectopic production and localization of beta-human chorionic gonadotropin in lymphoepithelioma-like carcinoma of the cervix: a case report. *International Journal of Gynecological Pathology.* 19:179-82.

Costa A, Zoppetti G, Benedetto C, Bertino E, Marozio L, Fabris C, Arisio R, Giraudi GF, Testori O, Ariano M, et al. (1993) Immunolike growth hormone substance in tissues from human embryos/fetuses and adults. *Journal of Endocrinology Investigation.* 16:625-33.

Courtois SJ, Lafontaine DA, Rousseau GG. (1992) Characterization of an alternative promoter in the human growth hormone gene. *Journal of Biological Chemistry.* 267:19736-43.

Coutelier JP, Kehrl JH, Bellur SS, Kohn LD, Notkins AL, Prabhakar BS. (1990) Binding and functional effects of thyroid stimulating hormone on human immune cells. *Journal of Clinical Immunology.* 10:204-

Crisanti P, Omri B, Hughes E, Meduri G, Hery C, Clauser E, Jacquemin C, Saunier B. (2001) The expression of thyrotropin receptor in the brain. *Endocrinology.* 142(2):812-22.

Crisp MS, Lane C, Halliwell M, Wynford-Thomas D, Ludgate M. (1997) Thyrotropin receptor transcripts in human adipose tissue. *Journal of Clinical Endocrinology and Metabolism.* 82:2003-5.

Croyle ML and Maurer RA. (1984) Thyroid hormone decreases thyrotropin subunit mRNA levels in rat anterior pituitary. *DNA.* 3:231-6.

Csaba G, Shahin MA, Dobozy O. (1980) The overlapping effect of gonadotropins and TSH on embryonic chicken gonads. *Anatomia Histologia Embryolia.* 63:31-38.

Daumerie C, Ludgate M, Costagliola S, Many MC. (2002) Evidence for thyrotropin receptor immunoreactivity in pretibial connective tissue from patients with thyroid-associated dermopathy. *European Journal of Endocrinology.* 146:35-8.

Decuypere E, Scanes CG. (1983) Variation in the release of thyroxine, triiodothyronine and growth hormone in response to thyrotrophin releasing hormone during development of the domestic fowl. *Acta Endocrinologica.* 102(2):220-3.

Delgado E, Finkel V, Baggiolini M, Mackay CR, Steinman RM, Granelli-Piperno A. (1998) Mature dendritic cells respond to SDF-1, but not to several beta-chemokines. *Immunobiology*. 198:490-500.

Delhase M, Vergani P, Malur A, Hooghe-Peters EL, Hooghe RJ. (1993) The transcription factor Pit-1/GHF-1 is expressed in hemopoietic and lymphoid tissues. *European Journal of Immunology*. 23:951-5.

DeNef C. (1986) Paracrine interactions in the anterior pituitary. *Clinical Endocrinology and Metabolism*. 15:1-32.

DeVito WJ, Connors JM, Hedge GA. (1985) Distribution and release of immunoreactive thyroid-stimulating hormone in the rat hypothalamus: effects of thyroidectomy, hypophysectomy and treatment with thyroid hormones. *Neuroendocrinology*. 41:23-30.

DeVito WJ. (1989) Thyroid hormone regulation of hypothalamic immunoreactive thyrotropin. *Endocrinology*. 125:1219-1223.

Dolle P, Castrillo JL, Theill LE, Deerinck T, Ellisman M, Karin M. (1990) Expression of GHF-1 protein in mouse pituitaries correlates both temporally and spatially with the onset of growth hormone gene activity. *Cell*. 60:809-20.

Drakakis P, Loutradis D, Milingos S, Michalas S, Kallianidis K, Bletsa R, Aravantinos D, Kiessling AA. (1995) A preliminary study of the effect of growth hormone on mouse preimplantation embryo development in vitro. *Gynecologic and Obstetric Investigation*. 40:222-6.

Drolet DW, Scully KM, Simmons DM, Wegner M, Chu KT, Swanson LW, Rosenfeld MG. (1991) TEF, a transcription factor expressed specifically in the anterior pituitary during embryogenesis, defines a new class of leucine zipper proteins. *Genes and Development*. 10:1739-53.

Drvota V, Janson A, Norman C, Sylven C, Haggblad J, Bronnegard M, Marcus C. (1995) Evidence for the presence of functional thyrotropin receptor in cardiac muscle. *Biochemical and Biophysical Research Communications*. 211:426-31.

Dutton CM, Joba W, Spitzweg C, Heufelder AE, Bahn RS. (1997) Thyrotropin receptor expression in adrenal, kidney, and thymus. *Thyroid*. 7:879-84.

Eberhardt NL, Jiang SW, Shepard AR, Arnold AM, Trujillo MA. (1996) Hormonal and cell-specific regulation of the human growth hormone and chorionic somatomammotropin genes. *Progress in Nucleic Acid Research*. 54:127-63.

Emanuele NV, Baker G, McDonald D, Kirsteins L, Lawrence AM. (1985) The impact of aging on luteinizing hormone (LH) and thyroid-stimulating hormone (TSH) in the rat brain. *Brain Research*. 352:179-183.

38

Emanuele NV, Jurgens JK, Halloran MM, Tentler JJ, Lawrence AM, Kelley MR. (1992) The rat prolactin gene is expressed in brain tissue: detection of normal and alternatively spliced prolactin messenger RNA. *Molecular Endocrinology.* 6:35-42.

Endo T, Ohno M, Kotani S, Gunji K, Onaya T. (1993) Thyrotropin receptor in non-thyroid tissues. *Biochemical and Biophysical Research Communications.* 190:774-9.

Endo T, Ohta K, Haraguchi K, Onaya T. (1995) Cloning and functional expression of a thyrotropin receptor cDNA from rat fat cells. *Journal of Biological Chemistry.* 270:10833-7.

Fernandez-Trujillo FJ, Prada A, Verastegui C. (1996) Thyrotropin-like immunoreactivity in human retina: immunoreactive co-localization in ganglion cells and perivascular fibers. *Neurochemistry International.* 28:381-4.

Foster DN, Kim SU, Enyeart JJ, Foster LK. (1991) Nucleotide sequence of the complementary DNA for turkey growth hormone. *Biochemical and Biophysical Research Communications.* 173:967-75.

Francis T, Burch HB, Cai WY, Lukes Y, Peele M, Carr FE, Wartofsky L, Burman KD. (1991) Lymphocytes express thyrotropin receptor-specific mRNA as detected by the PCR technique. *Thyroid.* 1:223-8.

Frawley LS, Boockfor FR. (1991) Mammosomatotropes: presence and functions in normal and neoplastic pituitary tissue. *Endocrine Reviews.* 12:337-55.

Fukaya T, Yamanaka T, Terada Y, Murakami T, Yajima A. (1998) Growth hormone improves mouse embryo development in vitro, and the effect is neutralized by growth hormone receptor antibody. *Tohoku Journal of Experimental Medicine.* 184:113-22.

Garcia-Aragon J, Lobie PE, Muscat GE, Gobius KS, Norstedt G, Waters MJ. (1992) Prenatal expression of the growth hormone (GH) receptor/binding protein in the rat: a role for GH in embryonic and fetal development? *Development.* 114:869-76.

Gasc JM, Sar M. (1981) Appearance of LH-immunoreactive cells in the Rathke's pouch of the chicken embryo. *Differentiation.* 20:77-80.

Gaylinn BD. (1999) Molecular and cell biology of the growth hormone-releasing hormone receptor. *Growth Hormone IGF research.* 9 Suppl A:37-44.

Geffner ME. (1996) The growth without growth hormone syndrome. *Endocrinology and Metabolism Clinics of North America.* 25:649-63.

Gellersen B, Kempf R, Telgmann R, DiMattia GE. (1995) Pituitary-type transcription of the human prolactin gene in the absence of Pit-1. *Molecular Endocrinology.* 9:887-901.

39

Gennick SE, Thomas CG Jr, Nayfeh SN. (1986) Characterization of the guinea pig adipocyte thyrotropin receptor. *Biochemical and Biophysical Research Communications.* 135:208-14.

Geris KL, Berghman LR, Kuhn ER, Darras VM. (1998) Pre- and posthatch developmental changes in hypothalamic thyrotropin-releasing hormone and somatostatin concentrations and in circulating growth hormone and thyrotropin levels in the chicken. *Journal of Endocrinology.* 159:219-25.

Germain S, Konoshita T, Philippe J, Corvol P, Pinet F. (1996) Transcriptional induction of the human renin gene by cyclic AMP requires cyclic AMP response element-binding protein (CREB) and a factor binding a pituitary-specific trans-acting factor (Pit-1) motif. *Biochemical Journal.* 316 (Pt 1):107-13.

Gilbert MT, Sun J, Yan Y, Oddoux C, Lazarus A, Tansey WP, Lavin TN, Catanzaro DF. (1994) Renin gene promoter activity in GC cells is regulated by cAMP and thyroid hormone through Pit-1-dependent mechanisms. *Journal of Biological Chemistry.* 269:28049-54.

Giordano M, Marchetti C, Chiorboli E, Bona G, Momigliano Richiardi P. (1997) Evidence for gene conversion in the generation of extensive polymorphism in the promoter of the growth hormone gene. *Human Genetics.* 100:249-55.

Gourdji D. (1965) Modifications des types cellularies hypophysaries impliques dans le cycle sexual annuel chez l'Ignicolore male. *General and Comparative Endocrinology.* 5:862.

Gregory CC and Porter TE. (1997) Cloning and sequence analysis of a cDNA for the β subunit of chicken thyroid-stimulating hormone. *General and comparative Endocrinology.* 107:182-90.

Gregory CC, Dean CE, Porter TE. (1998) Expression of chicken thyroid-stimulating hormone -subunit messenger ribonucleic acid during embryonic and neonatal development. *Endocrinology.* 139:474-478.

Guidon PT, Jr, Whitfield GK, Porti D, Kourides IA. (1988). The human thyrotropin β-subunit gene differs in 5' structure from murine TSH-β gene. *DNA.* 7:691-9.

Hamori J, Mess B, Szekely G. (1959) Onset of thyroidal ^{131}I accumulation in normal and decapitated chick embryos. *Acta Biologica Hungarica.* 10: 207-14.

Harada A, Hershman JM. (1978). Extraction of human chorionic thyrotropin (hCT) from term placentas: failure to recover thyrotropic activity. *Journal of Clinical Endocrinology and Metabolism.* 47:681-685.

Haraguchi K, Shimura H, Kawaguchi A, Ikeda M, Endo T, Onaya T. (1999) Effects of thyrotropin on the proliferation and differentiation of cultured rat preadipocytes. *Tyroid.* 9:613-9.

Harbour DV, Kruger TE, Coppenhaver D, Smith EM, Meyer WJ. (1989) Differential expression and regulation of thyrotropin (TSH) in T cell lines. *Molecular and Cellular Endocrinology.* 64:229-241.

Harrisson F, Van Hoof J, Vakaet L. (1982) The relationship between the folliculo-stellate network and the thyrotropic cells of the avian adenohypophysis. *Cell Tissue Research.* 226:97-111.

Harvey S, Davison TF, Chadwick A. (1979) Ontogeny of growth hormone and prolactin secretion in the domestic fowl (Gallus domesticus). *General and Comparative Endocrinology.* 39:270-3.

Harvey S, Hull KL, Fraser RA. (1993) Growth hormone: neurocrine and neuroendocrine perspectives. *Growth and Regulation.* 3:161-71.

Harvey S. (1995) Growth Hormone-Sevreting Cells. *In* "Growth Hormone" (Harvey S, Scanes CG, Daughaday WH, Eds), pp 39-54. Boca Raton, CRC Press, Inc.

Harvey S, Hull KL. (1997) Growth hormone. A paracrine growth factor? *Endocrine.* 7:267-79.

Harvey S, Johnson CD, Sharma P, Sanders EJ, Hull KL. (1998) Growth hormone: a paracrine growth factor in embryonic development? *Comparative Biochemistry and Physiology C: Pharmacology, Toxicology and Endocrinology.* 119:305-15.

Harvey S, Johnson CD, Sanders EJ. (2000a) Extra-pituitary growth hormone in peripheral tissues of early chick embryos. *Journal of Endocrinology.* 166:489-502.

Harvey S, Azumaya Y, Hull KL. (2000b) Pituitary and extrapituitary growth hormone: Pit-1 dependence? *Canadian Journal of Physiology and Pharmacology.* 78:1013-28.

Harvey S, Johnson CD, Sanders EJ. (2001a) Growth hormone in neural tissues of the chick embryo. *Journal of Endocrinology.* 169:487-98.

Harvey S, Lavelin I, Pines M. (2001b) Growth hormone (GH) action in early embryogenesis: expression of a GH-response gene in sites of GH production and action. *Anatomy and Embryology.* 204:503-10.

Hassan A. (1975) Histology and ultrastructure of certain endocrine glands in geese and ducks during sexual cycles. PhD thesis, The University of Verterinary Science, Budapest.

Haugen BR, Wood WM, Gordon DF, Ridgway EC. (1993) A thyrotrope-specific variant of Pit-1 transactivates the thyrotropin beta promoter. *Journal of Biological Chemistry.* 268:20818-24.

He X, Treacy MN, Simmons DM, Ingraham HA, Swanson LW, Rosenfeld MG. (1989) Expression of a large family of POU-domain regulatory genes in mammalian brain development. *Nature.* 340:35-41.

Henderson IW. (1987) The expanding endocrine system. *Journal of Endocrinology.* 115:195-7.

Hiromatsu Y, Sato M, Inoue Y, Koga M, Miyake I, Kameo J, Tokisawa S, Yang D, Nonaka K. (1996) Localization and clinical significance of thyrotropin receptor mRNA expression in orbital fat and eye muscle tissues from patients with thyroid-associated ophthalmopathy. *Thyroid.* 6:553-62.

Hojvat S, Emanuele N, Baker G, Connick E, Kirsteins L, Lawrence AM. (1982a) Growth hormone (GH), thyroid-stimulating hormone (TSH), and luteinizing hormone (LH)-like peptides in the rodent brain: non-parallel ontogenetic development with pituitary counterparts. *Brain Research.* 256:427-434.

Hojvat S, Baker G, Kirsteins L, Lawrence AM. (1982b) TSH in the rat and monkey brain. Distribution, characterization and effect of hypophysectomy. *Neuroendocrinology.* 34:327-332.

Hojvat S, Emanuele N, Baker G, Kirsteins L, Lawrence AM. (1985) Brain thyroid-stimulating hormone: effects of endocrine manipulations. *Brain Research.* 360:257-263.

Holdstock JG and Burrin JM. (1994) Regulation of glycoprotein hormone free alpha-subunit secretion and intracellular alpha-subunit content in primary pituitary cells. *Endocrinology.* 134:685-94.

Hsieh Y, Chatterjee A, Lee G, Yu JY. (2000) Molecular cloning and sequence analysis of the cDNA for thyroid-stimulating hormone β subunit of Muscovy duck. *General and Comparative Endocrinology.* 120:336-44.

Hu L, Lytras A, Bock ME, Yuen CK, Dodd JG, Cattini PA. (1999) Detection of placental growth hormone variant and chorionic somatomammotropin-L RNA expression in normal and diabetic pregnancy by reverse transcriptase-polymerase chain reaction. *Molecular and Cellular Endocrinology.* 157:131-42.

Hull KL, Thiagarajah A, Harvey S. (1996) Cellular localization of growth hormone receptors/binding proteins in immune tissues. *Cell and Tissue Research.* 286:69-80.

Hull KL, Harvey S. (1998) Autoregulation of central and peripheral growth hormone receptor mRNA in domestic fowl. *Journal of Endocrinology.* 156:323-9.

Hull KL, Murphy A, Harvey S. (2000) Avian somatotrophs: differentiation, morphology, distribution, and regulation. *Canadian Journal of Physiology and Pharmacology.* 78:994-1002.

Iguchi G, Okimura Y, Takahashi T, Mizuno I, Fumoto M, Takahashi Y, Kaji H, Abe H, Chihara K. (1999) Cloning and characterization of the 5'-flanking region of the human growth hormone-releasing hormone receptor gene. *Journal of Biological Chemistry.* 274:12108-14.

Indinnimeo M, Cicchini C, Memeo L, Stazi A, Ghini C, Ricci F, Reale MG, Mingazzini P. (2001) Plasma and tissue prolactin detection in colon carcinoma. *Oncology Report.* 8:1351-3.

Ingraham HA, Chen RP, Mangalam HJ, Elsholtz HP, Flynn SE, Lin CR, Simmons DM, Swanson L, Rosenfeld MG. (1988) A tissue-specific transcription factor containing a homeodomain specifies a pituitary phenotype. *Cell.* 55:519-29.

Ip SC, Zhang X, Leung FC. (2001) Genomic growth hormone gene polymorphisms in native Chinese chickens. *Experimental Biology and Medicine.* 226:458-62.

Iqbal A, Decuypere E, Kühn ER, Schneider R, Verheyen G, Huybrechts LM. (1987) The influence of methimazole on the thyrotrophic and peripheral activity of thyrotrophin and thyrotrophin-releasing hormone in the chick embryo and growing chicken. *Domestic Animal Endocrinology.* 4:291-298.

Ito M, Koide Y, Takamatsu N, Kawauchi H, Shiba T. (1993) cDNA cloning of the β subunit of teleost thyrotropin. *Proceedings of the National Academy of Science USA.* 90:6053-5.

Izadyar F, Van Tol HT, Hage WG, Bevers MM. (2000) Preimplantation bovine embryos express mRNA of growth hormone receptor and respond to growth hormone addition during in vitro development. *Molecular Reproduction and Development.* 57:247-55.

Jacobson G, Roos P. Wide L. (1977) Human pituitary thyrotropin. Characterization of five glycoproteins with thyrotropin activity. *Biochemica et Biophysica Acta.* 490:403-10.

Janson A, Rawet H, Perbeck L, Marcus C. (1998) Presence of thyrotropin receptor in infant adipocytes. *Pediatric Research.* 43:555-8.

Jin L, Qian X, Kulig E, Scheithauer BW, Calle-Rodrigue R, Abboud C, Davis DH, Kovacs K, Lloyd RV. (1997) Prolactin receptor messenger ribonucleic acid in normal and neoplastic human pituitary tissues. *Journal of Clinical Endocrinology and Metabolism.* 82:963-8.

43

Jozsa R, Scanes CG, Vigh S, Mess B. (1979) Functional differentiation of the embryonic chicken pituitary gland studied by immunohistological approach. *General and Comparative Endocrinology.* 39:158-63.

Kansaku N, Shimada K, Terada O, Saito N. (1994) Prolactin, growth hormone, and luteinizing hormone-beta subunit gene expression in the cephalic and caudal lobes of the anterior pituitary gland during embryogenesis and different reproductive stages in the chicken. *General and comparative Endocrinology.* 96:197-205.

Kato Y, Kato T, Tomizawa K, Iwasawa A. (1997) Molecular cloning of quail thyroid-stimulating hormone (THS) β subunit. *Endocrinology Journal.* 44:837-40.

Kato Y, Kato T, Tomizawa K, Kamiyoshi M, Iwasawa A. (1998) Complementary DNA sequence of chicken thyroid-stimulating hormone (THS) β subunit. *Endocrinology Journal.* 45:591-4.

Kolata G. (1982) New theory of hormones proposed. *Science.* 215:1383-4.

Konzak KE, Moore DD. (1992) Functional isoforms of Pit-1 generated by alternative messenger RNA splicing. *Molecular Endocrinology.* 6:241-7.

Kooijman R, Malur A, Van Buul-Offers SC, Hooghe-Peters EL. (1997a) Growth hormone expression in murine bone marrow cells is independent of the pituitary transcription factor Pit-1. *Endocrinology.* 138:3949-55.

Kooijman R, Berus D, Malur A, Delhase M, Hooghe-Peters EL. (1997b) Human neutrophils express GH-N gene transcripts and the pituitary transcription factor Pit-1b. *Endocrinology.* 138:4481-4.

Krishnan KA, Proudman JA, Bahr JM. (1994) Purification and partial characterization of isoforms of luteinizing hormone from the chicken pituitary gland. *Comparative Biochemistry and Physiology.* 108B:253-64.

Kühn ER, Decuypere E, Iqbal A, Luysterborgh D, Michielsen R. (1988) Thyrotropic and peripheral activities of thyrotrophin and thyrotropin-releasing hormone in the chick embryo and adult chicken. *Hormone and Metabolism Research.* 20:158-162.

Kuhnlein U, Ni L, Weigend S, Gavora JS, Fairfull W, Zadworny D. (1997) DNA polymorphisms in the chicken growth hormone gene: response to selection for disease resistance and association with egg production. *Animal Genetics.* 28:116-23.

Kumar RS, Ijiri S, Kight K, Swanson P, Dittman A, Alok D, Zohar Y, Trant JM. (2000) Cloning and functional expression of a thyrotropin receptor from the gonads of a vertebrate (bony fish): potential thyroid-independent role for thyrotropin in reproduction. *Molecular and Cellular Endocrinology.* 167:1-9.

Kurima K, Weatherly KL, Sharova L, Wong EA. (1998) Synthesis of turkey Pit-1 mRNA variants by alternative splicing and transcription initiation. *DNA Cell Biology.* 17:93-103.

Kyle CV, Evans MC, Odell WD. (1981) Growth hormone-like material in normal human tissues. *Journal of Clinical Endocrinology and Metabolism.* 53:1138-44.

Labudova O, Cairns N, Koeck T, Kitzmueller E, Rink H, Lubec G. (1999) Thyroid stimulating hormone-receptor overexpression in the brain of patients with Down syndrome and Alzheimer's disease. *Life Science.* 64:1037-1044.

Lacroix MC, Devinoy E, Servely JL, Puissant C, Kann G. (1996) Expression of the growth hormone gene in ovine placenta: detection and cellular localization of the protein. *Endocrinology.* 137:4886-92.

Lantinga-van Leeuwen IS, Mol JA, Kooistra HS, Rijnberk A, Breen M, Renier C, van Oost BA. (1999) Cloning of the canine gene encoding transcription factor Pit-1 and its exclusion as candidate gene in a canine model of pituitary dwarfism. *Mammalian Genome.* 11:31-6.

Law MS, Cheng KW, Fung TK, Chan YH, Yu KL, Chan KM. (1996) Isolation and characterization of two distinct growth hormone cDNAs from the goldfish, Carassius auratus. *Archives of Biochemistry and Biophysics.* 330:19-23.

Lee BJ, Jeong JK, Kim JH, Kang SG, Kim MO, Choi WS. (1996) Local expression of a POU family transcription factor, Pit-1, in the rat placenta. *Molecular and Cellular Endocrinology.* 118:9-14.

Lew D, Brady H, Klausing K, Yaginuma K, Theill LE, Stauber C, Karin M, Mellon PL. (1993) GHF-1-promoter-targeted immortalization of a somatotropic progenitor cell results in dwarfism in transgenic mice. *Genes and Development.* 7:683-93.

Lewis UJ, Bonewald LF, Lewis LJ. (1980) The 20,000-dalton variant of human growth hormone: location of the amino acid deletions. *Biochemical and Biophysical Research Communications.* 92:511-6.

Lewis UJ. (1984) Variants of growth hormone and prolactin and their posttranslational modifications. *Annual Reviews of Physiology.* 46:33-42.

Li S, Crenshaw EB 3rd, Rawson EJ, Simmons DM, Swanson LW, Rosenfeld MG. (1990) Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene pit-1. *Nature.* 347:528-33.

Lin C, Lin SC, Chang CP, Rosenfeld MG. (1992) Pit-1-dependent expression of the receptor for growth hormone releasing factor mediates pituitary cell growth. *Nature.* 360:765-8.

Lin SC, Li S, Drolet DW, Rosenfeld MG. (1994) Pituitary ontogeny of the Snell dwarf mouse reveals Pit-1-independent and Pit-1-dependent origins of the thyrotrope. *Development.* 120:515-22.

Liu N, Mertani HC, Norstedt G, Tornell J, Lobie PE. (1997) Mode of the autocrine/paracrine mechanism of growth hormone action. *Experimental Cell Research.* 237:196-206.

Lobie PE, Breipohl W, Lincoln DT, Garcia-Aragon J, Waters MJ. (1990a) Localization of the growth hormone receptor/binding protein in skin. *Journal of Endocrinology.* 126:467-71.

Lobie PE, Breipohl W, Waters MJ. (1990b) Growth hormone receptor expression in the rat gastrointestinal tract. *Endocrinology.* 126:299-306.

Lorens JB, Nerland AH, Aasland R, Lossius I, Male R. (1993) Expression of growth hormone genes in Atlantic salmon. *Journal of Molecular Endocrinology.* 11:167-79.

Lytras A, Bock ME, Dodd JG, Cattini PA. (1994) Detection of placental growth hormone variant and chorionic somatomammotropin ribonucleic acid expression in human trophoblastic neoplasms by reverse transcriptase-polymerase chain reaction. *Endocrinology.* 134:2461-7.

MacLeod JN, Lee AK, Liebhaber SA, Cooke NE. (1992) Developmental control and alternative splicing of the placentally expressed transcripts from the human growth hormone gene cluster. *Journal of Biological Chemistry.* 267:14219-26.

Maggiano N, Piantelli M, Ricci R, Larocca LM, Capelli A, Ranelletti FO. (1994) Detection of growth hormone-producing cells in human thymus by immunohistochemistry and non-radioactive in situ hybridization. *Journal of Histochemistry and Cytochemistry.* 42:1349-54.

Malagon MM, Garrido JC, Dieulois C, Hera C, Castrillo JL, Dobado-Berrios PM, Gracia-Navarro F. (1996) Expression of the pituitary transcription factor GHF-1/PIT-1 in cell types of the adult porcine adenohypophysis. *Journal of Histochemistry and Cytochemistry.* 44:621-7.

Malamed S, Gibney JA, Scanes CG. (1988) Immunogold identification of the somatotrophs of domestic fowl of different ages. *Cell and Tissue Research.* 251:581-5. Manger JA. (1989) Thyroid stimulating hormone: structure and function. *Advances in Experimental Medicine and Biology.* 261:27-103.

Malamed S, Gibney JA, Cain LD, Perez FM, Scanes CG. (1993) Immunocytochemical studies of chicken somatotrophs and somatotroph granules before and after hatching. *Cell and Tissue Research.* 272:369-74.

Marchand CR and Bugnon C. (1972) Characterisation et localisation des cellules "thyreoprive" l'adenohypophyse des Canards males et femelles Pekin, Barbarie et hybrides issues de croisement male Pekin x femelle Barbarie. *C.R. Academie de Science (Paris).* 274:2335-7.

Marchand CR and Bugnon C. (1973) La response "thyreoprive" de l'adenohypophyse des Canards males et femelles Barbarie, Pekin et hybrides (du croisement Canard Pekin x cane de Barbarie) comparee aux effects de la castration. *Bulletin de l'Association des anatomistes.* 57:157-164.

Marcus C, Ehren H, Bolme P, Arner P. (1988) Regulation of lipolysis during the neonatal period. Importance of thyrotropin. *Journal of Clinical Investigation.* 82:1793-7.

Martial JA, Hallewell RA, Baxter JD, Goodman HM. (1979) Human growth hormone: complementary DNA cloning and expression in bacteria. *Science.* 205:602-7.

Martin CR. (1985) What are hormones? What do they do? *In* "Endocrine Physiology". (Martin CR, Ed). pp 3-31. Oxford, Oxford University Press, Inc.

Martin SAM, Waller W, Youngson AF, Smith T. (1999) Differential expression of atlantic salmon thyrotropin β subunit and its cDNA sequence. *Journal of Fish Biology.* 54:757-66.

Martinez-Coria H, Lopez-Rosales LJ, Carranza M, Berumen L, Luna M, Aramburo C. (2002) Differential secretion of chicken growth hormone variants after growth hormone-releasing hormone stimulation in vitro. *Endocrine.* 17:91-102.

Matsuo S, Vitums A, King JR, Farner DS. (1969) Light-microscope studies of the cytology of the adenohypophysis of the white-crowned sparrow, *Zonotrichia leucophrys gambelii. Zeitschrift für Zellforschung und mikroskopische Anatomie.* 95:143-76.

Maurer RA, Croyle ML, Donalson JE. (1984) The sequence of cloned cDNA for beta subunit of bovine thyrotropin predicts a protein containing both NH_2- and COOH-terminal extensions. *Journal of Biological Chemistry.* 259:5024-7.

McCann-Levorse LM, Radecki SV, Donoghue DJ, Malamed S, Foster DN, Scanes CG. (1993) Ontogeny of pituitary growth hormone and growth hormone mRNA in the chicken. *Proceedings of the Society of Biological Medicine.* 202:109-13.

Melen L, Hennen G, Dullaart RP, Heinen E, Igout A. (1997) Both pituitary and placental growth hormone transcripts are expressed in human peripheral blood mononuclear cells (PBMC). *Clinical and Experimental Immunology.* 110:336-40.

Mertani HC, Pechoux C, Garcia-Caballero T, Waters MJ, Morel G. (1995) Cellular localization of the growth hormone receptor/binding protein in the human anterior pituitary gland. *Journal of Clinical Endocrinology and Metabolism.* 80:3361-7.

Mikami S, Kurosu T, Farner DS. (1975) Light- and electron-microscope studies on the secretory cytology of the adenohypophysis of the Japanese Quail, *Coturnix coturnix japonica. Cell and Tissue Research.* 159:147-165.

Mikami S, Takahashi H. (1987) Immunocytochemical studies on the cytodifferentiation of the adenohypophysis of the domestic fowl. *Nippon Juigaku Zasshi.*49:601-11.

Miller TL, Godfrey PA, Dealmeida VI, Mayo KE. (1999) The rat growth hormone-releasing hormone receptor gene: structure, regulation, and generation of receptor isoforms with different signaling properties. *Endocrinology.* 140:4152-65.

Misra-Press A, Cooke NE, Liebhaber SA. (1994) Complex alternative splicing partially inactivates the human chorionic somatomammotropin-like (hCS-L) gene. *Journal of Biological Chemistry.* 269:23220-9.

Mol JA, Henzen-Logmans SC, Hageman P, Misdorp W, Blankenstein MA, Rijnberk A. (1995a) Expression of the gene encoding growth hormone in the human mammary gland. *Journal of Clinical Endocrinology and Metabolism.* 80:3094-6.

Mol JA, van Garderen E, Selman PJ, Wolfswinkel J, Rijinberk A, Rutteman GR. (1995b) Growth hormone mRNA in mammary gland tumors of dogs and cats. *Journal of Clinical Investigation.* 95:2028-34.

Mol JA, van Garderen E, Rutteman GR, Rijnberk A. (1996) New insights in the molecular mechanism of progestin-induced proliferation of mammary epithelium: induction of the local biosynthesis of growth hormone (GH) in the mammary glands of dogs, cats and humans. *Journal of Steroid Biochemistry and Molecular Biolology.* 57:67-71

Morgello S, Schwartz E, Horwith M, King ME, Gorden P, Alonso DR. (1988) Ectopic insulin production by a primary ovarian carcinoid. *Cancer.* 61:800-5.

Mori T, Devlin RH. (1999) Transgene and host growth hormone gene expression in pituitary and nonpituitary tissues of normal and growth hormone transgenic salmon. *Molecular and Cellular Endocrinology.* 149:129-39.

Murakami M, Hosoi Y, Araki O, Morimura T, Imamura M, Ogiwara T, Mizuma H, Mori M. (2001) Expression of thyrotropin receptors in rat thymus. *Life Science.* 68:2781-7.

Nathanielsz PW (1976) Fetal endocrinology – an experimental approach. *Biomedical Press,* Amsterdam. 48-119.

Nguyen AP, Chandorkar A, Gupta C. (1996) The role of growth hormone in fetal mouse reproductive tract differentiation. *Endocrinology.* 137:3659-66.

Nickel BE, Kardami E, Cattini PA. (1990) Differential expression of human placental growth-hormone variant and chorionic somatomammotropin in culture. *The Biochemical Journal.* 267:653-8.

Ogilvie S, Buhi WC, Olson JA, Shiverick KT. (1990) Identification of a novel family of growth hormone-related proteins secreted by rat placenta. *Endocrinology.* 126:3271-3.

Ohta K, Shichiri M, Kameya T, Matsubara O, Imai T, Marumo F, Hirata Y. (2000) Thymic hyperplasia as a source of ectopic ACTH production. *Endocrine Journal.* 47:487-92.

Ottenweller JE, Hedge GA. (1982) Thyrotropin-like immunoreactivity in the pituitary and three brain regions of the female rat: diurnal variations and the effect of thyroidectomy. *Endocrinology.* 111:515-521.

Palmetshofer A, Zechner D, Luger TA, Barta A. (1995) Splicing variants of the human growth hormone mRNA: detection in pituitary, mononuclear cells and dermal fibroblasts. *Molecular and Cellular Endocrinology.* 113:225-34.

Pantaleon M, Whiteside EJ, Harvey MB, Barnard RT, Waters MJ, Kaye PL. (1997) Functional growth hormone (GH) receptors and GH are expressed by preimplantation mouse embryos: a role for GH in early embryogenesis? *Prceedings of the National Academy of Science* (USA). 94:5125-30.

Parks JS. (1989) Molecular biology of growth hormone. *Acta Paediatrica Scandinavica Suppl.* 349:127-35.

Peart WS. (1977) The kidney as an endocrine organ. *Lancet.* 2:543-8.

Peele ME, Carr FE, Baker Jr. JR, Wartofsky L, Burman KD. (1993) TSH beta subunit gene expression in human lymphocytes. *American Journal of Medical Science.* 305:1-7.

Pierce JG. (1971) The subunits of pituitary thyrotropin – their relationship to other glycoprotein hormones. *Endocrinology.* 89:1331-44.

Pierce JG, Parsons TF. (1981) Glycoprotein hormones: structure and function. *Annual Reviews of Biochemistry.* 50:465-95.

Porter TE, Couger GS, Dean CE, Hargis BM. (1995a) Ontogeny of growth hormone (GH)-secreting cells during chicken embryonic development: initial somatotrophs are responsive to GH-releasing hormone. *Endocrinology.* 136:1850-6.

Porter TE, Couger GS, Morpurgo B. (1995b) Evidence that somatotroph differentiation during chicken embryonic development is stimulated by a blood-borne signal. *Endocrinology.* 136:3721-8.

Porter TE. (1997) Regulation of somatotroph differentiation during chicken embryonic development: a review. *In* "Perspectives in Avian Endocrinology" (Harvey S, Etches RJ, Eds), pp 47-56. Bristol, Journal of Endocrinology Ltd.

Porter TE, Muchow M, Bossis I. (2000) Use of an antiserum to rat thyroid stimulating hormone beta (TSHβ) for studies of the TSHβ production in chickens. *In* "Abstracts: VII International Symposium on Avian Endocrinology" (Dawson A, Ed.), 8.05. Varanasi, India.

Prada JA, Verastegui C, Perez-Rios N, Gonzalez-Moreno M, Fdez-Trujillo FJ. (2000) Thyrotropin-like immunoreactivity in the developing chicken retina. *European Journal of Morphology.* 38:34-40.

Provinciali M, Di Stefano G, Fabris N. (1992) Improvement in the proliferative capacity and natural killer cell activity of murine spleen lymphocytes by thyrotropin. *International Journal of Immunopharmacology.* 14:865-70.

Prummel MF, Brokken LJ, Meduri G, Misrahi M, Bakker O, Wiersinga WM. (2000) Expression of the thyroid-simulating hormone receptor in the folliculo-stellate cells of the human anterior pituitary. *Journal of Clinical Endocrinology and Metabolism.* 85:4347-53.

