Endogenous thyroid hormones modulate pituitary somatotroph differentiation during chicken embryonic development

L Liu¹ and T E Porter^{1,2}

¹Department of Animal and Avian Sciences, University of Maryland, College Park, Maryland 20742, USA

²Molecular and Cell Biology Program, University of Maryland, College Park, Maryland 20742, USA

(Requests for offprints should be addressed to Email: TP44@UMAIL.UMD.EDU)

Abstract

Growth hormone cell differentiation normally occurs between day 14 and day 16 of chicken embryonic development. We reported previously that corticosterone (CORT) could induce somatotroph differentiation *in vitro* and *in vivo* and that thyroid hormones could act in combination with CORT to further augment the abundance of somatotrophs *in vitro*. The objective of the present study was to test our hypothesis that endogenous thyroid hormones regulate the abundance of somatotrophs during chicken embryonic development. Plasma samples were collected on embryonic day (e) 9–14. We found that plasma CORT and thyroid hormone levels increased progressively in mid-embryogenesis to e13 or e14, immediately before normal somatotroph differentiation. Administration of thyroxine (T₄) and triiodothyronine

(T₃) into the albumen of fertile eggs on e11 increased somatotroph proportions prematurely on e13 in the developing chick embryos *in vivo*. Furthermore, administration of methimazole, the thyroid hormone synthesis inhibitor, on e9 inhibited somatotroph differentiation *in vivo*, as assessed on e14; this suppression was completely reversed by T₃ replacement on e11. Since we reported that T₃ alone was ineffective *in vitro*, we interpret these findings to indicate that the effects of treatments *in vivo* were due to interactions with endogenous glucocorticoids. These results indicate that treatment with exogenous thyroid hormones can modulate somatotroph abundance and that endogenous thyroid hormone synthesis likely contributes to normal somatotroph differentiation.

Journal of Endocrinology (2004) **180**, 45–53

Introduction

The anterior pituitary gland differentiates from Rathke's pouch into five distinct hormone-secreting cell types. The first functionally mature cell type to appear is the corticotroph. Differentiation of the other cell types is not autonomous and is controlled by various hormones and hypothalamic peptides (Dubois & Hemming 1991, Dubois *et al.* 1997). We are studying the mechanisms regulating differentiation of growth hormone (GH)-secreting cells in the chicken anterior pituitary. Somatotrophs first become a significant population between embryonic day (e) 14 and e16 of chicken development (Porter *et al.* 1995a). Somatotroph differentiation does not occur *in vitro* without an extrapituitary signal, and glucocorticoids have been shown to induce somatotroph differentiation in chicken embryos and fetal rats *in vitro* and *in vivo* (Hemming *et al.* 1988, Nogami & Tachibana 1993, Nogami *et al.* 1995, 1997, Morpurgo *et al.* 1997, Dean *et al.* 1999, Bossis & Porter 2000, Porter *et al.* 2001, Liu *et al.* 2003).

Thyroid hormones play an important role in embryonic and neonatal development in mammals and birds. Studies with cultured fetal rat pituitary glands indicated that thyroid hormones exert their stimulatory action on fetal

GH gene expression and GH cell abundance only in the presence of glucocorticoids (Hemming *et al.* 1988, Nogami *et al.* 1997). Furthermore, Nogami *et al.* (1997) found that intraperitoneal injection of thyroxine (T₄) into pregnant rats, in the absence of concomitant dexamethasone treatment, failed to induce either GH cells or the accumulation of GH in the fetal pituitary gland. In contrast, adding methimazole (MMI) to the drinking water of pregnant rats suppressed GH cell number, GH content and GH mRNA level in the fetal pituitaries, and this suppression was reversed by additional intraperitoneal T₄ injections to the pregnant dams (Nogami *et al.* 1995). However, as treatments were administered to the dams rather than directly to the fetuses in these studies, the effect of fetal–maternal interactions or maternal thyroid hormones could not be ruled out. Furthermore, as GH immunostaining in histology sections of the pituitary glands was evaluated in this study, quantitative determination of the total abundance of fetal somatotrophs was not achieved.

We found that thyroid hormones could act in combination with corticosterone (CORT) to further augment the abundance of somatotrophs in cultures of dispersed, chick embryonic pituitary cells, while having little or no

effect on their own (Liu *et al.* 2003). However, the role played by thyroid hormones in modulating somatotroph differentiation *in vivo* during chicken or rat fetal development is not well understood.

In the present study, we measured plasma CORT and thyroid hormone levels during mid-embryogenesis to determine whether they increased prior to the substantial appearance of somatotrophs between e14 and e16. We then evaluated whether administration of thyroid hormones could increase somatotroph abundance *in vivo*. Finally, we examined whether inhibition of endogenous thyroid hormone synthesis could affect normal somatotroph differentiation.

