Identification of cis elements necessary for glucocorticoid induction of growth hormone gene expression in chicken embryonic pituitary cells

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Heuck-Knubel K, Proszkowiec-Weglarz M, Narayana J, Ellestad LE, Prakobsaeng N, Porter TE. Identification of cis elements necessary for glucocorticoid induction of growth hormone gene expression in chicken embryonic pituitary cells. Am J Physiol Regul Integr Comp Physiol 302: R606–R619, 2012.—Glucocorticoid (GC) treatment of rat or chicken embryonic pituitary (CEP) cells induces premature production of growth hormone (GH). GC induction of the GH gene requires ongoing protein synthesis, and the GH genes lack a canonical GC response element (GRE). To characterize cis-acting elements and identify trans-acting proteins involved in the process, we characterized the regulation of a luciferase reporter containing a fragment of the chicken GH gene (−1727/+48) in embryonic day 11 CEP cells. Corticosterone (Cort) increased luciferase activity and mRNA expression, and mRNA induction was blocked by protein synthesis inhibition. Through deletion analysis, we identified a GC-responsive region (GCRR) at −1045 to −954. The GCRR includes an ETS-1 binding site and a degenerate GRE (dGRE) half site. Nuclear proteins, including ETS-1, bound to a GCRR probe in electrophoretic mobility shift assays, and Cort regulated protein binding. Using chromatin immunoprecipitation, we found that ETS-1 and GC receptor (GR) were associated with the GCRR in CEP cells, and Cort increased GR recruitment to the GCRR. Mutation of the ETS-1 site or dGRE site in the −1045/+48 GH reporter abolished Cort responsiveness. We conclude that GC regulation of the GH gene during development requires cis-acting elements in the GCRR and involves ETS-1 and GR binding to these elements. Similar ETS-1 elements/dGREs are located in the 5′-flanking regions of GH genes in mammals, including rodents and humans. This is the first study to demonstrate involvement of ETS-1 in GC regulation of the GH gene during embryonic development in any species, enhancing our understanding of GH regulation in vertebrates.

somatotropin; development; corticosterone; ETS-1

ONSET OF GROWTH HORMONE (GH) secretion and differentiation of GH-producing cells (somatotrophs) in the anterior pituitary during embryonic development are processes still not wholly understood. Differentiation of somatotrophs naturally occurs during embryonic development, thus providing larger quantities of pituitary cells in vitro by pretreatment of anterior pituitary cells from embryonic rats or chickens with a protein synthesis inhibitor (4, 35), suggesting that one or more proteins must be first synthesized or that ongoing protein synthesis is necessary for GC induction of GH gene expression. The present study characterized GC regulation of the chicken GH gene in primary chicken embryonic pituitary (CEP) cells. The chicken is a unique model for studies of pituitary development, because there is little maternal interaction with the growing embryo. Embryonic development is easily timed and controlled, and the pituitary is relatively large compared with rodent pituitary at the same stage of development, thus providing larger quantities of pituitary cells at a single embryonic age, which are necessary for cell culture experiments aimed at defining molecular mechanisms. The objectives of the present study were to define the GC-responsive region (GCRR) of the GH gene through deletion/mutation analysis of chicken GH promoter reporter constructs and to test for binding of specific proteins to this region using EMSAs and chromatin immunoprecipitation (ChIP). uncovering the GC-inducible element in the GH gene and its associated trans-acting factors will aid in understanding the mechanisms regulating somatotroph differentiation and GH production in vertebrates.

MATERIALS AND METHODS

Reagents and materials. All reagents, including cell culture media, additives, transfection reagents, and enzymes, were purchased from Invitrogen (Carlsbad, CA), unless otherwise noted. Hormones, oligonucleotides, and other chemicals were obtained from Sigma Aldrich (St. Louis, MO).

Generation of deletion and mutant luciferase reporter constructs. Deletion constructs were made using the −1727/+48 chicken GH pGL3 luciferase reporter (a generous gift from Dr. Frederick C. Leung, School of Biological Sciences, The University of Hong Kong, Hong Kong, PR China) was used as the template in PCR amplification with a proofreading DNA polymerase. The primers and restriction enzymes are listed in Table 1. Mutant constructs were generated in the −1045/+48 GH reporter using the QuikChange II site-directed mutagenesis kit (Strategene, La Jolla, CA). After transformation of the pregnant rat by administration of dexamethasone (Dex) in the drinking water (36). Cort induction of somatotroph differentiation involves the glucocorticoid (GC) receptor (GR) (3), and GR mRNA levels peak at e14, concomitant with the normal differentiation of GH cells in chickens (21). GR protein is detected in pituitary extracts as early as e8, and GR is expressed in ~95% of all pituitary cells (3). However, examination of 10 kb upstream and 5 kb downstream of the chicken, rat, and human GH genes revealed no full-length consensus GC response element (GRE), only imperfect half sites (45, 49). Furthermore, induction of GH mRNA by Cort can be blocked in vitro by pretreatment of anterior pituitary cells from embryonic rats or chickens with a protein synthesis inhibitor (4, 35), suggesting that one or more proteins must be first synthesized or that ongoing protein synthesis is necessary for GC induction of GH gene expression. The present study characterized GC regulation of the chicken GH gene in primary chicken embryonic pituitary (CEP) cells. The chicken is a unique model for studies of pituitary development, because there is little maternal interaction with the growing embryo. Embryonic development is easily timed and controlled, and the pituitary is relatively large compared with rodent pituitary at the same stage of development, thus providing larger quantities of pituitary cells at a single embryonic age, which are necessary for cell culture experiments aimed at defining molecular mechanisms. The objectives of the present study were to define the GC-responsive region (GCRR) of the GH gene through deletion/mutation analysis of chicken GH promoter reporter constructs and to test for binding of specific proteins to this region using EMSAs and chromatin immunoprecipitation (ChIP). Uncovering the GC-inducible element in the GH gene and its associated trans-acting factors will aid in understanding the mechanisms regulating somatotroph differentiation and GH production in vertebrates.

