

# Glucocorticoid Induction of Lactotrophs and Prolactin Gene Expression in Chicken Embryonic Pituitary Cells: A Delayed Response Relative to Stimulated Growth Hormone Production

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We reported that corticosterone (CORT) induces GH cell differentiation in chicken embryonic pituitary cells in culture and in living embryos. The present study tested whether CORT could also induce prolactin (PRL) cell differentiation *in vitro*. CORT increased the percentage of GH cells in cultures of embryonic d (e) 13 pituitary cells after 1 d of treatment and PRL cells after 3 d of incubation. Dual immunofluorescence showed that the PRL cells induced by CORT did not contain GH, indicating they were separate cell populations. Similar PRL cell responses were also observed in cultures of e11, e15, and e17 pituitary cells. ZK98299, a glucocorticoid receptor (GR) antagonist, suppressed CORT effects, verifying involvement of GR. Northern blot analysis indicated that CORT increased GH mRNA levels after 1 d of treatment. In contrast, increases in PRL mRNA levels were delayed and observed after 3 d of treatment. Induction of a luciferase reporter driven by the PRL promoter was also delayed until 3 d of

CORT treatment. Dual-labeling immunofluorescence indicated that the majority of PRL cells induced by CORT were not labeled with bromodeoxyuridine, suggesting that lactotrophs induced by CORT do not result from cell proliferation. Proportions of pituitary-specific transcription factor (Pit-1)-containing cells and the total amount of Pit-1 protein spontaneously increased with increasing culture time. However, no effect of CORT on Pit-1 levels or the number of Pit-1-containing cells was observed. We conclude that CORT can induce lactotroph differentiation in culture and that longer CORT exposure is needed for lactotroph induction compared with somatotroph induction. The effects of CORT on PRL cell induction are GR dependent and involve an increase in PRL gene expression. PRL cell induction by CORT is not associated with an increase in Pit-1-containing cells. (*Endocrinology* 145: 1322–1330, 2004)

THE ANTERIOR PITUITARY contains five major hormone-secreting cell types: corticotrophs produce ACTH; gonadotrophs secrete FSH and LH; thyrotrophs secrete TSH; somatotrophs secrete GH, and lactotrophs secrete prolactin (PRL). Although morphological studies suggest that all of the cell types arise from a progenitor cell type in Rathke's pouch, differentiation of these five cell types does not happen at the same time during development. Lactotrophs usually differentiate after the differentiation of somatotrophs, and evidence suggests that there is a close relationship between somatotrophs and lactotrophs. Both somatotrophs and lactotrophs belong to the acidophil class of cells in the pituitary gland, and lactotrophs are derived from GH-producing progenitor cells in rodents (1–3). Both lactotrophs and somatotrophs express the pituitary-specific transcription factor (Pit-1), which is important for GH and PRL gene expression, and some pituitary acidophils appear to secrete GH only, PRL only, or both hormones simulta-

neously, depending on the physiologic state of the animal (4–8).

Our laboratory has been using the chicken embryo as a model to study somatotroph and lactotroph differentiation in the anterior pituitary. Chicken embryonic development is a good model because of the ease of access to the embryo and the capacity for manipulation without maternal interactions. In addition, the pattern of pituitary cell differentiation in chickens is comparable with that in mammals (9). During chicken development, somatotrophs first appear on embryonic day (e) 14 and become a significant population by e16 (10). A significant population of lactotrophs appears about 3 d after the appearance of somatotrophs, on e17 (11).

Glucocorticoids are steroid hormones mainly secreted from adrenal glands. The main form of glucocorticoids in rats and chickens is corticosterone (CORT). It is well documented that glucocorticoids have differentiative effects in many embryonic tissues (12, 13). In studies of rats and chickens, GH cell differentiation can be induced by glucocorticoids *in vivo* and *in vitro* (14–22). On the other hand, the repression of mammalian PRL gene expression by glucocorticoids in both pituitary and nonpituitary cell lines is well documented (23–28). Previous reports also indicate that glucocorticoids have suppressive effects on PRL secretion (29) and lactotroph differentiation (30) in rats. Considering the ontogenic association of somatotrophs with lactotroph differentiation and the inductive effect of glucocorticoids on GH cells, effects of

Abbreviations: BrdU, Bromodeoxyuridine; CORT, corticosterone; e, embryonic day; FITC, fluorescein isothiocyanate; GR, glucocorticoid receptor; GRE, glucocorticoid response element; ICC, immunocytochemistry; Pit-1, pituitary-specific transcription factor; PRL, prolactin; SFM, serum-free culture medium.

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glucocorticoids on lactotroph differentiation during chicken embryonic development warrant investigation. The objectives of this study were to determine glucocorticoid effects on lactotroph differentiation, explore the mechanism of glucocorticoid effects on lactotroph differentiation, and compare glucocorticoid induction of lactotrophs and somatotrophs.

## Materials and Methods

### Primary pituitary cell cultures

Cell culture reagents were purchased from Invitrogen Life Technologies, Inc. (Grand Island, NY). Hormones and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). CORT was first dissolved in 100% ethanol and further diluted to required concentrations with culture medium. Animals used in this study were Avian × Avian strain chicken embryos purchased from Allen's Hatchery (Seaford, DE). Eggs were incubated in a humidified incubator (G.Q.F. Manufacturing, Savannah, GA) at 37.5 C. At the end of incubation, the pituitary glands were isolated from chicken embryos under a dissecting microscope. Pituitary glands were pooled and then monodispersed as previously described (10, 11). One milliliter of pituitary cell suspension ( $1.5 \times 10^6$  cells/ml) in serum-free culture medium (SFM; a 1:1 mixture of phenol red-free medium 199 and Ham's F12, supplemented with 0.1% BSA, 5  $\mu$ g/ml bovine insulin, 5  $\mu$ g/ml human transferrin, and penicillin/streptomycin) was transferred into each well of a sterile 12-well tissue culture plate and cultured in a cell culture incubator at 37.5 C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. The medium was replaced with fresh medium on the third day.