Puy LA, Asa SL. (1996) The ontogeny of pit-1 expression in the human fetal pituitary gland. *Neuroendocrinology.* 63:349-55.

Querat B. (1994) Molecular evolution of the glycoprotein hormones in vertebrates. *In* "Perspectives in Comparative Endocrinology" (KG Davey, RE Peter, and SS Tobe, Eds.), pp. 27-35. National Research Council of Canada, Ottawa.

Radke WJ and Chiasson RB. (1974) TSH location in the chicken *pars distalis. Journal of Endocrinology.* 60:187-8.

Radke WJ and Chiasson RB. (1977) *In vitro* regulation of chicken thyrotropes. *General and Comparative Endocrinology.* 31:175-82.

Rahn HH. (1939) The development of the chick oituitary with special reference to the cellular differentiation of the pars buccalis. *Journal of Morphology.* 64:483-517.

Raiden S, Polack E, Nahmod V, Labeur M, Holsboer F, Arzt E. (1995) TRH receptor on immune cells: in vitro and in vivo stimulation of human lymphocyte and rat splenocyte DNA synthesis by TRH. *Journal of Clinical Immunology.* 15:242-9.

Ramesh R, Kuenzel WJ, Buntin JD, Proudman JA. (2000) Identification of growth-hormone- and prolactin-containing neurons within the avian brain. *Cell and Tissue Research.* 299:371-83.

Rand-Weaver M, Noso T, Muramoto K, Kawauchi H. (1991) Isolation and characterization of somatolactin, a new protein related to growth hormone and prolactin from Atlantic cod (Gadus morhua) pituitary glands. *Biochemistry.* 30:1509-15.

Recher S, Raccurt M, Lambert A, Lobie PE, Mertani HC, Morel G. (2001) Prenatal and adult growth hormone gene expression in rat lymphoid organs. *Journal of Histochemistry and Cytochemistry.* 49:347-54.

Render CL, Hull KL, Harvey S. (1995a) Neural expression of the pituitary GH gene. *Journal of Endocrinology.* 147:413-22.

Render CL, Hull KL, Harvey S. (1995b) Expression of growth hormone gene in immune tissues. *Endocrine.* 3:729-35.

Rodrigues CV, Guimaraes SE, Neto ED, Pinheiro LE. (1998) Identification of a novel polymorphism in the promoter region of the bovine growth hormone gene. *Animal Genetics.* 29:65-6.

Roselli-Rehfuss L, Robbins LS, Cone RD. (1992) Thyrotropin receptor messenger ribonucleic acid is expressed in most brown and white adipose tissues in the guinea pig. *Endocrinology.* 130:1857-61.

Sabharwal P, Varma S. (1996) Growth hormone synthesized and secreted by human thymocytes acts via insulin-like growth factor I as an autocrine and paracrine growth factor. *Journal of Clinical Endocrinology and Metabolism.* 81:2663-9.

Sakai T, Sakamoto S, Ijima K, Matsubara K, Kato Y, Inoue K. (1999b) Characterization of TSH-positive cells in foetal rat pars tuberalis that fail to express Pit-1 factor and thyroid hormone beta2 receptors. *Journal of Neuroendocrinology.* 11:187-93.

Sakai Y, Hiraoka Y, Ogawa M, Takeuchi Y, Aiso S. (1999a) The prolactin gene is expressed in the mouse kidney. *Kidney International.* 55:833-40.

Salmon C, Marchelidan J, Fontaine YA, Huet JC, Querat B. (1993) Cloning and sequence of thyrotropin beta-subunit of a teleost fish, the eel (*Anguilla anguilla* L). *C. R. Academy of Science Paris.* 316:749-53.

Saunier B, Pierre M, Jacquemin C, Courtin F. (1993) Evidence for cAMP-independent thyrotropin effects on astroglial cells. *European Journal of Biochemistry.* 218:1091-4.

Scanes CG. (1974) Some *in vitro* effects of synthetic thyrotropin releasing factor on the secretion of thyroid stimulating hormone from the anterior pituitary gland of the domestic fowl. *Neuroendocrinology.* 15:1-9.

Schanke JT, Conwell CM, Durning M, Fisher JM, Golos TG. (1997) Pit-1/growth hormone factor 1 splice variant expression in the rhesus monkey pituitary gland and the

rhesus and human placenta. *Journal of Clinical Endocrinology and Metabolism.* 82:800-7.

Schaub C, Bluet-Pajot MT, Szikla G, Lornet C, Talairach J. (1977) Distribution of growth hormone and thyroid-stimulating hormone in cerebrospinal fluid and pathological compartments of the central nervous system. *Journal of Neurological Science.* 31:123-31.

Schwartz J, Perez FM. (1994) Intercellular interactions in the anterior pituitary. *Journal of Endocrinology Investigation.* 17:459-70.

Schwartz J, Van de Pavert S, Clarke I, Rao A, Ray D, Vrana K. (1998) Paracrine interactions within the pituitary gland. *Annals of the New York Academy of Science.* 15;839:239-43

Schwarzler P, Untergasser G, Hermann M, Dirnhofer S, Abendstein B, Madersbacher S, Berger P. (1997) Selective growth hormone/placental lactogen gene transcription and hormone production in pre- and postmenopausal human ovaries. *Journal of Clinical Endocrinology and metabolism.* 82:3337-41.

Seeburg PH, Shine J, Martial JA, Baxter JD, Goodman HM. (1977) Nucleotide sequence and amplification in bacteria of structural gene for rat growth hormone. *Nature.* 270:486-94.

Sellitti DF, Hill R, Doi SQ, Akamizu T, Czaja J, Tao S, Koshiyama H. (1997) Differential expression of thyrotropin receptor mRNA in the porcine heart. *Thyroid.* 7:641-6

Sellitti DF, Akamizu T, Doi SQ, Kim GH, Kariyil JT, Kopchik JJ, Koshiyama H. (2000) Renal expression of two "thyroid-specific" genes: thyrotropin receptor and thyroglobulin. *Experimental Nephrology.* 8:235-43.

Shahin MA, Sudár F, Dobozy O. (1982a) Electron microscopic study of the overlapping effect of thyrotropin and gonadotropins on the Sertoli cells of chick embryos. *Zeitschrift für mikroskopisch-anatomische Forschung.* 96:1044-1068.

Shahin MA, Török O, Csaba G. (1982b) The overlapping effects of thryotropin and gonadotropins on chick embryo gonads *in vitro*. *Acta morphologica Academiae Scientiarum Hungaricae.* 30:109-125.

Sharpe PJ, Chiasson RB, el Tounsy MM, Klandorf H, Radke WJ. (1979) Localization of cells producing thyroid stimulating hormone in the pituitary gland of the domestic drake. *Cell and Tissue Research.* 198:53-63.

Simmons DM, Voss JW, Ingraham HA, Holloway JM, Broide RS, Rosenfeld MG, Swanson LW. (1990) Pituitary cell phenotypes involve cell-specific Pit-1 mRNA

translation and synergistic interactions with other classes of transcription factors. *Genes and Development.* 4:695-711.

Slominski A, Malarkey WB, Wortsman J, Asa SL, Carlson A. (2000) Human skin expresses growth hormone but not the prolactin gene. *Journal of Laboratory and Clinical Medicine.* 136:476-81.

Smith EM, Phan M, Kruger TE, Coppenhaver DH, Blalock JW. (1983) Human lymphocyte production of immunoreactive thyrotropin. *Proceedings of the National Academy of Science.* (USA). 80:6010-6013.

Sornson MW, Wu W, Dasen JS, Flynn SE, Norman DJ, O'Connell SM, Gukovsky I, Carriere C, Ryan AK, Miller AP, Zuo L, Gleiberman AS, Andersen B, Beamer WG, Rosenfeld MG. (1996) Pituitary lineage determination by the Prophet of Pit-1 homeodomain factor defective in Ames dwarfism. *Nature.* 384:327-33.

Spitzweg C, Joba W, Heufelder AE. (1999) Expression of thyroid-related genes in human thymus. *Thyroid.* 9:133-41.

Stolar MW, Amburn K, Baumann G. (1984) Plasma "big" and "big-big" growth hormone (GH) in man: an oligomeric series composed of structurally diverse GH monomers. *Journal of Clinical Endocrinology and Metabolism.* 59:212-8.

Sun J, Oddoux C, Gilbert MT, Yan Y, Lazarus A, Campbell WG Jr, Catanzaro DF. (1994) Pituitary-specific transcription factor (Pit-1) binding site in the human renin gene 5'-flanking DNA stimulates promoter activity in placental cell primary cultures and pituitary lactosomatotropic cell lines. *Circulation Research.* 75:624-9.

Szkudlinski MW, Thotakura NR, Weintraub BD. (1995) Subunit-specific functions of N-linked oligosaccharides in human thyrotropin: role of terminal residues of α- and β-subunit oligosaccharides I metabolic clearence and bioactivity. *Proceedings of the National Academy of Science USA.* 92:9062-6.

Takeuchi S, Haneda M, Teshigawara K, Takahashi S. (2001) Identification of a novel GH isoform: a possible link between GH and melanocortin systems in the developing chicken eye. *Endocrinology.* 142:5158-66.

Tanaka M, Hosokawa Y, Watahiki M, Nakashima K. (1992) Structure of the chicken growth hormone-encoding gene and its promoter region. *Gene.* 112:235-9.

Tanaka M, Yamamoto I, Ohkubo T, Wakita M, Hoshino S, Nakashima K. (1999) cDNA cloning and developmental alterations in gene expression of the two Pit-1/GHF-1 transcription factors in the chicken pituitary. *General and Comparative Endocrinology.* 114:441-8.

Tang F, Wong PY. (1984) Immunreactive TSH in the amniotic fluid of the rat. *Experientia.* 15;40:575-6.

Tatsumi K, Notomi T, Amino N, Miyai K. (1992) Nucleotide sequence of the complementary DNA for human Pit-1/GHF-1. *Biochimica Biophysica Acta.* 1129:231-4.

Theill LE, Hattori K, Lazzaro D, Castrillo JL, Karin M. (1992) Differential splicing of the GHF1 primary transcript gives rise to two functionally distinct homeodomain proteins. *EMBO Journal.* 11:2261-9.

Theodoropoulou M, Arzberger T, Grubler Y, Korali Z, Mortini P, Joba W, Heufelder AE, Stalla GK, Schaaf L. (2000) Thyrotropin receptor protein expression in normal and adenomatous human pituitary. *Journal of Endocrinology.* 167:7-13.

Thommes RC, Hylka VW. (1977) Plasma iodothyronines in the embryonic and immediate post hatch chick. *General and Comparative Endocrinology.* 32:417-22.

Thommes RC, Vieth RL, Levasseur S. (1977) The effects of hypophysectomy by means of surgical decapitation on thyroid function in the developing chick embryo. I. Plasma thyroxine. *General and Comparative Endocrinology.* 31:29-36.

Thommes RC, Hylka VW. (1978) Hypothalamo-adenohypophyseal-thyroid interrelationships in the chick embryo. I. TRH and TSH Sensitivity. *General and Comparative Endocrinology.* 34:193-200.

Thommes RC, Martens JB, Hopkins WE, Caliendo J, Sorrentino MJ, Woods JE. (1983) Hypothalamo-adenohypophyseal-thyroid interrelationships in the chick embryo. IV. Immunocytochemical demonstration of TSH in the hypophyseal pars distalis. *General and Comparative Endocrinology.* 51:434-443.

Thommes RC, Umporowicz DM, Leung FC, Woods JE. (1987) Ontogenesis of immunocytochemically demonstrable somatotrophs in the adenohypophyseal pars distalis of the developing chick embryo. *General and Comparative Endocrinology.* 67:390-8.

Tixier-Vidal A. (1954) Etude histophysiologique de l'hypophyse anterieur de l'embryon de poulet. *Archives d'Anatomie Microscopie et de Morphologie Experimentale.* 43:463-86.

Tixier-Vidal A. (1959) Etude chronologique *in vivo* et *in vitro* des correlations hypophyse-thyroide chez l'embryon de poulet. *Archives d'Anatomie Microscopie et de Morphologie Experimentale.* 45:236-53.

Tixier-Vidal A and Assenmacher I. (1961) Etude comparee de l'activite thyroidienne chez le Canard male normal castre ou maintenu a l'obscurite permanente *I. C.R. Societe Biologique (Paris)* 155:215-20; *II. C.R. Societe Biologique (Paris).* 155:286-90.

Tixier-Vidal A, Chandola A, Franquelin F. (1972) "Cellules de thyrodectomie" et "cellules de castration" chez la Caille japonaise, *Coturnix coturnix japonica*. Etude ultrastructurale et cytoenzymologi. *Zeitschrift für Zellforschung und mikroskopische Anatomie*. 125: 506-531.

Torner L, Nava G, Duenas Z, Corbacho A, Mejia S, Lopez F, Cajero M, Martinez de la Escalera G, Clapp C. (1999) Changes in the expression of neurohypophyseal prolactins during the estrous cycle and after estrogen treatment. *Journal of Endocrinology*. 161:423-32.

Torres AI, Pasolli HA, Maldonado CA, Aoki A. (1995) Changes in the thyrotrph and somatotroph cell populations induced by stimulation and inhibition of their secretory activity. *Histochemical Journal*. 27:370-9.

Tournier C, Gavaret JM, Jacquemin C, Pierre M, Saunier B. (1995) Stimulation of mitogen-activated protein kinase by thyrotropin in astrocytes. *European Journal of Biochemistry*. 228:16-22.

Tresguerres JA, Ariznavarreta C, Granados B, Costoya JA, Perez-Romero A, Salame F, Hermanussen M. (1999) Salivary gland is capable of GH synthesis under GHRH stimulation. *Journal of Endocrinology*. 160:217-22.

Tuggle CK, Yu TP, Helm J, Rothschild MF. (1993) Cloning and restriction fragment length polymorphism analysis of a cDNA for swine PIT-1, a gene controlling growth hormone expression. *Animal Genetics*. 24:17-21.

Untergasser G, Kranewitter W, Schwarzler P, Madersbacher S, Dirnhofer S, Berger P. (1997) *Molecular and Cellular Endocrinology*. 130:53-60.

Van As P, Buys N, Onagbesan OM, Decuypere E. (2000) Complementary DNA cloning and ontogenic expression of pituitary-specific transcription factor of chickens (Gallus domesticus) from the pituitary gland. *General and Comparative Endocrinology*. 120:127-36.

Vidal S, Horvath E, Kovacs K, Cohen SM, Lloyd RV, Scheithauer BW. (2000) Transdifferentiation of somatotrophs to thyrotrophs in the pituitary of patients with protracted primary hypothyroidism. *Virchows Archiv : an international journal of pathology*. 436:43-51.

Vidal S, Roman A, Oliveira MC, De La Cruz LF, Moya L. (1998) Simultaneous localization of Pit-1 protein and gonadotropins on the same cell type in the anterior pituitary glands of the rat. *Histochemistry and Cell Biology*. 110:183-8.
Wang JJ. (1989) Immunocytochemical demonstration of the binding of growth-related polypeptide hormones on chick embryonic tissues. *Histochemistry*. 93:133-41.

Wang J, Whetsell M, Klein JR. (1997) Local hormone networks and intestinal T cell homeostasis. *Science.* 275:1937-1939.

Waters MJ, Shang CA, Behncken SN, Tam SP, Li H, Shen B, Lobie PE. (1999) Growth hormone as a cytokine. *Clinical and Experimental Pharmacology and Physiology.* 26:760-4.

Weigent DA, Blalock JE. (1991) The production of growth hormone by subpopulations of rat mononuclear leukocytes. *Cellular Immunology.* 135:55-65.

Weigent DA, Blalock JE, LeBoeuf RD. (1991) An antisense oligodeoxynucleotide to growth hormone messenger ribonucleic acid inhibits lymphocyte proliferation. *Endocrinology.* 128:2053-7.

Werther GA, Haynes K, Waters MJ. (1993) Growth hormone (GH) receptors are expressed on human fetal mesenchymal tissues--identification of messenger ribonucleic acid and GH-binding protein. *Journal of Endocrinology and Metabolism.* 76:1638-46.

Whetsell M, Bagriacik EU, Seetharamaiah GS, Prabhakar BS, Klein JR. (1999) Neuroendocrine-induced synthesis of bone marrow-derived cytokines with inflammatory immunomodulating properties. *Cellular Immunology.* 192:159-66.

Wilson DB, Wyatt DP. (1993) Immunocytochemical effects of thyroxine stimulation on the adenohypophysis of dwarf (dw) mutant mice. *Cell and Tissue Research.* 274:579-85.

Wingstrand KG. (1951) The structure and development of the avian pituitary: from a comparative and functional viewpoint. London: *Hakan Ohissons Boktrycheri.*

Wong EA, Silsby JL, El Halawani ME. (1992) Complementary DNA cloning and expression of Pit-1/GHF-1 from the domestic turkey. *DNA Cell Biology.* 11:651-60.

Woods JE, Damianides-Keenan M, Thommes RC. (1991) FSH-and TSH-binding cells in the ovary of the developing chick embryo. *General and Comparative Endocrinology.* 82:487-494.

Wu H, Devi R, Malarkey WB. (1996) Localization of growth hormone messenger ribonucleic acid in the human immune system--a Clinical Research Center study. *Journal of Clinical Endocrinology and Metabolism.* 81:1278-82.

Yang BY, Greene M, Chen TT. (1999) Early embryonic expression of the growth hormone family protein genes in the developing rainbow trout, *Oncorhynchus mykiss.* *Molecular Reproduction and Development.* 53:127-34.

Yoshizato H, Fujikawa T, Soya H, Tanaka M, Nakashima K. (1998) The growth hormone (GH) gene is expressed in the lateral hypothalamus: enhancement by GH-releasing hormone and repression by restraint stress. *Endocrinology.* 139:2545-51.

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Zhang CZ, Li H, Young WG, Bartold PM, Chen C, Waters MJ. (1997) Evidence for a local action of growth hormone in embryonic tooth development in the rat. *Growth Factors.* 14:131-43.

Zhvirblis GS, Gorbulev VG, Rubtsov PM, Karapetian RV, Zhuravlev IV. (1987) Genetic engineering of peptide hormones. I. Cloning and primary structure of cDNA of chicken growth hormone. *Molecular Biology.* 21:1620-4.

Zouboulis CC. (2000) Human skin: an independent peripheral endocrine organ. *Hormone Research.* 54:230-42.

Chapter 2

Extrapituitary βTSH and GH in Early Chick Embryos

I. INTRODUCTION

The ontogenic appearance of pituitary somatotrophs occurs during mid-late

gestation in most mammalian fetuses and towards the end of the first trimester in human

pregnancy (Harvey *et al.,* 1997). Early embryonic and fetal growth is therefore

independent of pituitary growth hormone (GH). However, while fetal development is

thought to reflect a *growth-without-GH* syndrome (Geffner, 1996), GH and GH mRNA

are present in extrapituitary tissues of murine (Pantaleon *et al.,* 1997), piscine (Yang *et*

al., 1999) and avian (Harvey *et al.,* 2000a) embryos. It is therefore possible that

extrapituitary GH acts as a paracrine or autocrine growth factor during early

embryogenesis (Harvey *et al.,* 1997), especially as the distribution of GH overlaps the

distribution of the GH receptor (Pantaleon *et al.,* 1997; Harvey *et al.,* 2000a) and as GH

antibodies block the stimulatory effect of GH on the uptake of amino acids by murine

embryos (Pantaleon *et al.,* 1997). The widespread distribution of GH in peripheral

tissues of early chick embryos (Harvey *et al.,* 2000a) is also consistent with the presence

of GH in neural, immune, reproductive, digestive and respiratory systems of neonates and

adults (Wu *et al.,* 1996; Harvey and Hull, 1997; Tresguerres *et al.,* 1999; Allen *et al.,*

2000). It is therefore possible that other pituitary hormones in extrapituitary sites may

similarly be expressed prior to the differentiation of the pituitary gland and its cell-types.

Indeed, luteinizing hormone (LH)-immunoreactivity has been detected in the trachea,

lung, esophagus and stomach of ED3-ED7 chick embryos, prior to the differentiation of

pituitary gonadotropes on ED8 (Shirasawa *et al.,* 1996).

Thyrotropes appear ontogenically prior to somatotroph differentiation in

mammals (Burrows *et al.,* 1999) and birds (Porter, 1997). The expression of thyrotropin

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(βTSH) is not, however, confined to these cells in neonatal or adult mammals, since it also occurs in the brain (eg. Hojvat *et al.,* 1982a,b; 1985; DeVito *et al.,* 1985), in immune tissues (Smith *et al.,* 1983; Harbour *et al.,* 1989; Peele *et al.,* 1993; Bodey *et al.,* 2000) and in the gut (Wang *et al.,* 1997) and placenta (Harada and Hershman, 1978). The possibility that βTSH may be produced in extrapituitary tissues of the chick during early embryogenesis was therefore investigated in the present study, especially as βTSH- and GH- secreting cells are thought to be derived from the same cellular lineage, and as both βTSH and GH gene expression is thought to be dependent on the Pit-1 transcription factor (Burrows *et al.,* 1999).

II. MATERIALS AND METHODS

Tissues

Fertile White Leghorn eggs from the University of Alberta Poultry Unit were incubated at 37.5 °C in humidified air. The eggs were turned one quarter of a revolution each day during incubation. At embryonic day (ED) 7 (stage 31) (Hamburger and Hamilton, 1951) the embryos were collected into phosphate-buffered saline (PBS, pH 7.4). Rathke's pouch is present at this stage of the 21 day incubation period, and extrapituitary GH-immunoreactivity is widespread in central and peripheral tissues of these embryos (Harvey *et al.,* 2000a).

Immunohistochemistry

Tissues were fixed in freshly prepared Carnoy's (60% v/v ethanol, 30% v/v chloroform, 10% v/v acetic acid) overnight at 4 °C. They were then dehydrated in a

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graded series of ethanol (50%, 15-30 min; 70%, 30-60 min; 95%, 30-120 min) and

cleared with Hemo-de (Fisher Scientific, Edmonton, Alberta, Canada) for 30 min.

Tissues were then infiltrated with paraffin wax for 24-48 h at 60 °C, under vacuum.

Serial (8 μm) sections were taken using a microtome and mounted on charged slides

(Fisher Scientific). Transverse sections were taken through the head, and at the level of

the wingbud, lung, heart, liver and mesonephros. Immunocytochemical staining was

performed using the avidin-biotin-peroxidase (ABC) (Hsu *et al.,* 1981) method and

commercial reagents (Vector Laboratories, Burlingame, CA, USA). Sections were

incubated with specific polyclonal antisera raised in rabbits against native chicken (c) GH

(cGH1; Harvey & Scanes, 1977) or rat βTSH (βTSH; NIDDK-anti-rBeta βTSH-IC-I,

AFP-1274789 (rabbit), Bethesda, Maryland, USA). Both antibodies were diluted in PBS

(αcGH-1 at 1:1000; βTSH at 1:500) and incubations were overnight at room temperature.

After incubation the sections were washed 3 times for 15 min in PBS and then incubated

for 1 h at room temperature in biotinylated goat anti-rabbit immunoglobulin G (I_gG)

(Vector 1:500). The sections were then washed in PBS and incubated in ABC reagent for

1 h at room temperature and washed in PBS. Staining was visualized using the

chromogenic substrate diaminobenzidine tetrahydrochloride (DAB) (Sigma), which

resulted in a brown precipitate. The specificity of staining was determined by

preabsorbing the primary antibodies with recombinant cGH (Amgen, Thousand Oaks,

CA, USA; 1 mg/ml) or rat βTSH (Bachem, Torrance, CA, USA; 1 mg/ml) for 1 h prior to

section incubation. Antibody specificity was also demonstrated by the localization of

GH-immunoreactivity in the caudal lobe of adult chicken pituitary glands (Fig 2.1A) and

the localization of βTSH-immunoreactivity in the cephalic lobe (Fig 2.1B). Non-specific

staining was determined by replacing the primary antibodies with pre-immune rabbit serum or with PBS. Digital images were collected using a SPOT Digital Microscope camera (Carsen Group, Markam, Ontario, Canada) mounted on an Olympus Bx40 microscope.

III. RESULTS

Immunoreactivity in the head

Within the head, GH-immunoreactivity was widespread, but most intense in neural tissue. Strong GH labeling was found in the anterior (Fig 2.1C) and posterior (Fig 2.2E) diencephalon. Within the diencephalon, the infundibulum was stained (Fig 2.1C), as were ependymal and subependymal cells lining the diocoele (Fig 2.1C; 2.2E). The GH staining in the diencephalon was in discrete layers of cells that were separated by concentric layers of unstained cells. GH-immunoreactivity was also present in adjacent accessory ganglia and in the trigeminal nerve (Fig 2.1C). Intense GH-immunoreactivity was also present in some, but not all, ependymal cells in the mesencephalon (Fig 2.2A, C). The mantle layer of the mesencephalon was also strongly labeled for GH-immunoreactivity (Fig 2.2C). βTSH-immunoreactivity was also present in ependymal cells of the diencephalon (Fig 2.1D) and mesencephalon (Fig 2.2B, D), but it was not in the mesencephalon mantle (Fig 2.2D), or anterior diencephalon (Fig 2.1D). The accessory ganglia and trigeminal nerve were also devoid of βTSH staining (Fig 2.1D, H), although the otic vesicle was strongly stained (Fig 2.2B).

In addition to the brain, faint GH- and βTSH staining was also present in the large (lateral rectus) muscles below the eyes (Fig 2.1C, D). βTSH-immunoreactivity was also

present in the outer epidermis (Fig 2.2B, F) and in endothelial cells lining the ethmoidal

arteries (Fig 2.1D). These tissues were, however, devoid of GH staining (Fig 2.1C; 2.2A,

E). In contrast, GH-immunoreactivity was present in the dura mater (Fig 2.2E), which

lacked βTSH-staining (Fig 2.2F).

Rathke's pouch, the primordial pituitary gland, was devoid of specific GH or

βTSH staining (Fig 2.1E, F). Control sections incubated with preabsorbed antisera or

with NRS or PBS were also completely unstained (data not shown).

Immunoreactivity in the trunk

The outer epidermal cells of the trunk were again stained for βTSH but not for

GH (Fig 2.3A, B). Intense GH-immunoreactivity was, however, present in the ventral

and dorsal horns of the spinal cord and was particularly strong in the outer marginal layer

and in the basal plate mantle (Fig 2.3A). In contrast, βTSH-immunoreactivity was

restricted to ependymal cells lining the spinal canal (Fig 2.3B), which were devoid of

GH-immunoreactivity (Fig 2.3A). Strong GH staining was also present in the dorsal and

ventral root ganglia (Fig 2.3A), both of which lacked βTSH staining (Fig 2.3B).

In non-neural tissues, both GH- and βTSH- staining was present in the crop (Fig

2.3C, E). GH-immunoreactivity in this tissue was restricted to a single layer of

endodermal cells lining the alimentary canal, whereas βTSH-immunoreactivity was more

widespread, although not in every cell (Fig 2.3E). In the crop TSH-immunoreactivity

was present in most adluminal cells and in cells on the basement membrane, with a layer

of unstained cells between them (Fig 2.3E).

Within the developing lungs, βTSH-immunoreactivity was present in the cells lining the bronchial ducts (Fig 2.3F) (in adluminal cells and cells on the basement membrane) and in adluminal cells lining pleural cavities (Fig 2.4B; 2.5B, D). It was similarly present in cells lining the pericardial and hepatic cavities (Fig 2.4B; 2.5G). GH-immunoreactivity in contrast, was not present in these tissues (Fig 2.3D; 2.4A; 2.5C), but was intense in the surrounding intercostal muscle of the body wall (Fig 2.4A, C), which lacked βTSH staining (Fig 2.4B and E). Although the atria and ventricles lacked specific GH- or TSH-staining, squamous cells in the atrioventricular sulcus were stained for βTSH (Fig 2.4F), but not for GH (Fig 2.4D).

Immunoreactivity for GH was also intense in some cells in the Müllerian duct (Fig 2.5A), which lacked βTSH staining (Fig 2.5B). In the liver, hepatocytes were stained for both GH (Fig 2.5E) and βTSH (Fig 2.5F) immunoreactivity, in contrast to unstained erythrocytes within this tissue (Fig 2.5E, F). Intense staining for GH-immunoreactivity was also present in the right and left vagal nerve (Fig 2.5G), both of which lacked βTSH-immunoreactivity (Fig 2.5H). Control trunk sections incubated with preabsorbed antisera or with NRS or PBS were completely unstained (data not shown).

IV. DISCUSSION

These results clearly show, for the first time, the presence of βTSH-immunoreactivity in central and peripheral tissues of ED 7 chick embryos, prior to the differentiation of pituitary thyrotrophs. They also show that βTSH- and GH-immunoreactive cells are differentially located within embryonic tissues.

In the developing brain, βTSH-immunoreactivity was located in ependymal layers

of the mesencephalon, in scattered cells within the trigeminal ganglia and in the otic

vesicle. Although βTSH immunoreactivity has previously been detected by

radioimmunoassay in rodent (Otteweller and Hedge, 1982; Hojvat *et al.,* 1982a; De Vito

et al., 1985; De Vito, 1989) and monkey (Hojvat *et al.,* 1982b) brains, its cellular location

has not been shown. The predominately hypothalamic concentration of βTSH-

immunoreactivity in the mammalian brain differs, however, from its cellular distribution

in the embryonic chick brain. Since this immunoreactivity is present in the chick brain

prior to the differentiation of pituitary thyrotrophs, it is likely to reflect *de novo* synthesis

rather than sequestration from peripheral blood. The demonstration that the

concentration of βTSH in the rat brain increases after colchicine blockade of axonal

transport (De Vito, 1989) supports this view. This possibility is also supported by the

pituitary-independent changes in brain-βTSH concentration that occur in rats diurnally

(Ottenweller and Hedge, 1982), with growth (Emanuele *et al.,* 1985) and with endocrine

manipulations (Hojvat *et al.,* 1985; De Vito *et al.,* 1985). The ontogeny of brain βTSH in

the chick prior to the ontogeny of pituitary βTSH is in agreement with the earlier onset of

βTSH immunoreactivity in the brains of fetal rats (Hojvat *et al.,* 1982).

The presence of βTSH receptors in the human brain (Labudova *et al.,* 1999)

suggests roles for βTSH in neural function, especially as increased receptor abundance

has been correlated with neurodegenerative disease and programmed cell death

(Labudova *et al.,* 1999). Roles of βTSH in the chick brain are, as yet, unknown.

In the brain the localization of βTSH was principally in ependymal and sub-

ependymal layers surrounding the mesocoele. It was also restricted to ependymal cells in

the spinal cord. In the periphery, βTSH immunoreactivity was similarly found in cellular layers surrounding body cavities (eg. in pericardial endoderm surrounding pericardial space; in the crop, surrounding the alimentary canal, in bronchial endoderm surrounding bronchial ducts and the pleural cavity). The adluminal location of βTSH immunoreactivity in the chick embryo may suggest transport or secretory roles for βTSH at these tissue interfaces, although roles for βTSH in these tissues are unknown. βTSH-immunoreactivity has previously been detected in the gut of adult rats (Wang et al., 1997), but not in other embryonic tissues.

In addition to being produced in early chick embryos, βTSH is likely to have biological activity during early chick embryogenesis. Although receptors for βTSH have yet to be demonstrated in the chick embryo, the ability of exogenous (mammalian) βTSH to bind to the medullary cords of early chick embryonic (ED 6.5 – ED 11.5) ovaries (Woods et al., 1991) and to embryonic (ED 8 – ED 15) Sertoli cells (Shahin et al., 1982a,b) suggests actions for βTSH in the early embryo. Indeed, in early chick embryogenesis, βTSH promotes the migration of germ cells to the gonads (Shahin et al., 1982b), the growth and differentiation of the gonad (Csaba et al., 1980; Shahin et al., 1982a,b) and the mitosis of the oocyte (Csaba et al., 1980).

In addition to these extrathyroidal tissues, βTSH binding to "prefollicular" thyroid cells has also been determined in early (ED 5.5) chick embryos (Thommes et al., 1992). The extrathyroidal actions of βTSH may, however, occur ontogenically prior to its thyrotrophic action, since βTSH-induced thyroid function has been demonstrated only in late-stage (ED 15-ED 21) chick embryos (Thommes and Hylka, 1978; Iqbal et al., 1987;

Kühn *et al.,* 1988), when serum T_3 and T_4 levels are first detectable (Gregory *et al.,* 1998).

The ontogeny of extrapituitary βTSH in the chick embryo also appears to occur prior to its ontogeny in pituitary thyrotrophs. At ED 7, intense βTSH immunoreactivity was present in extrapituitary tissues of the chick embryo, although Rathke's pouch was devoid of βTSH staining. This suggests βTSH synthesis does not occur in the pituitary analogue during early ontogeny. This possibility is supported by the absence of βTSH mRNA in the pituitary glands of ED 11 embryos and by its minimal presence in the pituitary glands of ED 13 embryos (Gregory *et al.,* 1998). Although Thommes *et al.,* (1983) found immunoreactive βTSH in Rathke's pouch at ED 6.5; these cells were of low abundance until ED 11.5, when the caudal and cephalic lobes of the adenohypophysis were readily discernable. It is therefore likely that pituitary βTSH synthesis is minimal until the ontogenic differentiation of the thyrotroph cells.

The immunoreactivity for βTSH in peripheral tissues of the chick embryo was primarily in epithelial and endothelial cells surrounding body cavities or ductal systems. This luminal location is similar to the presence of LH-immunoreactivity in luminal cells of the esophagus, stomach, trachea and bronchi of ED 3-ED 7 chick embryos (Shirasawa *et al.,* 1996). It is therefore possible that these cells are derived from a similar glycoprotein hormone lineage. The βTSH immunoreactivity detected in the present study is not, however, due to cross-reactivity with LH, since the antibodies used have strict specificities and because the βTSH immunoreactivity is also in tissues devoid of LH immunoreactivity (eg. the brain and limb bud). The occurrence of βTSH and LH in the lung is, nevertheless, of interest, since other endocrine cells have been identified in the

chicken lung (Walsh and McLelland, 1978; Lopez *et al.,* 1983; Cook *et al.,* 1986), in

which bombesin and somatostatin have been located (Adriaensen *et al.,* 1994). The

location of these peptides differs, however, from the locations of βTSH (this study) and

LH (Shirasawa *et al.,* 1996), since they were distributed on the basement membrane of

the epithelium, in cells lacking direct contact with the luminal surface. It is also of

interest that chorionic gonadotropin has been demonstrated in human fetal lungs

(Yoshimoto *et al.,* 1977) and in benign lung neoplasms (Fukayama *et al.,* 1986).

The occurrence of GH-immunoreactivity in chick (Harvey *et al.,* 1997; 2000a)

and murine (Pantaleon *et al.,* 1997) embryos prior to the differentiation of the pituitary

gland is now well established. GH-immunoreactivity was present in the embryonic chick

brain, as expected from the immunocytochemical distribution of GH in the

metencephalon and diencephalon of adult turkeys and Ring doves (Ramesh *et al.,* 2000),

the presence of GH-immunoreactive proteins in the brains of adult chickens (Render *et*

al., 1995) and the neonatal expression of the GH gene in the chicken brain (Render *et al.,*

1995). The ontogeny of neural GH therefore occurs in chickens prior to the ontogeny of

pituitary somatotrophs (at ED 12-14, Jozsa *et al.,* 1979; Porter, 1997), as also observed in

rats (Hojvat *et al.,* 1982b).