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DH5α Max Efficiency Competent cells with the newly ligated plasmids, ampicillin-selected colonies were chosen. Plasmids were sequenced to confirm the sequence and orientation of inserts. Nucleo-bond Maxi plasmid purification columns (Clontech, Mountain View, CA) were used to purify plasmids for transfection.

Pituitary dissection, dispersion, cell culture, and transfection. Fertilized chicken eggs (Ross broiler strain) were set in a humidified incubator (60% humidity, 37.5°C), with day 1 of incubation denoted as e0. Pituitaries were dissected from e11 embryos and trypsin-dispersed, as previously described (41). Cells were counted and viability was assessed by the trypan blue dye-exclusion method (23).

cells were dispersed, plated at a density of 1 × 10⁶ cells per well in poly-L-lysine-coated 24-well plates and allowed to attach for 1 h. Plasmids to be transfected were diluted in sterile OptiMEM to a concentration of 1 μg per well for all luciferase mRNA, as noted.

The RNA was reverse-transcribed into cDNA and used in a quantitative PCR using an iCycler (Bio-Rad, Hercules, CA), as previously described (13). All primers used to quantify mRNA are listed in Table 2. Primers were designed to span an intron where appropriate to ensure amplification of mRNA, and not genomic DNA. Relative mRNA levels for each sample were calculated using the relative cycle threshold (Ct) method [mRNA level = 2^(-ΔΔCt)] of the no-RT control (Ct of the sample)]], as described previously (13, 15). Levels of mRNA were then normalized to levels of β-actin or Renilla luciferase mRNA, as noted.

### Table 1. Primers and restriction enzymes used for cloning chicken GH 5′-flanking region

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**GH**, growth hormone; **dGRE**, degenerate glucocorticoid response element.
Preparation of nuclear protein extracts. On e11, CEP cells (2.0 × 10⁶ per treatment) were cultured for 6 h in the presence or absence of Cort (1 nM). Cells were scraped on ice, collected into 15-ml centrifuge tubes, and rinsed once with PBS. The nuclear and cytoplasmic fractions were isolated according to the method described previously with modifications (11). The cells were washed once in hypotonic buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5 mM DTT], resuspended in hypotonic buffer, and incubated on ice for 10 min. The cells were then transferred to a 1-ml Dounce homogenizer fitted with a tight pestle and homogenized with 60 up-and-down strokes. An aliquot of cells was checked under a microscope for loss of cell membrane and retention of nuclei with trypan blue. The nuclei were then transferred to a microcentrifuge tube and centrifuged at 3,300 g for 15 min. The cytoplasmic fraction was removed and saved. The nuclear pellet was rapidly resuspended in low-salt buffer [20 mM HEPES (pH 7.9), 25% glycerol, 0.02 M KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.02 PMSF, and 0.05 mM DTT] and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore) for 30 min at 16 V and for 1 h with Tris-glycine buffer (25 mM Tris and 192 mM glycine) containing 0.1% SDS, and transferred to a polyvinylidene difluoride (PVDF) membrane for 5 min, resolved on a 10% gel by SDS-PAGE with Tris-glycine buffer (25 mM Tris and 192 mM glycine) containing 0.1% SDS, and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore) for 30 min at 16 V and for 1 h at 25 V using a Trans-Blot SD semi-dry electrophoretic transfer cell

EMSA. Single-stranded (sense and antisense) 5'-infrared dye-labeled (IRDye 700) probes designed for the proximal (−1042/−956) and distal (−1496/−1465) GCRR and exon 3 (+207/+237) of the chicken GH gene (Table 3) were purchased from Integrated DNA Technologies (Coralville, IA). Double-stranded probes were prepared by mixing equal amounts of the single-stranded cDNAs, heating to 95°C for 5 min, and slow cooling to room temperature. One microliter of annealed probe (50 nM final concentration) and 2.5 µg of nuclear extract proteins were added to the binding reaction. The binding reaction, which consisted of binding buffer (10 mM Tris, 50 mM NaCl, and 1 mM DTT, pH 7.5), 50 ng of sheared salmon sperm DNA, and 15 mM MgCl₂, 2.5 mM DTT, 0.25% Tween 20, and 0.2% NP-40, was incubated for 30 min on ice in darkness. After addition of orange loading dye (Li-Cor Biosciences, Lincoln, NE), the DNA-protein complexes were separated by electrophoresis (for 3 h at 70 V) on nondenaturating 7% polyacrylamide-Tris-borate-EDTA gels and scanned directly (intensity level 8) using the Odyssey infrared imaging system (Li-Cor Biosciences).