### Immunocytochemistry (ICC)

Cultured pituitary cells or cells immediately after isolation were washed and diluted in DMEM to a concentration of  $10^4$  cells/ml. The cells were then attached for 1 h to the bottom of poly-L-lysine-coated 24-well tissue culture plates and subjected to ICC using VECTASTAIN ABC Kits from Vector Laboratories (Burlingame, CA). The attached cells were fixed with 3.7% formaldehyde in PBS for 15 min. Cells were then permeabilized with 0.1% Tween 20/0.1% Triton X-100 for 8 min and then quenched with 0.3% H<sub>2</sub>O<sub>2</sub> for 5 min. Cells were then blocked with 1% normal goat serum for 30 min at room temperature. The cells were incubated with rabbit antichick PRL antiserum (1:8000 in PBS), rabbit antichick GH antiserum (1:4000 dilution in PBS), or rabbit antirat Pit-1 antiserum (1:1000 dilution in PBS) overnight at 4 C. Antisera for chicken GH and PRL have been described previously (10, 31). Rabbit antiserum against rat Pit-1 (no. 1603) was generously provided by Dr. S. J. Rhodes of Indiana University-Purdue University Indianapolis (Indianapolis, IN). Cells were further processed using rabbit ABC kits according to the manufacturer's guide (Vector Laboratories). Finally, PRL- or GH-positive cells were developed by incubation with VIP peroxidase substrate (Vector Laboratories). Data shown are the percentage of PRL, GH, or Pit-1-containing cells among at least 500 cells counted in each replicate.

### Dual immunofluorescence

Cells were washed and resuspended in DMEM and allowed to attach to the surface of poly-L-lysine-coated slides for 1 h in the cell culture incubator. The cells were fixed in 3.7% formaldehyde for 20 min at room temperature, washed (three times for 5 min each) in PBS, permeabilized in 0.1% Triton X-100/0.1% Tween 20 for 8 min, and blocked for 1 h in 5% normal goat serum at room temperature. Then the slides were incubated in a mixture of two primary antibodies (rabbit antichick PRL antiserum at 1:4000 and monoclonal mouse antichick GH antibody at 1:1000, diluted in 1% normal goat serum) overnight at 4 C in humidified conditions. The monoclonal antibody to chicken GH was generously provided by Dr. L. Berghman of Texas A&M University (College Station, TX). This antibody was characterized previously (32). The slides were rinsed (four times for 5 min each) in PBS and (one time for 10 min) in PBS with 0.1% Tween 20. The slides were then incubated in the dark for 1 h in a diluted second antibody solution, which was a mixture of rhodamine red-X-conjugated affinity pure goat antimouse IgG (1:50 dilution) and fluorescein (FITC)-conjugated affinity pure goat antirabbit

IgG (1:100 dilution) (Jackson ImmunoResearch Laboratories, West Grove, PA) in 2.5% normal goat serum with 0.025% Tween 20. The slides were washed (three times for 5 min each) in PBS and (one time for 5 min) in PBS with 0.05% Tween 20. After air-drying, the slides were mounted in VECTORSHIELD (Vector Laboratories) and observed under a fluorescence microscope. For dual immunofluorescence labeling for Pit-1 and PRL, a rabbit polyclonal antiserum against rat Pit-1 (no. 1603) and a monoclonal antichick PRL antibody were used, the latter of which was provided by Dr. L. Berghman and characterized previously (33).

### Analysis of lactotroph mitosis

To examine the mitotic activity of PRL cells induced by CORT, e13 pituitary cells were cultured for 4 d with or without CORT ( $10^{-9}$  M), and bromodeoxyuridine (BrdU; 10  $\mu$ M) was added on each day. BrdU was removed by replacing the medium on the following day. To detect BrdU-labeled PRL cells, dual labeling immunofluorescence was used to detect BrdU-labeled and PRL-positive cells simultaneously. DNA was denatured by incubating the cells with 4 N HCl for 30 min at 37 C after the cells were fixed and permeabilized as described above for dual labeling immunofluorescence. The pH was equilibrated through washing with PBS (four times for 5 min each). BrdU-labeled cells were detected by incubation with monoclonal anti-BrdU antibody (1:1000 dilution, Sigma) as the first antibody and rhodamine red-X-conjugated affinity pure goat antimouse IgG (1:50 dilution) as the second antibody. PRL cells were detected as described above.

### Total RNA isolation and Northern blotting analysis

Total RNA was extracted using TRIZOL Reagent (Invitrogen), according to the manufacturer's protocol. Five micrograms of total RNA from each sample were size-separated in 1% agarose gels containing formaldehyde and 3[N-morpholino]propanesulfonic acid, and the RNA was then transferred to nylon membranes. [<sup>32</sup>P]-labeled cDNA probes for chicken PRL and GH were generated by PCR using pGEM plasmids containing partial chicken PRL and GH cDNA inserts as templates [generously provided by Dr. H. M. Sang of the Agriculture and Food Research Council Institute of Animal Physiology and Genetics Research at Roslin (Midlothian, UK) and Dr. D. Foster of the University of Minnesota (Minneapolis, MN), respectively]. The primers used were designed against the M13 forward and reverse sequencing sites contained in the plasmids: 5'-AGCGATAACAATTTTCACACAGG-3' and 5'-CCCAGTCACGACGTTGTAAAACG-3'. The PCR conditions were: one cycle of 95 C for 5 min; 30 cycles of 94 C for 30 sec, 60 C for 30 sec and 72 C for 45 sec; and one cycle of 72 C for 5 min. The membranes were first prehybridized with hybridization buffer (PERFECTHYB PLUS, Sigma) for 2 h. Hybridization with cDNA probe was performed at 65 C overnight. The membranes were washed under stringent conditions (0.2× saline sodium citrate, 0.1% sodium dodecyl sulfate; 60 C). Filters were exposed to film for up to 7 d, and the bands were viewed by autoradiography. To normalize for loading efficiency, the membranes were stripped, and the blots were rehybridized with a 286-bp [<sup>32</sup>P]-labeled chicken  $\beta$ -actin cDNA probe, which was generated by PCR using a chicken  $\beta$ -actin template. The PCR conditions were: one cycle of 94 C for 4 min; 35 cycles of 94 C for 60 sec, 53 C for 60 sec and 72 C for 1.5 min; and one cycle of 72 C for 5 min. Chicken  $\beta$ -actin template was amplified by RT-PCR from chicken total RNA. The primers used in RT-PCR for chicken  $\beta$ -actin were: 5'-CGCATAAAACAAGACGAGATT-3' (sense), 5'-GTTTTTAAGGCGGAAGAT ACA-3' (antisense). Quantification of relative PRL and GH band intensity was normalized to the intensity of  $\beta$ -actin bands using the Scion computer program (Scion Corp., Frederick, MD).