Materials and Methods

Animals, egg injections and pituitary dispersions

All chicken embryos used in the present study were purchased from Allen's Hatchery (Seaford, DE, USA). All procedures with chicken embryos were approved by the Animal Care and Use Committee of the University of Maryland. Unless stated otherwise, all cell culture reagents were obtained from Gibco, and hormones and other chemicals were purchased from Sigma. Thyroid hormone stock solutions were prepared in 0.01 M NaOH, and then diluted with water. All media were supplemented with 0.1% BSA, 100 U/ml penicillin G and 100 µg/ml streptomycin sulfate. Fertile eggs were placed in a humidified incubator (GQF Manufacturing, Savannah, GA, USA) at 37.5 °C. The typical length of incubation for chickens is 21 days. On e9 or e11, 100 µl of the treatment solutions were injected 8 mm into the egg albumen through a hole punched in the small apical end of the egg. Embryos were removed on e13 or e14, and their anterior pituitary glands were isolated with the aid of a dissecting microscope. The pituitaries were then dissociated by a combination of trypsin digestion and mechanical agitation as described previously (Porter *et al.* 1995a,b, Morpurgo *et al.* 1997, Dean *et al.* 1999). The viability of the cells was assessed by the trypan blue dye exclusion method and was consistently greater than 95%. The dispersed cells were assayed for detection of GH-secreting or GH-containing cells by reverse hemolytic plaque assay (RHPA) or immunocytochemistry (ICC) respectively.

Plasma sample collection and RIAs for CORT, triiodothyronine (T₃) and T₄

Blood was collected from the chorioallantoic vessels of e9–e14 embryos. Blood was allowed to flow into capillary tubes coated with heparin and then centrifuged at 14 000 g for 10 min to isolate the plasma. Plasma was stored at –20 °C until subjected to double antibody RIA for CORT, T₃ and T₄. The tracers, [¹²⁵I]CORT, [¹²⁵I]T₃

and [¹²⁵I]T₄, were purchased from Diagnostic Products Corporation (Los Angeles, CA, USA). Primary antibody for CORT was purchased from Fitzgerald Industries International, Inc (Concord, MA, USA). Primary antibodies for T₃ and T₄ were purchased from Endocrine Sciences (Calabasas Hills, CA, USA). Rabbit immunoglobulin (IgG) and secondary antibody (sheep anti-rabbit IgG) were purchased from Sigma. Plasma was extracted with ether and stored at –20 °C until subjected to double antibody RIA for CORT; 40 µl plasma were incubated with anti-CORT antibody (1:800 in PBS containing 0.8% normal rabbit serum) and tracer at room temperature for 3 h. After incubation with the second antibody at 4 °C overnight, 1 ml cold PBS was added, the tubes were centrifuged and the supernatants were removed. The CORT radioactivity precipitated was measured by gamma counter. The T₃ and T₄ RIA were performed as described previously (McNabb & Hughes 1983). Plasma from e9 embryos was pooled at two embryos per sample. All other ages were evaluated as individual embryos. Plasma (12.5 µl for T₄, 25 µl for T₃) was incubated with primary antisera, carrier IgG, barbital buffer (Sigma) and tracer at 37 °C for 2 h. After incubation with the second antibody at 4 °C overnight, 10% polyethylene glycol-8000 was added, the tubes were centrifuged, and the supernatants were removed. Assay sensitivity was 2.5 ng/ml for CORT, 0.125 ng/ml for T₃ and 0.625 ng/ml for T₄. Intra-assay coefficients of variation were determined by assaying a pooled sample 3, 6 and 6 times in the CORT, T₃ and T₄ assays respectively. Intra-assay coefficients of variation were 14.3, 4.5 and 17.6% for CORT, T₃ and T₄ respectively.

Reverse hemolytic plaque assays (RHPA)

The assays were performed according to the protocol described in detail previously (Porter *et al.* 1995a). Briefly, the harvested pituitary cells (1.0 × 10⁵ cells/ml) were mixed with an equal volume of an 18% suspension of protein A-coated ovine erythrocytes and infused by capillary action into poly-L-lysine-coated Cunningham chambers. After cells were allowed to attach for 45 min (37.5 °C; 95% air–5% CO₂), chambers were rinsed with Dulbecco's modified Eagle's medium (DMEM) to remove unattached cells. DMEM containing GH antiserum (1:40) and human GH-releasing hormone (hGHRH, 10^{–7} M) was then added to the resulting monolayer of cells, and replicate chambers were incubated for 20 h (three chambers per treatment). The GH antiserum used was as described and validated previously (Porter *et al.* 1995a). Plaque formation was subsequently induced by a 45 min incubation with guinea pig complement (1:40, in DMEM). The cells were then fixed with 2% glutaraldehyde in 0.9% saline and stained with methyl green. Chambers were analyzed using a light microscope. GH-positive cells were defined as those surrounded by a

zone of hemolysis at least one lysed ovine erythrocyte in radius. Data shown are the percentage of all pituitary cells that formed plaques (i.e. secreted GH), with at least 200 pituitary cells counted per assay chamber.