Western blotting. To determine if Cort treatment affected expression of ETS-1 protein, e11 anterior pituitary cells (5 × 10⁵ cells per well) were cultured for 24 h and left untreated or treated with Cort (1 nM) for the final 1.5, 3, or 6 h of culture. Cells were placed on ice, gently rinsed once with ice-cold PBS, and incubated on ice with rocking for 5 min in nondenaturating cell lysis buffer containing protease and phosphatase inhibitors (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin, and 1 mM PMSE). Cells were then scraped, sonicated on ice (3 times for 10 s each), and centrifuged at 14,000 g and 4°C for 10 min, and the supernatant was stored at −80°C until analysis. Protein levels in each sample were quantified with the micro-bicinchoninic acid protein assay kit (Pierce). Extracts (10 µg of total cellular protein) were boiled in Laemmli sample buffer [60 mM Tris-HCl (pH 6.8), 100 mM DTT, 2% SDS, 10% glycerol, and 0.01% bromophenol blue] for 5 min, resolved on a 10% gel by SDS-PAGE with Tris-glycine buffer (25 mM Tris and 192 mM glycine) containing 0.1% SDS, and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore) for 30 min at 16 V and for 1 h at 25 V using a Trans-Blot SD semi-dry electrophoretic transfer cell with 0.1% SDS, and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore) for 30 min at 16 V and for 1 h at 25 V using a Trans-Blot SD semi-dry electrophoretic transfer cell
conical tubes. The dishes were washed twice with 2 ml of ice-cold water, and the cells were scraped into 50-ml tubes. The supernatant was then added for 5 min to quench the formaldehyde. The supernatant was then added to the cells in TBS-T containing 5% nonfat milk. Immunoreactive bands were detected using enhanced chemiluminescent detection reagents (ECL, Amersham Biosciences, Piscataway, NJ). Immunoreactive bands were detected as described above.

To identify proteins that bound to the GR CR, proteins from the acrylamide gel used for the EMSA were transferred to PVDF membranes, and immunoblotting for ETS-1 was performed as described above. For Western blotting of the GR, the membrane was incubated overnight at 4°C with the mouse monoclonal anti-rat GR antibody GR49-4, which recognizes chicken GR, as shown previously (17) (1:1,000 dilution; kindly provided by Dr. Lily Vardimon, The George S. Wise Faculty of Life Sciences, Tel Aviv, Israel, with permission from Dr. H. Westphal, Institute of Molecular Biology and Tumor Research, Marburg, Germany), and GR was detected using horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:2,000 dilution; Cell Signaling Technology, Danvers, MA) diluted in TBS-T containing 5% nonfat milk. Immunoreactive bands were detected using enhanced chemiluminescent detection reagents (ECL, Amersham Biosciences, Piscataway, NJ). Immunoreactive bands were detected as described above.

Pull-down assay. LMH cells (CRL-2117, American Type Culture Collection; 2 × 10^6) were transfected with OptiMEM-Lipofectamine 24 h according to the manufacturer’s protocol with 1 μg of chicken Ets1A (accession no. JQ086323) or Ets1B (accession no. JQ086324) cloned from e18 pituitary RNA samples into pSPORT expression system (Invitrogen). Aliquots of the quantified chromatin were used for each immunoprecipitation, 10 μg of sheared chromatin were precleared twice for a total of 24 h at 4°C, with rotation using prewashed protein A magnetic beads (New England Bio Labs) and 0.25 mg/ml normal rabbit serum. The cleared chromatin was transferred to a new tube, washed twice with 50 μl of the appropriate antibody were added, and the tube was incubated overnight at 4°C with rotation. Before antibody addition, 2% of the total volume was removed and set aside as “input control.” The following antibodies were used: normal rabbit serum (catalog no. 069019, CalBiochem, Darmstadt, Germany), rabbit polyclonal anti-human histone H3 COOH-terminal (catalog no. 39163, Active Motif, Carlsbad, CA), rabbit polyclonal anti-human GR-ETS-1 (1:1,000 dilution; catalog no. sc-112, Santa Cruz Biotechnology), and mouse monoclonal anti-rat GR antibody GR49-4, which recognizes chicken GR, as shown previously (17) (1:1,000 dilution).

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Cell culture and chromatin preparation. CEP cells (e11; 2.5 × 10^6 cells per treatment) were plated in 5 ml of DMEM-Ham’s F-12 medium (supplemented with 0.1% BSA, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml human insulin) in four 100 × 20 mm Corning cell culture petri dishes and allowed to recover overnight. The cells were then treated with vehicle or Cort (1 nM) for 1.5 or 6 h and fixed with formaldehyde (1.2% final concentration) on a rotator for 10 min at room temperature. Glycine (0.1 M final concentration) was then added for 5 min to quench the formaldehyde. The plates were placed on ice, and the cells were scraped into 50-ml conical tubes. The dishes were washed twice with 2 ml of ice-cold PBS-1 mM PMSF. The cells were collected by centrifugation at 3,000 g and the pellet was washed once with PBS-1 mM PMSF. The cells were resuspended in swelling buffer [25 mM HEPES (pH 7.8), 1.5 mM MgCl₂, 10 mM KCl, 0.1% NP-40, 1 mM DTT, 0.5 mM PMSF, and 1× Halt protease inhibitor cocktail (Thermo Scientific)] and incubated on ice for 10 min. The cell suspension was homogenized with a 15-ml Dounce homogenizer with 15 up-and-down strokes using a loose-fitting pestle, transferred to 15-ml conical tubes, and centrifuged at 1,800 g for 5 min. The nuclear pellet was resuspended in 2 ml of micrococcal nuclease buffer [0.32 M sucrose, 50 mM Tris-HCl (pH 7.4), 4 mM MgCl₂, 1 mM CaCl₂, 0.1 mM PMSF, and 1% SDS] and homogenized with a Polytron (model PT 1200 C, Kinematica, Bohemia, NY) for 30 s on ice. After addition of 10 μl of BSA and 7.5 μl of micrococcal nuclease (New England Bio Labs, Ipswich, MA) to each sample, the reaction was incubated at 37°C for 15 min. The reaction was stopped by addition of 100 μl of 0.5 M EDTA. The samples were stored overnight at −80°C. On the next day, the samples were sonicated in an ethanol-ice bath for 5 cycles (20-s continuous pulse, output at 60%, power setting of 6) using a Branson Sonifier 250 (Applio Ultrasorics). The samples were centrifuged at 16,000 g for 10 min, and the supernatant was collected into a new tube and quantified using a Quanti-it Picogreen DNA quantification kit (Invitrogen). Aliquots of the quantified chromatin were stored at −80°C. Gel electrophoresis confirmed that chromatin was sheared to 200–800 bp.