In addition to the brain, GH-immunoreactivity was also located in the white

matter of the spinal cord (in the marginal layer and the basal plate mantle) and in dorsal-

and ventral root ganglia of the chick embryo. It was also present in the trigeminal nerve

and in the vagal nerve. A more widespread distribution of GH-immunoreactivity was

previously observed in the neural tube of ED 3 embryos (Harvey *et al.,* 2000a),

suggesting developmental changes occur in the production or action of GH in this neural

tissue. As GH is obligatory for normal neural development and neural function (reviewed by Harvey *et al.,* 1997), the results of these studies suggest local actions for GH in these neural sites.

In the periphery, strong GH-immunoreactivity was present in the intracostal muscles. Although this is a novel finding, the presence of GH-immunoreactivity in other peripheral tissues of ED 7 chick embryos (liver, Müllerian duct, wingbud chondrocytes) has been shown previously (Harvey *et al.,* 2000a). The absence of GH staining in the embryonic heart (Fig 2.4A) contrasts with our previous finding of GH-immunoreactivity in the atria of ED 7 embryos (Harvey *et al.,* 2000a), although as this immunoreactivity was lost by ED 8 (Harvey *et al.,* 2000a), this may reflect small differences between the embryos in their stage of development.

The presence of GH-immunoreactivity in the liver is of interest, since the liver is rich in biotin. The GH staining in the liver is not, however, an artifact of endogenous biotin in the ABC immunocytochemical technique (Polak and Van Noorden, 1997), since no staining was seen in the preabsorbed, NRS or PBS controls. Moreover, although DAB may stain non-specifically to hemoglobin in erythrocytes, in which endogenous peroxidase activity could cause a non-specific reaction (Van Bogaert *et al.,* 1981), the GH staining in hepatocytes contrasts with the absence of staining in red blood corpuscles in the liver (Fig 2.5E, F). The presence of GH in the embryonic liver prior to somatotroph differentiation therefore suggests it is not just a target site of GH action, but also a site of GH synthesis or sequestration in early embryos.

The distribution of GH-immunoreactivity in the ED 7 chick embryo was clearly very different from the distribution of βTSH. Although GH- and βTSH-

immunoreactivity were sometimes found in the same tissues (eg. in the brain, spinal cord and lung) they were in different cellular locations, as also observed in the anterior pituitary gland. Extrapituitary GH- and βTSH-cells are thus likely to have a different cellular lineage, whereas pituitary thyrotropes and somatotrophs in rats are thought to be derived from the same lineage (Burrows *et al.,* 1999). Indeed, thyrotropes and somatotropes are both absent in Snell dwarf mice, reflecting the lack of the Pit-1 transcription factor that is normally required for the differentiation of these cell-types.

However, βTSH-positive cells that fail to express Pit-1 have been characterized in the pars tuberalis of the rat pituitary gland (Sakai *et al.,* 1999), demonstrating the existence of heterogeneous populations of βTSH cell types. The Pit-1 dependence of pituitary and extrapituitary βTSH in embryonic or neonatal chicks is currently unknown, although GH expression in pituitary somatotrophs is thought to be Pit-1 dependent (Harvey *et al.,* 2000b).

In summary, these results demonstrate the presence of GH- and βTSH-immunoreactive cells in extrapituitary tissues of early chick embryos prior to pituitary differentiation and show a differential distribution of these cell types.

Fig 2.1 Immunocytochemical localization of GH and βTSH.

Immunocytochemical localization of GH and βTSH in the adult chicken pituitary gland. (A) Localization of GH-immunoreactivity in the caudal lobe (Ca) of the adult chicken pituitary gland, (B) Localization of βTSH-immunoreactivity in the cephalic lobe (Ce) of the adult chicken pituitary gland. Magnification x100. The insets show higher magnifications (x1000) of the caudal and cephalic lobes.

Localization of GH-immunoreactivity in the head of a chick embryo at embryonic day (ED) 7. (C) GH-immunoreactivity is present in the diencephalon (d), infundibulum (i), trigeminal nerve (tg), accessory ganglia (ag), and the lateral rectus muscle (rem), but is not present in Rathke's pouch (Rp) or the endothelial cells of the ethmoidal artery (e). Magnification x100. The inset shows a higher magnification (x1000) of the ethmoidal artery. (E) Higher magnification (x1000) of Rathke's pouch. (G) Higher magnification (x1000) of the trigeminal nerve.

Localization of βTSH-immunoreactivity in the head of an ED 7 chick embryo. (D) Specific βTSH staining is present in the ependymal cells (ep) of the diencephalon (d) and in the endothelial cells of the ethmoidal artery (e). The mantle layers of the diencephalon (d), the trigeminal nerve (tg), accessory ganglia (ag) and Rathke's pouch (Rp) are unstained. Magnification x100. The inset shows a higher magnification (x1000) of the endothelial cells in the ethmoidal artery. (F) Higher magnification (x1000) of Rathke's pouch. (H) Higher magnification (x1000) of the trigeminal nerve.

A

Ce

Ca

B

Ca

Ce

C

tg

Rp

ag

e

ep

i

rem

D

tg

Rp

e

ag

ep

i

d

E

F

rem

G

H

72
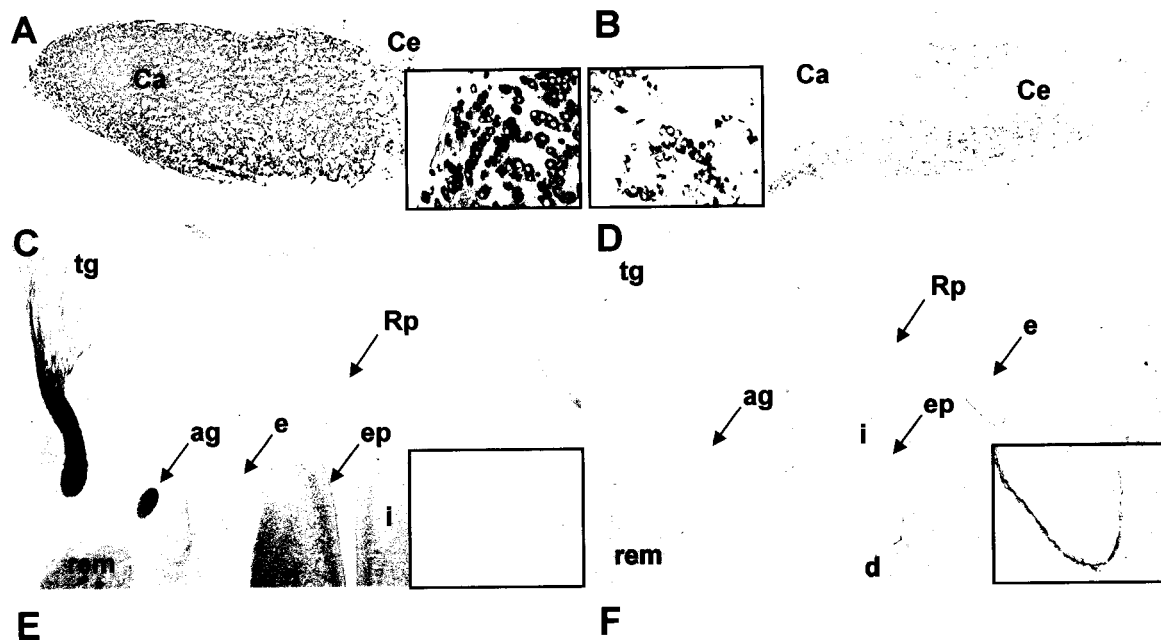
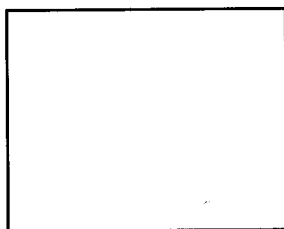**Fig 2.2 GH- and βTSH-immunoreactivity in the head of 7 day-old chick embryos.**

(A) GH-staining is shown in the ependymal cells (ep) of the mesencephalon (m), but not in the otic vesicle (ov). Magnification x100. The insert shows a higher magnification (x1000) of the otic vesicle. (B) βTSH staining in the ependymal cells (ep) of the mesencephalon (m). The inset shows a higher magnification (x1000) of the otic vesicle (ov). (C) GH-immunoreactivity in the mantle of the mesencephalon (m). Note the absence of staining in the ependymal cells (ep). Magnification x100. The inset is a higher magnification (x1000) of the mantle at the marked (+) area. (D) βTSH-immunoreactivity in the ependymal cells (ep) of the mesencephalon (m). Magnification x100. The inset is a higher magnification (x1000) of the marked (+) area. (E) GH-staining in the mantle region of the diencephalon (d) and in the dura mater (dm). Note the absence of staining in the epidermis (ed). Magnification x100. The inset shows a higher magnification (x1000) of the marked (+) area. (F) βTSH-staining in the mantle region of the diencephalon (d) and in the epidermis (ed). Magnification x100. Note the restriction of staining to the ependymal cells (ep) lining the diocele. Magnification x100. The inset shows a higher magnification (x1000) of the marked (+) area.

A

ov

ep

m

B

ov

ep

C

ep

m

D

ep

m
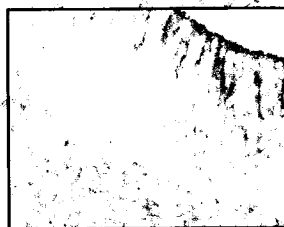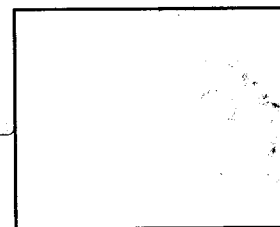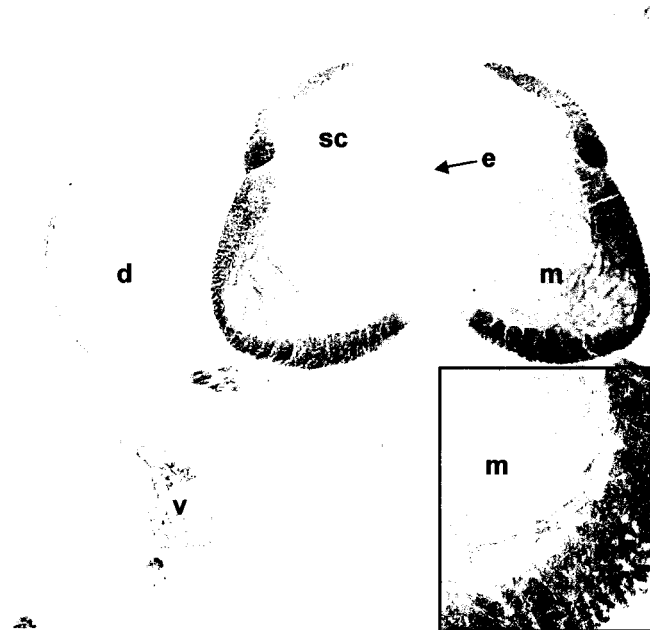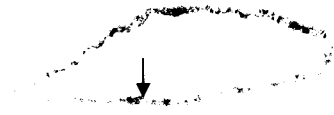
E

d

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Fig 2.3 GH- and βTSH-immunoreactivity in peripheral tissues of a 7-day-old chick embryo. (A) GH-staining in the mantle (m) and marginal layer (ml) of the spinal cord (sc) and in the dorsal- (d) and ventral- (v) root ganglia. Magnification x100. The inset shows a higher magnification (x1000) of the mantle and marginal layer. (B) βTSH-staining in the ependymal (e) cells of the spinal cord and in the epidermis (arrow). Note the absence of staining in other areas of the spinal cord and the lack of staining in the dorsal- (d) and ventral- (v) root ganglia. Magnification x100. The inset shows a higher magnification (x1000) of the ependymal cells surrounding the spinal canal. (C) GH-immunoreactivity in endothelial cells lining the crop (arrowed). Magnification x1000. (D) Absence of GH-staining in a bronchus of the developing lung. Magnification x1000. (E) βTSH-immunoreactivity in the crop. Magnification x1000. (F) βTSH-immunoreactivity in a bronchus of the developing lung. Magnification x1000.
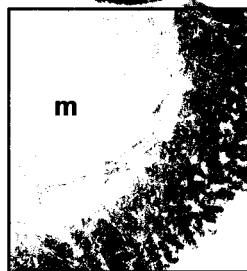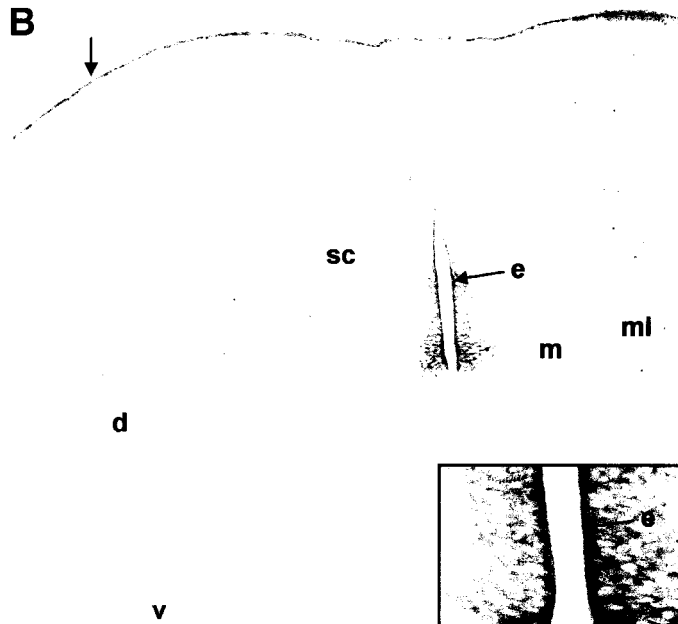
A ↓

sc
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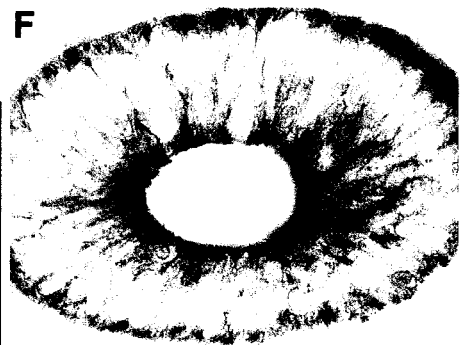v

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m
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Fig 2.4 GH-and βTSH-immunoreactivity in peripheral tissues of a 7-day-old chick embryo. (A) GH-staining in intracostal muscles (i) between developing ribs, surrounding the body cavity. Note the absence of GH-staining in the bronchi (b), dorsal aorta (d), atria (a) and the atrioventricular sulcus (avs). Magnification x100. (C) A higher magnification (x1000) of the intracostal muscle (i). (D) A higher magnification (x1000) of the atrioventricular sulcus. (B) βTSH-staining in the crop (c), and bronchi (b), and in cells lining pleural cavities (pc) and the pericardial space (ps) and in the atrioventricular sulcus (avs). Note the absence of βTSH-staining in the intracostal muscle (i). Magnification x100. (E) Higher magnification (x1000) of the intracostal muscle (i), and pericardial space. (F) Higher magnification (x1000) of the atrioventricular sulcus.
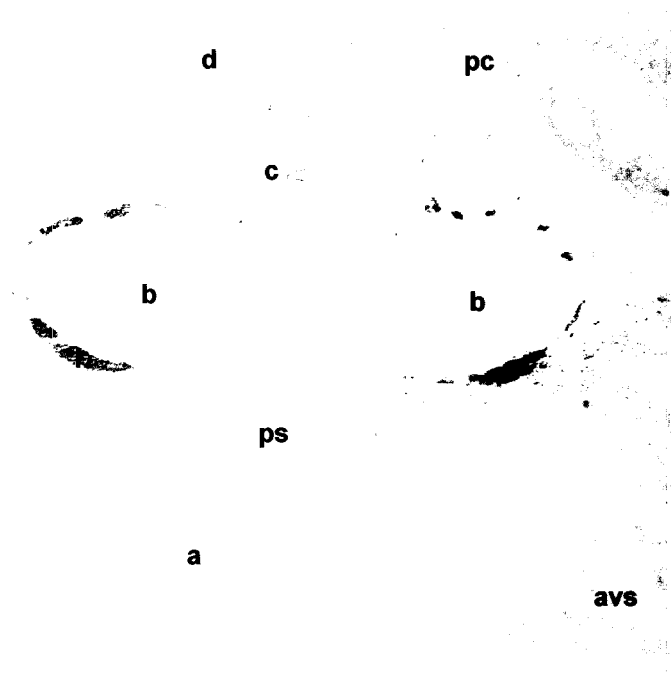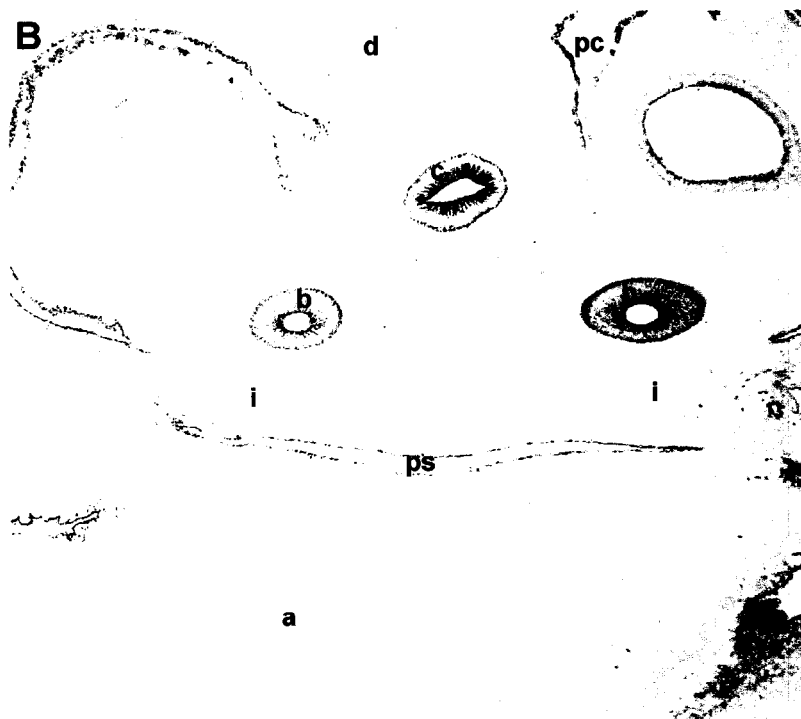
Fig 2.5 GH- and βTSH-immunoreactivity in peripheral tissues of 7-day-old chick embryos. GH- staining is present in (A) the Müllerian duct (md), (E) in liver hepatocytes and (G) the right (rv) and left (lv) vagal nerve, but not in (C) cells lining the pleural cavity (pc) (arrow), nor (E) in liver erythrocytes (arrow). βTSH-staining is in cells lining the pleural cavity (pc) (B,D) and hepatic cavity (hc) (H) and in liver hepatocytes (F), but not (B) in the Müllerian duct (md), (F) liver erythrocytes (arrow), or (H) vagal nerves. Magnification x1000.
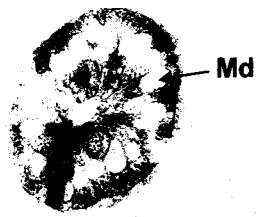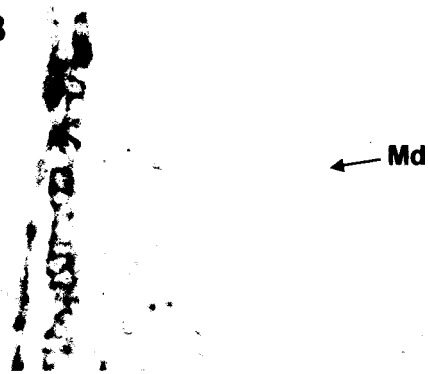
V. REFERENCES

Adriaensen D, Scheuermann DW, Gomi T, Kimura A, Timmermans JP, De Groodt-Lasseel MHA. (1994) The pulmonary neuroepithelial endocrine system in the quail, Coturnix coturnix. Light- and electron-microscopical immunocytochemistry and morphology. *Anatomy Records*. 239:65-74.
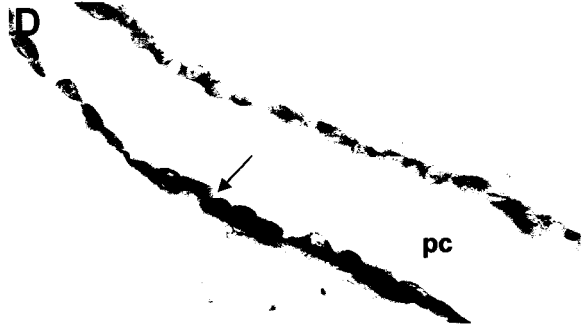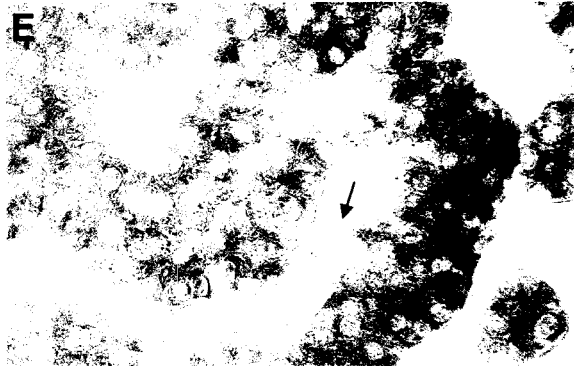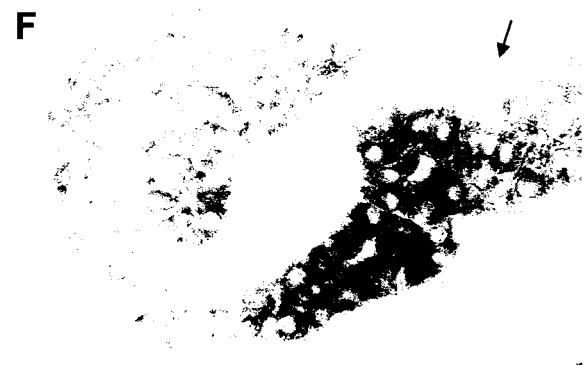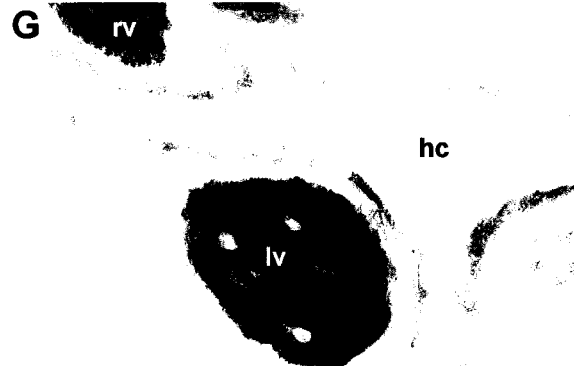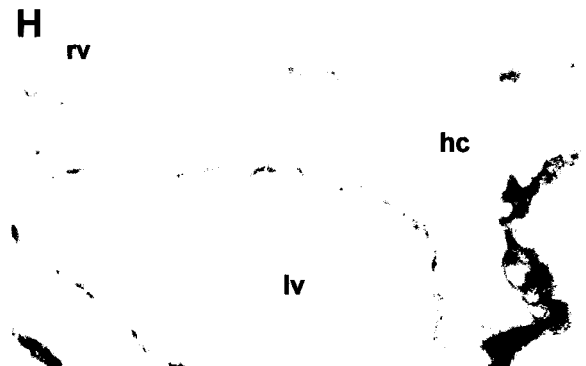
Allen JT, Bloor CA, Kedia RK, Knight RA, Spiteri MA. (2000) Expression of growth hormone-releasing factor, growth hormone, insulin-like growth factor-1 and its binding proteins in human lung. *Neuropeptides*. 34:98-107.

Bodey B, Bodey Jr. B, Siegel SE, Kaiser HE. (2000) The role of the reticulo-epithelial (RE) cell network in the immuno-neuroendocrine regulation of intrathymic lymphopoiesis. *Anticancer Research*. 20:1871-88.

Burrows HL, Douglas KR, Camper SA. (1999) Genealogy of the anterior pituitary gland: tracing a family tree. *Trends in Endocrinology and Metabolism*. 10:343-52.

Cook RD, Vaillant CR, King AS. (1986) The abdominal air sac ostium of the domestic fowl: a sphincter regulated by neuro-epithelial cells? *Journal of Anatomy*. 149:101-11.

Csaba G, Shahin MA, Dobozy O. (1980) The overlapping effect of gonadotropins and TSH on embryonic chicken gonads. *Anatomia Histologia Embryolia*. 63:31-38.

DeVito WJ, Connors JM, Hedge GA. (1985) Distribution and release of immunoreactive thyroid-stimulating hormone in the rat hypothalamus: effects of thyroidectomy, hypophysectomy and treatment with thyroid hormones. *Neuroendocrinology*. 41:23-30.

DeVito WJ. (1989) Thyroid hormone regulation of hypothalamic immunoreactive thyrotropin. *Endocrinology*. 125:1219-23.

Emanuele NV, Baker G, McDonald D, Kirsteins L, Lawrence AM. (1985) The impact of aging on luteinizing hormone (LH) and thyroid-stimulating hormone (TSH) in the rat brain. *Brain Research*. 352:179-83.

Fukayama M, Hayashi Y, Koike M, Hajikano H, Endo S, Okumura H. (1986) Human chorionic gonadotropin in lung and lung tumors: Immunohistochemical study on unbalanced distribution of subunits. *Laboratory Investigations*. 55:433-43.

Geffner ME. (1996) The growth without growth hormone syndrome. *Endocrinology and Metabolism Clinics of North America*. 25:649-63.

Gregory CC, Dean CE, Porter TE. (1998) Expression of chicken thyroid-stimulating hormone β-subunit messenger ribonucleic acid during embryonic and neonatal development. *Endocrinology*. 139:474-8.

Hamburger V & Hamilton H. (1951) A series of normal stages in the development of the chick embryo. *Journal of Morphology* 88:49-92.

Harada A, Hershman JM. (1978) Extraction of human chorionic thyrotropin (hCT) from term placentas: failure to recover thyrotropic activity. *Journal of Clinical Endocrinology and Metabolism.* 47:681-5.

Harbour DV, Kruger TE, Coppenhaver D, Smith EM, Meyer WJ. (1989) Differential expression and regulation of thyrotropin (TSH) in T cell lines. *Molecular and Cellular Endocrinology.* 64:229-41.

Harvey S, Scanes CG. (1977) Purification and radioimmunoassay of chicken growth hormone. *Journal of Endocrinology.* 73:321-9.

Harvey S, Hull KL. (1997) Growth hormone: a paracrine growth factor? *Endocrine.* 7:261-79.

Harvey S, Johnson CDM, Sharma P, Sanders EJ, Hull KL. (1997) Growth hormone: a paracrine growth factor in embryonic development? *Comparative Biochemistry and Physiology.* 119C:305-15.

Harvey S, Johnson CDM, Sanders EJ. (2000a) Extra-pituitary growth hormone in peripheral tissues of early chick embryos. *Journal of Endocrinology.* 166:489-502.

Harvey S, Azumaya Y, Hull KL. (2000b) Extrapituitary growth hormone: Pit-1 dependence? *Canadian Journal of Physiology and Pharmacology.* 78:1-16.

Hojvat S, Baker G, Kirsteins L, Lawrence AM. (1982a) TSH in the rat and monkey brain. Distribution, characterization and effect of hypophysectomy. *Neuroendocrinology.* 34:327-32.

Hojvat S, Emanuele N, Baker G, Connick E, Kirsteins L, Lawrence AM. (1982b) Growth hormone (GH), thyroid-stimulating hormone (TSH), and luteinizing hormone (LH) -like peptides in the rodent brain: non-parallel ontogenetic development with pituitary counterparts. *Brain Research.* 256:427-34.

Hojvat S, Emanuele N, Baker G, Kirsteins L, Lawrence AM. (1985) Brain thyroid-stimulating hormone: effects of endocrine manipulations. *Brain Research.* 360:257-63.

Hsu SM, Raine L, Fanger H. (1981) Use of avidin-peroxide complex ABC in immunoperoxidase techniques: a comparison between ABD and unlabeled antibody AAP procedures. *Journal of Histochemistry and Cytochemistry.* 29 ;577-80.

Iqbal A, Decuypere E, Kühn ER, Schneider R, Verheyen G, Huybrechts LM. (1987) The influence of methimazole on the thyrotrophic and peripheral activity of thyrotrophin and thyrotrophin-releasing hormone in the chick embryo and growing chicken. *Domestic Animal Endocrinology*. 4:291-8.

Józsa R, Scanes CG, Vigh S, Mess B. (1979) Functional differentiation of the embryonic chicken pituitary gland studied by immunohistological approach. *General and Comparative Endocrinology*. 39:58-163.

Kühn ER, Decuypere E, Iqbal A, Luysterborgh D, Michielsen R. (1988) Thyrotropic and peripheral activities of thyrotrophin and thyrotropin-releasing hormone in the chick embryo and adult chicken. *Hormone and Metabolic Research*. 20:158-62.

Labudova O, Cairns N, Koeck T, Kitzmueller E, Rink H, Lubec G. (1999) Thyroid stimulating hormone-receptor overexpression in the brain of patients with Down syndrome and Alzheimer's disease. *Life Science*. 64:1037-44.

López J, D'az de Rada O, Sesma P, Vázquez JJ. (1983) Silver methods applied to semithin sections to identify peptide-producing endocrine cells. *Anatomy Records*. 205:465-70.

Ottenweller JE, Hedge GA. (1982) Thyrotropin-like immunoreactivity in the pituitary and three brain regions of the female rat: diurnal variations and the effect of thyroidectomy. *Endocrinology*. 111:515-521.

Pantaleon M, Whiteside EJ, Harvey MB, Barnard RT, Waters MJ, Kaye PL. (1997) Functional growth hormone (GH) receptors and GH are expressed by preimplantation mouse embryos: a role for GH in early embryogenesis? *Prceedings of the National Academy of Science* (USA). 94:5125-30.

Peele ME, Carr FE, Baker Jr. JR, Wartofsky L, Burman KD. (1993) TSH beta subunit gene expression in human lymphocytes. *American Journal of Medical Science*. 305:1-7.

Polak JM and Van Noorden S. (1997) *Introduction to Immunocytochemistry*, 2nd Ed. Springer-Verlag.

Porter TE. (1997) Regulation of somatotroph differentiation during chicken embryonic development: a review. *In* "Perspectives in Avian Endocrinology" (Harvey S, Etches RJ, Eds), pp 47-56. Bristol, Journal of Endocrinology Ltd.

Ramesh R, Kuenzel WJ, Buntin JD, Proudman JA. (2000) Identification of growth-hormone- and prolactin-containing neurons within the avian brain. *Cell and Tissue Research*. 299:371-83.

Render CL, Hull KL, Harvey S. (1995) Neural expression of the pituitary GH gene. *Journal of Endocrinology*. 147:413-22.

Sakai T, Sakamoto S, Ijima K, Matsubara K, Kato Y, Inoue K. (1999) Characterization of TSH-positive cells in foetal rat pars tuberalis that fail to express Pit-1 factor and thyroid hormone beta2 receptors. *Journal of Neuroendocrinology*. 11:187-93.

Shahin MA, Sudár F, Dobozy O. (1982a) Electron microscopic study of the overlapping effect of thyrotropin and gonadotropins on the Sertoli cells of chick embryos. *Zeitschrift für mikroskopisch-anatomische Forschung*. 96:1044-1068.

Shahin MA, Török O, Csaba G. (1982b) The overlapping effects of thryotropin and gonadotropins on chick embryo gonads *in vitro*. *Acta morphologica Academiae Scientiarum Hungaricae*. 30:109-125.

Shirasawa N, Shiino M, Shimizu Y, Nogami H, Ishii S. (1996) Immunoreactive luteinizing hormone (ir-LH) cells in the lung and stomach of chick embryos. *Cell and Tissue Research*. 283:19-27.

Smith EM, Phan M, Kruger TE, Coppenhaver DH, Blalock JW. (1983) Human lymphocyte production of immunoreactive thyrotropin. *Proceedings of the National Academy of Science*. (USA). 80:6010-3.

Thommes RC, Hylka VW. (1978) Hypothalamo-adenohypophyseal-thyroid interrelationships in the chick embryo. I. TRH and TSH Sensitivity. *General and Comparative Endocrinology*. 34:193-200.

Thommes RC, Martens JB, Hopkins WE, Caliendo J, Sorrentino MJ, Woods JE. (1983) Hypothalamo-adenohypophyseal-thyroid interrelationships in the chick embryo. IV. Immunocytochemical demonstration of TSH in the hypophyseal pars distalis. *General and Comparative Endocrinology*. 51:434-443.

Thommes RC, Fitzsimons EJ, Davis M, Woods JE. (1992) Immunocytochemical demonstration of T_4 content and TSH-binding by cells of the thyroid of the developing chick embryo. *General and Comparative Endocrinology*. 85:79-85.

Tresguerres JA, Ariznavarreta C, Granados B, Costoya JA, Perez-Romero A, Salame F, Hermanussen M. (1999) Salivary gland is capable of GH synthesis under GHRH stimulation. *Journal of Endocrinology*. 160:217-22.

Van Bogaert LJ, Van Craynest MP, Quinones JA. (1981) Diaminobenzidine histochemistry in light microscopy. *Acta Histochemica*. 69:61-6.

Walsh C, McLelland J. (1978) The development of epithelium and its innervation in the avian extra-pulmonary respiratory tract. *Journal of Anatomy*. 125:171-82.

Wang J, Whetsell M, Klein JR. (1997) Local hormone networks and intestinal T cell homeostasis. *Science.* 275:1937-1939.

Woods JE, Damianides-Keenan M, Thommes RC. (1991) FSH-and TSH-binding cells in the ovary of the developing chick embryo. *General and Comparative Endocrinology.* 82:487-494.

Wu H, Devi R, Malarkey WB. (1996) Localization of growth hormone messenger ribonucleic acid in the human immune system--a Clinical Research Center study. *Journal of Clinical Endocrinology and Metabolism.* 81:1278-82.

Yang BY, Greene M, Chen TT. (1999) Early embryonic expression of the growth hormone family protein genes in the developing rainbow trout, *Oncorhynchus mykiss.* *Molecular Reproduction and Development.* 53:127-34.

Yoshimoto Y, Wolfsen AR, Odell WD. (1977) Human chorionic gonadotropin-like substance in non-endocrine tissues of normal subjects. *Science.* 197:575-7.

Chapter 3

Extrapituitary TSH in Early Chick Embryos: Pit-1 Dependence?

I. INTRODUCTION

Thyrotropin-stimulating hormone (TSH) is primarily produced in pituitary thyrotrophs, although it is also found in extrapituitary sites. TSH immunoreactivity is, for instance, present in the rat brain (Hojvat et al., 1982a,b, 1985), particularly in the hypothalamus (De Vito et al., 1985, 1989). The TSH gene is also thought to be expressed in the human immune system (Smith et al., 1983; Harbour et al., 1989; Peele et al., 1993; Bodey et al., 2000), gut (Wang et al., 1997) and placenta (Harada and Hershman, 1978). It was also recently detected in embryonic chicks, in discrete cells in the developing brain (in ependymal cells lining the diocoele and mesocoele and in the lining of the otic vesicle), spinal cord (in ependymal cells), liver (in hepatocytes), lungs (in the linings of the bronchi), gut (in the linings of the proventriculus) and cardiopulmonary system (in cells in the adluminal linings of the pleural and pericardial cavities) (Murphy and Harvey, 2001). In the pituitary, the expression of the TSH gene is thought to be dependent upon the pituitary-specific transcription factor, Pit-1 (Burrows et al., 1999). The presence of TSH in extrapituitary tissues therefore suggests a widespread distribution of Pit-1 or that extrapituitary TSH is independent of Pit-1 regulation. This possibility has been assessed in the present study, by comparing the distribution of TSH and Pit-1 in central and peripheral tissues of early chick embryos, prior to the ontogenic differentiation of the pituitary gland.