Immunocytochemistry (ICC)

The harvested pituitary cells were diluted in DMEM to a concentration of 1.0×10^4 cells/ml and were then attached to poly-L-lysine-coated 24-well tissue culture plates during a 1 h incubation period (37.5 °C; 95% air–5% CO₂). The plated cells were fixed with 3.7% formaldehyde in PBS for 20 min. Cells were permeabilized with 0.1% Tween-20/0.1% Triton-X100 (8 min), quenched with 0.3% H₂O₂ (5 min), blocked with 1% normal goat serum (15 min) and incubated overnight with rabbit anti-chicken GH (1:4000 in PBS) using the antiserum described above for the RHPA. The cells were rinsed with PBS, and incubated with biotinylated goat anti-rabbit IgG for 30 min. The cells were further processed using rabbit ABC kits according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA). VIP reagent (Vector) was used as substrate for the peroxidase. GH-containing cells were visually identified as exhibiting obvious staining using an inverted light microscope, and data shown are the percentage of the total pituitary cell population that contained GH.

Statistical analysis

Except for RIA results, the data reported are the means $\pm$ S.E.M. from at least three completely separate experiments, with the number of replicate experiments provided in the legend to each Figure. Percentile data were transformed by taking the log of the arcsine of the ratio of GH cells to all pituitary cells. Transformed data were analyzed by ANOVA using the MIXED models procedure of SAS (SAS Institute, Cary, NC, USA). Differences between treatments were tested using Tukey's Studentized range test and were considered significant at $P < 0.05$.

Results

Plasma CORT and thyroid hormone levels increase by e14

We demonstrated previously that significant somatotroph differentiation occurs between day 14 and day 16 of chicken embryonic development and that CORT could induce somatotroph differentiation *in vitro* and *in vivo*. We also found that thyroid hormones could act in combination with CORT to further augment the abundance of somatotrophs by 3 days in cultures of chick embryo pituitary cells, while having little or no effect on their own (Liu *et al.* 2003). To determine whether CORT and

Figure 1 Plasma CORT and thyroid hormone levels in mid-embryogenesis determined by RIA. Plasma samples were collected on e9–e14, and CORT (A), total T₃ (B) and total T₄ levels (C) were measured by RIA. Results are the least squares means of three or four independent replicates. Data were analyzed by analysis of variance, followed by Tukey's Studentized range test. Values denoted by different letters are significantly different ($P < 0.05$). Vertical bars represent the pooled S.E. of comparisons. Dashed lines denote the limit of detection in the RIAs. Note that levels of total T₄ on e9, e10 and e11 were at or below the limit of detection.

thyroid hormones increase in the systemic circulation prior to the time of somatotroph differentiation, plasma samples were collected from chorioallantoic vessels of embryos, and total CORT levels (Fig. 1A), total T₃ levels (Fig. 1B) and

total T_4 levels (Fig. 1C) were measured by RIA. Plasma total CORT could be detected at e11, e12 and e13 with levels of 6.9 ± 0.9 , 6.3 ± 1.3 and 14.5 ± 4.2 ng/ml respectively (mean $\pm$ pooled S.E.M.). However, total CORT levels increased by e14 to 27.9 ± 4.2 ng/ml. Data published on concentrations of adrenal hormones in plasma during development of the chick vary from several to several tens of ng/ml (Wise & Frye 1973, Kalliecharan & Hall 1974, Marie 1981), comparable to our current data. Plasma T_3 levels increased progressively from e9 to e14. The plasma total T_3 levels on e13 and e14 were significantly higher than those on e9, e10 and e11 ($P < 0.05$). Plasma T_4 levels showed a similar pattern to T_3 , although those on e9, e10 and e11 were at or below the level of sensitivity of the RIA. Furthermore, plasma total T_4 levels on e14 were statistically higher than those on e9, e10 and e11 ($P < 0.05$). Thus, plasma total CORT and total thyroid hormone levels increased just prior to normal somatotroph differentiation (e14) during chicken embryonic development.

Treatment with exogenous thyroid hormones increases somatotroph abundance in vivo

We found that both T_4 and T_3 could act with CORT to increase somatotroph abundance *in vitro*, although the effects of T_3 were biphasic, inhibiting CORT actions at higher concentrations (Liu *et al.* 2003). The following experiments were designed to examine the effect of thyroid hormones on somatotroph abundance *in vivo*. On e11, the albumen of fertile eggs was injected with T_3 (1.2 pg, 12 pg, 120 pg, 1.2 ng in 100 μ l water). The embryos were then allowed to continue developing until e13, when pituitary cells were isolated and subjected to RHPA to detect GH-secreting cells. Figure 2 presents the results from these trials. T_3 at 12 pg and 120 pg increased the proportions of GH-secreting cells respectively to 7.7 ± 2.1 and $8.3 \pm 2.2\%$ of all pituitary cells versus basal levels of $4.0 \pm 2.1\%$ and water injection controls of $3.8 \pm 2.1\%$. T_3 at 1.2 pg and 1.2 ng slightly increased the percentage of GH-secreting cells, although these effects were not statistically significant. We also conducted a single experiment to test the effect of T_3 on somatotroph abundance *in vivo* by ICC. Injection of T_3 on e11 at 12 pg, 120 pg, 1.2 ng and 12 ng increased the percentage of GH-containing cells on e13 to 7.4, 9.4, 12.6 and 8.0% respectively, versus the vehicle-injected control of 4.6%. Administration of T_3 to developing embryos could, therefore, augment somatotroph proportions prematurely *in vivo*.