### Cell transfection and luciferase reporter assays

Reporter plasmid pGL2-PRL (pGL2-Basic vector containing an insert of the chicken PRL promoter fragment spanning from -1366 to +33) was generously supplied by Dr. T. Ohkubo, Kagawa University (Kagawa, Japan) (34). Primary chicken embryonic pituitary cells ( $1 \times 10^6$ /ml) or the GH<sub>4</sub>C<sub>1</sub> rat pituitary cell line ( $5 \times 10^5$  cells/ml) were cultured in six-well culture plates and allowed to attach for 24 h in SFM. Transfection reagent FuGene (Roche, Basel, Switzerland) and DNA complex were prepared in OPTI-MEM I (Invitrogen) according to the manufacturer's guide. The cells were washed once with OPTI-MEM I before transfection. The cells in each well were then transiently transfected with 2  $\mu$ g of pGL2-PRL and 20 ng of pRL-SV40 (Promega, Madison, WI),

which served as an internal control to normalize for variations in transfection efficiency. Medium was changed 8 h later, and cells were cultured in SFM with or without CORT ( $10^{-9}$  M). Luciferase activity was measured 3 d later with the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

#### Western blotting for Pit-1

Nuclear proteins were extracted from treated cells using NE-PERTM Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL). Protein concentration was measured using the Micro BCA Protein Assay Reagent Kit (Pierce). Proteins were separated by electrophoresis in 10% sodium dodecyl sulfate-polyacrylamide gels. Equal amounts of protein (20  $\mu$ g) were applied to each lane, based on the results of the bicinchoninic acid assay. The separated proteins in the gel were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) and incubated with 2% (vol/vol) normal goat serum in PBS-0.1% Tween 20 at room temperature for 1 h with gentle shaking. The membrane was then incubated overnight at 4°C with rabbit antirat Pit-1 polyclonal antibody (no. 1603) diluted 1:1000 in PBS-0.1% Tween 20. Pit-1 staining was visualized using ABC and VIP kits (Vector Laboratories) according to the manufacturer's guides. The molecular weights of the bands were determined by comparison with the migration of molecular weight markers (Rainbow molecular weight markers, Amersham Pharmacia Biotech, Piscataway, NJ). Quantification of band intensity was measured using the Scion computer program.

#### Statistical analysis

All of the data were from at least three completely separate experiments and were analyzed by ANOVA using the MIXED models procedure of SAS (SAS Institute, Cary, NC). Differences between treatments were tested using Tukey's Studentized range test and were considered significant at  $P < 0.05$ .

## Results

### CORT induction of PRL cells in cultures of e13 pituitary cells

GH cells normally differentiate around e14, and this is followed by differentiation of a substantial lactotroph population on e17. We previously reported that CORT could induce GH cell differentiation in chicken embryonic pituitary cells in culture and in living embryos (14–16, 21, 22). To determine the effect of CORT on lactotroph differentiation, e13 embryonic pituitary cells were cultured *in vitro* with vehicle or CORT ( $10^{-9}$  M) for up to 4 d. PRL- and GH-containing cells were detected by ICC on each day, and the percentages of PRL and GH cells were counted (Fig. 1). Results in Fig. 1 demonstrate that CORT can induce both GH and PRL cell differentiation. The results in Fig. 1 also indicate the time course of CORT effects on GH and PRL cell differentiation. CORT significantly increased ( $P < 0.05$ ;  $n = 3$  independent experiments) the percentage of GH cells after 1 d of treatment to  $10.7 \pm 1.5\%$  of total cells, relative to controls ( $5.3 \pm 0.1\%$ ). CORT dramatically increased ( $P < 0.05$ ;  $n = 3$ ) the percentage of PRL cells after 3 d of treatment to  $11 \pm 1\%$ , compared with that in controls ( $1.0 \pm 0.1\%$ ). In contrast, lactotroph abundance did not increase during culture in the absence of CORT ( $P = 0.20$ ). Obviously, longer CORT exposure was needed for lactotroph induction compared with somatotroph induction. Because CORT can induce the differentiation of both somatotrophs and lactotrophs, the next question we addressed was whether those lactotrophs induced by CORT are the same group of cells induced by CORT to produce GH. Based on the results shown in Fig. 1, 4 d of incubation is long enough for CORT

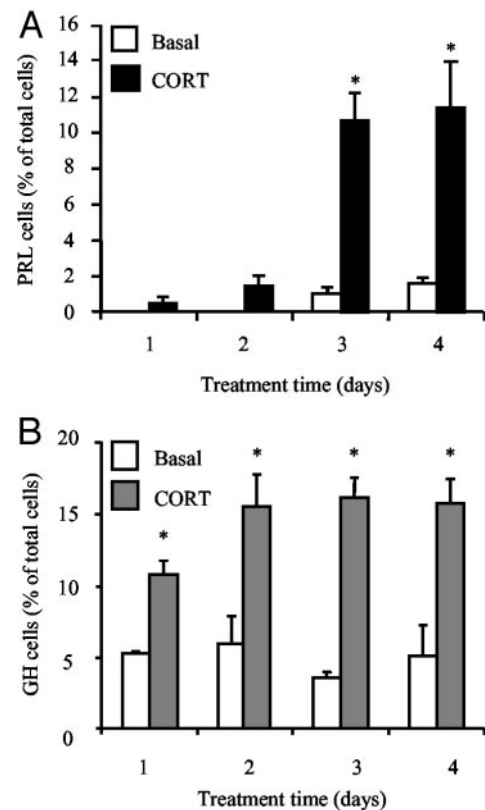


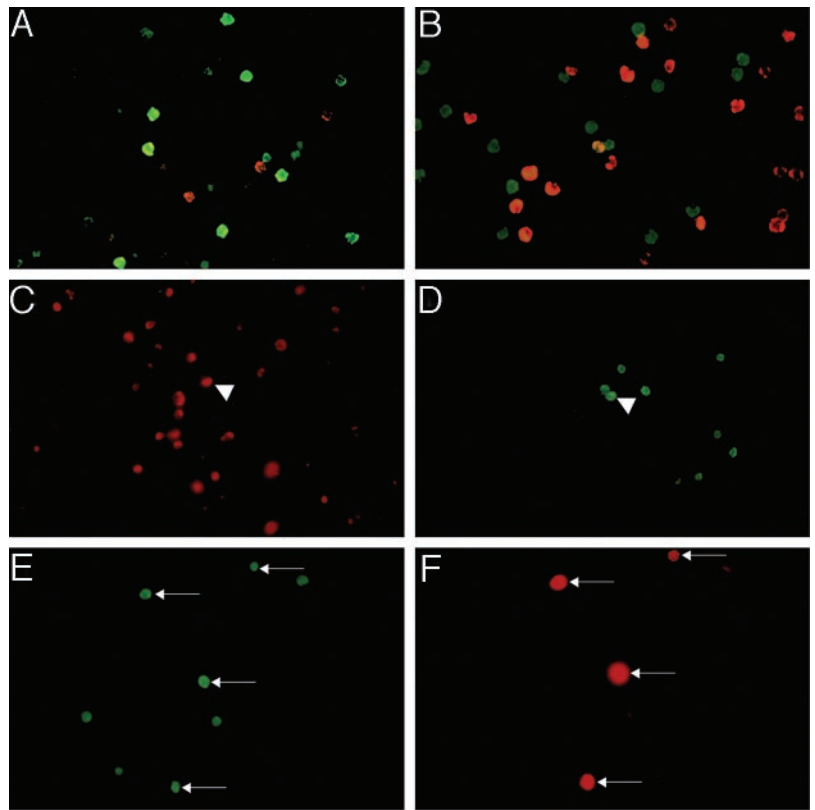
FIG. 1. Time course of CORT induction of PRL cells (A) and GH cells (B). E13 pituitary cells were cultured with vehicle or with  $10^{-9}$  M CORT up to 4 d. Then ICC was used to detect PRL and GH cells. The percentages of PRL and GH cells among 500 total cells on each day were determined using a light microscope. Values are expressed as means  $\pm$  SEM of three independent experiments. \*,  $P < 0.05$ , compared with control.