II. MATERIALS AND METHODS

Tissues

Fertile White Leghorn eggs from the University of Alberta Poultry Unit were

incubated at 38 °C in humidified air. At embryonic day (ED) 7 (stage 31) (Hamburger

and Hamilton, 1951), the embryos were collected into phosphate-buffered saline (PBS,

pH 7.4). Rathke's pouch, the pituitary precursor, is present at this stage of the 21-day

incubation period, and extrapituitary TSH immunoreactivity is widespread in central and

peripheral tissues of these embryos (Murphy and Harvey, 2001).

Immunocytochemistry

Tissues were fixed in freshly prepared Carnoy's (60% v/v EtOH, 30% v/v

chloroform, 10% v/v acetic acid) overnight at 4 °C. They were then dehydrated in a

graded series of ethanol (50%, 15-30 min; 70%, 30-60 min; 95%, 30-120 min; 100%, 30-

120 min) and cleared with Hemo-de (Fisher Scientific, Edmonton, Alberta, Canada) for

30 min. Tissues were then infiltrated with paraffin wax for 24-48 h at 60 °C, under

vacuum. Serial (8 μm) transverse sections were taken using a microtome and mounted on

charged slides (Fisher Scientific). Sections were taken through the head, and at the level

of the wing bud, lung, heart, liver and mesonephros. Immunocytochemical staining was

performed using the avidin-biotin-peroxidase (ABC) method (Hsu *et al.*, 1981) and

commercial reagents (Vector Laboratories, Burlingame, CA, USA). Adjacent sections

were incubated with specific polyclonal antisera raised in rabbits against rat βTSH

(αβTSH; NIDDK-anti-rBeta TSH-IC-I, AFP-1274789 (rabbit), Bethesda, MD, USA) or

human Pit-1 (αPit-1, #132; Voss *et al.*, 1991). Both antibodies were diluted in PBS

(αβTSH at 1:500; αPit-1 at 1:200) and incubations were overnight at 4 °C. After

incubation, the sections were washed three times for 15 min in PBS and then incubated

for 1 h at room temperature in biotinylated goat anti-rabbit immunoglobulin G (I_gG) (Vector, 1:500). The sections were then washed in PBS and incubated in ABC reagents for 1 h at room temperature and washed in PBS. Staining was visualized using the chromogenic substrate, diaminobenzide tetrahydrochloride (DAB) (Sigma), which resulted in a brown or blue precipitate. The specificity of TSH staining was determined by preabsorbing the primary antibody with rat βTSH (Bachem, Torrance, CA, USA; 1 mg/ml) for 1 h prior to section incubation, which completely abolished all staining. Antibody specificity was also demonstrated by the localization of TSH-immunoreactivity in the cephalic lobe of adult chicken pituitary glands (Fig. 3.1A). Pit-1 immunoreactivity was present in both the cephalic and caudal lobes of the anterior pituitary (Fig. 3.1B), presumably reflecting the presence of growth hormone (GH) in the caudal lobe (Murphy and Harvey, 2001) and TSH and prolactin in the cephalic lobe (Mikami, 1983). Staining for βTSH was restricted to the cytoplasm of pituitary cells, whereas Pit-1 immunoreactivity was mostly nuclear. Replacing the primary antibodies with preimmune rabbit serum or with PBS abolished all staining. Digital images were collected using a SPOT Digital Microscope camera (Carsen Group, Markam, Ontario, Canada) mounted on an Olympus Bx40 microscope. Some of the staining for TSH was reported previously (Murphy and Harvey, 2001) and is presented here for comparison.

Western Analysis

Pooled ED 7 chick embryo tissues (heads, headless trunks, wingbuds and whole eyes) and adult chicken pituitary glands were placed in protease inhibitor solution (Hepes, $MgCl_2$, EDTA, EGTA, aprotinin, leupeptin and pepstatin) plus 1% v/v PMSF

and quickly homogenized using a polytron homogenizer (Brinkman Instruments, Westbury, NY, USA). Sample homogenates were centrifuged for 5 min, the supernatant collected and a protein assay was done to determine protein concentration. 50 μg of embryo tissue protein and 10 μg of pituitary protein were added to loading buffer (10% glycerol, 5% 2-β-mercaptoethanol, 2% SDS, 0.001% bromophenol blue; pH 6.8) plus 10% DTT and denatured at 70 °C for 5 min prior to loading. Proteins were separated by SDS-polyacrylamide gel (15% w/v) electrophoresis under reducing conditions. Gels were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) and transferred electrophoretically (100V for 1 h at 4 °C) to Immobilon PVDF membranes (Millipore, Bedford, MA, USA). Non-specific binding sites were blocked by incubating the membrane with 5% skim milk in Tris buffered saline (25 nM TrisHCl, 0.5 M NaCl; pH 7.6) plus Tween® 20 (TBST) for 1 h at room temperature. Pit-1 immunoreactivity was detected using a polyclonal antibody raised in rabbits against human Pit-1 (αPit-1, #132; Voss et al., 1991) diluted 1:50 in TBST/5% skim milk. Membranes were washed with TBST (3 x 10 min), incubated for 1 h at room temperature with a biotinylated goat anti-rabbit immunoglobulin G (IgG) (Vector, 1:500), washed with TBST (3 x 10 min) and then incubated in ABC reagents (Vector) for 1 h at room temperature. After washing, blots were developed with an enhanced chemiluminescence detection system (ECL Kit, Amersham Pharmacia Biotech) and exposed to Kodak X-AR film (Kodak, Rochester, USA).

Reverse Transcription - Polymerase Chain Reaction

The presence of the Pit-1 gene was assessed using the reverse transcription polymerase chain reaction (RT-PCR). Total RNA from pooled ED 7 chick embryo tissues (heads, headless trunks, and wingbuds), adult chicken pituitary glands and muscle was isolated using the QIAGEN RNeasy Mini kit and protocol (QIAGEN, Mississauga, ON, Canada). The RNA was reverse transcribed in the presence of 10 pmol of the oligo-deoxythymine primer, Khu 14, 5'- GACTCGAGTCGACATCGTTTTTTTTTTTTT TTTT-3', (DNA Core Facility, University of Alberta), 5x First-Strand Buffer (Invitrogen Life Technologies, Carlsbad, CA, USA), excess deoxynucleotides (10 nM each deoxy-ATP, dCTP, dGTP and dTTP) (Invitrogen Life Technologies), the reverse transcription enzyme Superscript II (200 U, Invitrogen Life Technologies) and 0.1 M dithiothreitol (DTT). This was incubated for 1 h at 42 °C and inactivated for 10 min at 70 °C. The newly generated cDNA was diluted in 200 µl of double-distilled water. Transcribed pituitary RNA was used as a positive control and transcribed muscle RNA and RNA that was not transcribed (no Superscript II was added to the mixture) were used as negative controls.

The cDNA was amplified in the presence of oligonucleotide primers Pit-1(11), 5'-GCTCTAGACAGCAGGACTCTACTCT-3' and Pit-1 (12), 5'- GCTCTAGAGTCTGGT GCATGGTGTGAA-3', (DNA Core Facility, University of Alberta) and Ready-To-Go PCR Beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The samples were then placed in a Techne thermal cycler (Techne Incorporated, Princeton, NJ, USA), denatured at 95 °C for 5 min, amplified for 35 cycles of denaturing (95 °C for 1 min), annealing (57 °C for 1 min) and extension (72 °C for 2 min), followed by a final

extension of 10 min at 72 °C. The amplified cDNA (10 µl) was electrophoresed on a

1.5% (w/v) agarose ethidium bromide gel and visualized with UV light.

Southern Blotting

Pit-1 cDNA amplified by PCR was transfered to a nylon membrane using the

S&S TurboBlotter and Blotting Stack Assembly for Alkaline Transfers (Schleicher &

Schuell, Keene, NH, USA). After transfer, the DNA was fixed to the membrane by

baking at 80 °C for 30 min in a vacuum oven. The blot was prehybridized in 10 ml of

DIG Easy Hyb hybridization buffer (Roche Applied Science, Basel, Switzerland) for 1 h

at 50 °C. The DNA blot was then hybridized with 10 pmol digoxigenin (DIG) labeled

Pit-1 RNA probe/ml hybridization buffer overnight at 50 °C. The hybridization buffer

was poured off and the blot was washed 2 x 5 min in 2 x SSC (Roche Applied Science)

containing 0.1% sodium dodecyl sulfate (SDS) at room temperature, 2 x 5 min in 0.5 x

SSC containing 0.1% SDS at 65 °C and 1 x 1 min in 0.3% (v/v) Tween® 20 in maleic

acid buffer (10 mM maleic acid, 15 mM NaCl; pH 7.5) at room temperature. The blot

was incubated in 1 x Blocking Reagent (Roche Applied Science) for 1 h with gentle

shaking and then with alkaline phosphatase-conjugated anti-DIG antibody (Roche

Applied Science) diluted 1:5000 in 1 x Blocking Reagent. Colormetric detection of the

DIG labeled probe was carried out using NBT/BCIP (Roche Applied Science).

III. RESULTS

Immunoreactivity in the head

Within the head, TSH immunoreactivity was present in the linings of the otic vesicle and in the ependymal and subependymal lining of the mesocoele (Fig. 3.1C). Pit-1 immunoreactivity was also present in these sites, but was most abundant in the cells and fibres in the trigeminal nerve (Fig. 3.1H), in which TSH staining was slight and restricted to scattered cells. Immunoreactivity for TSH was also present in ependymal and subependymal cells lining the anterior and posterior diencephalon (Fig. 3.2A, C, E) and in infundibular cells (Fig. 3.2A). It was also present in the epidermis (Fig. 3.2C) and in the endothelial cell lining of the ethmoidal artery (Fig. 3.2C). In marked contrast, staining for Pit-1 was not seen in any of these sites (Fig. 3.2B, D, F), or in Rathke's pouch (Fig. 3.2B), which was also devoid of TSH immunoreactivity (Fig. 3.2A).

Immunoreactivity in the trunk

The epidermal cells of the trunk were strongly immunoreactive for TSH (Fig. 3.3A), but not for Pit-1 (Fig. 3.3B). TSH immunoreactivity was also intense in the ependymal and subependymal cells of the spinal cord and weakly present in the dorsal root ganglia (Fig. 3.3A). In contrast, Pit-1 immunoreactivity was intense in the upper marginal layer of the spinal cord and abundant in the dorsal root ganglia (Fig. 3.3B). A single adluminal layer of spinal cord ependymal cells also had Pit-1 immunoreactivity (Fig. 3.3E), as did cells in the myotome (Fig. 3.3F). Slight TSH immunoreactivity was present in a small number of cells in the marginal layer of the spinal cord, although most were not immunreactive (Fig. 3.3C). TSH immunoactivity was also absent in the myotome (Fig. 3.3D).

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At the level of the limb bud, TSH immunoreactivity was widespread and intense (Fig. 3.4A). It was particularly strong in a subepidermal layer of cells (Fig. 3.4C) and in pockets of the brachial nerve plexus (Fig. 3.4D), but it was also in the dorsal and ventral muscle blocks and in chondrocytes in the humerus (Fig. 3.4A). In marked contrast, there was no staining for Pit-1 in the limb bud (Fig. 3.4B, E, F). In the internal organs, heavy TSH immunostaining was present in the bronchi of the lungs (Fig. 3.5A) and in the proventriculus (crop) of the gastrointestinal tract (Fig. 3.5C). There was no Pit-1 immunoreactivity in the lung (Fig. 3.5B) and only a single layer of Pit-1 immunoreactive cells that lined the crop lumen (Fig. 3.5D). Similarly, while cells lining the pleural cavity had intense TSH immunoreactivity (Fig. 3.6A), these cells were completely devoid of Pit-1 immunostaining. In contrast, hepatocytes were strongly immunoreactive for both TSH and Pit-1 (Fig. 3.6C, D). No other peripheral tissues contained TSH or Pit-1 immunoreactivity.

Western Analysis

A Pit-1-like protein of approximately 33 kDa was detected using αPit-1 #132 in the adult chicken pituitary gland, ED 7 chick head, trunk, wingbud and whole eye (Fig. 3.9).

Reverse Transcription - Polymerase Chain Reaction

In the presence of oligonucleotide primers for pituitary Pit-1 cDNA (Pit-1 (11) and Pit-1 (12)), a moiety of approximately 279 bp was amplified from the adult chicken

pituitary gland as visualized with ethidium bromide staining (Fig. 3.8A). No bands were visible for ED7 chicken embryo tissues or the negative controls.

Southern Blotting

In the presence of a DIG labeled Pit-1 RNA probe, a moiety of approximately 279 bp was detected for amplified adult chicken pituitary and ED7 chick head, trunk and wingbud cDNA (Fig. 3.8B). No bands were visible in the negative controls.

IV. DISCUSSION

These results demonstrate, for the first time, the presence of Pit-1 immunoreactivity in pituitary and extrapituitary cells in the chicken. They also clearly show that Pit-1 immunoreactivity in the early chick embryo is present in tissues with and without TSH immunoreactivity. The extrapituitary expression of the TSH gene may thus be tissue-specific, and in some tissues appears to be Pit-1 independent.

This is the first demonstration of Pit-1 immunoreactivity in avian tissues, although the Pit-1 gene has been cloned and sequenced from chicken (Buys *et al.*, 1998; Tanaka *et al.*, 1999; Harvey *et al.*, 2000) and turkey (Wong *et al.*, 1992) pituitary glands. The most abundant transcript, Pit-1α, has sequence homology with mammalian Pit-1, but other avian-specific variants are also expressed, although in much smaller amounts (Tanaka *et al.*, 1999). It is therefore likely that the immunoreactivity detected in chicken tissues reflects the presence of Pit-1α or a very similar protein, especially as the antisera used has cross-reactivity with Pit-1-like proteins in more primitive vertebrates (Candiani and Pestarino, 1998a,b, 1999) and Western analysis revealed a protein of 33 kDa in

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embryonic chick tissues, the same molecular weight as pituitary Pit-1 and mammalian Pit-1 (Harvey et al., 2000). Indeed, the Pit-1 gene appears to have been conserved during phylogenetic evolution (Harvey et al., 2000) and Pit-1-like proteins are even present in sponges (Seimiya et al., 1997).

In the pituitary gland, Pit-1 is thought to regulate the transcription of the GH, prolactin and TSH genes (Harvey et al., 2000). The somatotrophs in the caudal lobe are spatially separated from the thyrotrophs and lactotrophs in the cephalic lobe of the chicken pituitary gland (Mikami, 1983), and Pit-1 immunoreactivity was, accordingly, found in both the caudal and cephalic lobes of the adenohypophysis. In addition to regulating the pituitary expression of these genes, Pit-1 is thought to be obligatory for the phenotypic differentiation of the GH-, prolactin- and TSH-secreting cells (Harvey et al., 2000). Indeed, these cells are thought to be derived from the same cellular lineage and are absent in Pit-1 deficient Snell dwarf mice (Burrows et al., 1999). It is therefore of interest that Pit-1 immunoreactivity was not detected in Rathke's pouch, which was also devoid of TSH immunoreactivity at this stage of embryonic development (Fig. 3.2). The ontogeny of pituitary TSH expression in the chick embryo therefore appears to occur after the ontogeny of extrapituitary TSH production. The absence of βTSH mRNA in the pituitary glands of ED 11 embryos (Gregory et al., 1998) supports this view. Thommes et al. (1983) previously found some immunoreactive βTSH in Rathke's pouch at ED 6.5, but these cells were of low abundance until ED 11.5, when the caudal and cephalic lobes of the adenohypophysis were readily discernable. It is therefore likely that pituitary TSH synthesis is minimal until the ontogenic differentiation of thyrotroph cells. The ontogeny of Pit-1 gene expression in the pituitary glands of embryonic chicks is similarly not

thought to occur until the second third of incubation (Geris *et al.,* 2002), even though extrapituitary Pit-1 mRNA is present in ED 2 embryos and the headless bodies of ED 6 and ED 8 embryos (Harvey *et al.,* 2000), and this study demonstrates Pit-1 mRNA in tissues of the ED 7 chick embryo (heads, headless and wingless trunks, wingbuds). Pit-1 is thus not pituitary-specific in its expression in embryonic chicks and its extrapituitary expression occurs before that in the pituitary, as also observed in rats (He *et al.,* 1989; Dolle *et al.,* 1990).

The so-called pituitary specificity of Pit-1 is based on early studies that indicated it was absent from the intestine, liver, lung, spleen, thyroid, thymus, pancreas, kidney, muscle, heart, testes, prostate, uterus, brain and neural retina, and was lacking in fibroblasts, hepatocytes and placental cells (reviewed by Harvey *et al.,* 2000). More recent studies have, however, indicated its presence in the rat (He *et al.,* 1989), mouse (Dolle *et al.,* 1990) and chicken (Yoshizato *et al.,* 1998) brain, as well as its presence in the placenta, spleen, thymus, bone marrow and kidney of mammals (Harvey *et al.,* 2000). The results of the present study extend the list of extrapituitary tissues that appear to express the Pit-1 gene. In addition to neural tissues, it was found in skin, muscle and bone, and was present in the liver, gastrointestinal tract and the cardiopulmonary system.

In some of these tissues (brain and spinal cord ependymal cells, neural cells in the otic vesicle, and liver hepatocytes), the distribution of Pit-1 immunoreactivity overlapped the distribution of immunoreactive TSH. This overlapping pattern of distribution may reflect a casual relationship between Pit-1 and TSH expression and indicates that extrapituitary TSH may be Pit-1 dependent. However, in other tissues (eg. the trigeminal nerve in the head and the marginal mantle layer of the spinal cord), Pit-1

immunoreactivity was intense, but TSH immunoreactivity was marginal. Conversely, other tissues (e.g. cells in the skin, blood vessels, limb bud, bronchus, proventriculus and cardiopleural cavities) had intense TSH staining but little, if any, Pit-1 immunoreactivity. The Pit-1 dependence of the TSH gene may therefore be tissue-specific. Sakai *et al.* (1999) similarly found that both Pit-1 and TSH were present in the rat pars distalis, but only TSH was present in the pars tuberalis.

The disparate distribution of Pit-1 and TSH in many extrapituitary tissues may thus indicate that it is not involved in extrapituitary TSH regulation. The disparate distribution of Pit-1 may, for instance, reflect its regulation of genes other than TSH, GH and prolactin, especially as it is known to be involved in the expression of the renin gene (Cantanzaro *et al.*, 1994; Gilbert *et al.*, 1994; Germain *et al.*, 1996) and the genes for the somatostatin receptor (Baumeister and Meyerhof, 1998), and the GH-releasing hormone receptor (Lin *et al.*, 1992; Gaylinn, 1999; Iguchi *et al.*, 1999; Miller *et al.*, 1999). Pit-1 binding sites have also been detected in the promoter regions of the gonadotropin-releasing hormone receptor (Fan *et al.*, 1995; Kakar, 1997), the thyrotropin-releasing hormone receptor, the secretogranin II gene (Jones *et al.*, 1996) and the somatolactin gene (Takayama *et al.*, 1991; Ono *et al.*, 1995). It is therefore possible that the presence of Pit-1 in extrapituitary tissues may reflect a variety of tissue-specific actions.

In summary, these results demonstrate that Pit-1 immunoreactivity is widespread in tissues of the chick embryo, although the expression of the pituitary TSH gene is thought to be Pit-1 dependent. The disparate location of Pit-1 and TSH immunoreactivities in these tissues suggests TSH expression in extrapituitary tissues may be Pit-1 independent.

Fig 3.1 Immunocytochemical localization of TSH and Pit-1. (A) Localization of TSH immunoreactivity in the cephalic (Ce) lobe of the adult chicken pituitary gland. (B) Localization of Pit-1 immunoreactivity in both the cephalic and caudal (Ca) lobes of the gland. Magnification x100. The insets show higher magnifications (x1000). (C) Localization of TSH immunoreactivity in the head of an ED 7 chick embryo. Staining is present in the ependymal and subependymal cells lining the mesocoele (mc) and in the otic vesicles (ov). Magnification x100. The inset shows a higher magnification (x1000) of the otic vesicles. (D) Localization of Pit-1 immunoreactivity in the head of the ED 7 chicken embryo. Staining is present in the otic vesicles and in the ependymal lining of the mesocoele. Magnification x100. The inset shows a higher magnification of the otic vesicle (x1000). (E) TSH immunoreactivity in ependymal mesencephalon (m). Magnification x100. (F) Pit-1 immunoreactivity in the trigeminal nerve (tg). (G) Higher magnification (x1000) of the TSH immunoreactivity in ependymal cells surrounding the mesocoele (boxed area in [E]). (H) Higher magnification (x1000) of the Pit-1 immunoreactivity in the trigeminal nerve (boxed area in [F]). TSH staining in (C), (D) and (E) is modified from data presented by Murphy and Harvey (2001) and is shown for comparison.
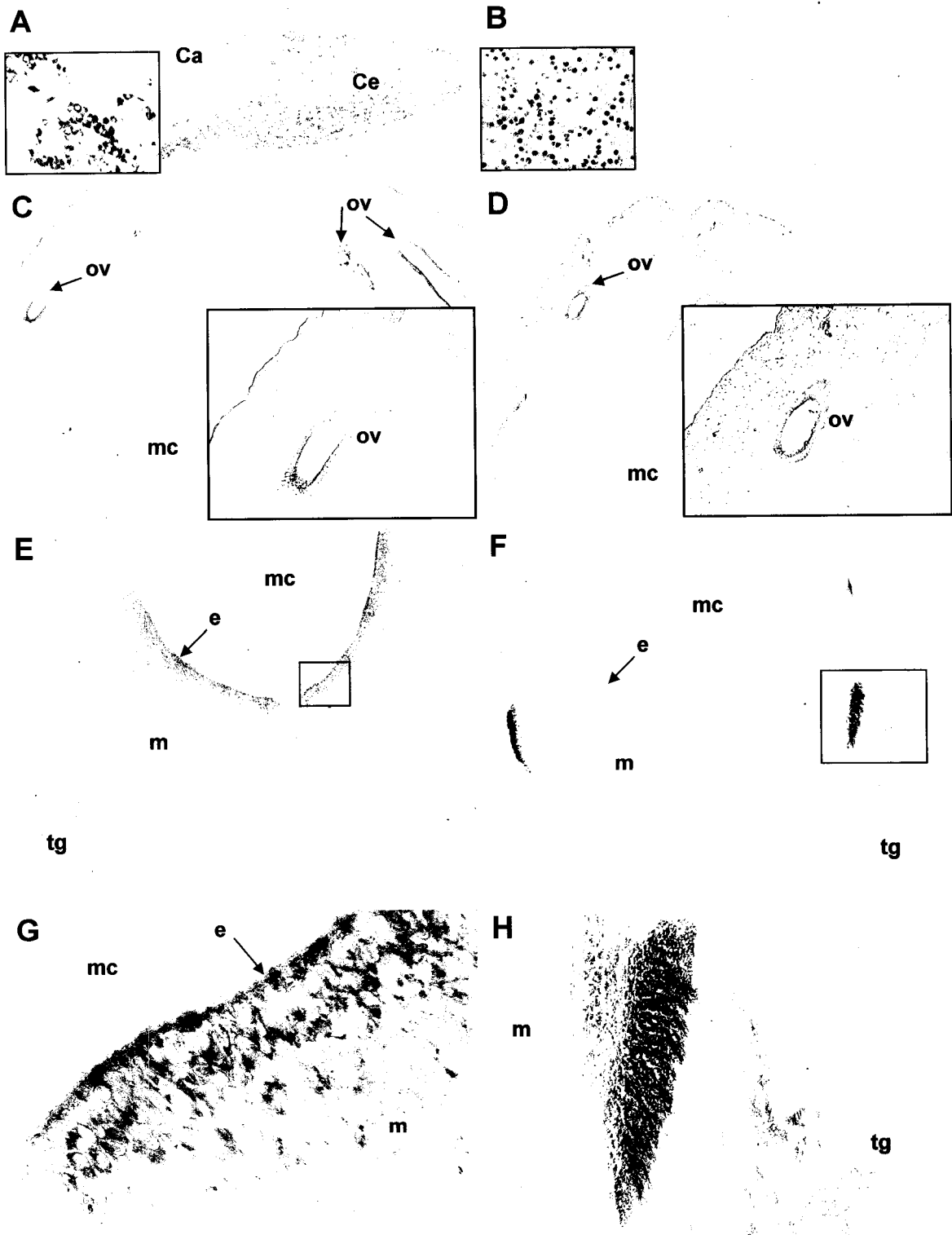
A   Ca   Ce

B

C   ov

ov

ov

mc   ov

D   ov

mc   ov

E   mc

e

m

tg

F   mc

e

m

tg

G   e

mc

m

H   m

tg

Fig 3.2 TSH and Pit-1 immunostaining of the ED 7 chick embryo head. (A) TSH immunoreactivity is present in ependymal and subependymal cells in the infundibular (i) region of the diencephalon and in endothelial cells (arrow) of the ethmoid artery (bv). Rathke's pouch (Rp) is devoid of TSH staining. Magnification x100. (B) Pit-1 immunoreactivity is not present in a similar section of the ED7 embryo head. Magnification x100. (C) TSH immunoreactivity in ependymal cells (e) lining the diocoele (dc) in the mantle margin of the diencephalon (d) and in the outer epidermis (ep) (arrow). Magnification x100. The inset shown in (E) shows TSH immunoreactivity in ependymal cells in the mantle region of the diencephalon at a higher (x1000) magnification. (D) and (F) Pit-1 immunoreactivity is not present in similar sections of the ED 7 embryo head. Magnification x100 and x1000, respectively. TSH immunoreactivity in (A), (C) and (E) is modified from data presented by Murphy and Harvey (2001) and is shown for comparison.
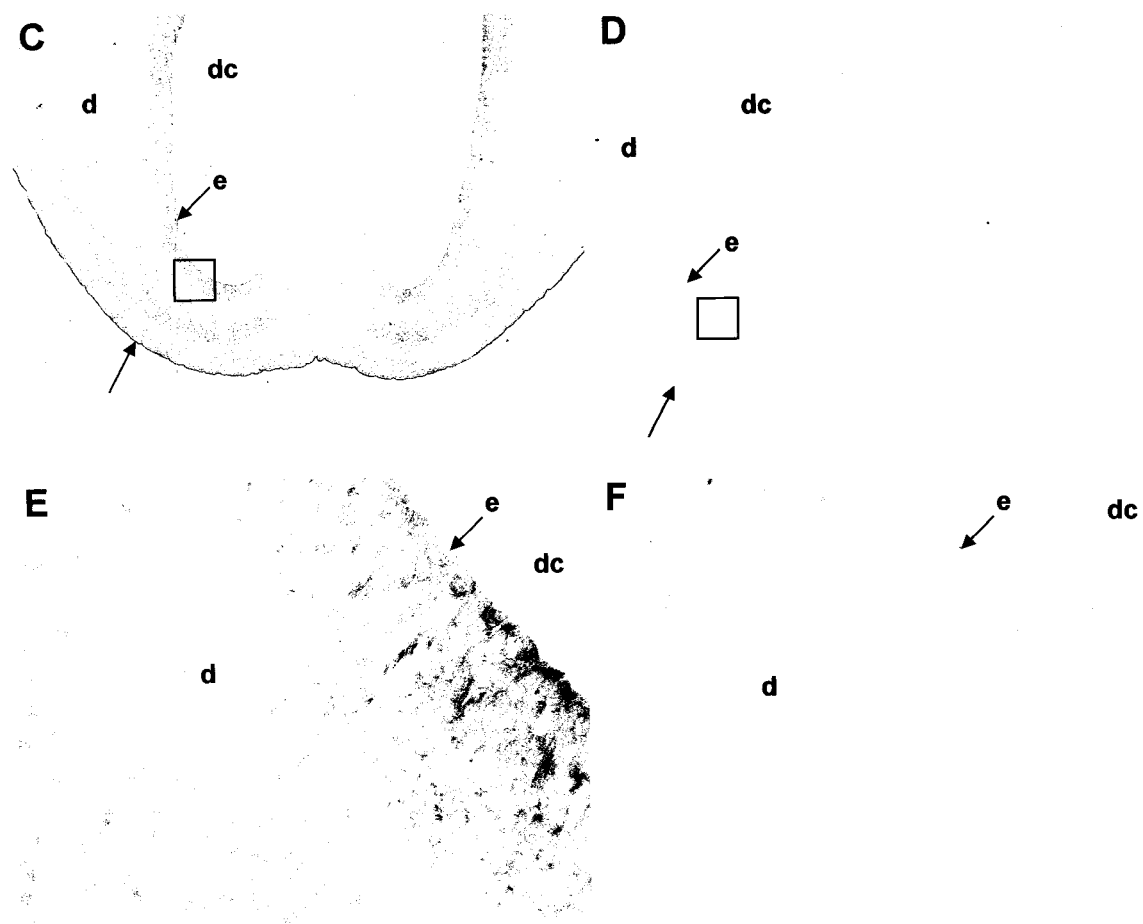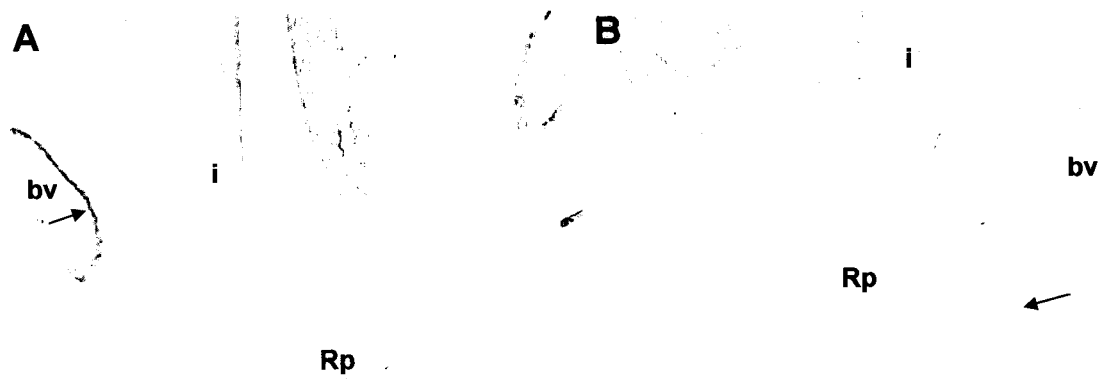
A

bv

i

Rp

B

i

bv

Rp

C

dc

d

e

D

dc

d

e

E

e

dc

d

F

e

dc

d

Fig 3.3 TSH and Pit-1 immunostaining in the trunk of ED 7 chick embryos. (A)
Intense TSH immunoreactivity in the ependymal cells (e) of the spinal cord (sc) and outer
epidermis (arrow). Faint TSH staining is also present in the dorsal root ganglia (d) but is
not present in marginal (ml) or mantle (m) layers of white matter, nor in the notochord
(n). Magnification x100, except in the insert (x1000). (B) Intense Pit-1 immunoreactivity
is present in the upper marginal layer (ml) of the spinal cord (sc) and its ependymal lining
(e), as well as in the dorsal root ganglia (d) and surrounding myotome (my). Pit-1
immunoreactivity is not present in the mantle (m) layer of the spinal cord, nor in the
notochord or the surrounding notochordal sheath. Magnification x100, except in the inset
(x1000). (C) Higher magnification (x1000) of the marginal layer (ml) and mantle (m) of
the spinal cord. (D) Faint TSH immunostaining in the dorsal root ganglia. Magnification
x1000. (E) and (F) Higher magnifications (x1000) of the ependyma and myotome shown
in (B). TSH immunoreactivity in (A) is modified from data presented by Murphy and
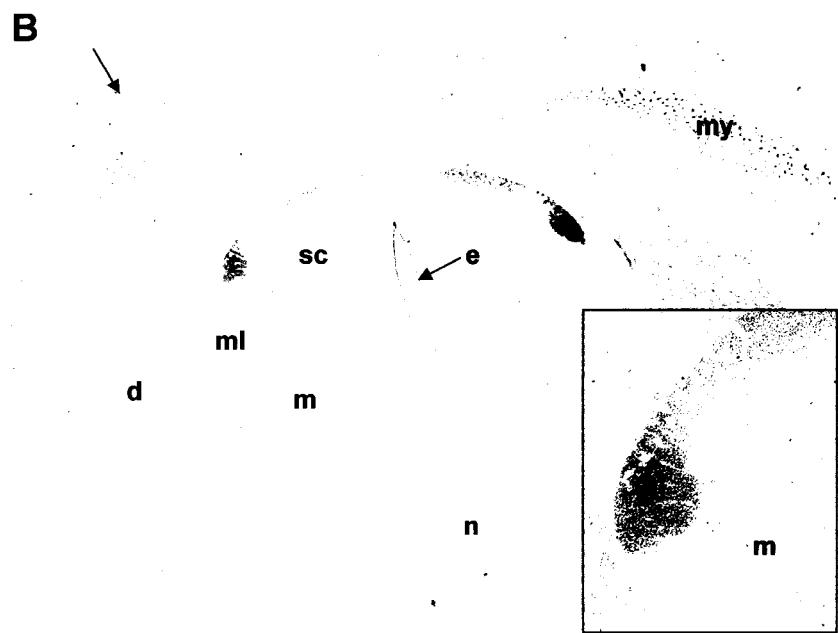Harvey (2001) and is shown for comparison.

A

my

sc

e

ml

m

d

n

C

ml

m

D

e

B

sc

e

ml

m

d

n

my

e

E

m

F

Fig 3.4 TSH and Pit-1 immunostaining in the trunk of ED 7 chick embryos. (A)

Widespread TSH immunoreactivity in present in most cells of the limb (wing) bud.

Labeling is most intense in pockets of the brachial nerve plexus (inset) and in the outer

epidermis (arrow), although the dorsal (d) and ventral (v) muscle masses of the wing and

chondrocytes in the humerus (h) are also immunostained. Magnification x100. (C) and

(D) show magnifications of the insets (x400, x1000, respectively). (B), (E) and (F) Pit-1

immunoreactivity is not present in similar sections of the wing bud.

A

d

h

v

B

d

h

v

C

D

E

F

Fig 3.5 TSH and Pit-1 immunostaining in the trunk of ED 7 chick embryos. (A)

Intense TSH immunoreactivity in a developing bronchus in the lung. Magnification

x1000. (B) Pit-1 immunoreactivity is not present in a lung bud bronchus. Magnification

x1000. (C) Intense TSH immunoreactivity in the proventriculus (crop) of the alimentary

canal. Magnification x1000. (D) Pit-1 immunoreactivity in a single layer of adluminal

cells in the proventriculus. Magnification x1000. TSH immunoreactivity in (A) and (C) is

modified from data presented by Murphy and Harvey (2001) and is shown for

comparison.