We also tested whether T_4 administration could modulate somatotroph abundance *in vivo*. As we found previously that conversion of T_4 to T_3 was necessary for the effect of T_4 on corticosterone induction of somatotrophs in culture (Liu *et al.* 2003), the albumen of fertile eggs was injected on e11 with T_4 (1.2 ng, 12 ng, 120 ng

Figure 2 The effect of T_3 on somatotroph abundance *in vivo* determined by RHPA. The albumen of fertile eggs was injected on e11 with T_3 (1.2 pg, 12 pg, 120 pg and 1.2 ng in 100 μ l water). The embryos were then allowed to continue developing until e13, when pituitary cells were isolated and subjected to RHPA for GH release. RHPAs were performed for 20 h in the presence of GHRH (10^{-7} M) to induce GH secretion. Three assay chambers were used for each group of treated cells. Results are reported as the percentage of GH-secreting cells and are the least squares means of four independent replicate trials. Percentile results were transformed by taking the log of the arcsine of the ratio of GH cells to all pituitary cells. Transformed data were then analyzed by analysis of variance, followed by Tukey's Studentized range test. Values denoted by an asterisk (*) are significantly different from the vehicle-injected control ($P < 0.05$). Vertical bars represent the pooled S.E. of comparisons.

and 1.2 μ g in 100 μ l water) at 10^3 -fold greater doses than T_3 . Pituitary cells were isolated on e13 and subjected to ICC for GH-containing cells (Fig. 3). The percentage of GH-containing cells was increased by all doses of T_4 ($P < 0.05$), with the greatest effect on GH cell abundance ($11.9 \pm 0.4\%$ of all cells) observed with 1.2 μ g T_4 , compared with $7.9 \pm 0.5\%$ for basal levels. Together with the results from T_3 injections, these data indicate that treatment with exogenous thyroid hormones can increase somatotroph abundance during chick embryonic development *in vivo*.

Inhibition of thyroid hormone synthesis suppresses normal somatotroph differentiation

The goitrogen MMI is a common anti-thyroid drug that inhibits thyroid hormone biosynthesis. In the present study, we established hypothyroidism during mid-embryogenesis by administration of MMI. First, we conducted preliminary experiments on the dose and timing of MMI treatment needed to suppress somatotroph abundance. Injection of 5 μ g MMI on e9, e10, e11 or e12 decreased the percentage of GH-containing cells on e14 to 1.8, 2.2, 2.4 and 3.4% respectively, versus the vehicle-injected control of 5.0%; this suggests that GH cells were most sensitive to MMI treatment on e9. Injection of 5 μ g

Figure 3 The effect of T₄ on somatotroph abundance *in vivo* determined by ICC. The albumen of fertile eggs was injected on e11 with T₄ (1.2 ng, 12 ng, 120 ng and 1.2 µg in 100 µl water). The embryos were then allowed to continue developing until e13, when pituitary cells were isolated and subjected to ICC for GH. Results are reported as the percentage of GH-containing cells and are the least squares means of three independent replicate trials. Transformed data were analyzed as in Fig. 2, with values denoted by an asterisk (*) being significantly different from vehicle-injected controls ($P < 0.05$). Vertical bars represent the pooled S.E. of comparisons.

MMI on e9 also reduced plasma T₃ levels on e14 to 0.47 ± 0.02 ng/ml (versus 0.59 ± 0.02 ng/ml for the vehicle-injected group, $P < 0.05$) and T₄ levels on e14 to 0.78 ± 0.13 ng/ml (versus 1.25 ± 0.35 ng/ml for the vehicle-injected group). It has been reported that continuous infusion of MMI (50 µg /0.1 ml) from e15 to e19 blocked the hatchability of chicken embryos (Iqbal *et al.* 1987). We also determined whether injection of 5 µg MMI on e9 was toxic to embryo growth by determining embryo body weights from e14 to 1 day after hatching. There were no significant differences in body weights between MMI-treated and control embryos (data not shown). We next evaluated the effect of MMI on somatotroph differentiation during embryonic development. We conducted four replicate trials in which 5 µg MMI or vehicle were injected on e9 and somatotroph abundance was assessed on e14 by ICC (Fig. 4). MMI decreased the abundance of GH-containing cells on e14 from 13.3 ± 1.3 to $7.6 \pm 1.3\%$ of all cells ($P < 0.05$). These results suggest that the thyroid hormone synthesis inhibitor, MMI, suppressed somatotroph differentiation during embryonic development, although it did not affect embryo growth.