to induce both somatotrophs and lactotrophs, so e13 embryonic pituitary cells were cultured with vehicle or CORT ( $10^{-9}$  M) for 4 d. Dual-labeling immunofluorescence was used to detect GH and PRL producing cells simultaneously. Monoclonal antichick GH antibody and rabbit antichick PRL antiserum were used. Then rhodamine red-X-conjugated affinity pure goat antimouse IgG and FITC-conjugated affinity pure goat antirabbit IgG were used to show GH cells and PRL cells, respectively. Comparing Fig. 2, A and B, we can see that CORT not only increased the percentage of GH cells (red fluorescence) but also increased the percentage of PRL cells (green fluorescence). However, most PRL cells and GH cells were stained separately. Results from different dual-labeling immunofluorescence experiments, in which antichick PRL monoclonal antibody and rabbit antichick GH antiserum were used, were consistent with the results in Fig. 2, A and B (data not shown). Consequently, CORT-induced lactotrophs and somatotrophs are two different groups of cells.

### CORT effects on lactotroph induction are not restricted to e13 pituitary cells

To determine whether the lactotroph response to CORT is restricted to e13, cells from e11, e15, and e17 pituitaries were cultured with vehicle or CORT ( $10^{-9}$  M) up to 4 d. PRL-containing cells were detected by ICC on each day, and the

FIG. 2. Dual-labeling immunofluorescence results. (A, B) E13 pituitary cells were cultured for 4 d with vehicle (A) or with  $10^{-9}$  M CORT (B). GH and PRL cells were identified using monoclonal antichickken GH antibody and rabbit antichickken PRL antiserum. Secondary antibodies to mouse anti-GH were conjugated to rhodamine red, whereas antibodies to rabbit anti-PRL were conjugated to FITC (green). Exposure time for B was shorter than that for A due to the increased number of GH and PRL cells in the presence of CORT. C and D, E13 pituitary cells were cultured for 3 d with  $10^{-9}$  M CORT. In the last 24 h, BrdU was added. Dual-labeling immunofluorescence was used to show BrdU-labeled cells (red fluorescence, C) and PRL-producing cells (green fluorescence, D), same field as in C). Arrowheads show cells labeled with both red and green fluorescence. E and F, Dual immunofluorescent colocalization of Pit-1 and PRL in e13 pituitary cells cultured for 3 d with  $10^{-9}$  M CORT. Pit-1 producing cells labeled with green fluorescence are shown in (E). F, Same field in (E) showing PRL cells labeled with red fluorescence. Arrows indicate PRL cells also producing Pit-1.



percentage of PRL cells was counted (Fig. 3). Lactotroph abundance did not increase under control conditions during the 4-d culture period for cells derived from e11 ( $P = 0.58$ ) or e17 ( $P = 0.66$ ), although there was an apparent increase with e15 cells that did approach significance ( $P = 0.06$ ). In contrast, CORT increased lactotroph abundance in cultures from all ages tested. CORT significantly increased ( $P < 0.05$ ;  $n = 3$ ) the percentage of PRL cells from e11 pituitaries after 3 d of treatment to  $6.3 \pm 1.1\%$ , compared with that in controls ( $0.9 \pm 0.9\%$ ). For e15 pituitary cells, CORT significantly increased ( $P < 0.05$ ;  $n = 3$ ) the percentage of PRL cells after 3 d of treatment to  $12.3 \pm 0.7\%$ , compared with that in controls ( $6.0 \pm 1.6\%$ ). Four days were needed for CORT to significantly increase the percentage of PRL cells from e17 pituitaries to  $17.4 \pm 0.5\%$ , compared with that in controls ( $11.4 \pm 0.3$ ). Consequently, cells from e11, e15, and e17 have similar responses to CORT as cells from e13. Moreover, CORT induction of lactotroph differentiation in cultures from different ages all needed at least 3 d of treatment.

#### The effects of CORT on PRL-cell induction are glucocorticoid receptor (GR) dependent

CORT receptors belong to the steroid receptor superfamily. There are two types of receptors potentially used by CORT, the GRs, and the mineralocorticoid receptors. This experiment was designed to determine whether GR are involved in the induction of PRL cells. Treatment with the GR antagonist, ZK 98299, suppressed ( $P < 0.05$ ;  $n = 3$ ) the effects of CORT on the percentage of PRL-producing cells to  $1.8 \pm 1.2\%$  after CORT plus ZK98299 treatment for 3 d, relative to

that in the CORT-treated group ( $8.4 \pm 2.4\%$ ), verifying involvement of GR (Fig. 4).

#### Involvement of PRL gene expression in lactotroph induction by CORT

Northern blots were used to evaluate PRL and GH mRNA levels in e13 pituitary cell cultures treated with or without CORT ( $10^{-9}$  M) for 1–4 d. Total RNA was extracted from cells and subjected to Northern blotting. The autoradiograph in Fig. 5A is a representative result of three independent experiments. Quantification of relative band intensity was normalized to the intensity of  $\beta$ -actin bands. The cumulative results from three independent trials showing relative PRL and GH mRNA levels are presented in Fig. 5, B and C, respectively. Results indicate that CORT significantly increased GH mRNA levels after 1 d of treatment ( $1.9 \pm 0.1$ -fold, relative to control;  $n = 3$ ). In contrast, increases in PRL mRNA levels by CORT were delayed. Significant increases in PRL mRNA levels were observed after 3 d of treatment ( $2.0 \pm 0.2$ -fold, relative to control;  $n = 3$ ) and continued through 4 d of treatment ( $2.5 \pm 0.6$ -fold, relative to control;  $n = 3$ ). The results demonstrate that an increase in PRL gene expression contributes to the induction of PRL cell differentiation by CORT. Although CORT can increase gene expression for both GH and PRL, the mechanisms are probably different because of the delayed induction of PRL mRNA relative to GH mRNA.