A
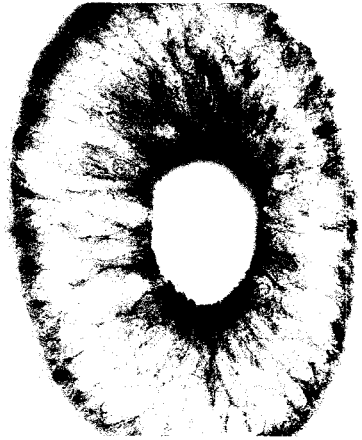
B
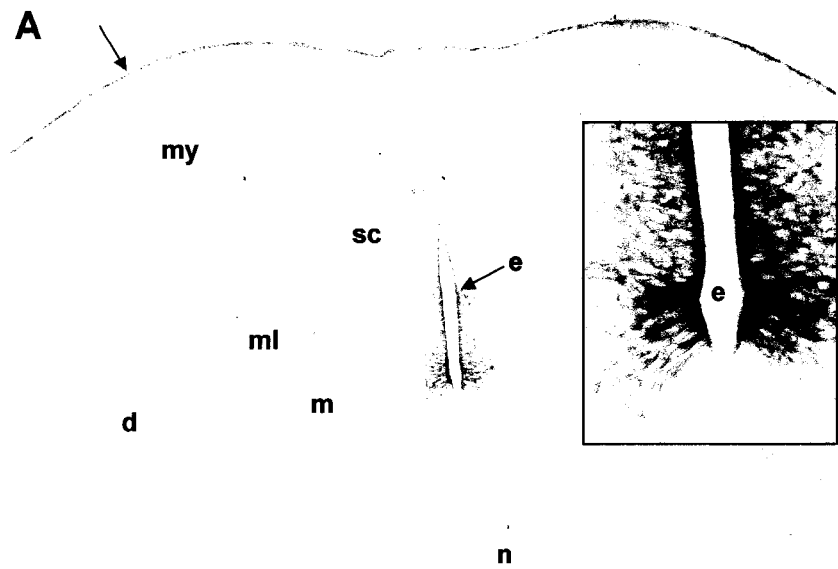
C
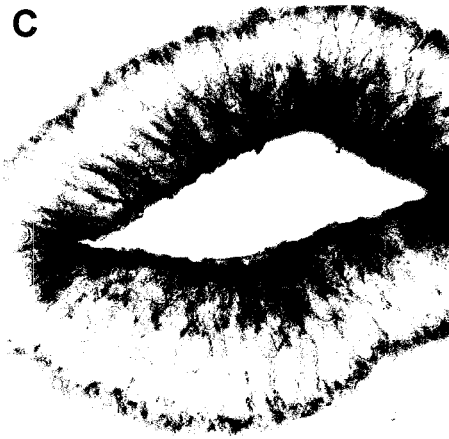
D
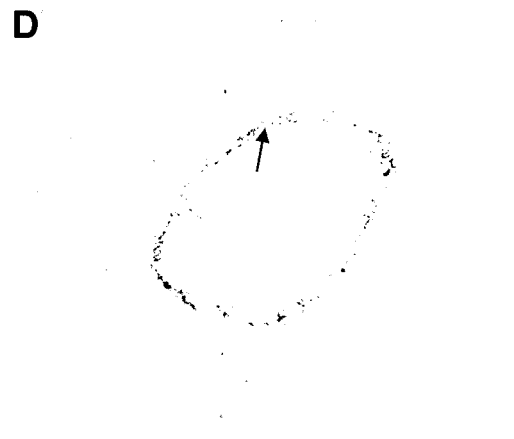
Fig 3.6 TSH and Pit-1 immunostaining in the trunk of ED 7 chick embryos. (A) Intense TSH immunoreactivity in a single layer (arrow) of cells lining the pleural cavity (pc). Magnification x1000. (B) Pit-1 immunoreactivity is not present in a similar section of the pleural cavity. Magnification x1000. (C) TSH immunolabeling of hepatocytes in the liver. Immunostaining was not present in blood cells (arrow). Magnification x1000. (D) Pit-1 immunoreactivity in liver hepatocytes but not blood cells (arrow). Magnification x1000. TSH immunoreactivity in (A) and (C) is modified from data presented by Murphy and Harvey (2001) and is shown for comparison.
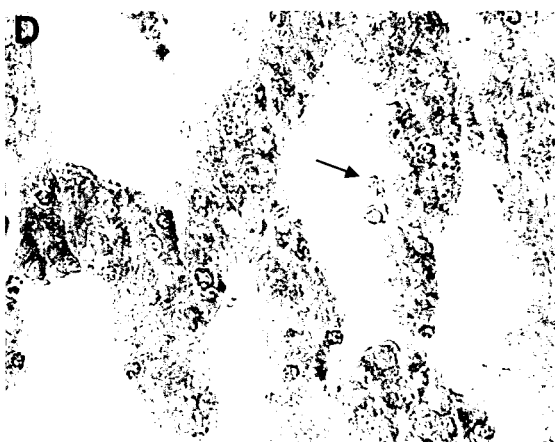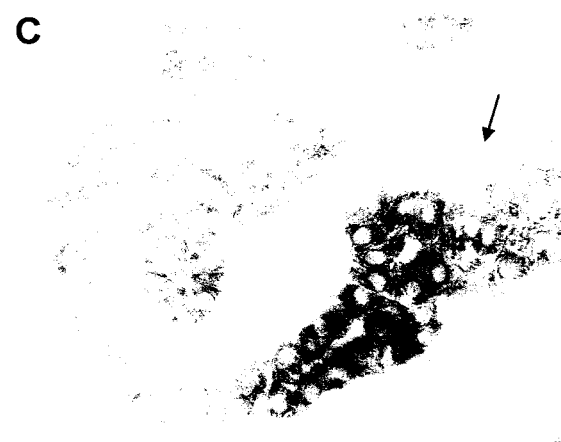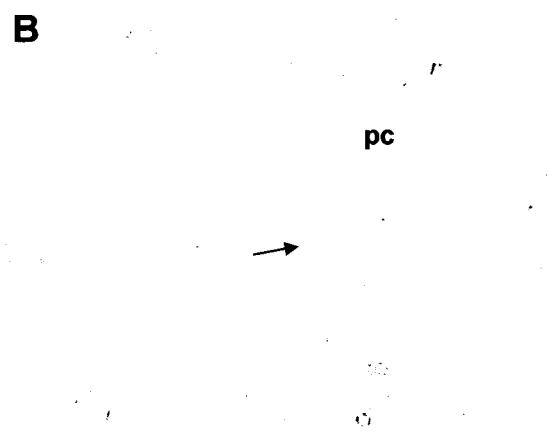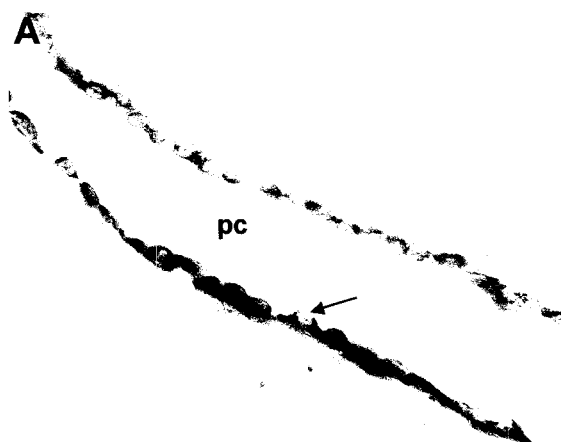
Fig 3.7 Western analysis of Pit-1-like proteins in tissues of ED 7 chick embryos.

Homogenates of adult chicken pituitary, ED 7 head, trunk, wingbud and whole eye were subjected to Western blotting. Pit-1-like proteins of appropriate size (about 33 kDa) are present in all tissues of the ED 7 chick embryo and correspond to the 33 kDa band seen in the adult chicken pituitary (positive control). Protein homogenates were obtained from a pool of at least 5 embryos.

Adult Pituitary   ED 7 Head   ED 7 Trunk   ED 7 Wingbud   ED 7 Whole Eye

~33 kDa

Fig 3.8 RT-PCR and Southern Blotting for Pit-1 in ED 7 chick embryos. (A) RT-PCR of Pit-1 mRNA in ED 7 chick embryos. Total tissue RNA was extracted and reverse transcribed. The cDNA was then amplified in the presence of oligonucleotide primers (t11 and t12) designed to amplify a 279 bp fragment of chicken pituitary Pit-1 cDNA (Harvey *et al* 2000). This fragment was amplified from reverse transcribed RNA (+) extracted from the ED 7 head, trunk and wingbud and adult chicken pituitary (positive control), but was only visible in the pituitary with ethidium bromide staining. This fragment was not evident when PCR was performed with RNA in the absence of reverse transcriptase (-) or in the adult chicken muscle (negative control). (B) Southern blotting of PCR products amplified in the presence of oligonucleotide primers designed to amplify a 279 bp fragment of chicken pituitary Pit-1 cDNA, using a DIG-labeled Pit-1 RNA probe. RNA from ED 7 head, trunk and wingbud produced a 279 bp Pit-1 cDNA after RT-PCR and Southern blotting (+), but not in the absence of reverse transcriptase (-).

A

Adult Pituitary ED 7 Head + ED 7 Trunk + ED 7 Wing + DNA Mass Ladder Adult Muscle ED 7 Head - ED 7 Trunk - ED 7 Wing -

279 bp

B

Adult Pituitary ED 7 Head + ED 7 Trunk + ED 7 Wing + Adult Muscle ED 7 Head - ED 7 Trunk - ED 7 Wing -

279 bp
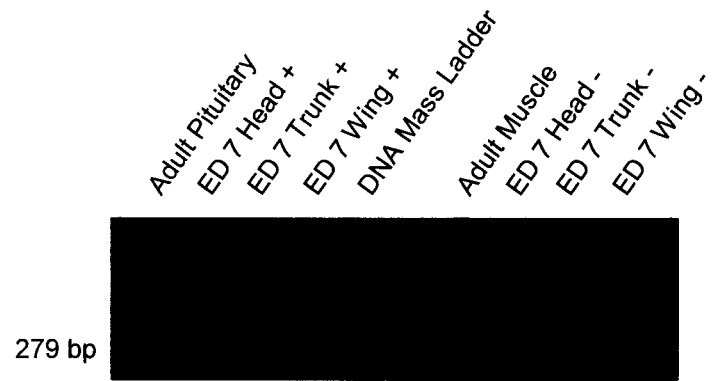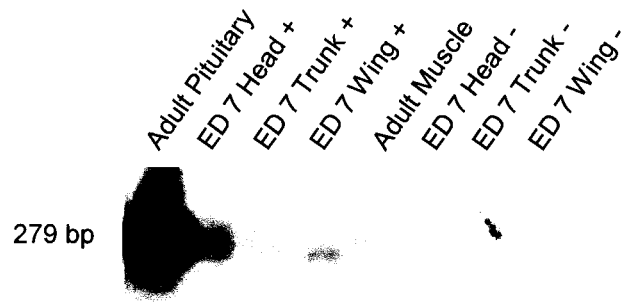
V. REFERENCES

Baumeister H, Meyerhof W. (1998) Involvement of a Pit-1 binding site in the regulation of the rat somatostatin receptor gene expression. *Annals of the New York Academy of Science.* 865:390-2.

Bodey B, Bodey B Jr, Siegel SE, Kaiser HE. (2000) The role of the reticulo-epithelial (RE) cell network in the immuno-neuroendocrine regulation of intrathymic lymphopoiesis. *Anticancer Research.* 20:1871-88.

Burrows HL, Douglas KR, Camper SA. (1999) Genealogy of the anterior pituitary gland: tracing a family tree. *Trends in Endocrinology and Metabolism.* 10:343-52.

Buys N, Van As P, Volchaert G, Decuypere E. (1998) Chicken pituitary-specific transcription factor-1 (Pit-1) cDNA cloning and analysis of sequence variation. *Biotechnologie, Agronomie Societe et Environement.* 2:20.

Candiani S, Pestarino M. (1998a) Evidence for the presence of the tissue-specific transcription factor Pit-1 in lancelet laevae. *Journal of Comparative Neurology.* 400:310-6.

Candiani S, Pestarino M. (1998b) Expression of the tissue-specific transcription factor Pit-1 in the lancelet, *Branchiostoma lanceolatum. Journal of Comparative Neurology.* 392:343-51.

Candiani S, Pestarino M. (1999) The tissue-specific transcription factor Pit-1 is expressed in the spinal cord of the lancelet *Branchiostoma lanceolatum. Neuroscience Letters.* 260:25-8.

Cantanzaro DF, Sun J, Gilbert MT, Yan Y, Black T, Sigmund C, Gross KW. (1994) A Pit-1 binding site in the human renin gene promoter stimulates activity in pituitary, placental and juxtaglomerular cells. *Kidney International.* 46:1513-151.

De Vito WJ (1989) Thyroid hormone regulation of hypothalamic immunoreactive thyrotropin. *Endocrinology.* 125:1219-23.

De Vito WJ, Connors JM, Hedge GA; (1985) Distribution and release of immunoreactive thyroid-stimulating hormone in the rat hypothalamus: effects of thyroidectomy, hypophysectomy and treatment with thyroid hormones. *Neuroendocrinology.* 41:23-30.

Dolle P, Castrillo JL, Theill LE, Deerinck T, Ellisman M, Karin M. (1990) Expression of GHF-1 protein in mouse pituitaries correlates both temporally and spatially with the onset of growth hormone gene activity. *Cell.* 60:809-20.

Fan NC, Peng C, Krisinger J, Lung PC. (1995) The human gonadotropin-releasing hormone receptor gene: complete structure including multiple promoters, transcription initiation sites and polyadenylation signals. *Molecular and Cellular Endocrinology.* 107:R1-R8.

Gaylinn BD. (1999) Molecular and cell biology of the growth hormone releasing hormone receptors on thymocytes and splenocytes from rats. *Growth Hormone IGF Research.* 9 (Suppl. A):37-44.

Germain S, Konoshita T, Phillippe J, Corvol P, Pinet F. (1996) Transcrptional induction of the human renin gene by cyclic AMP requires cyclic AMP response element-binding protein (CREB) and a factor binds a pituitary-specific trans-active factor (Pit-1) motif. *Biochemical Journal.* 316:107-13.

Geris KL, Van Os P, De Groof B, Hickey GJ, Kuhn ER, Millar RP, Decuypere E, Darras VM. (2000) Control of growth hormone secretion in the chicken: role of hypothalamic and hypophyseal agents. *Abstract V11 Internationl Symposium on Avian Endocrinology.* Varanassi, India.

Gilbert MT, Sun J, Yan Y, Oddux C, Lazarus A, Tansey WP, Lavey TN Catanzaro DF. (1994) Renin gene promoter activity in GC cells is regulated by cAMP and thyroid hormone through Pit-1 dependent mechanisms. *Journal of Biological Chemistry.* 269:28 049-28 054.

Gregory CC, Dean CE, Porter TE. (1998) Expression of chicken thyroid-stimulating hormone β-subunit messenger ribonucleic acid during embryonic and neonatal development. *Endocrinology.* 139:474-8.

Hamburger V & Hamilton H. (1951) A series of normal stages in the development of the chick embryo. *Journal of Morphology* 88:49-92.

Harada A, Hershman JM. (1978) Extraction of human chorionic thyrotropin (hCT) from term placentas: failure to recover thyrotropic activity. *Journal of Clinical Endocrinology and Metabolism.* 47:681-5.

Harbour DV, Kruger TE, Coppenhaver D, Smith EM, Meyer WJ. (1989) Differential expression and regulation of thyrotropin (TSH) in T cell lines. *Molecular and Cellular Endocrinology.* 64:229-41.

Harvey S, Azumaya Y, Hull KL. (2000) Extrapituitary growth hormone: Pit-1 dependence? *Canadian Journal of Physiology and Pharmacology.* 78:1013-28.

He XI, Treacy MN, Simmons DM, Ingraham HA, Swanson LW, Rosenfeld MG. (1989) Expression of a large family of POU domain regulatory genes in mammalian brain development. *Nature (London)* 340:35-42.

Hojvat S, Baker G, Kirsteins L, Lawrence AM. (1982a) TSH in the rat and monkey brain. Distribution, characterization and effect of hypophysectomy. *Neuroendocrinology.* 34:327-32.

Hojvat S, Emanuele N, Baker G, Connick E, Kirsteins L, Lawrence AM. (1982b) Growth hormone (GH), thyroid-stimulating hormone (TSH), and luteinizing hormone (LH) -like peptides in the rodent brain: non-parallel ontogenetic development with pituitary counterparts. *Brain Research.* 256:427-34.

Hojvat S, Emanuele N, Baker G, Kirsteins L, Lawrence AM. (1985) Brain thyroid-stimulating hormone: effects of endocrine manipulations. *Brain Research.* 360:257-63.

Hsu SM, Raine L, Fanger H. (1981) Use of avidin-peroxide complex ABC in immunoperoxidase techniques: a comparison between ABD and unlabeled antibody AAP procedures. *Journal of Histochemistry and Cytochemistry.* 29 ;577-80.

Iguchi G, Okimura Y, Takahashi T, Mizuno I, Fumoto M, Takahashi Y, Kaji H, Abe H, Chihera K. (1999) Cloning and characterization of the 5'-flanking region of the human growth hormone-releasing hormone receptor gene. *Journal of Biological Chemistry.* 274:12 108-12 114.

Jones LC, Day RN, Pittler SJ, Valentine CL, Scammell JG. (1996) Cell-specific expression of the rat secretogranin II promoter. *Endocrinology.* 137:3815-22.

Kakar SS. (1997) Molecular structure of the human gonadotropin-releasing hormone receptor gene. *European Journal of Endocrinology.* 137:183-92.

Lin CR, Lin SC, Chang CP, Rosenfeld MG. (1992) Pit-1 dependent expression of the receptor for growth hormone releasing factor mediates pituitary cell growth. *Nature (London).* 360:765-8.

Mikami SI. (1983) Avian adenohypophysis: recent progress in immunocytochemical studies. In. *Avian Endocrinology: Environmental and Ecological Perspectives* Edit S-I Mikami.pp 38-56. Springer-Varlag. Berlin .

Miller TL, Godfrey PA, Dealmeida VI, Mayo KE. (1999) The rat growth hormone-releasing hormone receptor gene: structure, regulation, and generation of receptor isoforms with different signalling properties. *Endocrinology.* 140:4152-65.

Murphy AE, Harvey S. (2001) Extrapituitary TSH and GH in early chick embryos. *Molecular and Cellular Endocrinology.* 185:161-71.

Ono M, Mochizuki E, Mori Y, Aizawa A, Harigai T. (1995) The regulatory region and transcription factor required for the expression of rat and salmon pituitary hormone-encoding genes shows cell-type and species specificity. *Gene.* 153:267-71.

Peele ME, Carr FE, Baker JR Jr, Wartofsky L, Burman KD. (1993) TSH beta subunit gene expression in human lymphocytes. *American Journal of Medical Science*. 305:1-7.

Sakai T, Sakemoto S, Ijima K, Matzubara K, Kato Y, Inoue K. (1999) Characterization of TSH positive cells in foetal rat pars distalis that fail to express Pit-1 factor and thyroid hormone beta 2 receptors. *Journal of Neuroendocrinology*. 11:187-93.

Seimiya M, Watanabe Y, Kurosawa Y. (1997) Identification of POU-class homeoboxgenes 1-freshwater sponge and the specific expression of these genes during differentiation. *European Journal of Biochemistry*. 243:31-37.

Smith EM, Phan M, Kruger TE, Coppenhaver DH, Blalock JW. (1983) Human lymphocyte production of immunoreactive thyrotropin. *Proceedings of the National Academy of Science. (USA)* 80:6010-3.

Takayama Y, Rand-Wenner M, Kawauchi H, Ono M. (1991) Gene structure of chum salmon somatolactin, a presumed pituitary hormone of the growth hormone/prolactin family. *Molecular Endocrinology*. 5:778-86.

Tanaka M, Yamamoto I, Ohkubo T, Akita M, Hoshino S, Nakashima K. (1999) cDNA cloning and developmental alterations in gene expression of the two Pit-1/GHR-1 transcription factors in the chicken pituitary. *General and Comparative Endocrinology*. 114:441-8.

Thommes RC, Martens JB, Hopkins WE, Caliendo J, Sorrentino MJ, Woods JE. (1983) Hypothalamo-adenohypophyseal-thyroid interrelationships in the chick embryo. IV. Immunocytochemical demonstration of TSH in the hypophyseal pars distalis. *General and Comparative Endocrinology*. 51:434-43.

Voss JW, Yao TP, Rosenfeld MG. (1991) Alternative translation initiation site usage results in two structurally distinct forms of Pit-1. *Journal of Biological Chemistry*. 266:12832-5.

Wang J, Whetsell M, Klein JR. (1997) Local hormone networks and intestinal T cell homeostasis. *Science*. 275:1937-9.

Wong EA, Silsby JL, El Halawani ME. (1992) Complementary DNA cloning and expression of Pit-1/GHF-1 from the domestic turkey. *DNA Cell Biology*. 11:651-60.

Yoshizato H, Fujikawa T, Soya H, Tanaka M, Nakashima K. (1998) The growth hormone (GH) gene is expressed in the lateral hypothalamus: enhancement by GH-releasing hormone and repression by restraint stress. *Endocrinology*. 139:2545-51.

Chapter 4

Extrapituitary Growth Hormone: Under-estimation of Immunohistochemical

Staining by Carnoy's Fixation

I. INTRODUCTION

Growth hormone (GH) gene expression primarily occurs in pituitary somatotrophs, although numerous extrapituitary sites of GH expression have been documented. Indeed, in domestic fowl, GH mRNA has been identified in the brain (Render *et al.*, 1995a), in the thymus, bursa and spleen (Render *et al.*, 1995b), in the testes and vas deferens (Harvey *et al.*, 2002; Murphy *et al.*, 2002), in the ovary (Luna *et al.*, 2000), in the heart (Takeuchi *et al.*, 2001) and in the eye (Takeuchi *et al.*, 2001). In mammalian species, GH gene expression has additionally been determined in the placenta (Schwarler *et al.*, 1997), mammary gland (Mol *et* al., 1995a, b), liver (Recher *et* al., 2001), lung (Allen *et* al., 2000), salivary gland (Tresguerres *et al.*, 1999), skin (Palmetshofer *et al.*, 1995; Slominski *et al.*, 2000) and in smooth muscle and endothelial cells of blood vessels (Wu *et al.*, 1996; Recher *et al.*, 2001). Proteins with GH-immunoreactivity are also present in these sites and in other peripheral tissues (eg. in teeth, kidney and gut; Kyle *et al.*, 1981; Costa *et al.*, 1993; Zhang *et al.*, 1997). Nevertheless, with the exception of limited studies on the nervous (Lechan *et al.*, 1981; Ramesh *et al.*, 2000, Takeuchi *et al.*, 2001) and immune (Kao *et al.*, 1992; Maggiano *et al.*, 1994) systems, this immunoreactivity has been determined by radioimmunoassay of tissue extracts and its cellular location is largely unknown. Two immunohistochemical studies on the localization of GH in peripheral tissues of embryonic chicks have, however, been published (Harvey *et al.*, 2000; Murphy & Harvey, 2001).

In one study, GH-immunoreactivity was found to be abundant and widespread in the bodies of embryonic tissues. This immunoreactivity was thought to be specific, since it was detectable by three different GH antibodies (two polyclonals, one monoclonal) and

was completely lost following pretreatment of the primary antibodies with excess recombinant GH. A different tissue distribution of GH immunoreactivity was, however, observed in a more recent study (Murphy & Harvey, 2001), in which the cellular distribution of GH was restricted to discrete cells or tissue layers. The main difference between these two studies was the fixative used for immunohistochemistry: 4% paraformaldehyde (Harvey *et al.*, 2000) and Carnoy's fixative (Murphy & Harvey, 2001). Since different fixatives can result in quantitative and qualitative changes in protein detection (Chiu *et al.*, 1994; Vince *et al.*, 1997; Hemmer *et al.*, 1998; Giaccone *et al.*, 2000), the influence of fixation on the immunocytochemical detection of GH in chick embryos was therefore empirically assessed.

II. MATERIALS AND METHODS

Tissues

Fertile White Leghorn chicken eggs from the University of Alberta Poultry Unit were incubated at 37 °C in humidified air. The eggs were turned one quarter of a revolution each day during the incubation period. Whole embryos at embryonic day 7 (ED 7) of the 21 day period of embryogenesis were dissected and decapitated in phosphate-buffered saline (PBS, pH 7.4) prior to fixation. ED 7 embryos were used since pituitary somatotrophs do not differentiate until ED 12 to ED 14 (Porter, 1997) and hence tissue GH-immunoreactivity could not be due to the sequestration of pituitary GH. Peripheral tissues were similarly used to ensure that GH-immunoreactivity was not due to pituitary GH detection.

Fixation

Tissues were collected into freshly prepared paraformaldehyde (4% w/v, in PBS) (Sigma, Mississauga, Ontario, Canada) or Carnoy's fluid (60% ethanol v/v; 30% chloroform v/v; 10% acetic acid v/v). Paraformaldehyde is a cross-linking fixative that forms links (hydroxyl-methylene bridges) between reactive end-groups of adjacent protein chains, whereas Carnoy's is a precipitant fixative that denatures proteins by destroying the hydrophobic bonds that hold together the tertiary structures of large protein molecules.

Immunohistochemistry

The headless embryos were fixed, overnight, at 4 °C. They were then dehydrated in a graded series of alcohol (50% 15 min; 70% 15 min; 95% 30 min; 100% 2x30 min) and cleared with Hemo-de (Fisher Scientific, Edmonton, Alberta, Canada) for 2x30 min. The tissues were then infiltrated with paraffin wax to 24 h at 60 °C under normal atmospheric pressure. Serial transverse (8 μm) sections were then taken using a microtome and mounted into charged slides (Fisher Scientific). Immunocytochemical staining was performed with commercial reagents (Vector Laboratories, Burlingame, CA, USA; Sigma) using the avidin-biotin-peroxidase (ABC) method (Hsu *et al.*, 1981). Sections were incubated overnight at 4 °C with a specific polyclonal antisera raised in rabbits against native (pituitary) chicken GH (Harvey *et al.*, 2000), diluted 1:1000 in 1% normal goat serum. After incubation, the slides were washed three times for 5 min in PBS. Sections were then incubated for 1 h at room temperature in biotinylated goat anti-rabbit immunoglobin G (IgG) (Sigma; 1:500). The slides were then washed in PBS and

incubated in ABC reagent for 1 h at room temperature and washed in PBS. Staining was

visualized using the chromogenic substrate, diaminobenzidine tetrahydrochloride (DAB)

(Sigma), which resulted in a brown coloration. The specificity of staining was determined

by preabsorbing the GH antisera with recombinant chicken GH (Amgen, Thousand Oaks,

CA, USA; 1 mg/ml) for 1 h prior to section incubation. All tissues collected in

paraformaldehyde or Carnoy's fluid were processed together under identical conditions

with the same reagents. Digital images were collected using a SPOT digital Microscope

Camera (Carsen Group, Markham, Ontario, Canada) mounted on an Olympus B

microscope.

III. RESULTS

After Carnoy's fixation, intense immunocytochemical staining for GH was seen

in the spinal cord, although restricted to the marginal cells of the white matter, to the

ventral horn and mantle, to spinal nerves, and to the ventral root nuclei (Fig. 4.1A, B, C).

The surrounding tissue was, however, only faintly stained or unstained. In contrast,

strong staining was seen throughout the spinal cord following paraformaldehyde fixation,

including the ependymal cells surrounding the spinal canal (Fig. 4.1C, D). In contrast to

the tissues fixed in Carnoy's, the tissues fixed in paraformaldehyde had intense GH-

immunoreactivity in both the ventral and dorsal root ganglia (Fig. 4.1C, D). Similarly,

while GH-immunoreactivity was abundant and widespread in the notochord, notochordal

sheath and in the myotome of paraformaldehyde-fixed tissues (Fig. 4.2B, D), GH-

immunoreactivity was not detectable in these tissues following Carnoy's fixation (Fig.

4.2A, C).

A single layer of epithelial cells lining the esophagus (crop) had GH-immunoreactivity following Carnoy's fixation (Fig. 4.3A), although intense GH-immunoreactivity was seen in numerous cells in the mucosal epithelium, submucosa and muscular layers of this structure following paraformaldehyde fixation (Fig. 4.3B). Strong GH staining was, likewise, seen in the bronchus after fixation in paraformaldehyde (Fig. 4.3D), but only faint staining was seen after fixation in Carnoy's (Fig. 4.3C). Similar differences in GH staining were also seen in the heart, in which intense staining was present in the myocardium of the atria and in atrial cushions (Fig. 4.4B) and ventricles (Fig. 4.5C, D) fixed in paraformaldehyde, but not in the atria (Fig. 4.4A) or ventricles (Fig. 4.5A, B) fixed in Carnoy's. Strong GH-immunoreactivity was also seen in the cells lining the pericardial cavity and in the body wall of embryos fixed in paraformaldehyde (Fig. 4.4C, D), but not in embryos fixed in Carnoy's (Fig. 4.4A, B). Although intense staining was present in the myocardial cells of the heart following paraformaldehyde fixation, only faint, diffuse staining was present in blood cells in the atria and ventricles and in the pericardial space (Fig. 4.4C, D, Fig. 4.5C, D). The atrio-pulmonary septum surrounding the ventricles was also only slightly stained following paraformaldehyde fixation (Fig. 4.5C, D). In marked contrast, strong and discrete GH staining was localized in the extensor nerve of the anterior limb bud fixed in Carnoy's (Fig. 4.6A), whereas GH-immunoreactivity was widespread in the limb bud fixed in paraformaldehyde. Following paraformaldehyde fixation, GH-immunoreactivity was particularly abundant in chondrocytes of the humerus (Fig. 4.6F) (as in chondrocytes in the ribs, Fig. 4.1D) and in a layer of outer epidermal cells (Fig. 4.6E), which were faintly stained or unstained after Carnoy's fixation (Fig. 4.6B, C). Intense GH-immunoreactivity is present in the dorsal

and ventral muscle mass of the wing bud following paraformaldehyde fixation (Fig. 4.6D), but not after Carnoy's (Fig. 4.6A).

In all tissues fixed in Carnoy's or paraformaldehyde, the GH staining was specific and completely lost following preabsorption of the primary antibody with excess recombinant GH (data not shown).

IV. DISCUSSION

These results clearly show the presence of GH-like proteins in peripheral tissues of early chick embryos, but demonstrate tissue-specific differences in staining following paraformaldehyde or Carnoy's fixation.

The distribution of GH-immunoreactivity in the peripheral tissues of paraformaldehyde-fixed ED 7 chicks was widespread, but particularly striking in the spinal cord, dorsal and ventral root ganglia, notochord, myotome, epidermis, heart and the humerus. These findings extend preliminary observations by Harvey *et al.* (2000) using paraformaldehyde-fixed tissues from ED 6 and ED 7 embryos. The cellular localization of GH staining in the neural tube, dorsal and ventral root ganglia, notochord, heart and limb bud is, however, much clearer in this study and presented at high magnification for the first time. The staining for GH in these tissues is clearly cellular and within the cytoplasm, whereas it could not be clearly differentiated from background staining in the earlier study (Harvey *et al.*, 2000). The presence of GH staining in the esophagus (crop), lung (bronchus), myotome and the anterior extensor nerve following paraformaldehyde staining are novel observations.

A widespread distribution of GH-immunoreactivity might be expected, since the headless bodies of ED 6 and ED 8 embryos readily express the GH gene (Harvey *et al.*, 2000). A widespread cellular distribution of GH-immunoreactivity might also be expected in view of the finding, in mammals, of GH mRNA and immunoassayable GH in extracts of most peripheral tissues (eg. Kyle *et al.*, 1981; Costa *et al.*, 1993; Wu *et al.*, 1996; Recher *et al.*, 2001), particularly in perinatal animals (Costa *et al.*, 1993; Recher *et al.*, 2001). A widespread distribution of GH-immunoreactivity might also be expected, since GH may act as an autocrine or paracrine growth factor during embryonic development (Harvey & Hull, 1997) and GH receptors (Harvey *et al.*, 2000) and a GH responsive gene (Harvey *et al.*, 2001a) are ubiquitous in embryonic tissues.

The distribution of GH-immunoreactivity in ED 7 tissues fixed in Carnoy's was noticeably different from that observed following paraformaldehyde fixation. After Carnoy's, the immunohistochemical staining of GH was more discrete (mainly restricted to marginal and mantle layers of the spinal cord, the ventral root ganglia, to spinal nerves and to the anterior extensor nerve). These results are similar to those published by Murphy and Harvey (2001), in which GH staining was also observed in the trigeminal nerve and vagal nerve following Carnoy's fixation. This suggests that Carnoy's fixative preferentially detects GH-immunoreactivity in neural tissues. The discrete presence of GH in the extensor nerve is a novel finding of this study, as is the absence of GH staining in chondrocytes and cardiac cells fixed in Carnoy's fluid. Indeed, apart from the neural derivatives, GH-immunoreactivity is barely detectable in the peripheral tissues of embryos fixed in Carnoy's. It is therefore of interest that the GH staining in the heads of ED 7 chicks fixed in Carnoy's (Murphy & Harvey, 2001) was also much less than in

heads fixed in paraformaldehyde (Harvey *et al.*, 2001b). This is particularly evident for

Rathke's pouch (a non-neural pharyngeal derivative that differentiates into the pituitary

gland), which is almost devoid of GH-immunoreactivity following Carnoy's fixation

(Murphy & Harvey, 2001) but abundantly stained for GH-immunoreactivity following

paraformaldehyde fixation (Harvey *et al.*, 2001b). The controversy in the literature

(Porter, 1997) on the ontogeny of pituitary somatotrophs (ED 4.5, Thommes *et al.*, 1987

(Bouin's fixation); ED 10, Malamed *et al.*, 1993 (Zenker's fixative); ED 12, Jozsa *et al.*,

1979; Allaerts *et al.*, 1999 (Zamboni's fluid); ED 12, Porter *et al.*, 1995 (B-5: formalin-

mercuric chloride)), might therefore partly reflect differences in the fixatives used.

Qualitative differences in the distribution of GH staining in other tissues of embryonic

chicks may similarly reflect differences in tissue fixation. For instance, while Harvey *et*

al. (2001b) observed widespread staining for GH in neural retinas fixed in

paraformaldehyde, Takeuchi *et al.* (2001) found GH-immunoreactivity only in pigmented

retinal epithelial cells following fixation in Bouin's (a picric acid-based fixative).

Paraformaldehyde is a cross-linking fixative and is particularly useful for fixing

smaller proteins and peptides (Polak & Van Noorden, 1997). Carnoy's, in contrast, is a

precipitant fixative and more useful for the fixation of large proteins. Carnoy's fluid is a

better fixative than paraformaldehyde in preserving tissue architecture and in facilitating

tissue sectioning (Polak & Van Noorden, 1997), but it reduces specific staining for GH-

immunoreactivity. Carnoy's fixative has similarly been found to reduce antigen staining

in other immunocytochemical studies, in comparison with formalin or paraformaldehyde

(e.g. Cammer *et al.*, 1985; Schutte *et al.*, 1987; Bos *et al.*, 2000). It is therefore possible

that Carnoy's and paraformaldehyde fixatives may differentially fix the different GH

moieties present in neural and non-neural tissues. It is now well established that pituitary GH comprises a family of size and charge variants that may result from differential gene transcription or post-translational modifications (Aramburo *et al.*, 2000, 2001a, b; Martinez-Coria *et al.*, 2002). This may include oligomerization (into dimmers, trimers, quadramers and pentamers) and proteolytic cleavage (into fragments of 15 kDa and 7 kDa) of the monomer (26 kDa) moiety (Aramburo *et al.*, 2001b). Small GH moieties of 14 kDa to 17 kDa have also been identified in pituitary extracts and, interestingly, these are preferentially produced in embryonic chicks (Aramburo *et al.*, 2000). GH size heterogeneity has also been identified in the testes of embryonic and adult chickens (Luna *et al.*, 2000, 2002), in which the submonomer variants are the most abundant moieties, particularly in embryonic tissues. Submonomer GH variants are also present in the eyes of chick embryos, but not in the eyes of neonatal chicks (Takeuchi *et al.*, 2001). Submonomer GH moieties, therefore, appear to be more abundant in peripheral extrapituitary tissues than in the pituitary and more abundant in embryos than in neonatal or adult chickens. It is, therefore, possible that these smaller moieties are not fixed by Carnoy's fluid, under-estimating the GH-immunoreactivity that is detected by the cross-linking of small and large GH proteins during paraformaldehyde fixation. Fixation with Carnoy's fluid does, however, appear to selectively label GH in neural derivatives, and it may be more useful than paraformaldehyde for immunohistochemical studies on neural GH. This selectivity may, interestingly, reflect the fact that no submonomer GH moieties are present in Western blots of hypothalamic and extrahypothalamic brain tissues (Render *et al.*, 1995a), whereas submonomer GH moieties are abundantly present in the other non-neural tissues (Render *et al.*, 1995a; Luna *et al.*, 2000).