ChIP. For each immunoprecipitation, 10 μg of sheared chromatin were precleared twice for a total of 24 h at 4°C, with rotation using prewashed protein A magnetic beads (New England Bio Labs) and 0.25 mg/ml normal rabbit serum. The cleared chromatin was transferred to a new tube, washed twice with 50 μl of the appropriate antibody were added, and the tube was incubated overnight at 4°C with rotation. Before antibody addition, 2% of the total volume was removed and set aside as “input control.” The following antibodies were used: normal rabbit serum (catalog no. 069019, CalBiochem, Darmstadt, Germany), rabbit polyclonal anti-human histone H3 COOH-terminal (catalog no. 39163, Active Motif, Carlsbad, CA), rabbit polyclonal anti-human GR-ETS-1 (1:1,000 dilution; catalog no. sc-112, Santa Cruz Biotechnology), and mouse monoclonal anti-rat GR antibody GR49-4, which recognizes chicken GR, as shown previously (17) (1:1,000 dilution).

For each experiment, 10 μg of sheared chromatin were precleared twice for a total of 24 h at 4°C, with rotation using prewashed protein A magnetic beads (New England Bio Labs) and 0.25 mg/ml normal rabbit serum. The cleared chromatin was transferred to a new tube, incubated overnight at 4°C with rotation. Before antibody addition, 2% of the total volume was removed and set aside as “input control.” For each immunoprecipitation, 10 μg of sheared chromatin were precleared twice for a total of 24 h at 4°C, with rotation using prewashed protein A magnetic beads (New England Bio Labs) and 0.25 mg/ml normal rabbit serum. The cleared chromatin was transferred to a new tube, incubated overnight at 4°C with rotation. Before antibody addition, 2% of the total volume was removed and set aside as “input control.”

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RESULTS

The −1727/+48 insert responds robustly to Cort in CEP cells. It was reported previously that Dex treatment produced a twofold increase in promoter activity of a luciferase reporter construct containing 1,775 bp of the 5′-flanking region of the chicken GH gene when transfected into rat GH4Cl cells (24). In that report, deletion of the −1,727-bp insert to −1,467 bp ablated Dex induction of promoter activity (24). Therefore, it is possible that a nonclassical GRE exists between −1,727 and −1,467 bp of the chicken GH gene. To confirm Cort responsiveness of the −1727/+48 chicken GH reporter in homologous pituitary cells, this reporter was transfected into e11 CEP cells. The −1727/+48 region of the GH gene increased basal luciferase activity by twofold (Fig. 1A), while the −1467/+48 GH reporter resulted in a 50% increase in basal luciferase activity. Cort did not affect luciferase activity of the empty vector. In contrast, Cort increased luciferase activity of the −1727/+48 GH reporter by sixfold. However, the −1467/+48 GH reporter was not Cort-responsive (n = 3, P > 0.05; Fig. 1A). Thus, the −1727/+48 GH reporter was Cort-responsive in GH4Cl cells (data not shown) and CEP cells, and this response was lost with deletion down to −1467. Inspection of −1272 to −1467 bp of the GH gene revealed no GRE. However, a half site of a canonical GRE is located in intron 1 (+302 to +320 bp) of the chicken GH gene. To address whether the purported GRE half site in intron 1 is functional, −1727 to +1004 bp of the GH gene was cloned into a luciferase reporter and tested in CEP cells (Fig. 1B). Cort treatment of the −1727/+48 GH reporter resulted in a 12-fold increase in luciferase activity.

Table 4. Primer sequences used for quantitative PCR on chromatin immunoprecipitation samples

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</table>

Fig. 1. Mean relative luciferase activity. At embryonic day 11 (e11), chicken embryonic pituitary (CEP) cells were transfected with −1727/+48 growth hormone (GH) reporter, −1467/+48 GH reporter, or basic empty vector and assayed for luciferase activity in response to corticosterone (Cort) treatment (A), −1727/+48 GH reporter, −1727/+1004 GH reporter, or basic empty vector and assayed for luciferase activity in response to Cort (B), or −1727/+48 GH reporter or basic empty vector and assayed for luciferase activity in response to various steroids (100 nM; C). For clarity, results were normalized to those for cells transfected with basic vector under basal conditions. Values are least-squared means (LSMeans) and SE of 3 separate experiments. Values denoted by different letters (a, b, c) are significantly different at P < 0.05.
However, Cort treatment of the −1727/+1004 GH reporter resulted in only a sixfold increase in luciferase activity (n = 4; P < 0.05) in these trials. Thus, inclusion of intron 1 and the GRE half site did not increase responsiveness to Cort. Next, e11 CEP cells were treated with vehicle or various steroids, Cort, aldosterone, testosterone, estradiol, and progesterone (100 nM), to define the specificity of the response to Cort. Cort treatment induced the −1727/+48 GH reporter by 10-fold (n = 3, P < 0.05; Fig. 1C), whereas treatment with aldosterone, testosterone, or estradiol did not produce a significant increase. Progesterone resulted in a partial increase in luciferase activity that was significantly lower than the response to Cort. Therefore, the −1727/+48 GH reporter response was relatively specific to Cort.