T₃ replacement reverses the reduction in somatotrophs induced by MMI

Based on the experiments described above, we determined (by ICC) that 1.2 ng was the most effective dose of T₃ administration to increase somatotroph abundance. We

Figure 4 The effect of MMI on somatotroph differentiation *in vivo* as determined by ICC. The albumen of fertile eggs was injected on e9 with 5 µg MMI or vehicle (100 µl). The embryos were then allowed to continue developing until e14, when pituitary cells were isolated and subjected to ICC for GH. Results are reported as the percentage of GH-containing cells and are the least squares means of four independent replicate trials. Transformed data were analyzed as in Fig. 2, with the asterisk (*) indicating a significantly different value from the vehicle-injected controls ($P < 0.05$). Vertical bars represent the pooled S.E. of comparisons.

next tested whether T₃ could reverse the effect of MMI on somatotroph abundance. We injected 5 µg MMI on e9 and 1.2 ng T₃ on e11 into the same eggs, and determined somatotroph abundance on e14 by ICC. Figure 5 presents the results from these trials. Compared with basal levels of somatotrophs, MMI significantly decreased the proportion of GH-containing cells, while T₃ alone augmented somatotroph abundance ($P < 0.05$). Interestingly, T₃

Figure 5 The effect of T₃ replacement on the reduction in somatotroph abundance induced by MMI. Fertile eggs were injected on e9 with 5 µg MMI and/or on e11 with 1.2 ng T₃ in 100 µl water. On e14, pituitary cells were isolated and subjected to ICC to identify GH-containing cells. Results are reported as the percentage of GH-containing cells and are the least squares means of four independent replicate trials. Data were analyzed as in Fig. 2, with values denoted by different letters being significantly different from water-injected controls ($P < 0.05$). Vertical bars represent the pooled S.E. of comparisons.

treatment overcame the reduction in GH cell abundance induced by the thyroid hormone synthesis inhibitor, MMI ($P < 0.05$, relative to MMI alone). Thus, endogenous thyroid hormone synthesis may contribute to normal somatotroph differentiation.

Discussion

During chicken embryonic development, which lasts 21 days, somatotrophs are rare before e12, and significant somatotroph differentiation occurs between e14 and e16 (Porter *et al.* 1995a). We and others have shown that GH-secreting cells do not differentiate spontaneously without an extrapituitary signal, and glucocorticoids were found to increase the abundance of GH mRNA and induce somatotroph differentiation in chicken embryos and fetal rats both *in vitro* and *in vivo* (Hemming *et al.* 1988, Nogami & Tachibana 1993, Morpurgo *et al.* 1997, Nogami *et al.* 1997, Dean *et al.* 1999, Bossis & Porter 2000, 2003, Porter *et al.* 2001). Low levels of CORT can be detected in the circulation by day 10 of chicken embryonic development (Wise & Frye 1973, Kalliecharan & Hall 1974), and our current study and other reports showed a substantial increase in plasma CORT levels around e13–e14 (Woods *et al.* 1971, Marie 1981, Tanabe *et al.* 1986), just prior to somatotroph differentiation at e14–e16 (Porter *et al.* 1995a). It is also noted that the levels of transcortin, the circulating glucocorticoid-binding protein, increase significantly around e15 (Gasc & Martin 1978). Taken together, these findings suggest a close correlation between increased adrenal CORT production and somatotroph differentiation.

In the developing chicken embryo, the ontogeny and maturation of the hypothalamo-adenohypophyseal-thyroid (HAT) axis has been studied extensively. Thyrotropin-releasing hormone (TRH) is immunocytochemically demonstrable in the infundibulum as early as e4.5, with a gradual increase in the number of TRH-positive perikarya, as well as the amount of immunoreactive TRH per cell, from e4.5 to e19.5 (Thommes *et al.* 1985). Similarly, immunoreactive thyroid-stimulating hormone (TSH) is found in the pituitary by e6.5 (Thommes *et al.* 1992), and thyrotrophs are responsive to TRH at this same age, as evidenced by heightened plasma T_4 levels following TRH treatment (Thommes & Hylka 1978). However, it is between e10.5 and e11.5 that there is a marked increase in both the number of TSH-immunoreactive cells and the amount of immunoreactive material per cell (Thommes *et al.* 1983). Immunoreactive T_3 was first detected at e9 in the colloid of primordial thyroid follicles and gradually increased with embryo age (Kameda *et al.* 1986). Our data indicate that plasma thyroid hormones steadily increase from e9 to e14, with significantly higher concentrations at e13 (T_3) or e14 (T_4) than e9–e11. Although the yolk of the hen's egg contains

both T_4 and T_3 as the maternal contribution of thyroid hormones to the developing embryo (Prati *et al.* 1992), the thyroidal origin of plasma thyroid hormone has been established by the finding that both thyroidectomy and thiourea treatment markedly reduce circulating T_4 levels at day 10.5 of incubation (Thommes & Tonetta 1979, Thommes *et al.* 1984). Furthermore, hypophysectomy and hormone replacement studies show that functionality of the HAT axis occurs between e10.5 and e12.5 of development (Thommes *et al.* 1977, Thommes 1987). Thus, circulating thyroid hormones in the embryo are principally derived from the embryonic thyroid gland, under the control of the hypothalamo-pituitary axis, by the time of somatotroph differentiation. In contrast to large mammals, birds possess only two types of thyroid hormone-binding plasma proteins, i.e. albumin and transthyretin, and not thyroxine-binding globulin. There are no reports describing the ontogeny of thyroid hormone-binding proteins during chicken embryonic development. However, similar patterns of circulating T_3 and T_4 concentrations have been observed in quail embryos (McNabb & Hughes 1983). Total serum protein and T_4 binding increased significantly from e11 to e16, while T_3 binding was essentially equal to adult levels. Most T_3 and T_4 in the circulation was bound to plasma proteins. Thus, free T_3 and free T_4 available to somatotrophs may be reduced. However, the affinity of albumin and transthyretin for thyroid hormones is relatively low.