#### CORT effects on PRL promoter activity

To test whether PRL gene transcription is regulated by CORT, e13 pituitary cells and GH<sub>4</sub>C<sub>1</sub> cells, a rat pituitary cell

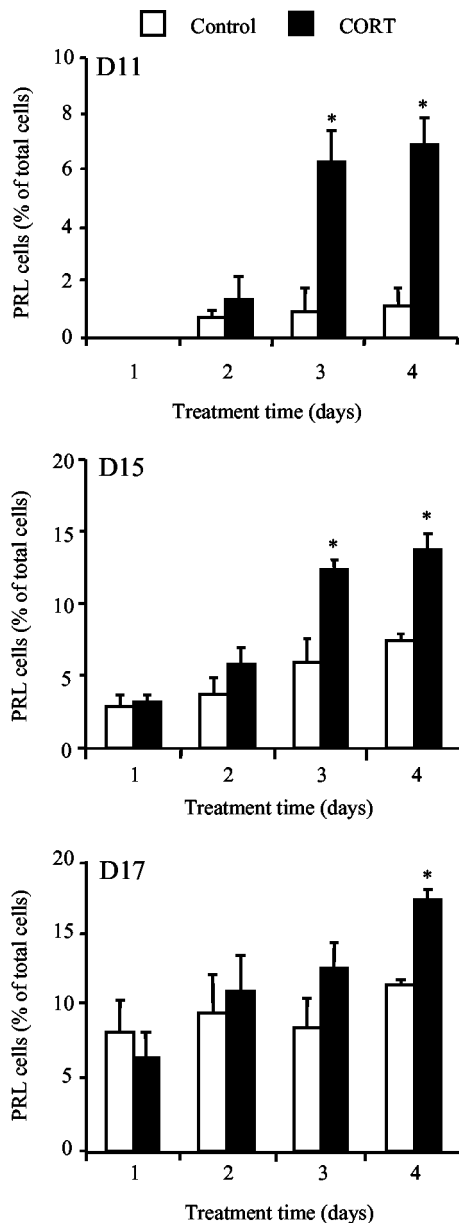


FIG. 3. CORT induction of lactotrophs in cells from e11, e15, and e17. Pituitary cells were cultured with vehicle or  $10^{-9}$  M CORT for up to 4 d. ICC was used to detect PRL cells. The percentages of all cells that contained PRL were counted on each day. Values are expressed as means  $\pm$  SEM of three independent experiments. \*,  $P < 0.05$ , compared with control.

line, were transiently transfected with empty luciferase reporter plasmid pGL2-Basic, or pGL2-PRL luciferase reporter plasmid containing the chicken PRL promoter region (–1366 to +33). Cells were cotransfected with renilla luciferase reporter plasmid pRL-SV40, which served as an internal control to normalize for variations in transfection efficiency. Reporter activity was measured after cells were treated with or without CORT ( $10^{-9}$  M) for 3 d. The cumulative results from six independent experiments are presented in Fig. 6. In both chicken embryonic pituitary cells and GH<sub>4</sub>C<sub>1</sub> cells, CORT significantly increased the expression of the luciferase reporter gene from the construct containing the PRL pro-

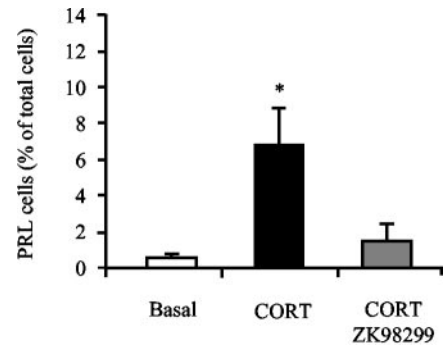


FIG. 4. ZK98299 inhibition of CORT induction of PRL cells. E13 pituitary cells were cultured with vehicle,  $10^{-9}$  M CORT, or  $10^{-9}$  M CORT plus ZK98299 (1  $\mu$ g/ml) for 3 d. ICC was used to detect PRL cells. Values are expressed as means  $\pm$  SEM of three independent experiments. \*,  $P < 0.05$ , compared with control;  $n = 3$ .

motor region. CORT treatment for 1 or 2 d had no effect on reporter gene expression compared with control (data not shown).

#### Involvement of mitosis in CORT induction of lactotrophs

To assess the mitotic activity of PRL cells induced by CORT, dual labeling immunofluorescence was used to detect BrdU-labeled DNA in PRL-containing cells. BrdU (10  $\mu$ M) was added to the culture medium on d 1, 2, or 3, and medium was changed back to BrdU-free medium in each case on the following day. Representative pictures of dual-labeling immunofluorescence are shown in Fig. 2, C and D, which are the same microscopic field showing *red* fluorescence (indicating BrdU-labeled cells) and *green* fluorescence (indicating PRL cells), respectively. Cells shown in Fig. 2, C and D, were e13 pituitary cells, cultured with CORT ( $10^{-9}$  M) for 3 d, and BrdU was added into the medium in the last 24 h. Comparing Fig. 2, C and D, we can see that most PRL cells and BrdU cells are labeled separately. Similar results were obtained from samples labeled with BrdU on the first or second 24 h (data not shown). *Arrows* in Fig. 2, C and D, show one cell dual labeled with both *red* and *green* fluorescence. The percentage of BrdU-labeled PRL cells were counted, and results are shown in Fig. 7. Mitotic activity of cultured pituitary cells was not changed by CORT treatment, and 97% of PRL cells were not BrdU positive, suggesting that PRL cells induced by CORT do not result from proliferation.

#### CORT effects on Pit-1

Pit-1 is a pituitary-specific transcription factor. Although Pit-1 proteins are expressed in lactotrophs, somatotrophs, and thyrotrophs in rats, the expression of Pit-1 in chicken lactotrophs has not been confirmed yet. Dual-labeling immunofluorescence was used to detect Pit-1- and PRL-containing cells simultaneously, after e13 pituitary cells were cultured with  $10^{-9}$  M CORT for 3 d. Results are shown in Fig. 2, E and F. Figure 2E shows Pit-1 cells (labeled with *green* fluorescence), whereas Fig. 2F shows PRL cells (labeled with *red* fluorescence) in the same field shown in Fig. 2E. *Arrows* in Fig. 2, E and F, show that PRL cells were also producing Pit-1. Next, we addressed whether Pit-1 is regulated by CORT. Pituitary cells from e13 embryos were cultured for 1,