In summary, qualitative and quantitative differences in tissue GH-immunoreactivity are evident in immunohistochemical studies employing paraformaldehyde and Carnoy's fixatives. The presence of GH-immunoreactivity in the embryonic chick is under-estimated by fixation in Carnoy's fluid, and caution should be used in interpreting immunohistochemical data obtained with this fixative.

Fig 4.1 GH –immunoreactivity in the spinal cord of ED 7 chick embryos. Following fixation in Carnoy's fluid (A, B, C), intense staining is present in the marginal layer (ml), spinal nerves (spn) and the ventral root nuclei (vr). Light staining is also present in the ventral horn (vh) and the mantle region (m). Magnification: A x100; B x400; C x400. Following fixation in 4% (w/v) paraformaldehyde, GH staining is present through the spinal cord, including the ependymal cells (e) surrounding the spinal canal and the dorsal root nuclei (dr) (D, E, F). GH-immunoreactivity is also present in the ventral root nuclei (vr) and the surrounding myotome (my), vertebral arch (arch), notochord (n), notochordal sheath (ns) and the head of a rib (r). Magnification: D x100; E x400; F x400.
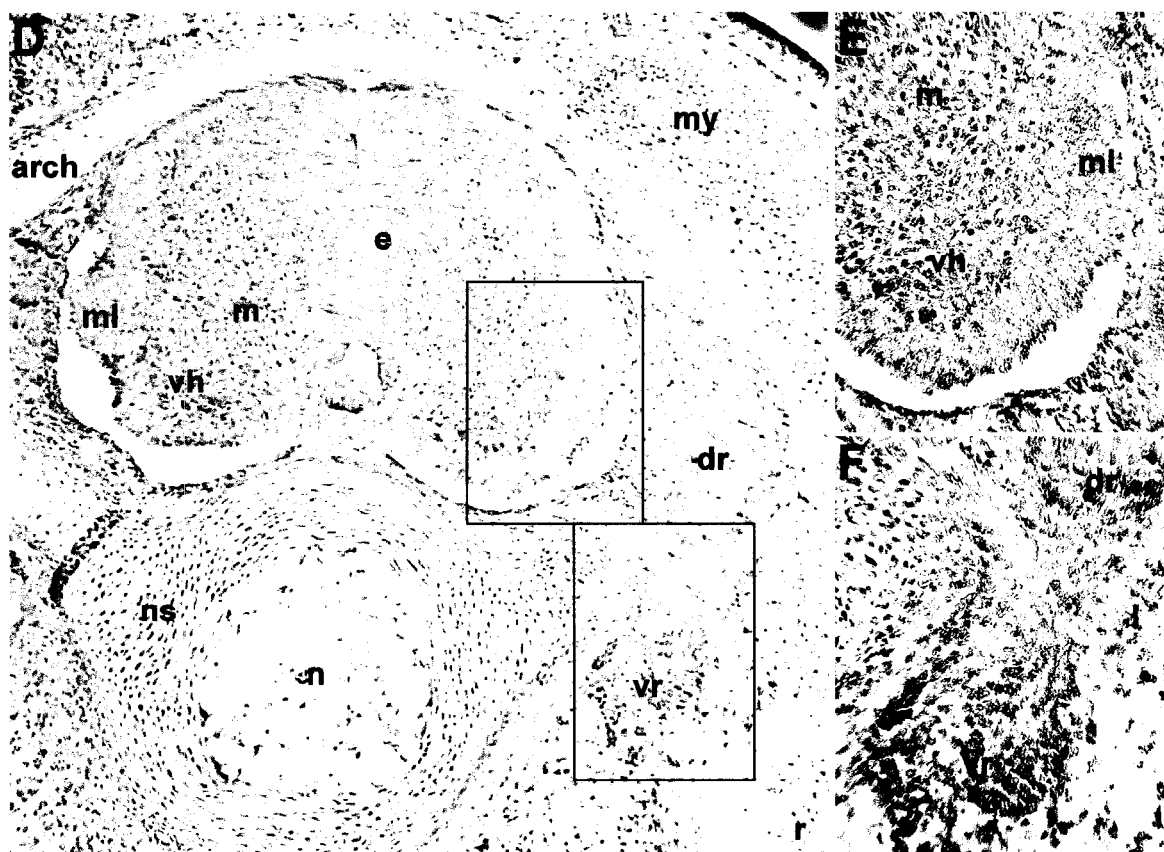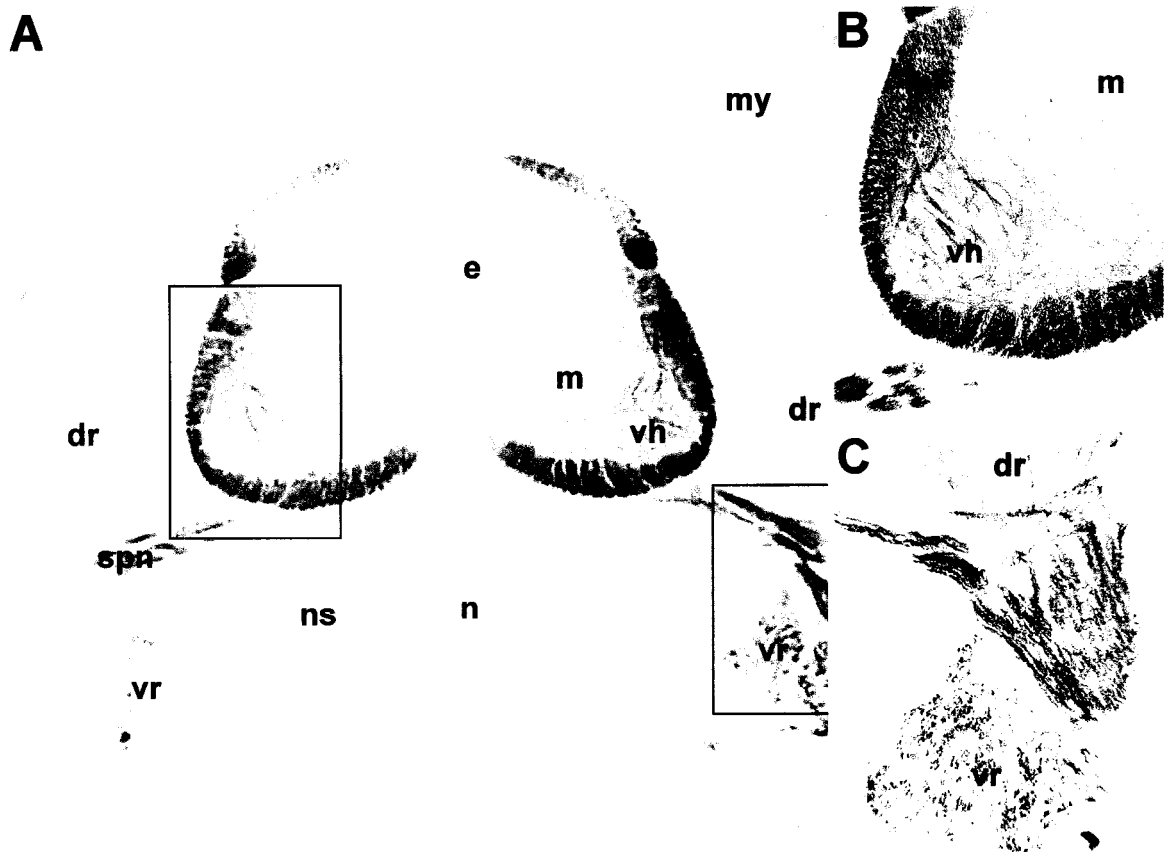
Fig 4.2 GH-immunoreactivity in the notochord and myotome of ED 7 chick embryos. Growth hormone (GH) –immunoreactivity in the notochord (n), notochordal sheath (ns; B) and myotome (my; D) of embryonic chicks following fixation in 4% (w/v) para-formaldehyde, in comparison with comparable tissues fixed in Carnoy's fluid (A, C). Magnification x400.

A
ns

n

B
ns

n

C

my

D

my
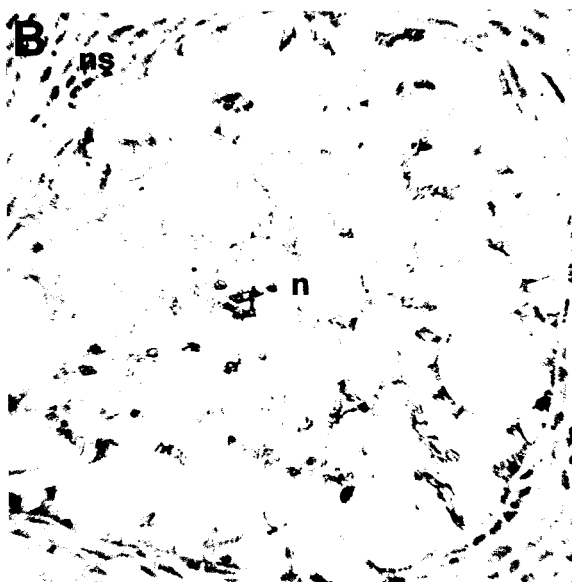
Fig 4.3 GH-immunoreactivity in the esophagus and bronchus of ED 7 chick embryos. GH –immunoreactivity in the esophagus (B) and bronchus (D) of embryonic chicks following fixation in 4% (w/v) paraformaldehyde, in comparison with tissues fixed in Carnoy's fluid (A, C): Abbreviations: e, epithelium; s, submucosa; m, esophageal muscularis. Magnification x400. Only a single layer of esophageal epithelial cells (arrowed) was GH-immunoreactive following Carnoy's fixation, whereas GH-immunoreactivity was widespread following paraformaldehyde fixation.

A

m

s

e

C

e

B

s

e

D
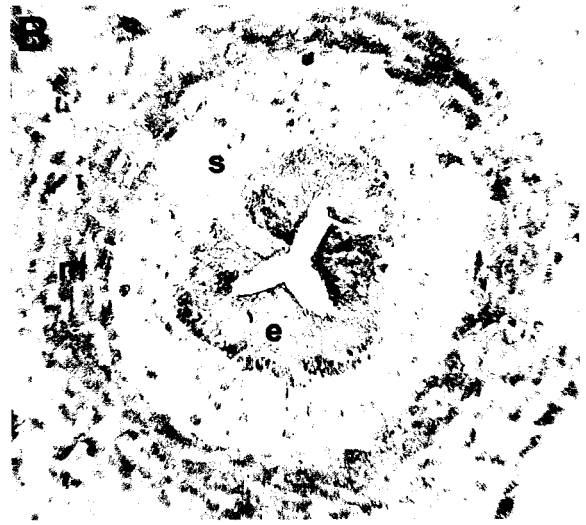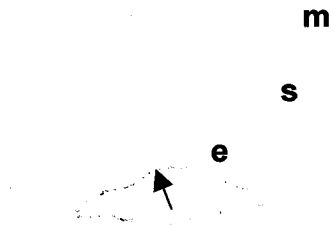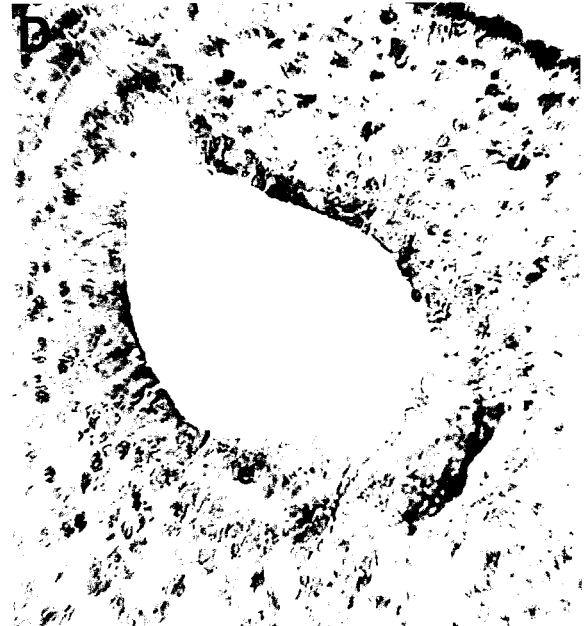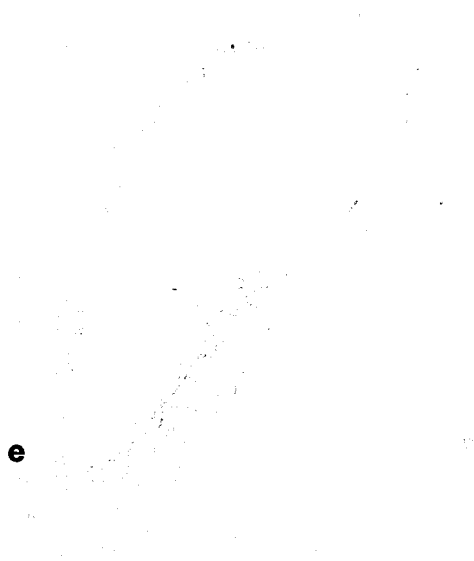
Fig 4.4 GH-immunoreactivity in the heart atria of ED 7 chick embryos. GH –

immunoreactivity in the atria of embryonic chicks following fixation in 4% (w/v)

paraformaldehyde (C, D), in comparison with atria fixed in Carnoy's fluid (A, B).

Intense GH-immunoreactivity is present throughout the myocardium (mc) of the right

atria (ra), atrial cushion (ac) and the body wall (bw) following fixation in

paraformaldehyde (C, D). Only faint, diffuse staining to blood cells (arrowed) is seen in

the atrium and pericardial cavity. Magnification: A, C x100; B, D x400. Abbreviation:

avc, atrio-ventricular canal.

A

bw

pc

ra

mc
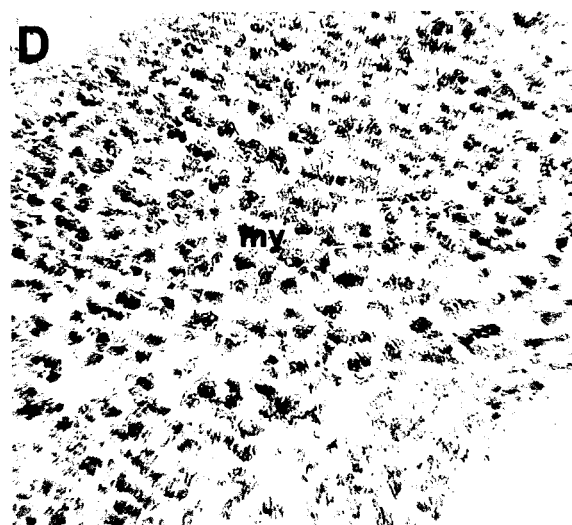
avc

B

bw

pc

mc

C

bw

pc

ra

ac

D

pc
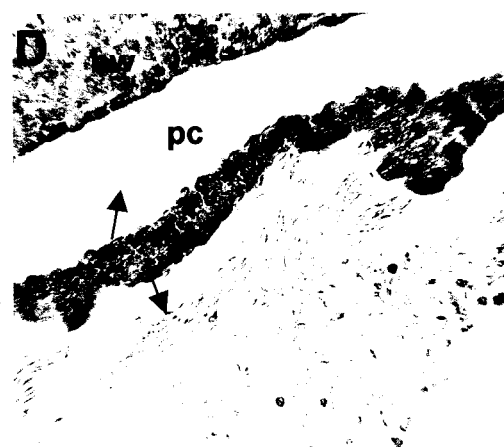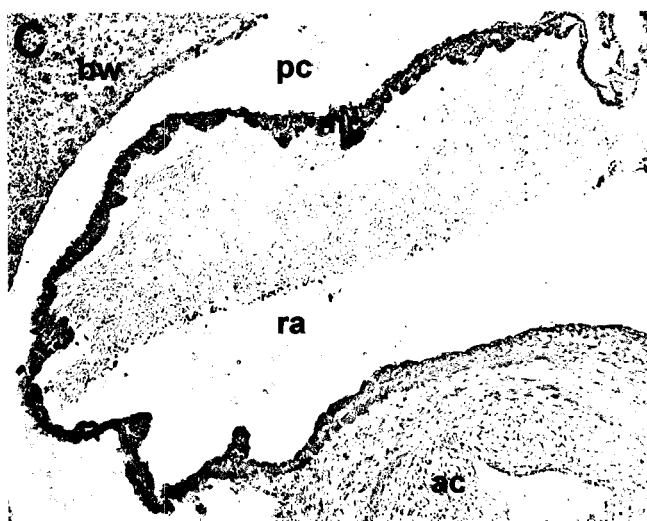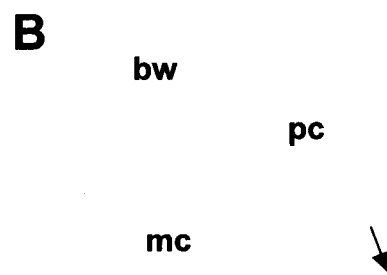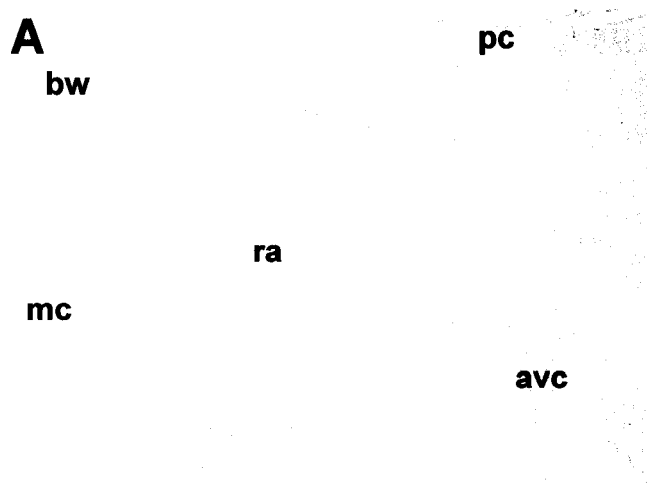
Fig 4.5 GH-immunoreactivity in the heart ventricles of ED 7 chick embryos. GH –
immunoreactivity in the ventricles of chick embryos following fixation in 4% (w/v)
paraformaldehyde (C, D), in comparison with ventricles fixed in Carnoy's fluid (A, B).
Intense GH-immunoreactivity is present throughout the ventricular myocardium (mc) of
tissues fixed in paraformaldehyde, whereas only faint GH staining is present in the
aortico-pulmonary septum (aps) or to blood cells (arrowed) in the ventricular lumen. In
contrast, GH-immunoreactivity is not seen in ventricular tissue fixed in Carnoy's (A, B).
Abbreviations: ra, right atrium; avc, atrio-ventricular canal; ivs, intraventricular septum;
bw, body wall; pc, pericardial cavity. Magnification: A, C x100; B, D x400.

A

pc

bw

B

ivs

lv

mc

mc

C

ra

pc

av

mc

rv

aps

D
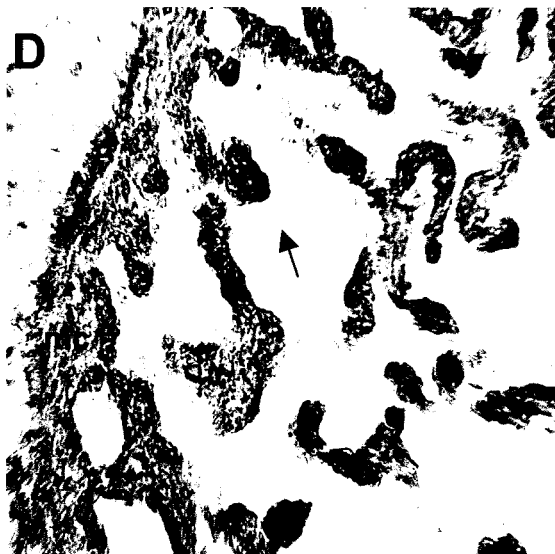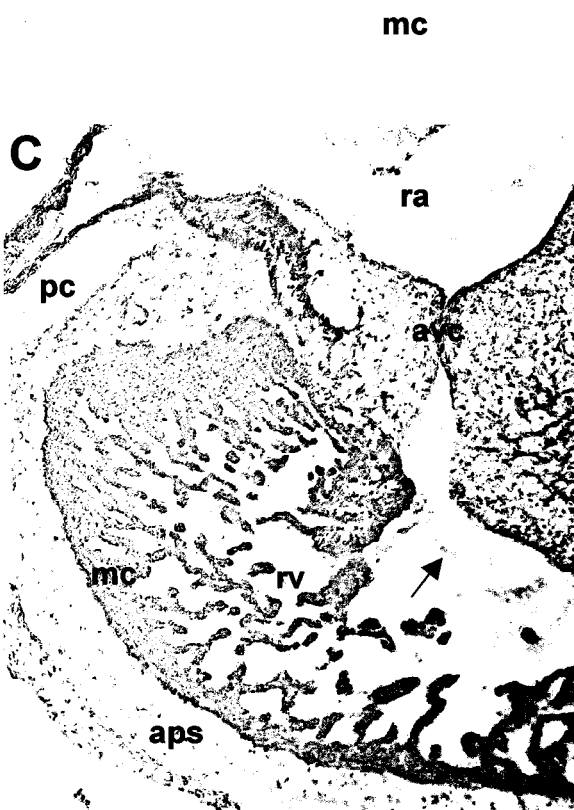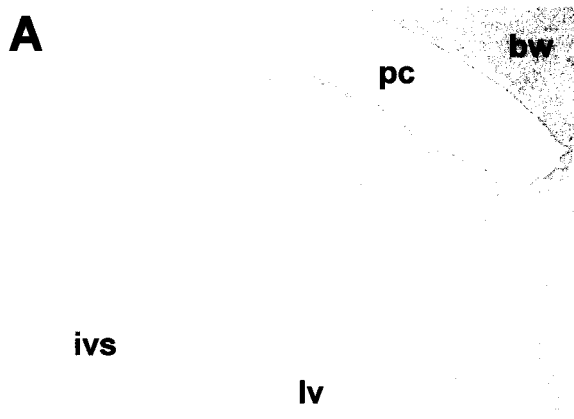
Fig 4.6 GH-immunoreactivity in the wing bud of ED 7 chick embryos. GH –

immunoreactivity in the chick embryo wingbud following fixation in Carnoy's fluid (A, B, C) or

4% (w/v) paraformaldehyde (D, E, F). Intense GH staining is present in the extensor nerve (exn,

and arrows) following fixation in Carnoy's (A, B), although cells in the dorsal (dm) and ventral

(vm) muscle masses, the epidermis (ep) and the humerus (h) were unstained. In contrast, strong

GH-immunoreactivity is seen throughout the wingbud following paraformaldehyde fixation, but

is particularly striking in the extensor nerve (arrows), muscle masses, epidermis and humerus (D,

E, F). Magnification: A, D x100; B, C, E, F x400. The insets show tracts of extensor nerve at

x1000 magnification.

A

B

dm

exn

ep

h

vm

C

ep

h

D

ep

dm

h

vm

ep

F

h
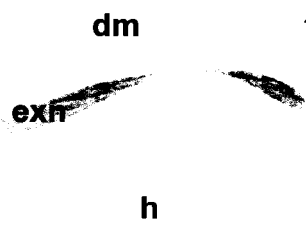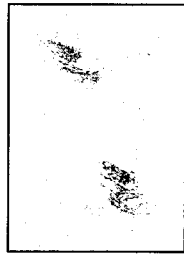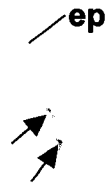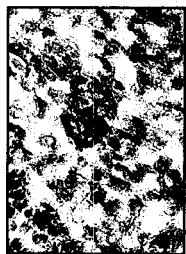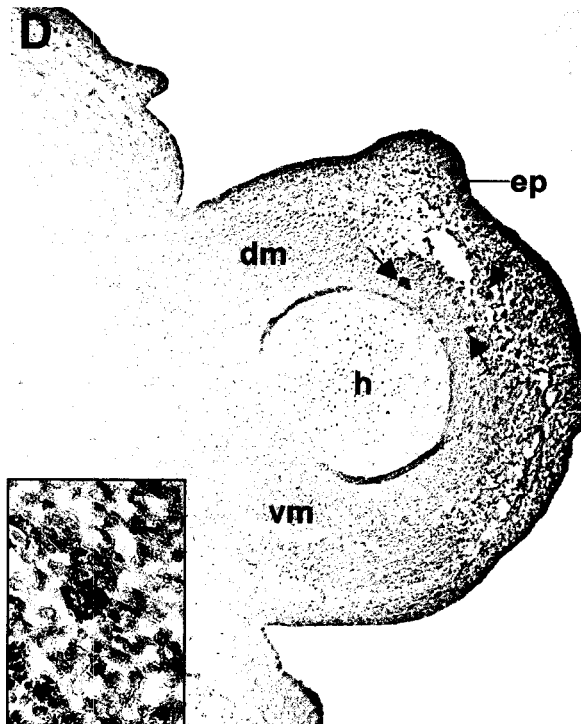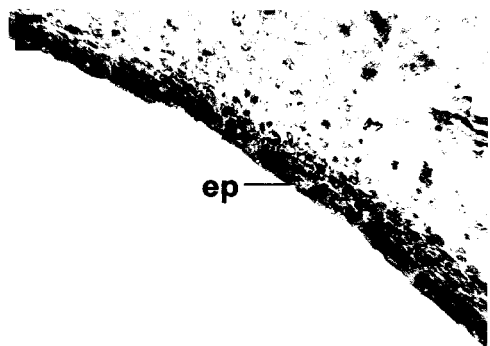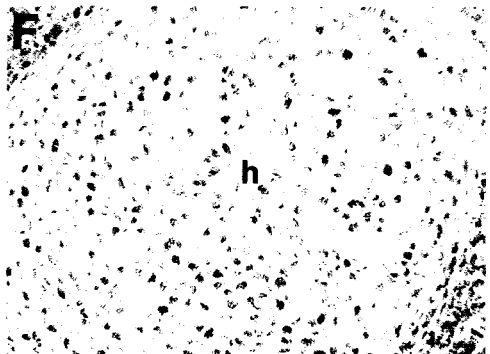
V. REFERENCES

Allaerts W, Boonstra-Blom AG, Peeters K, Janse EM, Berghman LR, Jeurissen SH. (1999) Prenatal development of hematopoietic and hormone-producing cells in the chicken adenohypophysis. *General and Comparative Endocrinology* 114:213-224.

Allen JT, Bloor CA, Kedia RK, Knight RA, Spiteri MA. (2000) Expression of growth hormone-releasing factor, growth hormone, insulin-like growth factor-1 and its binding proteins in human lung. *Neuropeptides* 34:98-107.

Aramburo C, Carranza M, Martinez-Coria H, Reyes M, Berumen L, Lopez-Rosales LJ, Pascacio H, Huerta L, Luna M. (2001a) Molecular and functional heterogeneity of growth hormone. in *Avian Endocrinology* (eds Dawson A & Chaturvedi CM) New Delhi, Narosa Publishing House, pp 273-286.

Aramburo C, Carranza M, Reyes M, Luna M, Martinez-Coria H, Berumen L, Scanes CG. (2001b) Characterization of a bioactive 15 kDa fragment produced by proteolytic cleavage of chicken growth hormone. *Endocrine* 15:231-240.

Aramburo C, Luna M, Carranza M, Reyes M, Martinez-Coria H, Scanes CG. (2000) Growth hormone size variants: changes in the pituitary during development of the chicken. *Proceedings of the Society for Experimental Biology and Medicine* 223:67-74.

Bos PK, van Osch GJ, van der Kwast T, Verwoerd-Verhoef HL, Verhaar JA. (2000) Fixation-dependent immunolocalization shift and immunoreactivity of intracellular growth factors in cartilage. *Histochemical Journal* 32:391-396.

Cammer W, Sacchi R, Sapirstein V. (1985) Immunocytochemical localization of carbonic anhydrase in the spinal cords of normal and mutant (shiverer) adult mice with comparisons among fixation methods. *Journal of Histochemistry and Cytochemistry*. 33:45-54.

Chiu KY, Loke SL, Ho FC. (1994) Immunohistochemical demonstration of c-erbB-2 oncoprotein in gastric adrenocarcinoma: comparison of cryostat and paraffin wax sections and effect of fixation. *Journal of Clinical Pathology* 47:117-121.

Costa Z, Zoppetti G, Benedettok C, Bertino E, Marozio L, Fabris C, Ariso R, Giraudi GH, Testori O, Ariano M, Maula V, Bertini E. (1993) Immunolike growth hormone substance in tissues from huhman embryos/fetuses and adults. *Journal of Endocrinological Investigation* 16:325-633.

Giaccone G, Canciani B, Puoti G, Rossi G, Goffredo D, Iussich S, Fociani P, Tagliavini T, Bugiani O. (2000) Creutzfeldt-Jakob disease: Carnoy's fixative improves the immunohisto-chemistry of the proteinase K-resistant prior protein. *Brain Pathology* 10:31-37.

Harvey S and Hull KL. (1997) Growth hormone: a paracrine growth factor? *Endocrine* 7:267-279.

Harvey S, Johnson CDM, Sanders EJ. (2000) Extra-pituitary growth hormone in peripheral tissues of early chick embryos. *Journal of Endocrinology* 166:489-502.

Harvey S, Johnson CDM, Sanders EJ. (2001b) Growth hormone in neural tissues of the chick embryo. *Journal of Endocrinology* 169:487-498.

Harvey S, Lavelin I, Pines M. (2001a) Growth hormone (GH) action in early embryogenesis: expression of a GH-response gene in sites of GH production and action. *Anatomy and Embryology* 204:503-510.

Harvey S, Murphy AE, Hull KL, Luna M, Aramburo C. (2002) Testicular growth hormone (GH) and GH mRNA. *Growth Hormone and IGF-1 Research* (in press).

Hemmer MJ, Courtney LA, Benson WH. (1998) Comparison of three histological fixatives on the immunoreactivity of mammalian P-glycoprotein antibodies in the sheepshead minnow, *Cyprinodon variegates. Journal of Experimental Zoology* 291:251-259.

Hsu SM, Raine L, Fanger H. (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *Journal of Histochemistry and Cytochemistry.* 39:159-63.

Jozsa R, Scanes CG, Vigh S, Mess B. (1979) Functional differentiation of the embryonic chick pituitary gland studies by immunohistological approach. *General and Comparative Endocrinology* 39:159-163.

Kao T-L, Supowit SC, Thompson EA, Meyer WJ III. (1992) Immunoreactive growth hormone production by human lymphocyte cell lines. *Cellular and Molecular Neurobiology* 12:483-498.

Kyle CV, Evans MC, Odell WD. (1981) Growth hormone-like material in normal human tissues. *Journal of Clinical Endocrinology and Metabolism* 53:1138-1144.

Lechan RM, Nestler JL, Molitch ME. (1981) Immunohistochemical identification of a novel substance with human growth hormone-like immunoreactivity in rat brain. *Endocrinology* 109:1948-1962.

Luna M, Huerta L, Harvey S, Aramburo C. (2002) Testicular growth hormone: heterogeneity and changes during ontogeny and maturation. *Journal of Endocrinology* (submitted).

Luna M, Martinez-Coria H, Carranza M, Huerta L, Harvey S, Aramburo C. (2000) Characterization of growth hormone molecular variants and gene expression in extra-

pituitary tissues in the chicken. *Proceedings of the European Society for Comparative Endocrinology*, Portugal, p 63.

Maggiano N, Piantelli M, Ricci R, Larocca LM, Capelli A, Ranelletti FO. (1994) Detection of growth hormone-producing cells in human thymus by immunohisto-chemistry and non-radioactive *in situ* hybridization. *Journal of Histochemistry and Cytochemistry* 42:1349-1354.

Malamed S, Gibney JA, Cain LD, Perez FM, Scanes CG. (1993) Immuno-ytochemical studies of chicken somatotrophs and somatotrophs granules before and after hatching. *Cell and Tissue Research* 272:369-374.

Martinez-Coria H, Lopez-Rosales LJ, Carranza M, Berumen L, Luna M, Aramburo C. (2002) Differential secretion of chicken growth hormone variants after growth hormone-releasing hormone stimulation *in vitro*. *Endocrine* 17:91-102.

Mol JA, Henzen-Logmans SC, Hageman WP, Misdorp W, Blankenstein MA, Rujnberk A. (1995a) Expression of the gene encoding growth hormone in the human mammary gland. *Journal of Clinical Endocrinology and Metabolism* 80:3094-3096.

Mol JA, van Garderen E, Selman PJ, Wolfswinkel J, Rijnberk A, Rutteman GR. (1995b) Growth hormone mRNA in mammary gland tumors of dogs and cats. *Journal of Clinical Investigation* 95:2028-2034.

Murphy AE and Harvey S. (2001) Extrapituitary βTSH and GH in early chick embryos. *Molecular and Cellular Endocrinology* 185:161-171.

Murphy AE, Hull KL, Luna M, Aramburo C, Harvey S. (2002) Growth hormone (GH) and GH-receptor mRNA in the testis and male reproductive tract: evidence for an autocrine/ paracrine role of GH in avian reproduction. *Journal of Endocrinology*, (submitted).

Palmetshofer A, Zechner D, Luger TA, Barta A. (1995) Splicing variants of the human growth hormone mRNA: detection in pituitary, mononuclear cells and dermal fibroblasts. *Molecular and Cellular Endocrinology* 113:225-234.

Polak JM and Van Noorden S. (1997) *Introduction to Immunocytochemistry*, 2nd Ed. Springer-Verlag.

Porter TE. (1997) Regulation of somatotrophs differentiation during chicken embryonic development: a review. in *Perspectives in Avian Endocrinology* (eds Harvey S & Etches RJ). Bristol, Society for Endocrinology, pp 47-56.

Porter TE, Couger GS, Dean CE, Hargis BM. (1995) Ontogeny of growth hormone (GH)-secreting cells during chick embryonic development: initial somatotrophs are responsive to GH-releasing hormone. *Endocrinology* 136:1850-1856.

Ramesh R, Kuenzel WJ, Bruntin JD, Proudman JA. (2000) Identification of growth hormone and prolactin containing neurons within the avian brain. *Cell and Tissue Research* 299:371-383.

Recher S, Raccurt M, Lambert A, Lobie PE, Mertani HC, Morel G. (2001) Prenatal and adult growth hormone gene expression in rat lymphoid organs. *Journal of Histochemistry and Cytochemistry* 49:347-354.

Render C, Hull KL, Harvey S. (1995a) Neural expression of the pituitary GH gene. *Journal of Endocrinology* 147:413-422.

Render C, Hull KL, Harvey S. (1995b) Expression of the growth hormone gene in immune tissues. *Endocrine* 3:729-735.

Schutte B, Reynders MM, Bosman FT, Blijham GH. (1987) Effect of tissue fixation on anti-bromodeoxyuridine immunohistochemistry. *Journal of Histochemistry and Cytochemistry* 35:1343-1345.