Multiple studies suggest that thyroid hormones can activate GH synthesis in adult rats and synergistically stimulate somatotroph abundance with glucocorticoids in rat pituitary cell lines (Hervas *et al.* 1975, Shapiro *et al.* 1978, Dobner *et al.* 1981, Spindler *et al.* 1982, Yaffe & Samuels 1984). In contrast, thyroid hormones appear to require glucocorticoids in order to regulate GH gene expression and somatotroph differentiation in fetal animals. Dexamethasone treatment of pregnant rats via their drinking water induced GH and GH mRNA accumulation in the fetal pituitary gland on day 17 or 18 of gestation, when substantial GH expression had not yet occurred in the control fetuses. Additional T_4 injections into the peritoneum of the pregnant females enhanced the effect of dexamethasone, whereas T_4 exhibited no effect when given alone (Nogami *et al.* 1995). Furthermore, GH immunoreactivity could be induced in the fetal rat pituitary primordia *in vitro* with cortisol. T_3 , which was individually ineffective, acted synergistically with cortisol to increase GH cell density (Hemming *et al.* 1988). As in mammals, thyroid hormones are thought to be involved in the control of growth and development of a number of organs, including the brain, liver and skeleton in early and mid-embryonic development of birds (Scanes *et al.* 1987). However, there have been no reported studies on the effects of thyroid hormones on growth hormone synthesis and somatotroph differentiation during chicken embryonic development. We reported previously that T_3 or T_4 alone

had minimal effects on the percentage of somatotrophs in e11 pituitary cell cultures, while both T_4 and T_3 could act in combination with CORT to increase somatotroph abundance. In that study, the effects of T_3 were biphasic, inhibiting CORT actions at higher concentrations (Liu *et al.* 2003). In the present study, we demonstrated that treatment with exogenous thyroid hormones on e11 increased somatotroph abundance on e13. The ineffectiveness of the highest dose of T_3 tested in the present study may reflect the biphasic actions of T_3 noted in cell culture previously (Liu *et al.* 2003). The comparable effectiveness of all doses of T_4 tested in our current investigation may reflect a limited capacity for conversion of T_4 to T_3 within the embryonic pituitary gland, as indicated in our previous report (Liu *et al.* 2003). In our previous study, we found that the deiodinase inhibitor iopanoic acid blocked the effect of T_4 but not T_3 *in vitro*. Thus, we believe that conversion of T_4 to T_3 is necessary for the somatotroph response. As plasma CORT levels increased around e13–e14, and thyroid hormones alone are individually ineffective in culture, we conclude that the induction of somatotrophs by exogenous thyroid hormones requires interaction with endogenous glucocorticoids *in vivo*. In contrast, T_4 injection failed to induce either GH cells or the accumulation of GH in fetal rat pituitary glands on e17 of gestation (Nogami *et al.* 1995). However, that study did not assess the effect of T_4 on day 18 or 19 of gestation, the time that somatotroph differentiation begins. Species differences could also explain the discrepancy between the current study in chickens and the rats used in Nogami *et al.* (1995).

Plasma concentrations of thyroid hormones were profoundly depressed, both in chick embryos and growing chickens, after surgical thyroidectomy or MMI treatment (Harvey *et al.* 1983, Leung *et al.* 1985, Iqbal *et al.* 1987). Consequently, reduction of physiological levels of thyroid hormones impairs the growth and development of the chick embryo. This is evident with MMI treatment, which significantly reduces body weight gain and bone growth during the second half of embryogenesis, and delays the onset of pipping and hatching (King & May 1984, Iqbal *et al.* 1987). This effect of MMI on growth can be reversed by T_4 treatment *in ovo* (King & May 1984). In the present study, we also used MMI to suppress thyroid hormone synthesis. To eliminate confounding effects, a dose of MMI was chosen that did not affect body weight. As a result, suppression of thyroid hormone levels in our study was not as great as that reported previously (King & May 1984, Iqbal *et al.* 1987). However, we did find that injection of MMI significantly suppressed normal somatotroph differentiation, while T_3 treatment overcame the reduction in GH cell abundance induced by MMI. These results strongly suggest that thyroid hormone synthesis plays a significant role in the regulation of normal somatotroph differentiation *in vivo*. Similarly, treatment of pregnant female rats with MMI resulted in marked

reductions in the number of GH cells, pituitary content of GH, and GH mRNA levels in the gestating rat fetuses, and this suppression was partially reversed by T_4 replacement (Morreale de Escobar *et al.* 1993, Nogami *et al.* 1995). However, no evidence indicated whether the circulating thyroid hormones in the rat fetuses originated from the fetuses or their mother. Thus, the possibility that thyroid hormones modulated somatotroph differentiation indirectly in the fetuses through maternal effects could not be eliminated in the earlier rat studies. In our current study, we demonstrate direct effects of thyroid hormones on somatotroph differentiation in chicken embryos in the absence of maternal–fetal interactions.