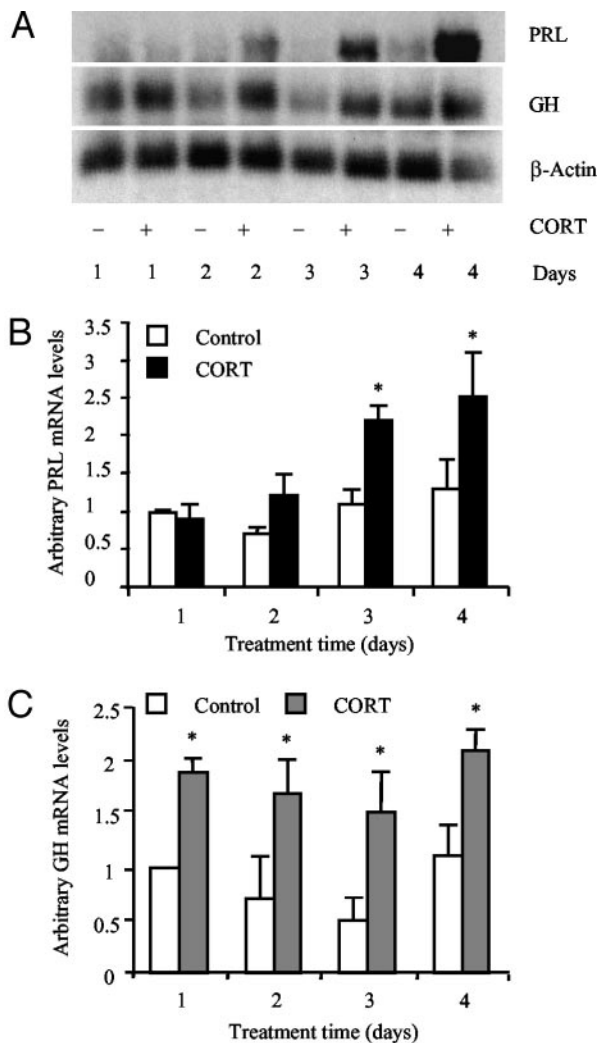


FIG. 5. Northern blotting for PRL and GH mRNA levels. E13 chicken pituitary cells were cultured with  $10^{-9}$  M CORT or with vehicle for 1–4 d. Five micrograms of total RNA from each sample were size-separated in a 1% agarose gel with formaldehyde and 3[*N*-morpholinol]propanesulfonic acid, and the RNA was then transferred to a nylon membrane.  $^{32}$ P-labeled cDNA probes for chicken PRL, chicken GH, and chicken  $\beta$ -actin were generated by PCR. GH and PRL mRNA levels on d 1 without CORT were set as one for normalization of responses. \*,  $P < 0.05$ , compared with control for that day.

2, and 3 d in SFM with or without CORT ( $10^{-9}$  M). The cells were then subjected to ICC for Pit-1, or the nuclear proteins were extracted and subjected to Western blotting for Pit-1. Results from ICC indicated that proportions of Pit-1 expressing cells spontaneously increased with increased culture duration (Fig. 8). However, there were no significant differences between control and CORT-treated cultures. Western blotting results shown in Fig. 9A are representative of three independent experiments. The cumulative results of band intensity quantification from three independent trials are presented in Fig. 9B. Results indicate that the 40-kDa isoform of Pit-1 is the most abundant one produced in cultured e13 pituitary cells; the other isoforms of Pit-1 were barely detected. Results shown in Fig. 9 are consistent with the results from ICC. Consequently, CORT does not induce lactotrophs through induction of Pit-1 in additional cells.

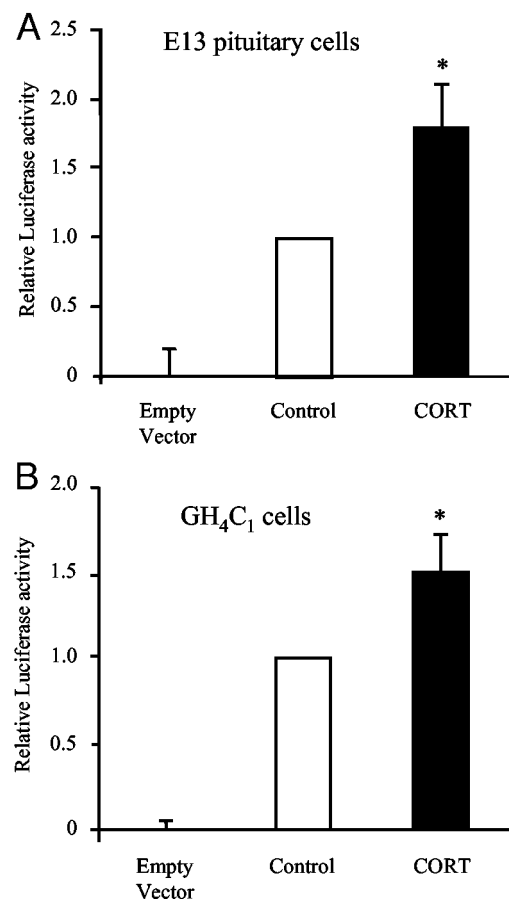


FIG. 6. Responsiveness of PRL promoter to CORT in cultures of primary embryonic pituitary cells and GH<sub>4</sub>C<sub>1</sub> cells. A, E13 chicken pituitary cells were transiently transfected with empty reporter plasmid pGL2-Basic or pGL2-PRL reporter plasmid. Cells were co-transfected with pRL-SV40, which served as an internal control to normalize the variations in transfection efficiency. PRL promoter reporter activity was measured after cells were treated with or without CORT ( $10^{-9}$  M) for 3 d. Levels of firefly luciferase (PRL reporter) were divided by levels of renilla luciferase (transfection control) in each sample to determine relative luciferase levels. B, GH<sub>4</sub>C<sub>1</sub> cells were transiently transfected according to the methods in (A). \*,  $P < 0.05$ , compared with control; n = 6.

## Discussion

The present studies demonstrate that glucocorticoids induce the differentiation of lactotrophs *in vitro*. We used multiple approaches to assess PRL production at both cellular and molecular levels. Our results indicate that CORT can increase the proportions of lactotrophs in cultured pituitary cells, increase PRL mRNA levels, and increase the expression of a reporter driven by the PRL promoter region. CORT effects on lactotroph induction were also compared with CORT induction of somatotrophs. Lactotroph induction by CORT required more time of incubation compared with somatotroph induction by CORT, suggesting that these two inductions by CORT might occur through different mechanisms. Glucocorticoid induction of GH cells has been well defined by our lab and others (14–22). Its effects on PRL cell induction were also studied to a limited extent in rats. As mentioned previously, glucocorticoids usually suppress human and rat PRL gene expression and can inhibit lactotroph