The −1727/+48 GH reporter recapitulates Cort induction of the endogenous GH gene. Induction of GH mRNA in CEP cells by GC can be blocked with the addition of CHX, a protein synthesis inhibitor, possibly indicating the necessity of an unknown protein in Cort induction of GH. Therefore, induction of the −1727/+48 GH reporter by GC and subsequent inhibition of that induction by CHX would be necessary for the reporter to recapitulate the natural system. To this end, mRNA levels of firefly luciferase and Renilla luciferase in response to Cort and CHX alone and in combination were quantified using quantitative RT-PCR. Firefly luciferase mRNA was normalized to Renilla luciferase mRNA as a control for transfection efficiency (Fig. 2A). Firefly luciferase mRNA transcibed off the −1727/+48 GH reporter increased 80-fold in response to Cort, and this response to Cort was blocked by pretreatment with CHX. GH mRNA, normalized to β-actin, was quantified in the same samples (Fig. 2B). Cort induced GH mRNA, regardless of the transfected reporter (−1727/+48 vs. empty vector), and Cort induction of GH mRNA was blocked by pretreatment with CHX, as expected. Thus, ongoing protein synthesis is required for Cort induction of the −1727/+48 GH reporter and the endogenous GH gene.

Deletion constructs reveal two potential GCRRs. For identification of the GCRR within the 5′-flanking region of the chicken GH gene, the −1727/+48 GH reporter was used as template to generate progressively shorter deletion constructs. Eleven deletion constructs were cloned into the luciferase vector and tested for Cort responsiveness (Fig. 3A). The −1467/+48 GH reporter remained unresponsive to Cort, while the −1477 clone was minimally responsive. The shorter −1430/+48, −1398/+48, −1201/+48, and −1045/+48 GH reporter constructs were responsive to Cort (P < 0.05, n = 3), while the −954/+48, −807/+48, and −382/+48 GH reporters were not responsive to Cort (P > 0.05, n = 3; Fig. 3). Therefore, a GC inhibitory region (GC-IR) was identified between −1477 and −1430, and a proximal GCRR was identified between −1045 and −954 of the chicken GH gene.

The GCRR is orientation-independent but context-dependent. We next sought to further characterize the −1045 to −954 GCRR. To this end, 10 additional constructs were made to test whether the proximal GCRR is orientation- and/or context-independent (Fig. 4). The GCRR was placed into other deletion constructs in the forward and reverse directions. Constructs were transfected into CEP cells and treated with Cort. The GCRR exhibited greater Cort responsiveness when placed in the reverse orientation (n = 4, P < 0.05; Fig. 4). Addition of the reverse GCRR to the −1727/+48 and −954/+48 constructs resulted in an average 35-fold increase in luciferase activity, but only in a 13-fold increase in luciferase activity when placed in front of the −650/+48 construct. Placement of the GCRR in the forward orientation linked to the −650/+48 construct resulted in an apparent twofold response to Cort, but this was not significant. The −1727/+48 construct, with −953 to −650 deleted but the GCRR intact, did not respond to Cort. Similarly, when the GCRR, in either orientation, was linked to the −382/+48 GH reporter, Cort induction of luciferase activity was lost (data not shown). This suggests that intervening sequences between −650 and −382 bp of the GH gene, including the distal Pit-1 site (−541/−528), are required for Cort induction of GH promoter activity. Taken together, these results indicate that responsiveness to Cort through the GCRR (−1045/−954) requires cis-acting elements between −650 and −382 and additional elements between −953 and −650. However, responsiveness to Cort was conferred in constructs containing −953/+48 when the GCRR was included in its forward or reverse orientation. Therefore, Cort responsiveness of the GCRR is orientation-independent but context-depen-
dent. With use of the vertebrate database in the MatInspector (43) and JASPAR CORE (44) programs, putative transcription factor binding sites were identified between $\text{H}11002\text{1045}$ and $\text{H}11002\text{954}$ of the chicken GH gene. Interestingly, a degenerate GRE half site (dGRE) and an ETS-1 site were identified by both programs.

Nuclear proteins bind to the GCRR in a Cort-regulated manner. Next, using EMSA, we sought to determine if the putative transcription factor binding sites within the GCRR were functional in vitro. Nuclear proteins extracted from Cort- or vehicle-treated CEP cells incubated with the $\text{H}11002\text{1045}$ to $\text{H}11002\text{954}$ GCRR probe (Table 3) produced a noticeable shift in mobility of the probe (Fig. 5). One high-molecular-weight and one low-molecular-weight shift were observed upon incubation of nuclear extracts with the GCRR probe. Cort treatment increased binding of high- and low-molecular-weight nuclear proteins to the GCRR probe. The high-molecular-weight band was not seen in all experiments, however, so we focused our attention on the lower-molecular-weight band. Quantification of the mean integrated intensity of the shifted band showed that Cort treatment significantly increased binding of proteins to the GCRR probe ($P < 0.05$; Fig. 5). Nuclear proteins incubated with a probe corresponding to a region within exon 3 ($\text{H}11002\text{207}/\text{H}11002\text{237}$) or a probe designed within a more distal segment of the 5' flanking region ($\text{H}11002\text{1566}$ to $\text{H}11002\text{1467}$) did not produce an observable shift in mobility of either control probe. Serial dilution of the GCRR probe used in the gel shift assays (diluted 1:4) demonstrated that binding was concentration-dependent (data not shown). Similarly, when the amount of nuclear protein incubated with the probe was serially diluted 1:1, intensity of the shifted band was reduced (data not shown).