Schwarzler P, Untergasser G, Hermann M, Dirnhofer S, Abendstein B, Madersbacher S, Berger P. (1997) Selective growth hormone/placental lactogen gene transcription and hormone production in pre- and postmenopausal human ovaries. *Journal of Clinical Endocrinology and metabolism.* 82:3337-41.

Slominski A, Malarkey WB, Wortsman J, Asa SL, Carlson A. (2000) Human skin expresses growth hormone but not the prolactin gene. *Journal of Laboratory and Clinical Medicine* 136:476-481.

Takeuchi S, Haneda M, Teshigawara K, Takahashi S. (2001) Identification of a novel GH isoform: a possible link between GH and melanocortin systems in the developing chicken eye. *Endocrinology* 142:5158-5166.

Thommes RC, Umporowicz DM, Leung FCD, Woods JE. (1987) Ontogenesis of immunocyto-chemically demonstrable somatotrophs in the adenohypophyseal pars distalis of the developing chick embryo. *General and Comparative Endocrinology* 67:390-398.

Tresguerres JA, Ariznavarreta C, Granados B, Costoy JA, Perez-Romero A, Salame F, Hermanussen M. (1999) Salivary gland is capable of GH synthesis under GHRH stimulation. *Journal of Endocrinology* 160:217-222.

Vince DG, Tbakhi A, Gaddipati A, Cothren RM, Cornhill JF, Tubbs RR. (1997) Quantitative comparison of immunohistochemical staining intensity in tissues fixed in formalin and Histochoice. *Anatomy and Cell Pathology* 15:119-129.

Wu H, Devi R, Malarkey WB. (1996) Localization of growth hormone messenger ribonucleic acid in the human immune system – a clinical research study. *Journal of Clinical Endocrinology and Metabolism* 81:1278-1282.

Zhang CZ, Li H, Young WG, Bartold PM, Chen C, Waters MJ. (1997) Evidence for a local action of growth hormone in embryonic tooth development in the rat. *Growth Factors* 14:131-143.

Chapter 5

Growth Hormone (GH) and GH-receptor mRNA in the Male Reproductive Tract:

Evidence for Autocrine/Paracrine Roles of GH in Avian Reproduction

I. INTRODUCTION

Growth hormone (GH) is primarily produced in the pituitary gland, although it is well established that some extrapituitary tissues express the GH gene (Harvey and Hull, 1997). Amongst these, the testis is thought to be a site of GH synthesis, since GH gene transcripts are present in the human testis (Untergasser *et al.,* 1996; 1997; 1998; Berger *et al.,* 1999). However, while the pituitary (GH-N) gene is transcribed in the human testis, 'placental' GH variants (GH-V) are preferentially expressed (Untergasser *et al.,* 1997; 1998; Berger *et al.,* 1999). The possibility that GH gene expression occurs in the testis of other species has yet to be determined, although proteins with GH-immunoreactivity have been demonstrated in the testes of fetal mice (Nguyen *et al.,* 1996) and the Wolffian ducts of embryonic chickens (Wang, 1989; Harvey *et al.,* 2000a). The possible expression of the GH gene in the male reproductive tract was therefore examined in the domestic fowl, in which GH mRNA has previously been identified in the brain (Render *et al.,* 1995a), spleen, thymus and bursa (Render *et al.,* 1995b), heart and eye (Takeuchi *et al.,* 2001). In addition, as roles for GH in male reproduction have been described (Hull and Harvey, 2000b), the possible presence of GH-receptor (GHR) mRNA in the male reproductive tract was also assessed.

II. MATERIALS AND METHODS

Adult male White Leghorns from the University of Alberta Poultry Station (Edmonton) were killed by cervical dislocation and the testes, upper vas deferens (1 cm adjacent to the testis), the middle vas deferens and the cloaca were rapidly collected and frozen in liquid nitrogen. The liver and pituitary glands were collected from some birds

as negative and positive controls for the presence of GH mRNA (Render *et al.,* 1995a) and as positive controls for the presence of GHR mRNA (Hull *et al.,* 1995).

RT-PCR

The presence of GH and GHR mRNA in reproductive tissues was assessed by RT-PCR. Total cellular RNA was extracted using commercial reagents (Trizol, Gibco, BRL, Burlington, Ontario, Canada) and analyzed for purity and content, respectively, by spectrophotometry at 260 nm and electrophoresis in ethidium-bromide-stained 1.5% (w/v) agarose minigels (Render *et al.,* 1995a; 1995b). RNA samples (1 μg) were reverse-transcribed using superscript (100 U; BRL) in the presence of an oligo dt primer and excess deoxynucleotides (Gibco-BRL) during a 1 h incubation at 37 °C and a 30 min incubation at 42 °C. cDNA aliquots (1 μl) were then amplified for 30 cycles in a Thermal Cycler (Fisher Scientific, Edmonton) in a mixture containing 10x PCR buffer (80 mmol KCl/l; 16 mmol Tris-HCI/l, pH 8.4, 1.5 mmol Mg CI$_2$/l; 0.1% (v/v) Triton X-100), deoxynucleotides (200 μm of each), 25 pmol oligonucleotide primers and 5 U Taq DNA polymerase (Boehringer Mannheim). Amplifications were achieved by cycles of denaturation (94 °C for 1 min), annealing (50 °C for 1 min) and extension (72 °C for 2 min), followed by a final period of extension (72 °C for 10 min). The PCR products were resolved by electrophoresis in 1.5% (w/v) agarose gels and were visualized by ethidium bromide staining.

Amplifications were in the presence of oligonucleotide primers CLR3 (5[l]-CGTTCAAGCAACACCTGAGCAACTCTCCCG-3[l]) and CLR5 (5[l]-GCCTCAGATGGTGCAGTTGCTCTCTCCGAA-3[l]), designed to amplify a 689 bp

fragment of the pituitary GH cDNA (Render *et al.,* 1995a), or in the presence of primers

khu9 (5'-CCTCGATTTGGATACCATATTGTGTTAAGC-3') and khu10 (5'-

CTGTTACGGCCAGCCCACACACTCCGAAG-3') designed to generate a 500 bp

fragment of the chicken GHR cDNA coding for the extracellular domain of the chicken

GHR (Hull *et al.,* 1995) or in the presence of primers khu11 (5'-

CTGCGGCCGCAGGACCAGTCCAAAGATTAA-3') and khu12 (5'-

AAGCGGCCGCGGCAGTAGTGGTAAGGCTTTC-3') designed to generate a 800 bp

fragment of the chicken GHR cDNA coding for the intracellular domain of the GHR

(Hull *et al.,* 1995). The possible presence of Pit-1 mRNA was similarly determined using

oligonucleotide primers t15 (5'-GCACTCGTGGTGC TCC TTGAT AATAGA -3') and

t14 (5'-ATCCTCATGCG TTTTCTTACCAGTCCCG -3'), designed to generate a 975 bp

fragment of chicken Pit-1 cDNA (Harvey *et al.,* 2000b). As a control, the presence of a

housekeeping gene (Gap DH) in the RNA extracts was also assessed, using

oligonucleotide primers based on the chicken Gap DH sequence: cGAP 5 (5'-

CTGGTGTCTTCACCACCATG-3') and cGAP3 (5'-CAGCAGCCTTCACTA CCCTC-

3') (Stone *et al.,* 1985).

cDNA Cloning and Sequencing

PCR products were purified using the High Pure™ PCR Product Purification Kit

(Roche Diagnostics, Indianapolis, IN, USA) and then ligated into pCR®II-TOPO®

vectors (Invitrogen Life Technologies Carlsbad, CA, USA). The ligated vector was then

transformed into TOP10F' One Shot® chemically competent *E. coli* cells (Invitrogen Life

Technologies Carlsbad, CA, USA). The cells were then plated onto Luria-Bertani (LB)

medium (1% tryptone, 0.5% yeast extract, 1% NaCl; pH 7.0) agar plates containing 50

μg/ml ampicillin and 40 μl X-gal (400 mg X-gal in 10 ml dimethylformamide) for

blue/white screening and incubated at 37 °C overnight. The next day, 3-4 white colonies

were selected, placed into 5 ml LB medium and incubated at 37 °C with vigorous shaking

for 8 h. The cell cultures were centrifuged, excess medium poured off and the wet pellet

of cells was purified with a QIAprep Spin Miniprep Kit (QIAGEN, Mississauga, ON,

Canada) to isolate plasmid DNA. The inserts were isolated by digestion with *EcoRI*

(Gibco, Invitrogen Life Technologies Carlsbad, CA, USA) and visualized by

electrophoresis in 1.5% (w/v) agarose gels and ethidium bromide staining. The inserts

were then sequenced using Sp6 and T7 or M13 forward primers (DNA Core Facility,

University of Alberta) and sequences were analyzed using BLAST software (NCBI,

Bethesda, Maryland, USA). The testicular GH cDNA was compared with a consensus

chicken pituitary GH cDNA based on Zhvirblis *et al.* (1987) (Gene Bank Accession #

211808), Lamb *et al.* (1988) (Gene Bank Accession # 63406), Baum *et al.* (1990) (Gene

Bank Accession # 62909), Tanaka *et al.* (1992) (Gene Bank Accession # 222822), and Ip

et al. (2001) (Gene Bank Accession # 9858171). This consensus sequence was identical

to that reported by Tanaka *et al.* (1992).

Southern Blotting

      cRNA Probe Synthesis: The 689 bp GH cDNA resulting from RT-PCR

amplification of template cDNA with the oligonucleotide primers CLR3 and CLR5 was

ligated into a PCR®II-TOPO® vector (Invitrogen Life Technologies Carlsbad, CA, USA)

and cloned as detailed above. The plasmid containing the PCR product generated from

the GH specific primers was linearized with *BamH1* (Gibco, Invitrogen Life Technologies Carlsbad, CA, USA) and then purified by phenol/chloroform extraction and ethanol precipitation. The pellet was resuspended in 10 mM Tris-HCl, pH 8.0. The purified, linearized plasmid DNA was then digoxigenin (DIG) labeled by *in vitro* transcription of the DNA with DIG RNA labeling mix with the reagents and protocol from Roche Applied Science (Basel, Switzerland). Probe yield was estimated using DIG Quantification Teststrips (Roche Applied Science, Basel, Switzerland).

Blotting: GH cDNA amplified by PCR was transferred to a nylon membrane using the S&S Turbo Blotter and Blotting Stack Assembly for Alkaline Transfers (Schleicher & Schuell, Keene, NH, USA). After transfer, the DNA was fixed to the membrane by baking at 80 °C for 30 min in a vacuum oven. The blot was then prehybridized in 10 ml of DIG Easy Hyb hybridization buffer (Roche Applied Science, Basel, Switzerland) for 1 h at 50 °C. The DNA blot was then hybridized with 10 pmol DIG labeled RNA probe/ml hybridization buffer overnight at 50 °C. The hybridization buffer was poured off and the blot was washed 2 x 5 min in 2 x SSC (Roche Applied Science, Basel, Switzerland) containing 0.1% sodium dodecyl sulfate (SDS) at room temperature, 2 x 5 min in 0.5 x SSC containing 0.1% SDS at 65 °C and 1 x 1 min in 0.3% (v/v) Tween® 20 in maleic acid buffer (10 mM maleic acid, 15 mM NaCl; pH 7.5) at room temperature. The blot was incubated in 1 x Blocking Reagent (Roche Applied Science, Basel, Switzerland) for 1 h with gentle shaking and then with alkaline phosphatase-conjugated anti-DIG antibody (Roche Applied Science, Basel, Switzerland)

diluted 1:5000 in 1 x Blocking Reagent. Colormetric detection of the DIG labeled probe was carried out using NBT/BCIP (Roche Applied Science, Basel, Switzerland).

Northern Blotting

Total cellular RNA was extracted from the pooled testes of three adult birds and subjected to Northern blot analysis using a DIG-labeled 689 bp GH cDNA, as detailed previously (Render *et al.*, 1995a). Briefly, the RNA was electrophoresed in 1% (w/v) agarose and 3.1% (w/v) formaldehyde gels and transferred to nylon membranes by capillarity. After transfer the RNA was immobilized in 60% (w/v) formamide (containing 0.75 mol NaCl/P, mmol pipes/P and 25 mmol EDTA/P, 0.2% (w/v) SDS, 1 X Denhart's reagent (0.1% w/v) ficoll, 0.1% (w/v) BSA, 0.1% (w/v) polyvinylpyrrolidine and 100 μg salmon sperm DNA/P, pH 6.8) in the presence of the probe (10 pmol/ml hybridization buffer) for 12 h at 55 °C. Following a brief rinse in 2 x SSC, the nylon membranes were washed at room temperature in 0.1% (w/v) SDS containing 2 x SSC and subsequently at 75 °C in 1% (w/v) SDS containing 0.1 x SSC. The blot was then incubated in Blocking Reagent (Roche Applied Science) for 1 h with gentle shaking and then with alkaline phosphatase-conjugated anti-DIG antibody (Roche Applied Science), diluted 1:5000 in 1 x Blocking Reagent. Colormetric detection of the DIG-labeled probe was carried out using BNT/BCIP (Roche Applied Science). Total cellular RNA from pooled adult pituitary glands was similar analyzed, for comparison.

III. RESULTS

GH mRNA: RT-PCR and Southern Blotting

As expected, RT-PCR of pituitary mRNA with oligonucleotide primers CLR3 and

CLR5 generated a 689 bp cDNA fragment, which was not amplified with liver mRNA

(Fig. 5.1A). A 689 bp fragment was, however, produced following RT-PCR of mRNA

from the testis and the upper and middle vas deferens. This moiety was not produced

with mRNA that had not been reverse-transcribed. The cDNA moieties generated from

pituitary, testicular and vas deferens mRNA also hybridized with a DIG-labeled 689 bp

riboprobe probe for GH mRNA, following southern blotting (Fig. 5.1B).

cDNA Sequencing

Sequencing of the 689 bp testicular GH cDNA showed it had >99.5% homology

with the consensus published sequence for chicken pituitary GH cDNA. The sequence

included 36 bps of the 5' untranslated region (UTR), the coding region (648 bp) for the

25 amino acid signal peptide and the 191 amino acid apoprotein, as well as 6 bps of the

3' UTR. This sequence only differed from the consensus pituitary GH cDNA by three

base-pair substitutions (Fig. 5.2): a base substitution at residue 102 (137 of the cDNA

fragment) (a T for C substitution), a base substitution at position 460 (496 of the cDNA

fragment) (a G for A substitution) and a base substitution at position 587 (623 of the

cDNA fragment) (an A for G substitution). The substitutions at residues 102 and 587

would not, however, change the amino acid sequence of the predicted protein, which

would only differ from pituitary GH by a single amino acid (glycine for aspartate), at

154

position 154 of the 216 amino acid prohormone (amino acid position 129 of the 191

amino acid hormone) (99.5% homology of the predicted amino acid sequences).

Northern Blotting

As expected (Render *et al.,* 1995a), the 689 bp DIG-labeled GH riboprobe

hybridized strongly to a 0.8 kb mRNA in the chicken pituitary gland (Fig. 5.3). This

probe did not, however, hybridize to total mRNA extracted from the testis (Fig. 5.3).

Pit-1 mRNA: RT-PCR

RT-PCR of mRNA from the testis and upper vas deferens with oligonucleotides

t14 and t15 for Pit-1 cDNA generated a 975 bp moiety identical to that in the pituitary

gland (Fig. 5.4A). This moiety was not, however, generated with reverse transcribed

mRNA from the liver (Fig. 5.4A), although a 500 bp cDNA moiety was generated from

this in the presence of Gap DH primers, as with RNA from the other tissues (Fig. 5.4B).

GHR mRNA: RT-PCR

RT-PCR of mRNA from the testis with oligonucleotide primers for the

extracellular (khu9 and khu10) and intracellular domains (khu11 and khu12) of the

chicken GHR cDNA and for the full-length receptor (khu9 and khu12) amplified cDNA

moieties identical in size (500 bp, 800 bp and 1350 bp respectively) to those amplified

from hepatic mRNA (positive control) (Fig. 5.5). RT-PCR of reverse transcribed mRNA

from the upper vas deferens with the same primer sets similarly generated 500 bp

(khu9/khu10) and 800 bp (khu11/khu12) fragments, as also amplified with hepatic

155

mRNA (positive control) (Fig. 5.6A, B). These moieties were not generated with mRNA

that had not been reverse-transcribed (data not shown). Endonuclease digestion with *Hae*

III cleaved the 800 bp GHR cDNA moieties generated from liver and vas deferens

mRNA into fragments of 470 bp and 330 bp (Fig. 5.6C).

IV. DISCUSSION

These results clearly demonstrate the presence of GH and GHR mRNA in the

male reproductive tract and suggest autocrine/paracrine roles for GH in avian

reproduction.

Although GH transcripts are present in the human testis, this is the first

demonstration of GH mRNA in the testis of another species. Moreover, while GH gene

expression occurs in the human testis, it is primarily the expression of the placental GH

variant (hGH-V) rather than the pituitary GH gene (hGH-N) (Untergasser *et al.*, 1997,

1998; Berger *et al.*, 1999). The GH cDNA in the chicken testis, in contrast, closely

resembles that in the pituitary gland.

The nucleotide sequence of chicken pituitary GH cDNA has been reported by

several groups (Souza *et al.*, 1984; Zhvirblis *et al.*, 1987; Lamb *et al.*, 1988; Baum *et al.*,

1990; Tanaka *et al.*, 1992). Although all the published sequences have close homology

(mostly >98%), a number of base-pair differences exist (Fig. 5.2) which may be species

or strain related (Baum *et al.*, 1990), as numerous intronic DNA polymorphisms have

also been identified in different chicken strains (Kuhnlein *et al.*, 1997; Feng *et al.*, 1997;

Ip *et al.*, 2001). The three base-pair substitutions in testicular GH cDNA are not,

however, species or strain related, since they are unique and, in the same birds, are not

present in pituitary GH cDNA, brain GH cDNA, bursal GH cDNA, thymic GH cDNA, or splenic GH cDNA (Render *et al.*, 1995a,b). It is therefore likely that GH gene transcription is tissue-specific in the domestic fowl. The recent discovery of a novel GH cDNA in the chicken retina (Takeuchi *et al.*, 2001) supports this possibility, as does the differential transcription of the GH gene family in the human testis (Untegasser *et al.*, 1997; Berger *et al.*, 1999). The cDNA of testicular GH also differs from the retinal GH cDNA, since the base-pair substitutions in testicular GH (at positions 460 and 587) are not present in retinal GH cDNA (Takeuchi *et al.*, 2001).

The predicted amino acid sequence coded by chicken testicular GH cDNA only differs from pituitary GH by a single residue (glycine for aspartate at position 154 of the 216 amino acid prohormone, position 129 of the mature hormone). The 191 amino acid apoprotein has 4 conserved cysteine residues (at positions 53, 164, 181 and 189) that are thought to be responsible for the formation of 2 intramolecular or intermolecular disulfide bridges and the structural folding of the molecule for receptor recognition. It also has the conserved domains between amino acids 82-87, 115-120, and 153-162 that are also thought to be important for receptor binding or for specific biological functions (Foster *et al.*, 1990). It is therefore highly likely that the coded protein would have biological activity.

The GH message in the chicken testis is of low abundance, since it could not be detected by northern blotting, although this technique readily detects pituitary GH mRNA. The testicular GH transcript is, however, likely to be translated, since proteins in the chicken testis with GH-immunoreactivity have been reported in preliminary studies (Harvey *et al.*, 2002), although GH has yet to be determined in the testis of other species.

Immunoreactive GH has, however, been located in the Wolffian ducts of fetal mice (Nguyen *et al.*, 1996) and the Wolffian ducts of embryonic chicks (Wang, 1989; Harvey *et al.*, 2000a). It is therefore of interest that GH mRNA was also detected in the vas deferens and the cloaca, indicating that they too are sites of GH synthesis. Moreover, as GHR transcripts are present throughout the male reproductive tract, GH may have local autocrine/paracrine actions in these tissues. The presence of GH in the reproductive tracts of embryonic chicks prior to the differentiation of the pituitary gland and the appearance of GH in plasma (Harvey *et al.*, 2000a) strongly supports this possibility.

It is now well established that the testis and the male reproductive tract are target sites for GH action (Hull and Harvey, 2000a, 2000b). Indeed, GH is thought to be essential for testicular growth and development, since GH deficiency is associated with abnormally small testes (Spiteri-Grech and Nieschlag, 1992). The differentiation of the Wolffian duct is similarly thought to be GH-dependent, since GH immunoneutralization blocks Wolffian duct differentiation in fetal mice, whereas GH administration restores normal development of the reproductive tract (Nguyen *et al.*, 1996). Numerous actions of GH in gametogenesis (Gravance, 1997; Ovesen *et al.*, 1998) and steroidogenesis (Shohan *et al.*, 1992; Kanzaki and Morris, 1999) have also been documented and GH deficiency is thought to be causally linked to male infertility (Shohan *et al.*, 1992; Shimonovitz *et al.*, 1993).

These actions of GH are likely to be receptor mediated, especially as GHR immunoreactivity and GHR mRNA are abundantly present in the Wolffian ducts, epididymis, vas deferens, seminal vesicles, prostate and testis (Leydig cells, Sertoli cells, spermatogonia, spermatocytes) of fetal and adult rats (Lobie *et al.*, 1990; Garcia-Aragon

et al., 1992; Reiter *et al.,* 1999). The demonstration of GHR mRNA in the testis, and vas

deferens of male chickens, for the first time, similarly suggests testicular and extra -

testicular actions of GH in avian reproductive function. As some of these are sites of GH

production and GH action, it is possible that GH has local autocrine/paracrine roles in the

male reproductive tract, as in many other peripheral tissues (Harvey and Hull, 1997;

Harvey *et al.,* 1998).

The expression of the GH gene in the pituitary is thought to be dependent upon

the Pit-1 transcription factor (Harvey *et al.,* 2000b). It is therefore of interest that Pit-1

mRNA was also detected in the chicken testes and vas deferens. This may, therefore,

indicate a role for Pit-1 in testicular GH expression. Pit-1 is, however, also present in

placental tissue but it has no role in the expression of the placental GH variant. Indeed,

the factors regulating GH expression in extrapituitary sites are largely unknown (Harvey

and Hull, 1997) and the presence of GH-releasing hormone (Matsubara *et al.,* 1995;

Olchovsky *et al.,* 1996), thyrotropin-releasing hormone (Wilber and Xu, 1998; Li *et al.,*

2002), ghrelin (Tanaka *et al.,* 2001; Tena-Sempere *et al.,* 2002), somatostatin (Pekary *et

al.,* 1984; Zhu *et al.,* 1998) and insulin-like growth factors (Morera *et al.,* 1987; Baker *et

al.,* 1996) in testicular tissue may also be unrelated to GH regulation (Campbell and

Scanes, 1995), in contrast with the roles of these factors in pituitary GH secretion.

In summary, the results of these studies demonstrate the presence of GH-, GHR-

and Pit-1 mRNA in the male reproductive system, in which GH may have local

autocrine/paracrine roles in reproductive function.

Fig 5.1 RT-PCR and Southern Blotting for GH in male chicken reproductive

tissues. (A) RT-PCR of growth hormone (GH) mRNA in the testis and reproductive tract

of male chickens. Total tissue RNA was extracted and amplified in the presence of

oligonucleotide primers (CLR3 and CLR5) designed to amplify a 689 bp fragment of

pituitary GH cDNA (Render *et al.*, 1995a). This fragment was amplified with reverse

transcribed RNA (+) extracted from the testis, lower vas deferens, middle vas deferens

and cloaca and stained by ethidium bromide. This fragment was not evident when PCR

was performed with RNA in the absence of reverse transcriptase (-). The presence and

absence of this fragment in amplified pituitary and liver cDNA is shown for comparison.

(B) Southern blotting of PCR products amplified in the presence of oligonucleotide

primers designed to amplify a 689 bp fragment of pituitary GH cDNA, using a DIG-

labeled 689 bp pituitary GH riboprobe. RNA from the testis, and the upper and middle

vas deferens produced a 689 bp GH cDNA after reverse transcription (+), but not in the

absence of reverse transcriptase (-).

A.

689bp —

Pituitary (+) Liver (+) Testis (+) Testis (-) Middle Vas Deferens (+) Middle Vas Deferens (-) Upper Vas Deferens (+) Upper Vas Deferens (-)

B.

689bp —

Pituitary (+) Testis (+) Testis (-) Middle Vas Deferens (+) Middle Vas Deferens (-) Upper Vas Deferens (+) Upper Vas Deferens (-)
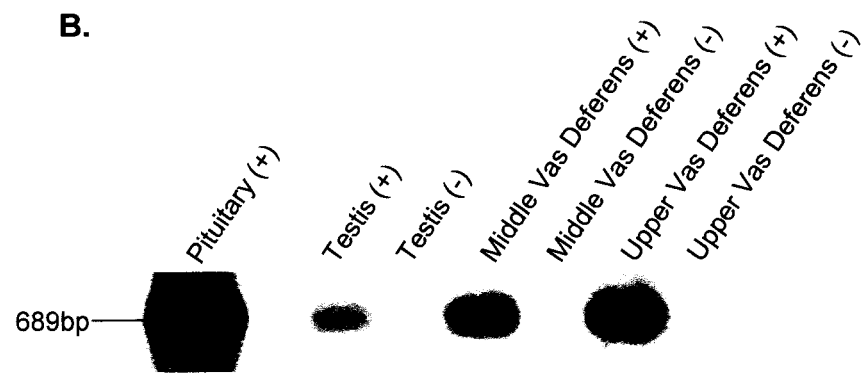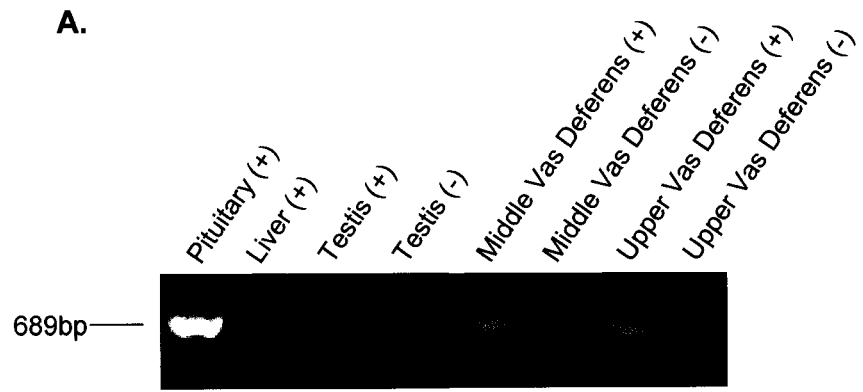
Fig 5.2 Comparison of testicular cGH nucleotide sequence with pituitary cGH.

Nucleotide and amino acid sequence of testicular growth hormone (GH) cDNA (tGH), in comparison with pituitary GH cDNA (Pit-GH) (Tanaka *et al.*, 1992). The initiation site is the ATG codon for methionine. The 5' flanking untranslated region is indicated as -36 to -1. The first 25 amino acids are the signal sequence for the 216 amino acid prohormone. Base-pair substitutions are indicated in the boxed codons. The base-pair substitution at position 460 of the coding region would result in a GGC codon and code for glycine rather than aspartate. In comparison with the sequence reported by Tanaka *et al.*, (1992), the pituitary GH cDNA reported by Lamb *et al.*, (1988) differs by base-pair substitutions at positions153 (T for C), 162 (C for T) and 302 (C for T). The pituitary GH cDNA reported by Baum *et al.*, (1990) differs by base-pair substitutions at positions 336 (G for C), 465 (A for C), 466 (T for A), 467 (C for G), 468 (A for C) and 504 (T for C). The pituitary GH cDNA reported by Zhvirblis *et al.*, (1987) differs by base-pair substitutions at position 336 (G for C) and 339 (T for C). None of these substitutions were present in testicular GH cDNA.

```
                            -30            -20            -10             1              10            20
                             |              |              |              |              |             |
tGH          gttcaagcaacacctgagcaactctcccggcaggaatggctccaggctcgtggttttctc 25
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PitGH        gttcaagcaacacctgagcaactctcccggcaggaatggctccaggctcgtggttttctc
                                                          M   A   P   G   S   W   F   S


tGH       26 ctctcctcatcgctgtggtcacgctgggactgccgcaggaagctgctgccaccttccctg 85
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PitGH        ctctcctcatcgctgtggtcacgctgggactgccgcaggaagctgctgccaccttccctg
           9 P   L   L   I   A   V   V   T   L   G   L   P   Q   E   A   A   A   T   F   P

                                    N
                                   ┌───┐
tGH       86 ccatgcccctctcc│aat│ctgtttgccaacgctgtgctgagggctcagcacctccacctcc 145
             |||||||||||||||  |||||||||||||||||||||||||||||||||||||||||||||
PitGH        ccatgcccctctcc│aac│ctgtttgccaacgctgtgctgagggctcagcacctccacctcc
          29 A   M   P   L   S   N   L   F   A   N   A   V   L   R   A   Q   H   L   H   L


tGH      149 tggctgccgagacatataaagagttcgaacgcacctatattccggaggaccagaggtaca 205
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PitGH        tggctgccgagacatataaagagttcgaacgcacctatattccggaggaccagaggtaca
          49 L   A   A   E   T   Y   K   E   F   E   R   T   Y   I   P   E   D   Q   R   Y


tGH      206 ccaacaaaaactcccaggctgcgttttgttactcagaaaccatcccagctcccacggggga 265
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PitGH        ccaacaaaaactcccaggctgcgttttgttactcagaaaccatcccagctcccacggggga
          69 T   N   K   N   S   Q   A   A   F   C   Y   S   E   T   I   P   A   P   T   G


tGH      266 aggatgacgcccagcagaagtcagacatggagctgcttcggttttcactggttctcatcc 325
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PitGH        aggatgacgcccagcagaagtcagacatggagctgcttcggttttcactggttctcatcc
          89 K   D   D   A   Q   Q   K   S   D   M   E   L   L   R   F   S   L   V   L   I


tGH      326 agtcctggctcaccccccgtgcaatacctaagcaaggtgttcacgaacaacttggttttttg 385
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PitGH        agtcctggctcaccccccgtgcaatacctaagcaaggtgttcacgaacaacttggttttttg
         109 Q   S   W   L   T   P   V   Q   Y   L   S   K   V   F   T   N   N   L   V   F


tGH      386 gcacctcagacagagtgtttgagaaactaaaggacctggaagaagggatccaagccctga 445
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PitGH        gcacctcagacagagtgtttgagaaactaaaggacctggaagaagggatccaagccctga
         129 G   T   S   D   R   V   F   E   K   L   K   D   L   E   E   G   I   Q   A   L

                                     G
                                    ┌───┐
tGH      446 tgagggagctggag│ggc│cgcagccgcgggggcccgcagctcctcagacccacctacgaca 505
             ||||||||||||||  ||||||||||||||||||||||||||||||||||||||||||||
PitGH        tgagggagctggag│gac│cgcagccgcgggggcccgcagctcctcagacccacctacgaca
         149 M   R   E   L   E   D   R   S   P   R   G   P   Q   L   L   R   P   T   Y   D


tGH      506 agttcgacatccacctgcgcaacgaggacgccctgctgaagaactacggcctgctgtcct 565
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PitGH        agttcgacatccacctgcgcaacgaggacgccctgctgaagaactacggcctgctgtcct
         169 K   F   D   I   H   L   R   N   E   D   A   L   L   K   N   Y   G   L   L   S
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163

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                                              K
tGH     566 gcttcaagaaggatctgca|caaa|gtggagacctacctgaaggtgatgaagtgccggcgct 625
            |||||||||||||||||||| ||| ||||||||||||||||||||||||||||||||||||
PitGH       gcttcaagaaggatctgca|caag|gtggagacctacctgaaggtgatgaagtgccggcgct
        189 C  F  K  K  D  L  H  K  V  E  T  Y  L  K  V  M  K  C  R  R


tGH     626 tcggagagagcaactgcaccatctgaggc 654
            |||||||||||||||||||||||||||||
PitGH       tcggagagagcaactgcaccatctgaggc
        209 F  G  E  S  N  C  T  I  ^^^
```

Fig 5.3 Northern blotting for growth hormone (GH) mRNA in the pituitary and testis of adult chickens. Hybridization of a 689 bp DIG-labeled GH riboprobe is shown for pituitary RNA, but not for testicular RNA.

Fig 5.4 RT-PCR for Pit-1 and Gap DH in male chicken reproductive tissues. (A) Pit-1 mRNA in the testis and vas deferens of chickens. Total tissue RNA was extracted and amplified by RT-PCR in the presence of oligonucleotide primers t14 and t15, designed to generate a 975 bp fragment of chicken Pit-1 cDNA (Harvey *et al.,* 2000). The generation of this cDNA with pituitary RNA and its absence following RT-PCR with liver RNA acted as positive and negative controls, respectively (Harvey *et al.,* 2000).

(B) The specificity of the Pit-1 PCR is indicated by the generation of 500 bp Gap DH cDNA fragments following PCR of reverse transcribed (+) RNA from the testis, vas deferens and liver. No signal was observed in the absence of reverse transcriptase (-).

A.

Pituitary (+) Testis (+) Upper Vas Deferens (+) Liver (+)

975bp—

B.

Testis (+) Testis (-) Upper Deferens (+) Upper Vas Deferens (-) Liver (+) Liver (-)

500bp—
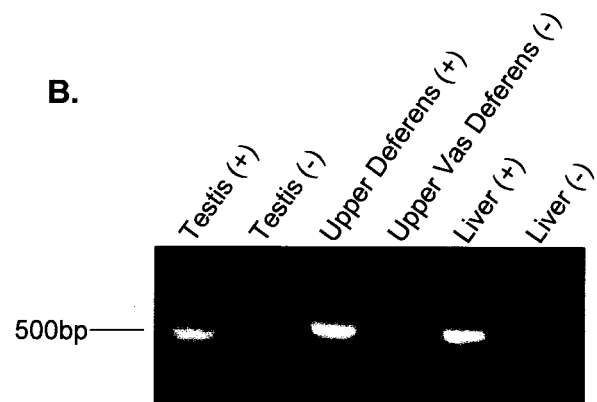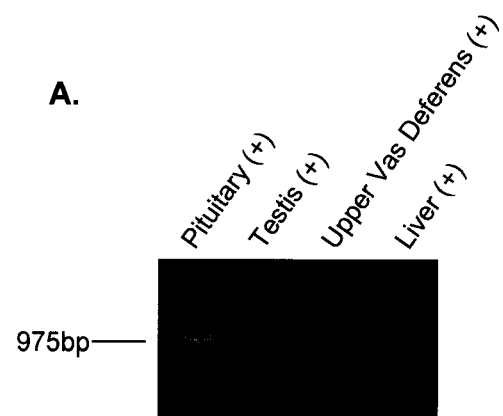
Fig 5.5 Growth hormone receptor (GHR) mRNA in the testis of male chickens. Total tissue RNA from the testis was amplified by RT-PCR in the presence of oligonucleotide primers khu9 and khu10, khu11 and khu12 or khu9 and khu12, designed to generate 500 bp, 800 bp and 1350 bp fragments, respectively, of the chick GHR cDNA (Hull *et al.,* 1995). The generation of similar cDNA fragments from reverse transcribed liver RNA is shown as a positive control.
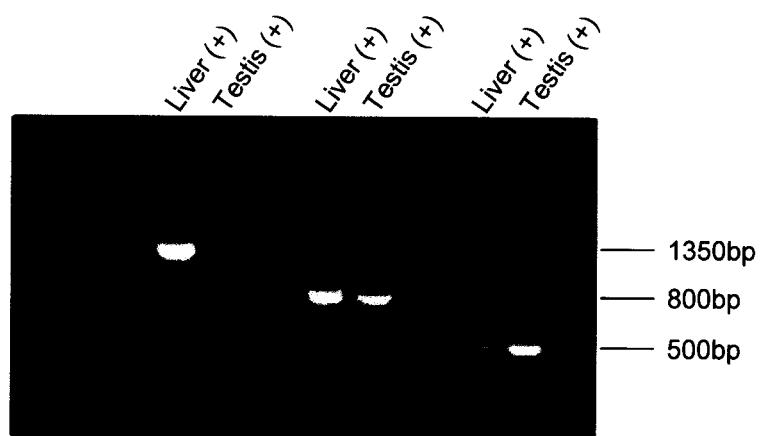
Fig 5.6 Growth hormone receptor (GHR) mRNA in the reproductive tract of male chickens. Total tissue RNA from the upper vas deferens was amplified by RT-PCR in the presence of oligonucleotide primers khu9 and khu10 or khu11 and khu12 (Hull *et al.*, 1995), designed to generate a 500 bp fragment of the GHR cDNA coding for the extracellular domain of the GHR (A) or with primers khu11 and khu12, designed to generate a 800 bp fragment of the GHR cDNA coding for the intracellular domain of the GHR (Hull *et al.*, 1995) (B). The generation of these fragments with hepatic cDNA provided a positive control. (C) Endonuclease digestion of growth hormone receptor (GHR) cDNA. The GHR cDNAs generated from the RT-PCR of hepatic and vas deferens mRNA (with oligonucleotide primers for the intracellular domains of the chicken GHR cDNA) were digested with *Hae III.* Fragments of appropriate size (470 bp and 330 bp) indicated sequence similarly with the full length chicken GHR cDNA (Hull *et al.*, 1995).