Our results indicate that normal somatotroph ontogeny involves peripheral thyroid hormone production. We have shown previously (Liu *et al.* 2003) that thyroid hormones and glucocorticoids can interact in cell cultures to induce GH cell differentiation, suggesting a direct mode of action within the pituitary. We have also shown that the effects of glucocorticoids on induction of GH mRNA are indirect within the pituitary and require synthesis of some other protein(s) (Bossis and Porter 2003). One possible explanation for the observed effects of thyroid hormones is that thyroid hormone receptors and glucocorticoid receptors interact directly to regulate production of the unknown intermediate protein, which in turn induces GH gene expression. A second possibility is that thyroid hormone receptors interact with the glucocorticoid-induced protein(s) to augment GH gene expression. Evaluation of these possibilities awaits identification of the glucocorticoid-induced intermediate protein(s).

In conclusion, the present findings indicate that treatment with exogenous thyroid hormones can modulate somatotroph abundance and that endogenous thyroid hormone synthesis contributes to normal somatotroph differentiation during chicken embryonic development. Since we reported that T_3 alone was ineffective *in vitro*, we interpret these findings to indicate that the effects of thyroid hormone treatments *in vivo* were due to interactions with endogenous glucocorticoids.

Funding

This research was supported by competitive grant 00–35206–9463 (to T. E. Porter) from the USDA–NRI program.

References

- Bossis I & Porter TE 2000 Ontogeny of corticosterone-inducible growth hormone-secreting cells during chick embryonic development. *Endocrinology* **141** 2683–2690.
- Bossis I & Porter TE 2003 Evaluation of glucocorticoid-induced growth hormone gene expression in chicken embryonic pituitary cells using a novel *in situ* mRNA quantitation method. *Molecular and Cellular Endocrinology* **201** 13–23.