To determine if the observed shift with the GCRR probe was specific, three unlabeled double-stranded DNA competitors were made: one corresponded to the immediate upstream region ($\text{H}11002\text{1201}$ to $\text{H}11002\text{1046}$) of the GH gene, one to the 5' half of the GCRR probe, and one to the 3' half of the GCRR probe.
Nuclear extracts were preincubated with the 5′ competitor, the 3′ competitor, or the upstream competitor in 100-fold molar excess for 30 min prior to addition of the GCRR probe. Extracted nuclear proteins from CEP cells produced an observable shift of the GCRR probe. Addition of the 5′ or 3′ competitor resulted in reduced protein binding to the probe, while addition of the upstream competitor did not affect protein binding to the probe (n = 4; data not shown). Thus, nuclear protein binding to the GCRR probe is Cort-regulated, and binding can be competed off with addition of a 5′ competitor or a 3′ competitor. Because the 5′ and 3′ competitors reduced binding to the probe and did not provide additional insight into the region bound by proteins, another competitor was made. An unlabeled competitor corresponding to the center 34 bp of the GCRR probe and spanning the putative ETS-1 and GR binding sites was tested with the nuclear extracts (Fig. 6B). Nuclear extracts were preincubated with the upstream competitor or the centered competitor in 100-fold molar excess for 30 min prior to addition of the GCRR probe. Addition of the centered competitor abolished protein binding to the GCRR probe. Addition of the upstream competitor did not affect protein binding to the probe (n = 4; Fig. 6B). Taken together, the GCRR probe binds nuclear proteins in a Cort-regulated manner, and this binding can be competed-off with competitor DNA corresponding to the central 34 bp spanning the putative ETS-1 site and dGRE half site. To confirm specificity of protein binding to the GCRR, the central 34 bp of the GCRR probe were scrambled twice, using a free-source randomized-sequence program (http://workbench.sdsc.edu) and reinserted into the full-length GCRR probe sequence (Fig. 7A, Table 3). The wild-type and mutant probes were incubated with increasing amounts of nuclear proteins extracted from basal or Cort-treated e11 pituitary cells (Fig. 7B). The shifted band intensity with the GCRR probe increased with increasing amounts of protein. A shifted band of the same molecular weight was also observed with the mutant probe; however, intensity of the shifted band with the mutated probe was significantly reduced (Fig. 7C; n = 3, P < 0.05). These results indicate that the central 34 bp of the GCRR containing the putative ETS-1 site and dGRE half site are important for nuclear protein binding.

ETS-1 binds to the GCRR probe in vitro. Next, we tested whether ETS-1 expression was responsive to Cort in CEP cells. ETS-1 was evaluated, because it has been shown to be involved in regulation of prolactin and GH gene expression in other species (5, 51). We found that Cort induces GH mRNA after 6 h of treatment, but it does not affect ETS-1 mRNA levels (Fig. 8A). Consistent with this, we found that ETS-1 protein expression was not affected by Cort (1 nM) treatment of e11 CEP cells for 1.5, 3, or 6 h (Fig. 8B). Next, using EMSA and pull-down assays, we tested for ETS-1 binding to the GCRR probe. To identify proteins that bind the GCRR probe,
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Fig. 8. Quantification of ETS-1 mRNA and identification of ETS-1 in the EMSA shift. A: ETS-1 mRNA was quantified in response to Cort treatment. GH mRNA was quantified from the same samples. Values are means ± SE; n = 3. *Significant response to Cort (P < 0.05). B: ETS-1 and α-tubulin (α-Tub) protein levels after 0, 1.5, 3, and 6 h of Cort treatment. Top: representative Western blots for ETS-1 and α-tubulin. Bottom: quantification from 2 independent replicate experiments (n = 2). Blot for α-tubulin was spliced to show corresponding molecular weight markers for the same samples shown in the ETS-1 blot. Values are means ± SD. C: nuclear extracts from basal and Cort-treated cells were tested for binding to the GCRR probe. EMSA gel was transferred to a polyvinylidene difluoride membrane, which was blotted for ETS-1 protein. Blots represent results from 4 replicate experiments. Molecular weight protein ladder (L) is shown as the original image and a bright image. D: binding of total cellular protein from LMH cells overexpressing chicken ETS-1A or ETS-1B to the biotinylated GCRR or biotinylated mutated GCRR probes was tested using pull-down assays. Proteins pulled down by the biotinylated probes were identified using Western blotting with an ETS-1-specific antibody.