A. Liver (+)   Upper Vas Deferens (+)

—500bp

B. Liver (+)   Upper Vas Deferens (+)

—800bp

C. Liver (+)   Upper Vas Deferens (+)

—470bp

—330bp
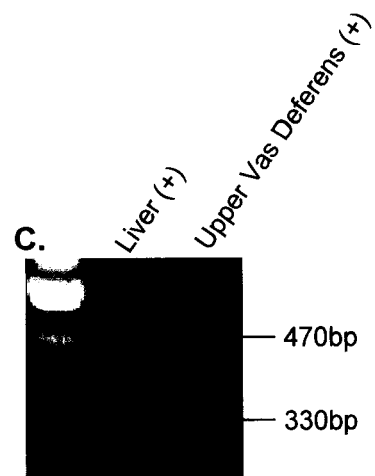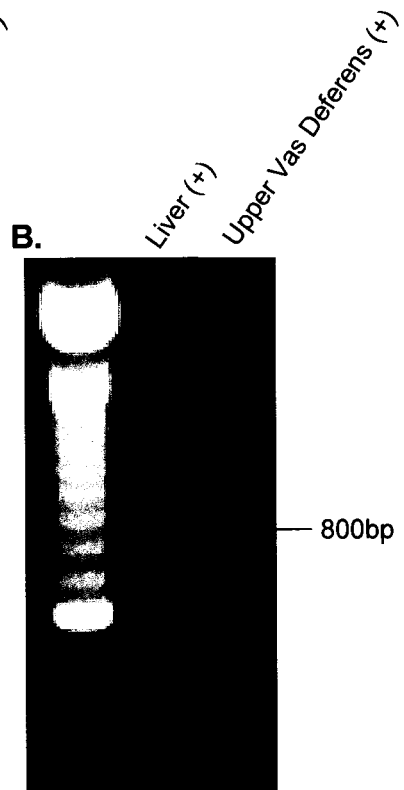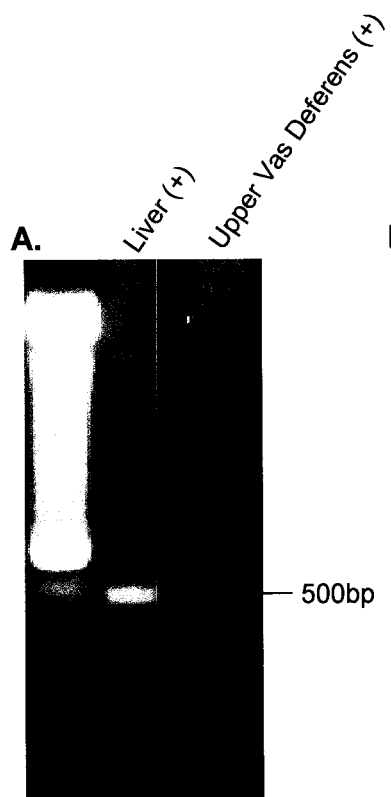
V. REFERENCES

Baker J, Hardy MP, Zhou J, Bondy C, Lupu F, Believe AR, Efstratiadis A. (1996) Effects of an Igfl gene null mutation on mouse reproduction. *Molecular Endocrinology* 10:903-918.

Baum D, Grasser G, Heib M, Krampitz, G. (1990) Chicken growth hormone: cDNA-synthesis and base sequence. *Comparative Biochemistry and Physiology* 96B:491-495.

Berger P, Untergasser G, Hermann M, Hittmair A, Madersbacher S Dirnhofer S. (1999) The testis-specific expression pattern of the growth hormone/placental lactogen (GH/PL) gene cluster changes with malignancy. *Human Pathology* 30:1201-1206.

Campbell RM and Scanes CG. (1995) Endocrine peptide 'moonlighting' as immune modulators: roles for somatostatin and GH-releasing factor. *Journal of Endocrinology* 147:383-396.

Feng XP, Kuhnlein U, Aggrey SE, Gavora JS, Zadworny D. (1997) Trait association of genetic markers in the growth hormone and the growth hormone receptor gene in a White Leghorn strain. *Poultry Science* 76:1770-1775.

Foster DN, Kim SU, Enyeart JJ, Foster LK. (1990) Nucleotide sequence of the complimentary DNA for turkey growth hormone . *Biochemical and Biophysical Research Communications* 173:967-975.

Garcia-Aragon J, Lobie PE, Muscat GEO, Gobius KS, Norstedt G, Waters MJ. (1992) Prenatal expression of growth hormone (GH) receptor/binding protein in the rat: a role for GH in embryonic and fetal development? *Development* 114:869-876.

Gravance CG, Breier BH, Vickers MH, Casey PJ. (1997) Impaired sperm characteristics in postpubertal growth-hormone-deficient dwarf (dw/dw) rats. *Animal Reproduction Science* 49:71-76.

Harvey S and Hull KL. (1997) Growth hormone: a paracrine growth factor? *Endocrine* 7:267-279.

Harvey S, Johnson CDM, Sharma P, Sanders EJ, Hull KL. (1998) Growth hormone: a paracrine growth factor in embryonic development. *Comparative Biochemistry and Physiology* 119C:305-315.

Harvey S, Johnson CDM, Sanders EJ. (2000a) Extrapituitary growth hormone in peripheral tissues of early chick embryos. *Journal of Endocrinology* 166:489-502.

Harvey S, Azumaya Y, Hull KL. (2000b) Pituitary and extrapituitary growth hormone: Pit-1 dependence? *Canadian Journal of Physiology and Pharmacology* 78:1013-1028.

Harvey S, Murphy AE, Hull KL, Luna M, Aramburo C. (2002) Testicular growth hormone (GH) and GH mRNA. *GH and IGF Research* (Abstract in press).

Hull KL, Janssens WCJ, Baumbach WR, Harvey S. (1995) Thyroid glands: novel sites of growth hormone action. *Journal of Endocrinology* 146:449-458.

Hull KL and Harvey S. (2000a) Growth hormone: a reproductive endocrine-paracrine regulator? *Reviews in Reproduction,* 5:175-182.

Hull KL and Harvey S. (2000b) Growth hormone: roles in male reproduction . *Endocrine* 13:243-250.

Ip SC, Zhang X, Leung FC. (2001) Genomic growth hormone gene polymorphisms in native Chinese chickens. *Experimental Biological Medicine,* 226:458-462.

Kuhnlein U, Ni L, Weigend S, Gavora JS, Fairfull W, Zadworny D. (1997) DNA polymorphisms in the chicken growth hormone gene: response to selection for disease resistance and association with egg production. *Animal Genetics,* 28:116-123.

Lamb IC, Galehouse DM, Foster DN. (1988) Chicken growth hormone cDNA sequence. *Nucleic Acids Research,* 16:9339.

Li Z, Zhang Y, Lui X, Xu R. (2002) Correlation of expression of preprothyrotropin-releasing hormone and receptor with rat testis development. *Chinese Medical Journal,* 115:6-12.

Lobie PE, Breiphol W, Aragon JG, Waters MJ. (1990) Cellular localization of the growth hormone receptor/binding protein in the male and female reproductive systems. *Endocrinology* 126:2214-2221.

Matsubara S, Sato M, Mizobuchi M, Niimi M, Takahara J. (1995) Differential gene expression of growth hormone (GH)-releasing hormone (GRH) and GRH receptor in various rat tissues. *Endocrinology* 136:4147-4150.

Morea AM, Chauvin MA, de Peretti E, Broux M, Benahmed M. (1987) Somatomedin C/insulin-like growth factor 1: an intracellular differentiative factor of Leydig cells. *Hormone Research* 28:50-57.

Nguyen AP, Chandorkar A, Gupta C. (1996) The role of growth hormone in fetal mouse reproductive function. *Endocrinology* 137:3659-3666.

Olchovsky D, Bruno JF, Berelowitz M. (1996) Growth hormone-releasing factor expression is discordantly regulated in the hypothalamus and testis of streptozotocin-diabetic rats. *Journal of Endocrinology* 148:189-182.

Ovesen P, Jorgensen JO, Ingerslev J, Orskov H, Christiansen JS. (1998) Growth hormone treatment of men with reduced sperm quality. *Ugeskrift For Laeger* 160:176-180.

Pekary AE, Yameda T, Sharp B, Basin S, Swerdloff RS, Hershman J. (1984) Somatostatin-14 and -28 in the male rat reproductive system. *Life Sciences* 34:939-945.

Reiter E, Hunnuy B, Bryninx M, Cornet A, Klug M, McNamara M, Closset J, Hennen G. (1999) Effects of pituitary hormones on the prostate. *Prostate* 38:159-165.

Render CL, Hull KL, Harvey S. (1995a) Neural expression of the pituitary GH gene. *Journal of Endocrinology* 147:413-422.

Render CL, Hull KL, Harvey S. (1995b) Expression of the growth hormone gene in immune tissues. *Endocrine* 3:729-735.

Shimonovitz S, Zacut D, Benchetrit A, Ron M. (1993) Growth hormone status in patients with maturation arrest of spermatogenesis *Human Reproduction* 8:919-921.

Shohan Z, Homburg R, Owen EJ, Conway SS, Ostergaard H, Jacobs HS. (1992) The role of treatment with growth hormone in infertile patients. *Baillieres Clinical Obstetrics and Gynecology* 6:267.

Souza LM, Boone TC, Murdock D, Langley K, Wypech J, Fenton D, Johnson S, Lai PH, Everett R, Hsu H-Y, Bosselman R. (1984) Application of recombinant DNA techniques to studies on chicken growth hormone. *Journal of Experimental Zoology* 232:465-473.

Spiteri-Grech J, Nieschlog E. (1992) The role of growth hormone and insulin-like growth factor I in the regulation of male reproductive function. *Hormone Research* 38:Supplement 1:22-27.

Stone EM, Rothblum KN, Alevy MC, Kuo TM, Schwartz RJ. (1985) Complete sequence of the chicken glyceraldehydes-3-phosphate dehydrogenase gene. *Proceedings National Academy of Science* 82:1628-1632.

Takeuchi S, Haneda M, Teshigawara K, Takahashi S. (2001) Identification of a novel GH isoform: a possible link between GH and melanocortin systems in the developing chicken eye. *Endocrinology* 142:5158-5166.

Tanaka M, Hosokawa Y, Watahiki M, Nakashima K. (1992) Structure of the chicken growth hormone-encoding gene and its promoter region. *Gene* 112:235-239.

Tanaka M, Hayashida Y, Nakao N, Nakai N, Nakashima K. (2001) Testis-specific and developmentally induced expression of a ghrelin gene-derived transcript that encodes a novels polypeptide in the mouse. *Biochemica et Biophysica Acta* 1522:62-65.

Tena-Sempere M, Barreiro ML, Gonzalez LC, Gaytan F, Zhang FP, Camino JE, Pinilla L, Casaneuva FF, Dieguez C, Aguilar E. (2002) Novel expression and functional role of ghrelin in rat testis. *Endocrinology* 143:717-725.

Untergasser G, Kranewitter W, Walser F, Madersbacher S, Dirnhofer S, Berger P. (1996) The testis as eutopic production site of human growth hormone, placental lactogen and prolactin, possible autocrine/paracrine effects on testicular function. *Wien Klin. Wochen.* 108:541-546.

Untergasser G, Kranewitter W, Schwarzler P, Madersbacher S, Dirnhofer S, Berger P. (1997) Organ-specific expression pattern of the human growth hormone/placental lactogen gene-cluster in the testis. *Molecular and Cellular Endocrinology* 130:43-60.

Untergasser G, Hermann M, Rumpold H, Berger P. (1998) Complex alternative splicing of the GH-V gene in the human testis. *European Journal of Endocrinology* 139:424-427.

Wang JJ. (1989) Immunocytochemical demonstration of the binding of growth-related polypeptide hormones on chick embryonic tissues. *Histochemistry* 93:133-141.

Wilber JF and Xu AH. (1998) Thyrotropin-releasing hormone gene 1998: cloning, characterization and transcriptional regulation in the central nervous system, heart and testis. *Thyroid* 8:897-901.

Zhu LJ, Krempels K, Bardin KW, O'Carroll AM, Mezey E. (1998) The localization of messenger ribonucleic acids for somatostatin receptors 1, 2, and 3 in rat testis. *Endocrinology* 139:350-357.

Zhvirblis GS, Gorbulev VG, Rubtsov PM, Karapetyan RB, Zhuravlev IV, Fisinin VI, Skryabin KG, Baev AA. (1987) Genetic engineering of peptide hormones: Cloning and primary structure of cDNA of chicken growth hormone. *Molecular Biology* 21:1324-1328.

Chapter 6

General Discussion

I. OVERVIEW

The results of these studies clearly demonstrate the presence of pituitary hormones (GH and TSH) in extrapituitary tissues of the chick embryo, prior to the differentiation of the pituitary gland.

These results confirm and extend similar results for GH that were recently published by Harvey *et al.* (1998, 2000, 2001a). These results more clearly identify the location of GH immunoreactivity within central and peripheral tissues and demonstrate, using two fixatives, that this is not an immunohistochemical artifact. The recent finding of GH expression in the heart of ED 7 chick embryos (Takeuchi *et al.*, 2001) and the headless bodies of ED 2-ED 7 embryos (Harvey *et al.*, 2001b) strongly suggests the presence of GH in early chick embryos arises from the local expression of a GH gene.

Although the presence of GH in extrapituitary tissues of the chick embryo was expected, it served as a positive control for immunohistochemical studies on TSH localization. This is the first study to demonstrate TSH immunoreactivity in central and peripheral extrapituitary tissues during embryogenesis, although it confirms previous reports of TSH in the brain (Hojvat *et al.*, 1982a,b) and gut (Wang *et al.*, 1997) of fetal and adult rats. Interestingly, the results of these studies clearly show tissue-specific differences in the distribution of TSH and GH in the chick embryo, possibly reflecting differences in gene regulation, hormone action or tissue function. The distribution of GH and TSH also reflects the distribution of Pit-1 in some tissues but not in others. The extrapituitary expression of the GH and TSH genes is therefore unlikely to be Pit-1 dependent, and Pit-1 is clearly not a pituitary-specific transcription factor in early embryos. Indeed, prolactin immunoreactivity is also widely present in ED 7 embryos

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(unpublished observation) and this too is likely to be Pit-1 independent, in contrast to the expression of GH, TSH and prolactin in the mammalian pituitary gland (Burrows *et al.*, 2001). The finding of TSH in extrapituitary tissues during embryogenesis is also consistent with the localization of a pituitary glycoprotein, LH, in chick embryos (Shirasawa *et al.*, 1996), although LH-immunoreactivity was of much less abundance and restricted to specific cells in the lung and alimentary tract.

The extrapituitary presence of GH in some tissues (e.g. the heart, liver and mesonephros, Harvey *et al.*, 1998; e.g. the eye, Takeuchi *et al.*, 2001) is thought to diminish during embryogenesis, although it persists in the brain (Render *et al.*, 1995a) and immune tissues (Render *et al.*, 1995b) of adult chickens. The results of these studies also demonstrate that GH expression persists in the gonad and reproductive tract of male domestic fowl. Furthermore, these results demonstrate the presence of a testis-specific variant of the GH gene, whereas the GH gene in the chicken brain, thymus, liver and spleen is identical to the pituitary GH gene (Render *et al.*, 1995a,b). This finding is, however, consistent with the presence of a novel GH gene variant in the eyes of embryonic chicks (Takeuchi *et al.*, 2001), although this variant was not detected in the male reproductive tract (unpublished observations).

II. THE EXPANDING ENDOCRINE SYSTEM

The presence of pituitary hormones in extrapituitary tissues of the chick is consistent with numerous studies that have now demonstrated this phenomenon throughout the vertebrates (Roth *et al.*, 1982; Kreiger, 1983; Smith *et al.*, 1983; Mandrekar *et al.*, 1990; Harvey *et al.*, 1993). Moreover, although *ectopic hormone*

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syndromes were once thought to be a manifestation of malignancy (Morgello *et al.*, 1988; Coleman *et al.*, 2000; Indinnimeo *et al.*, 2001) many peripheral hormones are now known to be widely expressed physiologically (Roth *et al.*, 1982; Harvey *et al.*, 1993; Hull *et al.*, 1998; Wu *et al.*, 1999; Huang *et al.*, 2001). Furthermore, every cell in an individual contains the same genome and, therefore, it is not surprising that most tissues have "endocrine" function, particularly during early development. Indeed, many endocrine systems appear to be developmentally regulated and this may provide a mechanism to limit or restrict hormone production to specific sites. The presence of GH, for instance, in fetal tissues is far greater than in the tissues of adults (Wu *et al.*, 1996; Recher *et al.*, 2000).

The presence of GH and TSH in the chick embryo occurs prior to the ontogenic differentiation of the pituitary gland. GH and GH mRNA have similarly been found at ED 2 and ED 3 (Harvey *et al,*, 2000; 2001a), prior to the differentiation of the cardiovascular system. The presence of GH in early embryos is therefore unlikely to have endocrine roles during development. Indeed, GH and GH mRNA have recently been found in zygotes from the two-cell stage and in the morulla and blastocyst (Pantaleon *et al.*, 1997; Yang *et al.*, 1999; Kolle *et al.*, 2001), preceding the development of endocrine communication. Moreover, although GH is produced in many extrapituitary tissues (reviewed by Harvey and Hull, 1997), extrapituitary GH is unlikely to significantly contribute to GH in the systemic circulation, since plasma GH levels are undetectable following pituitary ablation or hypophysectomy (Harvey and Hull, 1997). It is therefore likely that GH (and TSH) in extrapituitary tissues acts locally in autocrine or paracrine ways. The widespread presence of GH-receptors and GH-responsive genes in

tissues of early chick embryos (Harvey *et al.*, 1998, 2000, 2001a,b) supports this view, as does the demonstration of endogenous GH actions in preimplantation murine and bovine embryos that are blocked by GH immunoneutralization (Pantaleon *et al.*, 1997; Izadyar *et al.*, 2000). These actions (e.g. the stimulation of glucose uptake and hatching of preimplantation blastocysts; Pantaleon *et al.*, 1997; Kolle *et al.*, 2001), may be tissue and temporally specific and unrelated to the actions of endogenous GH in later development. Harvey and Hull (1997), in contrast, have suggested that the actions of GH locally may reflect an "emergency" mechanism that accelerates cellular pathways that are "strategically" regulated by pituitary GH in an endocrine way. It is, therefore, pertinent that GH and other "pituitary" hormones have been detected in invertebrates lacking pituitary glands (Tsushima *et al.*, 1974; Roth *et al.*, 1982; Verhaert *et al.*, 1986; Kawauchi *et al.*, 1990) and hence lacking the "endocrine" roles that these hormones have in vertebrates. It is thus likely that GH (and TSH) evolved phylogenetically as local messenger molecules, with roles dissimilar from those associated with pituitary gland function in vertebrates. The naming of a messenger molecule with respect to a biological function that it induces is therefore inappropriate, especially as it encourages dogmas that often hinder scientific enquiry (Kastin *et al.*, 1984).

III. LIMITATIONS OF THE STUDY

The methodologies of the present study have a number of limitations. Immunohistochemistry is a widely used and accepted technique. However, as Chapter 4 clearly indicates, the method of fixation can drastically affect the results in the same tissue with the same antibody and identical processing. In this study Carnoy's solution

(an alcohol based, precipitating fixative) was shown to mask the GH antigen in ED 7 chick embryo tissues and drastically reduce the amount of visible specific staining compared to 4% paraformaldehyde fixed sections. Therefore, it is difficult to conclude that a peptide is not present when using immunohistochemistry because the method of fixation may hide the antigen in question. Therefore, the presence of βTSH and Pit-1 should be re-evaluated in 4% paraformaldehyde fixed sections to ensure their presence was not underestimated. Antibodies can, alternatively, bind non-specifically to other antigens in tissues, leading to an artefactual overestimation of staining. However, this is likely not the case in this study. The high titre of the GH antibody and the high dilution (1:1000) used suggests it is unlikely to bind non-specifically to other proteins. In addition, Harvey and Scanes (1977) determined by cross reactivity studies that the antibody used for GH detection was specific for GH, and did not cross-react with other pituitary hormones, even at concentrations of 1 mg/ml. The Pit-1 antibody used in this study could, however, have been more specific if it was against chicken Pit-1 instead of rat Pit-1. However, this antibody has been used successfully in other species and the results of the Western analysis indicate it was reacting with a peptide of the appropriate size (33 kDa), and was therefore probably specific for chicken Pit-1. The anti-βTSH antibody was also directed against rat βTSH. The βTSH staining would be more reliable if an antibody directed against chicken βTSH had been used, or if Western analysis had been performed to ensure the antibody was detecting the correct size of protein. However, the anti-TSH antibody does not cross react with LH and FSH, so it is thought to be specific for TSH.

Although RT-PCR is a very sensitive method , rare transcripts may not be sufficiently abundant in some tissue extracts to be visibly seen on an agarose gels following RT-PCR amplification.. This methodological limitation probably accounts for the apparent absence of the Pit-1 transcript in embryo tissues subjected to RT-PCR and ethidium bromide staining, since the transcript was detectable following Southern blotting. As Southern blotting is far more sensitive than ethidium bromide staining, this procedure should, therefore be routinely used in conjunction with RT-PCR for the screening of transcript distribution. Comprehensive controls (tissue positive and negative controls) should also be routinely used with this technique, to ensure there is no genomic DNA contamination of the extracts and that the amplified cDNA fragments are the same size as those in the positive controls. Sequencing of the PCR products should also be undertaken to ensure the primers actually amplify the gene of interest.

GH, TSH and Pit-1 may be transiently expressed in the chick embryo at different times of development. Therefore, different stages of chick embryos, throughout embryonic development, should be used for immunohistochemical studies to determine if these peptides are present throughout development and whether the tissue distribution of the proteins changes with embryonic age.

IV. FUTURE STUDIES

This study clearly demonstrates the presence of GH, βTSH and Pit-1 in the ED 7 chick embryo, and suggests that the extrapituitary distribution of pituitary hormones is a common occurrence. Indeed, Shirasawa *et al.* (1996) identified LH in the developing lung of chick embryos and it would, therefore, be of interest to perform

immunohistochemistry (IHC) for the anterior pituitary hormones not fully studied in this thesis (LH, FSH, PRL and ACTH) to determine their distribution in the ED 7 chick embryo. This method localizes the protein in the tissue and, therefore, *in situ* hybridization could be performed in the same sections to localize the gene transcript for these hormones. IHC and *in situ* hybridization could be done on the same sections at the same time to co-localize the gene transcript and the protein, which could indicate whether the protein is actually synthesized in the tissue of localization, or simply sequestered from the blood.

This study has demonstrated the presence of GH and TSH in embryonic chick tissues, but provides no evidence that these hormones are secreted from these tissues. The reverse hemolytic plaque assay could be used to determine which tissues secrete the hormone since it can detect hormone secretion from individual cells (Neill and Frawley, 1983), and has previously been used by Porter (1997) to detect GH secretion from embryonic chick pituitary glands. Radioimmunoassays or ELISAs could also be used to investigate hormone secretion from whole tissues using different stimulatory or inhibitory factors to investigated secretory control. If GH and βTSH were secreted from cultured embryonic tissues in a regulated way, it would support the idea that these hormones have autocrine/paracrine roles in embryogenesis.

If these hormones are produced and secreted from extrapituitary tissues of the embryo, it would be of interest to determine what functions they have during embryogenesis and, similarly, what roles GH might have in the adult male reproductive tract. Functional studies would involve investigation into the hormones' mechanisms of action, and the intracellular sites of action. Sense oligonucleotides or

immunoneutralization could be used to block functional GH and TSH or their receptors and determine what occurs in the embryo or reproductive tract when these local hormones are unavailable. However, since many factors are likely to regulate the transcriptional activity of growth and differentiation, GH or TSH knockout may not impair early embryonic development. In fact, Snell dwarf mice, having a Pit-1 mutation and a lack of pituitary GH and TSH, have normal early embryonic development (Gage *et al.*, 1996), as do GH-receptor knockout mice (Bartke *et al.*, 1999). Another useful method for determining a particular hormone's action in a particular embryonic tissue would be the construction of a replication-defective retrovirus that carries an inserted gene of interest (Tickle, 1992). The viruses can be injected into specific embryonic tissues and will spread locally as development progresses. Dominant mutant receptor genes for GH and βTSH that result in non-functional receptors could be introduced to study the effects of GH and βTSH on individual embryonic tissues.

V. SUMMARY

In summary, this study has demonstrated the presence of GH-, βTSH- and Pit-1-like proteins in the ED 7 chick embryo by immunohistochemistry. Western analysis revealed that a Pit-1-like protein of identical molecular size to pituitary Pit-1 is present in some tissues of the ED7 chick embryo. RT-PCR and Southern blotting also reveal the presence of Pit-1 mRNA in embryo tissues the same size as in the pituitary. These results strongly indicate the presence of GH, βTSH and Pit-1 in the ED 7 embryo and raises questions concerning their sites of synthesis and potential actions. Also, RT-PCR has revealed the presence of GH, GHR and Pit-1 mRNA in the adult male chicken

reproductive tract and raises questions concerning GH synthesis and actions in chicken

reproductive physiology. The identification of a novel GH transcript in the adult chicken

testis indicates a possible novel role for testicular GH in the chicken. Together these

results suggest pituitary hormones are widely distributed in extrapituitary tissues of chick

embryos and adults and may act locally as autocrines or paracrines.

VI. REFERENCES

Bartke A, Chandrashekar V, Turyn D, Steger RW, Debeljuk L, Winters TA, Mattison JA, Danilovich NA, Croson W, Wernsing DR, Kopchick JJ. (1999) Effects of growth hormone overexpression and growth hormone resistance on neuroendocrine and reproductive functions in transgenic and knock-out mice. *Proceedings of the Society for Experimental Biology and Medicine.* 222:113-23.

Burrows HL, Douglas KR, Camper SA. (1999) Genealogy of the anterior pituitary gland: tracing a family tree. *Trends in Endocrinology and Metabolism.* 10:343-52.

Coleman RL, Lindberg G, Muller CY, Miller DS, Hameed A. (2000) Ectopic production and localization of beta-human chorionic gonadotropin in lymphoepithelioma-like carcinoma of the cervix: a case report. *International Journal of Gynecological Pathology.* 19:179-82.

Gage PJ, Brinkmeier ML, Scarlett LM, Knapp LT, Camper SA, Mahon KA. (1996) The Ames dwarf gene, df, is required early in pituitary ontogeny for the extinction of Rpx transcription and initiation of lineage-specific cell proliferation. *Molecular Endocrinology.* 10:1570-81.

Harvey S, Scanes CG. (1977) Purification and radioimmunoassay of chicken growth hormone. *Journal of Endocrinology.* 73:321-9.

Harvey S, Hull KL, Fraser RA. (1993) Growth hormone: neurocrine and neuroendocrine perspectives. *Growth and Regulation.* 3:161-71.

Harvey S, Hull KL. (1997) Growth hormone. A paracrine growth factor? *Endocrine.* 7:267-79.

Harvey S, Johnson CD, Sharma P, Sanders EJ, Hull KL. (1998) Growth hormone: a paracrine growth factor in embryonic development? *Comparative Biochemistry and Physiology C: Pharmacology, Toxicology and Endocrinology.* 119:305-15.

Harvey S, Johnson CD, Sanders EJ. (2000) Extra-pituitary growth hormone in peripheral tissues of early chick embryos. *Journal of Endocrinology.* 166:489-502.

Harvey S, Johnson CD, Sanders EJ. (2001a) Growth hormone in neural tissues of the chick embryo. *Journal of Endocrinology.* 169:487-98.

Harvey S, Lavelin I, Pines M. (2001b) Growth hormone (GH) action in early embryogenesis: expression of a GH-response gene in sites of GH production and action. *Anatomy and Embryology.* 204:503-10.

Hojvat S, Baker G, Kirsteins L, Lawrence AM. (1982a) TSH in the rat and monkey brain. Distribution, characterization and effect of hypophysectomy. *Neuroendocrinology.* 34:327-332.

Hojvat S, Emanuele N, Baker G, Connick E, Kirsteins L, Lawrence AM. (1982b) Growth hormone (GH), thyroid-stimulating hormone (TSH), and luteinizing hormone (LH)-like peptides in the rodent brain: non-parallel ontogenetic development with pituitary counterparts. *Brain Research.* 256:427-434.

Huang W, Yao B, Sun L, Pu R, Wang L, Zhang R. (2001) Immunohistochemical and in situ hybridization studies of gonadotropin releasing hormone (GnRH) and its receptor in rat digestive tract. 68:1727-34.

Hull KL, Fathimani K, Sharma P, Harvey S. (1998) Calcitropic peptides: neural perspectives. *Comparative Biochemistry and Physiology: C.* 119:389-410.

Indinnimeo M, Cicchini C, Memeo L, Stazi A, Ghini C, Ricci F, Reale MG, Mingazzini P. (2001) Plasma and tissue prolactin detection in colon carcinoma. *Oncology Report.* 8:1351-3.

Izadyar F, Van Tol HT, Hage WG, Bevers MM. (2000) Preimplantation bovine embryos express mRNA of growth hormone receptor and respond to growth hormone addition during in vitro development. *Molecular Reproduction and Development.* 57:247-55.

Kastin AJ, Zadina JE, Banks WA, Graf MV. (1984) Misleading concepts in the field of brain peptides. *Peptides.* 5 Suppl 1:249-53.

Kawauchi H, Yasuda A, Rand-Weaver M. (1990) Evolution of prolactin and growth hormone family. *Progress in Clinical Biological Research.* 342:47-53.

Kolle S, Stojkovic M, Prelle K, Waters M, Wolf E, Sinowatz F. (2001) Growth hormone (GH)/GH receptor expression and GH-mediated effects during early bovine embryogenesis. *Biology and Reproduction.* 64:1826-34.

Krieger DT. (1983) Brain peptides: what, where, and why? *Science.* 222:975-85.

Mandrekar PS, Sheth AR, Doctor VM, Zaveri JP, Sheth NA. (1990) Immunocytochemical localization of follicle stimulating hormone in normal human stomach. *Anatomical Record.* 227:334-9.

Morgello S, Schwartz E, Horwith M, King ME, Gorden P, Alonso DR. (1988) Ectopic insulin production by a primary ovarian carcinoid. *Cancer.* 61:800-5.

Neill JD, Frawley LS. (1983) Detection of hormone release from individual cells in mixed populations using a reverse hemolytic plaque assay. *Endocrinology.* 112:1135-7.

Pantaleon M, Whiteside EJ, Harvey MB, Barnard RT, Waters MJ, Kaye PL. (1997) Functional growth hormone (GH) receptors and GH are expressed by preimplantation mouse embryos: a role for GH in early embryogenesis? *Prceedings of the National Academy of Science* (USA). 94:5125-30.

Porter TE. (1997) Regulation of somatotroph differentiation during chicken embryonic development: a review. *In* "Perspectives in Avian Endocrinology" (Harvey S, Etches RJ, Eds), pp 47-56. Bristol, Journal of Endocrinology Ltd.

Recher S, Raccurt M, Lambert A, Lobie PE, Mertani HC, Morel G. (2001) Prenatal and adult growth hormone gene expression in rat lymphoid organs. *Journal of Histochemistry and Cytochemistry.* 49:347-54.

Render CL, Hull KL, Harvey S. (1995a) Neural expression of the pituitary GH gene. *Journal of Endocrinology.* 147:413-22.

Render CL, Hull KL, Harvey S. (1995b) Expression of growth hormone gene in immune tissues. *Endocrine.* 3:729-35.

Roth J, LeRoith D, Shiloach J, Rosenzweig JL, Lesniak MA, Havrankova J. (1982) The evolutionary origins of hormones, neurotransmitters, and other extracellular chemical messengers: implications for mammalian biology. *New England Journal of Medicine.* 306:523-7.

Shirasawa N, Shiino M, Shimizu Y, Nogami H, Ishii S. (1996) Immunoreactive luteinizing hormone (ir-LH) cells in the lung and stomach of chick embryos. *Cella nd Tissue Research.* 283:19-27.

Smith EM, Phan M, Kruger TE, Coppenhaver DH, Blalock JW. (1983) Human lymphocyte production of immunoreactive thyrotropin. *Proceedings of the National Academy of Science.* (USA). 80:6010-6013.

Takeuchi S, Haneda M, Teshigawara K, Takahashi S. (2001) Identification of a novel GH isoform: a possible link between GH and melanocortin systems in the developing chicken eye. *Endocrinology.* 142:5158-66.

Tickle C. (1992) A tool for transgenesis. *Nature.* 358:188-9.

Tsushima T, Friesen HG, Chang TW, Raben MS. (1974) Identification of sparganum growth factor by a radioreceptor assay for growth hormone. *Biochemistry and Biophysics Research Communications.* 59:1062-8.

Verhaert P, Van Mellaert H, Swinnen K, De Loof A. (1986) Vertebrate somatotropin-like peptides in insects. *Progress in Clinical Biological Research.* 217A:359-64.

Wang J, Whetsell M, Klein JR. (1997) Local hormone networks and intestinal T cell homeostasis. *Science.* 275:1937-1939.

Wu H, Devi R, Malarkey WB. (1996) Localization of growth hormone messenger ribonucleic acid in the human immune system--a Clinical Research Center study. *Journal of Clinical Endocrinology and Metabolism.* 81:1278-82.

Wu P, Liang X, Dai Y, Liu H, Zang Y, Guo Z, Zhang R, Lai W, Zhang Y, Liu Y. (1999) Aldosterone biosynthesis in extraadrenal tissues. *Chinese Medical Journal.* 112:414-8.

Yang BY, Greene M, Chen TT. (1999) Early embryonic expression of the growth hormone family protein genes in the developing rainbow trout, *Oncorhynchus mykiss*. *Molecular Reproduction and Development.* 53:127-34.