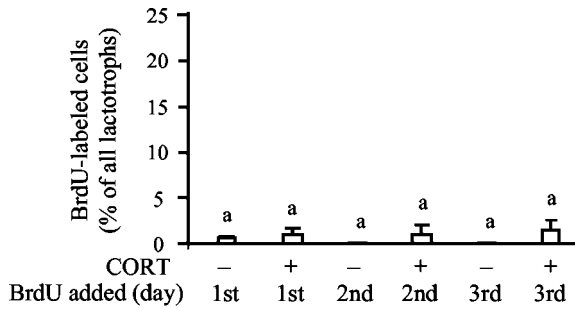


FIG. 7. Mitotic activity of PRL cells induced by CORT. E13 pituitary cells were cultured for 3 d with vehicle (–) or with  $10^{-9}$  M CORT (+). BrdU ( $10 \mu\text{M}$ ) was added into the culture medium on the first day (1st), the second day (2nd), or the third day (3rd). The percentage of BrdU-labeled cells among all cells and the percentage of BrdU-labeled cells among PRL cells were counted after 3 d of culture. BrdU-labeled cells and PRL-containing cells were identified using monoclonal anti BrdU antibody and rabbit antichick PRL antiserum, respectively. Secondary antibodies to monoclonal anti BrdU antibody were conjugated to rhodamine red, whereas secondary antibodies to rabbit antichick PRL antiserum were conjugated to FITC. Results for BrdU-labeled PRL cells are expressed as the percentage of all PRL cells and are the means  $\pm$  SEM of three independent experiments. Means identified with different letters are significantly different ( $P < 0.05$ ).

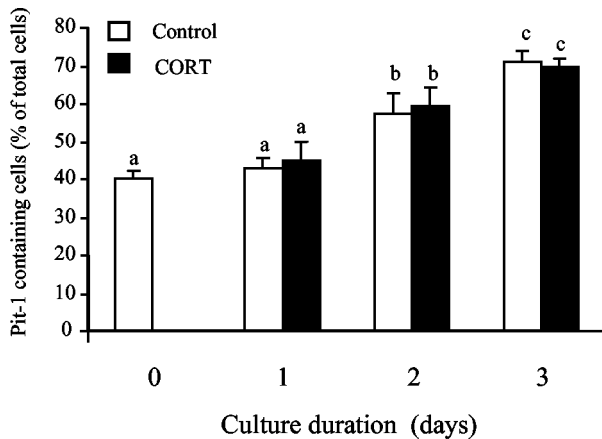


FIG. 8. CORT effects on proportions of Pit-1-containing cells. Pituitary cells from e13 embryos were cultured for 0, 1, 2, and 3 d in SFM with or without CORT ( $10^{-9}$  M). The cells were then subjected to ICC for Pit-1. Means with different letters (a–c) are significantly different ( $P < 0.05$ ).

differentiation, while increasing GH expression and somatotroph differentiation in rats (23–30). Our results for the first time demonstrate that glucocorticoids have a different effect on PRL expression in chicken embryos, which is to increase both GH and PRL expression in cultures of embryonic pituitary cells. To confirm CORT effects on PRL cell induction, several techniques were used. For example, when our initial results indicated that CORT increased the proportion of PRL cells as assessed by ICC, we used different antibodies in different combinations in dual-labeling immunofluorescence to confirm our ICC results. This was warranted because PRL shares approximately 40% homology with GH, and it is not impossible that one antibody used in ICC to detect PRL might cross-react with GH. Our results ruled out such a possibility because of the consistency in results with different antibodies and techniques. Using Smith Waterman local

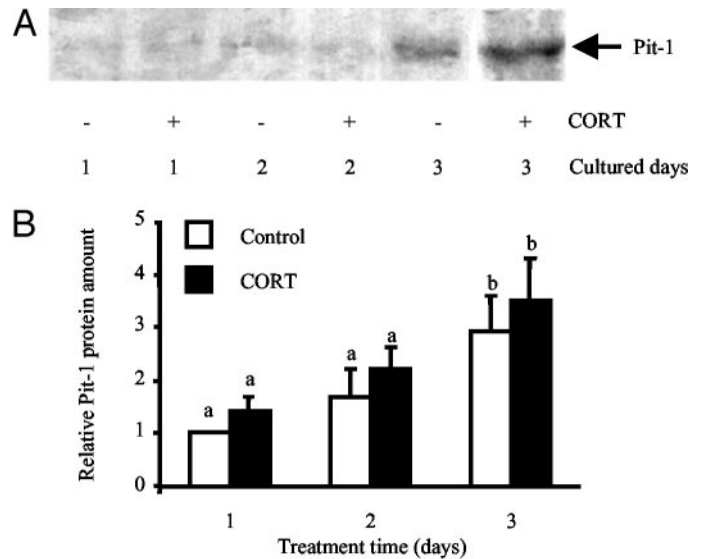


FIG. 9. CORT effects on Pit-1 protein level. Pituitary cells from e13 embryos were cultured for 1, 2, and 3 d in SFM with or without CORT ( $10^{-9}$  M). Nuclear proteins were extracted and quantified. Equal amounts of nuclear protein ( $20 \mu\text{g}$ ) for each sample were loaded into polyacrylamide gels and transferred to nitrocellulose membranes. Western blotting was used to detect Pit-1 protein levels. The molecular weights of the bands were determined by comparison with the migration of molecular weight markers. A, Representative Western blotting result of three independent experiments. B, The cumulative results of band intensity quantification from three independent trials. The intensities of bands from controls cultured for 1 d were set as one in all three trials. Values are expressed as means  $\pm$  SEM. Means with different letters are significantly different ( $P < 0.05$ ).

alignment tools in the San Diego Supercomputer Center Biology Workbench (San Diego, CA), the chicken PRL promoter region (–2192 to –1; GenBank accession no. GBVRT: 15294318) was compared with the rat PRL gene promoter region (–2192 to –1; GenBank accession no. GBROD: 312167). No extended regions of similarity were found between the two 5'-flanking regions. That might explain why the CORT effect on PRL cell induction in chickens is different from its effect in rats. An additional possibility is that the effects of glucocorticoids are biphasic, stimulating PRL gene expression at low physiologic levels while inhibiting PRL production at high pharmacologic levels. In the present study, we found that physiological concentrations ( $10^{-9}$  M) of the endogenous glucocorticoid CORT induced lactotroph differentiation and PRL gene expression. In contrast, prior reports of negative effects of glucocorticoids on rat and human PRL expression used much higher pharmacologic concentrations ( $10^{-7}$  M or higher) of the synthetic glucocorticoid dexamethasone (23–29) or cortisol (30). In addition, these negative effects of glucocorticoids on mammalian PRL gene expression were noted after 48 h or less of dexamethasone treatment of transfected cells, with the exception of one study (30) in which pituitary primordial tissue was cultured for 8 d with  $2.75 \times 10^{-7}$  M cortisol. In that study, tissue explants cultured with high levels of cortisol had fewer PRL cells than explants cultured for 8 d in basal medium. In the present study, a positive effect of physiologic concentrations of CORT ( $10^{-9}$  M) on PRL gene expression and lactotroph dif-

ferentiation in chicken embryonic pituitary cells required 72 h of treatment.