**DISCUSSION**

GC can induce expression of GH mRNA and protein in pituitary cells of embryonic chickens (40) and fetal rats (34). The objective of the present study was to identify *cis*-acting
elements and trans-acting factors underlying GC regulation of the GH gene. To this end, a luciferase reporter containing −1727/+48 bp of the 5′-flanking region of the chicken GH gene was used to analyze Cort responsiveness of the GH gene in CEP cells. We determined that the −1727/+48 GH reporter is an appropriate model for GC regulation of the endogenous GH gene, because luciferase mRNA transcribed off the −1727/+48 GH reporter in response to Cort required ongoing protein synthesis, as did induction of endogenous GH mRNA. GC induction of the GH gene during fetal rat development also requires ongoing protein synthesis (35). Thus, it is possible that the proteins required for this response are conserved between rats and chickens. Our findings indicate that the −1727/+48 GH reporter recapitulates the endogenous gene and is an appropriate model for use in studies of GC regulation of the chicken GH gene.

Through deletion analysis of the −1727/+48 GH reporter, two GCRR were identified in the present study: a proximal GCRR and a distal GCRR. This is the second study to show that the promoter of the chicken GH gene was responsive to GC. The −1727/+48 GH reporter exhibited a twofold increase in response to Dex when transfected into a rat pituitary cell line (24). This previous study characterizing the chicken GH gene concluded that a nonclassical GRE was located between −1727 and −1467 (24). The present study further defined this region, and we propose that there is a repressor region located between −1477 and −1430. However, further studies are necessary to determine the underlying mechanism of regulation by this region. The human GH gene has a GRE half site in the first intron that bound the GR complex in an exonuclease III protection/foot-printing assay (45). The gene devoid of its GRE was nonresponsive to Dex treatment, and the intact gene containing its native GRE responded sixfold to Dex treatment (45). Also, a putative GRE half site is located at +302/+320 with the sequence of TGTTCT in intron I of the chicken GH gene. However, the −1727/+1004 construct containing intron I and the putative GRE half site was less responsive than the −1727/+48 construct. Thus, we conclude that the putative GRE half site in intron I of the chicken GH gene is not necessary for Cort induction of the GH gene in CEP cells.

Our findings indicate that GC regulation of the GH gene is not dependent on the orientation of the proximal GCRR located between −1045 and −954. However, the GCRR was context-dependent. GC regulation of the GH gene in CEP cells is
Regulated protein binding to the GCRR was demonstrated using EMSA, and specificity of this binding to the central region of the GCRR was shown using unlabeled competitors and a mutant probe. The central portion of the GCRR contains putative binding sites for ETS-1 and GR. The 5' region of the GCRR contains a putative binding site for E47/CTCF, while the 3' region of the GCRR contains a putative binding site for RUSH/SMARCA3, a chromatin remodeling enzyme. These two proteins could also be involved in GC regulation of the GH gene. CCCCTC binding factor (CTCF) is a zinc finger protein with ~100% conservation of the central DNA-binding domain between mouse, chicken, and human. CTCF has been implicated as a transcriptional repressor, activator, and insulator (39). Interactions of CTCF with nuclear receptors are well characterized (1, 7, 9, 30). However, there is no evidence that CTCF interacts with GR.

This is the first study to demonstrate that GR and ETS-1 are associated with the promoter of the GH gene during embryonic development. We also demonstrated nuclear protein binding to the GCRR probe in vitro. One of the proteins contained in the protein-probe complex was identified as ETS-1 by Western blotting, and binding of chicken ETS-1 to the GCRR was confirmed by pull-down assays. ETS-1 mRNA and protein were not regulated by Cort treatment; however, the phosphorylation state of ETS-1 was not explored in this study. ETS-1 binds to DNA when its serine-rich region is phosphorylated (28, 31, 42). ETS-1 and GR were shown to be associated with the GCRR by ChIP assays. Therefore, GC regulation of the GH gene during chicken embryonic development involves recruitment of proteins, including GR and ETS-1, to the GCRR.

A requirement for GR binding to the GCRR is supported by our finding that mutation of the dGRE half site in the −1045/+48 GH reporter abolished the Cort response. There is mounting evidence that GREs are more degenerate than previously thought. Two recent studies demonstrated functional GR binding to a dGRE, and not to a full-length classical GRE (46, 47). The dGRE identified in the GCRR, although not a classical GRE, fits the classification described in those studies. Mutation of the ETS-1 site also rendered the −1045/+48 GH reporter unresponsive to Cort. The ETS family of transcription factors consists of 10 family members that bind to a core sequence of CCGGAA (48). It was also shown that functional redundant binding sites for all ETS-1 family members were more likely to occur close to the transcription start site of housekeeping-type genes, while more specific, nonredundant functional binding sites for individual ETS-1 family members occur farther away from the transcription start site of more specialized genes (22). ETS-1 has been implicated as the other required factor in the regulation of transcription of the prolactin gene in the rat (5). Pit-1 is necessary, but not sufficient, to direct GH expression in the pituitary in the rat, chicken, mouse, and human. Pit-1 is also necessary, but not sufficient, to direct prolactin expression in the rat pituitary. ETS-1 physically interacts with Pit-1 at a composite cis element to direct prolactin expression. The phosphorylation state of Pit-1 regulates its interaction with ETS-1 (2). Furthermore, binding of Pit-1 and ETS-1 to the composite site in the proximal promoter of the prolactin gene is necessary for Ras-MAPK activation of the prolactin promoter (12). ETS-1 is also involved in regulation of the human GH gene through its locus control region (LCR). Activator protein-1 and the ETS family member Elk-1 were found to bind in hypersensitive site (HS) III and HS IV, respectively, of the human GH gene LCR (26). Binding of these two transcription factors was associated with hyperacetylation of pituitary chromatin. This was the first study to implicate ETS-1 or an ETS family member in GH induction. In a follow-up study, it was shown that Pit-1 binds to HS I and HS II of the human GH normal (hGH-N) LCR, but it cannot bind to HS III alone (51). This requires interaction with ETS-1 via Pit-1’s POU homeodomain and a composite DNA binding element. ETS-1 and another ETS-1 family member, Elk-1, can be immunopre-