- Dean CE, Morpurgo B & Porter TE 1999 Induction of somatotroph differentiation *in vivo* by corticosterone administration during chick embryonic development. *Endocrine* **11** 151–156.
- Dobner PR, Kawasaki ES, Yu LY & Bancroft FC 1981 Thyroid or glucocorticoid hormone induces pre-growth-hormone mRNA and its probable nuclear precursor in rat pituitary cells. *PNAS* **78** 2230–2234.
- Dubois PM & Hemming FJ 1991 Fetal development and regulation of pituitary cell types. *Journal Electron Microscopy Techniques* **19** 2–20.
- Dubois PM, el Amraoui A & Heritier AG 1997 Development and differentiation of pituitary cells. *Microscopy Research and Techniques* **39** 98–113.
- Gasc JM & Martin B 1978 Plasma corticosterone binding capacity in the partially decapitated chick embryo. *General and Comparative Endocrinology* **35** 274–279.
- Harvey S, Sterling RJ & Klandorf H 1983 Concentrations of triiodothyronine, growth hormone, and luteinizing hormone in the plasma of thyroidectomized fowl (*Gallus domesticus*). *General and Comparative Endocrinology* **50** 275–281.
- Hemming FJ, Aubert ML & Dubois PM 1988 Differentiation of fetal rat somatotropes *in vitro*: Effects of cortisol, 3,5,3'-triiodothyronine, and glucagon, a light microscopic and radioimmunological study. *Endocrinology* **123** 1230–1236.
- Hervas F, Morreale de Escobar G & Escobar Del Rey F 1975 Rapid effects of single small doses of L-thyroxine and triiodo-L-thyronine on growth hormone, as studied in the rat by radioimmunoassay. *Endocrinology* **97** 91–101.
- Iqbal A, Decuyper E, Kuhn 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.
- Kalliecharan R & Hall BK 1974 A developmental study of the levels of progesterone, corticosterone, cortisol, and cortisone circulating in plasma of chick embryos. *General and Comparative Endocrinology* **24** 364–372.
- Kameda Y, Udatsu K, Horino M & Tagawa T 1986 Localization and development of immunoreactive triiodothyronine in thyroid glands of dogs and chickens. *Anatomy Research* **214** 168–176.
- King DB & May JD 1984 Thyroidal influence on body growth. *Journal of Experimental Zoology* **232** 453–460.
- Leung FC, Taylor JE & Van Iderstine A 1985 Effects of dietary thyroid hormones on growth, plasma T3 and T4, and growth hormone in normal and hypothyroid chickens. *General and Comparative Endocrinology* **59** 91–95.
- Liu L, Dean CE & Porter TE 2003 Thyroid hormones interact with glucocorticoids to affect somatotroph abundance in chicken embryonic pituitary cells *in vitro*. *Endocrinology* **144** 3836–3841.
- McNabb FM & Hughes TE 1983 The role of serum binding proteins in determining free thyroid hormone concentrations during development in quail. *Endocrinology* **113** 957–963.
- Marie C 1981 Ontogenesis of the adrenal glucocorticoids and of the target function of the enzymatic tyrosine transaminase activity in the chick embryo. *Journal of Endocrinology* **90** 193–200.
- Morpurgo B, Dean CE & Porter TE 1997 Identification of the blood-borne somatotroph-differentiating factor during chicken embryonic development. *Endocrinology* **138** 4530–4535.
- Morreale de Escobar G, Calvo R, Escobar del Rey F & Obregon MJ 1993 Differential effects of thyroid hormones on growth and thyrotrophic hormones in rat fetuses near term. *Endocrinology* **132** 2056–2064.
- Nogami H & Tachibana T 1993 Dexamethasone induces advanced growth hormone expression in the fetal rat pituitary gland *in vivo*. *Endocrinology* **132** 517–523.
- Nogami H, Yokose T & Tachibana T 1995 Regulation of growth hormone expression in fetal rat pituitary gland by thyroid or glucocorticoid hormone. *American Journal of Physiology* **268** E262–E267.
- Nogami H, Inoue K & Kawamura K 1997 Involvement of glucocorticoid-induced factor(s) in the stimulation of growth hormone expression in the fetal rat pituitary gland *in vitro*. *Endocrinology* **138** 1810–1815.
- 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** 1850–1856.
- Porter TE, Couger GS & Morpurgo B 1995b Evidence that somatotroph differentiation during chicken embryonic development is stimulated by a blood borne signal. *Endocrinology* **136** 3721–3728.
- Porter TE, Dean CE, Piper MM, Medvedev KL, Ghavam S & Sandor J 2001 Somatotroph recruitment by glucocorticoids involves induction of growth hormone gene expression and secretagogue responsiveness. *Journal of Endocrinology* **169** 499–509.
- Prati M, Calvo R, Morreale G & Morreale de Escobar G 1992 L-Thyroxine and 3,5,3'-triiodothyronine concentrations in the chicken egg and in the embryo before and after the onset of thyroid function. *Endocrinology* **130** 2651–2659.
- Scanes CG, Hart LE, Decuyper E & Kuhn ER 1987 Endocrinology of the avian embryo: an overview. *Journal of Experimental Zoology, Supplement* **1** 253–264.
- Shapiro LE, Samuels HH & Yaffe BM 1978 Thyroid and glucocorticoid hormones synergistically control growth hormone mRNA in cultured GH1 cells. *PNAS* **75** 45–49.
- Spindler SR, Mellon SH & Baxter JD 1982 Growth hormone gene transcription is regulated by thyroid and glucocorticoid hormones in cultured rat pituitary tumor cells. *Journal of Biological Chemistry* **257** 11627–11632.
- Tanabe Y, Saito N & Nakamura T 1986 Ontogenetic steroidogenesis by testes, ovary, and adrenals of embryonic and postembryonic chickens (*Gallus domesticus*). *General and Comparative Endocrinology* **63** 456–463.
- Thommes RC 1987 Ontogenesis of thyroid function and regulation in the developing chick embryo. *Journal of Experimental Zoology, Supplement* **1** 273–279.
- 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 & Tonetta SA 1979 Hypothalamo-adenohypophyseal-thyroid interrelationships in the chick embryo. II. Effects of thiourea treatment on plasma total thyroxine levels and thyroidal ¹²⁵I uptake. *General and Comparative Endocrinology* **37** 167–176.
- Thommes RC, Vieth RL & Levasseur S 1977 The effects of hypophysectomy by means of surgical decapitation on thyroid function in the developing embryo. *General and Comparative Endocrinology* **31** 29–36.
- 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, Clark NB, Mok LL & Malone S 1984 Hypothalamo-adenohypophyseal-thyroid interrelationships in the chick embryo. V. The effects of thyroidectomy on T4 levels in blood plasma. *General and Comparative Endocrinology* **54** 324–327.
- Thommes RC, Caliendo J & Woods JE 1985 Hypothalamo-adenohypophyseal-thyroid interrelationships in the developing chick embryo. VII. Immunocytochemical demonstration of thyrotrophin-releasing hormone. *General and Comparative Endocrinology* **57** 1–9.
- Thommes RC, Fitzsimons EJ, Davis M & Woods JE 1992 Immunocytochemical demonstration of T4 content and TSH-binding by cells of the thyroid of the developing chick embryo. *General and Comparative Endocrinology* **85** 79–85.
- Wise PM & Frye BE 1973 Functional development of the hypothalamo-hypophyseal-adrenal cortex axis in the chick embryo, *Gallus domesticus*. *Journal of Experimental Zoology* **185** 277–292.

Woods JE, De Vries GW & Thommes RC 1971 Ontogenesis of the pituitary–adrenal axis in the chick embryo. *General and Comparative Endocrinology* **17** 407–415.

Yaffe BM & Samuels HH 1984 Hormonal regulation of the growth hormone gene. Relationship of the rate of transcription to the level of nuclear thyroid hormone–receptor complexes. *Journal of Biological Chemistry* **259** 6284–6291.

Received 27 June 2003

Accepted 26 September 2003

Made available online as an

Accepted Preprint 1 October 2003