Previous studies report that lactotroph differentiation occurs between d 15 and 19 of chicken embryonic development (11, 35, 36). A previous study from our laboratory (11) indicated that VIP can increase the percentage of PRL-secreting cells after 4 d of incubation, and lactotroph differentiation may be stimulated *in vitro* by VIP as early as e13. Based on that report, we chose pituitary cells from e13 and treated the cells for 4 d with CORT. To determine whether or not the lactotroph response to CORT is restricted to e13, cells from e11, e15, and e17 pituitaries were also tested. Our results indicated that cells from e11, e15, and e17 had the same response to CORT as cells from e13. Moreover, CORT induction of lactotroph differentiation in cultures from different ages all needed at least 3 d of treatment. Consequently, CORT can induce PRL cells during embryonic development of the chicken, and this response requires at least 3 d of glucocorticoid exposure.

After CORT effects on lactotroph induction were confirmed, we next defined the mechanisms of CORT effects on PRL cell induction. Glucocorticoids regulate various biological processes, including transcription, metabolism, development and reproduction, cell growth, and proliferation. We addressed whether the lactotrophs induced by CORT arose from mitosis of existing lactotrophs. We labeled mitotic cells with BrdU. Results indicate that less than 3% of PRL cells induced by CORT were labeled, and CORT did not increase mitosis of lactotrophs or pituitary cells in general compared with control. These results indicate that PRL cells induced by CORT do not derive from proliferation. CORT increased PRL mRNA levels in the cultured e13 pituitary cells as detected by Northern blot, and the increase in PRL mRNA in response to CORT was due at least in part to activation of PRL transcription because reporter expression from a PRL promoter-containing construct was induced by CORT. Moreover, this response involves GR because inclusion of the GR antagonist ZK98299 blocked lactotroph induction. Comparison of the chicken PRL promoter region used in the present study (–1366 to +33) with the consensus glucocorticoid response element (GRE, 5'-AGAACAnnnTGTTCT-3') identified a region between –1078 and –1065 that has 87% similarity with the consensus GRE. That region might be involved in glucocorticoid effects on PRL gene expression. However, because glucocorticoid stimulation of PRL promoter (–1366 to 33) activity was minimal, other additional GRE sites might exist outside the examined promoter region. A second region with 93% identity to the GRE (one mismatch) is located between –2126 and –2112 of the chicken PRL gene (GenBank accession no. GBVRT:15294318). Interestingly, no sites with significant similarity to the consensus GRE are found within the 2192 bp of the 5'-flanking region of the rat PRL gene (GBROD:312167). CORT induction of PRL in chick embryonic pituitary cells may also involve stabilization of PRL mRNA. The present studies did not assess this possibility. However, our finding that the GR antagonist, ZK98299, suppressed CORT effects on lactotroph induction implies that GRE and gene transcription are involved. Previous results from our lab indicated that treatment of e12 pituitary cells with CORT in the presence of the protein synthesis inhibitor

cycloheximide failed to increase GH mRNA levels, suggesting that glucocorticoid mediated induction of GH gene expression is indirect and requires active protein synthesis (37). Our current results indicate that PRL cell induction by CORT requires at least 3 d of treatment. This length of time is too long to evaluate effects of protein synthesis inhibition, but more than sufficient for the production of intermediate factors that might in turn induce PRL cell differentiation. Therefore, CORT might also have indirect effects on PRL gene expression. Taken together, our findings suggest that in addition to direct effects on PRL gene expression CORT effects on PRL cell induction might also be indirect and need the involvement of other intermediate factors. However, further work is required to determine whether this is truly the case.

Pit-1 is a pituitary-specific transcription factor, mainly localized in somatotrophs, lactotrophs, and thyrotrophs and is known to be necessary for the transcription of GH and PRL genes. Pit-1 has been identified in several species, including chickens. Ohkubo *et al.* (34) reported distal Pit-1 binding sites in the chicken PRL promoter region, between –1314 and –1128, and a putative proximal Pit-1 binding site localized at –123 to –98. In chickens, mRNA for Pit-1 was first detected in the pituitary from e5 (38). We have identified Pit-1 expression in chicken embryonic pituitaries as early as e8 by fluorescence ICC and in greater than 30% of all pituitary cells by e10 (our unpublished results). That expression of Pit-1 in the pituitary precedes the expression of GH, PRL, and TSH $\beta$  was also determined in rodents (39, 40). Although a previous report indicated that Pit-1 was not detected in prolactin cells from the turkey (41), our results from dual labeling immunofluorescence clearly demonstrate the colocalization of Pit-1 and PRL in chicken embryonic pituitary cells. In the turkey, three Pit-1 mRNA species were identified. The length of these three transcripts of turkey Pit-1 are 1008, 1092, and 984 bp, which encode for 335, 363, and 327 amino acids, respectively (38, 42). In our study, the 40-kDa isoform of Pit-1 appears to be the most abundant one produced in cultured e13 pituitary cells. The other isoforms of Pit-1 were barely detectable by Western blotting. Intriguingly, results from both ICC and Western blotting indicate that proportions of Pit-1-containing cells and overall levels of Pit-1 protein spontaneously increased with increasing culture time, regardless of whether e13 pituitary cells were cultured with or without CORT. This spontaneous increase in Pit-1 expressing cells may reflect the normal ontogeny of Pit-1 cells through e17 or it may be an artifact of pituitary dissociation and culture. Neither possibility was excluded in the present study. However, there were no significant differences between control and CORT-treated groups, indicating that CORT does not increase the production of Pit-1 to induce lactotroph differentiation. That does not exclude the involvement of Pit-1 because Pit-1 is likely required to act synergistically with GR or other factors induced by CORT to activate the PRL promoter. Lactotrophs were not induced by CORT after e13 pituitary cells were cultured for 3 d without any treatment followed by 1 d of vehicle or CORT ( $10^{-9}$  M) incubation (data not shown). That means that the spontaneous increase in Pit-1 is not sufficient to allow a more rapid PRL response to CORT. A full 3 d of CORT exposure was required, suggesting a complex mechanism involving a cascade of gene induction events.

In summary, glucocorticoids can induce lactotroph differentiation in culture, and longer CORT exposure is needed for lactotroph induction compared with somatotroph induction. The effects of glucocorticoids on lactotroph induction are GR dependent and involve an increase in PRL gene expression. PRL cell induction by glucocorticoids is not associated with an increase in Pit-1-containing cells or lactotroph mitosis.

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