![Image](https://example.com/figure10.png)
cipated from human pituitary extracts with a Pit-1 antibody, and overexpression of Elk-1 or Pit-1 alone or together could increase hGH-N expression in HEK-293 cells (51). This was the first study demonstrating that Elk-1 could induce the GH gene. Elk-1 is an immediate downstream target of ERK. Pharmacological inhibition of ERK signaling blocks Cort induction of the chicken GH gene (unpublished data). Elk-1 belongs to the ternary complex family, a subfamily of the ETS family (6). Members are downstream targets of the ras-Raf-MAPK kinase signaling pathway. This implies that the ETS-1 binding site in the GCRR is a necessary and essential element for GC regulation of the GH gene in CEP cells and suggests that ETS-1 may be interacting with Pit-1 in this regulation.

The ETS-1 site and dGRE half site in the GCRR of the chicken GH gene are separated by only 17 bp. This begs the following question: Do ETS-1 and GR physically interact? The rat tyrosine aminotransferase gene is induced by GC, and ETS-1 participates in this response (16). The promoter of the cytochrome P-450 c27 multifunctional enzyme is GC-responsive and contains a functional GR binding site and an ETS-like site that binds ETS-2 in a GR-dependent manner that appears to synergistically activate the gene (33). GR and ETS-2 were shown to coimmunoprecipitate in that study. A fusion protein consisting of only the GR DNA binding domain and the ETS-2 transcriptional activation domain recapitulated activation of the cytochrome P-450 c27 promoter, suggesting a novel synergy between these two proteins (33). In the cytochrome P-450 c27 promoter, a GR binding site is flanked by two ETS-like binding sites and a CTCF binding site. The sites span a 50-bp region. The ETS-1 and dGRE putative sites in the chicken GH promoter span a region of 34 bp. Combinatorial transcription factor binding sites may allow for the integration of signaling from multiple external stimuli. Additional studies are necessary to determine if GR and ETS-1 physically interact during Cort induction of the chicken GH gene.

At first glance, 6-h ChIP results and the 6-h EMSA data from the present study do not agree. The EMSA results indicate that Cort increased protein binding, while the ChIP results indicate that GR and ETS-1 binding to the GCRR were diminished at 6 h after Cort treatment. However, there are inherent differences between EMSA and ChIP methodologies and the questions that the methods are able to answer. It is possible that another protein or protein complex is necessary to maintain association of ETS-1 and GR with the GCRR during GC regulation of the GH gene. Regardless, the ChIP results do agree with the mutant luciferase construct results. We showed that GR and ETS-1 are recruited to the proximal GCRR and that the dGRE and ETS-1 sites are necessary for GC regulation of the GH gene during chicken embryonic development. Furthermore, EMSA and ChIP results implicate binding of GR and ETS-1 to the GCRR of the GH gene in chickens. Although the time-specific recruitment of GR and ETS-1 does not agree on the basis of the EMSA and ChIP studies, we can speculate that GR and ETS-1 are recruited to specific sequences located in the GCRR of the endogenous GH gene and that they, in turn, recruit other proteins, including nucleosome remodeling enzymes, bridging proteins, and basal transcriptional machinery. This is followed by the unwinding of nucleosomes and the formation of an “open” chromatin conformation that is conducive to a high rate of gene transcription. Once gene transcription has moved into the elongation phase, it is possible that the bridging complex, consisting of GR, ETS-1, and cofactors, falls off the gene, so that maximum transcription can ensue. From our studies, GH transcription is maximal at 6 h after Cort treatment of e11 CEP cells (unpublished data).

In conclusion, we identified a functional dGRE half site and a functional ETS-1 site upstream of the GH gene that mediate GC responsiveness in the chicken. Collectively, our results indicate that ETS-1 is associated with its response element located at 1014 to 1009 upstream from the chicken GH gene under basal and Cort-treated conditions. GR is also associated with its response element located at 995 to 985, and on Cort treatment, GR recruitment is increased. Both cis-acting elements are required for GC induction of the GH gene. Additional elements contained in the 650 to +48 region, such as Pit-1, are necessary for GC regulation. We demonstrated that each of these proteins binds to its own response element, and not to a composite element. We showed that ETS-1 is not a GC-regulated gene; however, it is not known whether ETS-1 or GR is phosphorylated in response to GC to affect their binding to the GCRR or interactions with other transcription factors, including Pit-1. The cumulative results from these studies raise new questions. Are bridging complexes or accessory factors necessary for GC induction of the GH gene? What is the target of CHX during GC regulation of the GH gene? It is unlikely to be ETS-1, GR, or Pit-1, inasmuch as Cort treatment did not increase expression of any of these factors. Finally, do GR, ETS-1, and Pit-1 physically interact to mediate GC induction of the GH gene?

Perspectives and Significance

We have known for decades that GCs can induce GH expression in cultures of pituitary cells from fetal rats and embryonic chickens (20, 32, 37). However, the mechanism underlying this response has remained elusive. In the present study, we took advantage of the ability to isolate large numbers of precisely timed CEP cells to identify a region of the GH gene required for the GC response, the GCRR, and two transcription factors that bind to the GCRR and function in the GC response, GR and ETS-1. These findings have implications for the regulation of GH production and somatotroph ontogeny during embryonic development in vertebrates.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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