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Transcriptional regulation of the chicken *CRHR2* gene by pituitary transcription factors

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ABSTRACT

Corticotropin-releasing hormone (CRH) is known to act as a potent thyrotropin-releasing factor in non-mammalian species such as chicken and bullfrog. This interaction is mediated by type 2 CRH receptors (CRHR2) expressed by the thyrotropes in the pituitary gland. However, the response elements (REs) and their corresponding transcription factors (TFs) that control *CRHR2* expression in thyrotropes are not known. Since thyrotrope-specific expression of the β-subunit of thyrotropin is synergistically stimulated by the co-expression of POU1F1 and GATA2, we hypothesised that in non-mammalian vertebrates like chicken, *CRHR2* expression is controlled by the same TFs and that their REs are present in the chicken *CRHR2* gene promoter. In situ hybridisation and immunohistochemistry suggest that chicken thyrotropes, like those of mammals, express the mRNAs for the TFs GATA2, POU1F1 and PITX1, but not NR5A1. Using luciferase reporter assays, we show that both GATA2 and PITX1 can activate the promoter of *CRHR2*, but PITX1 requires a functional GATA2 RE to be present. POU1F1 alone did not affect promoter activity, but synergistically increased the effect of GATA2. Promoter deletion analysis and mutagenesis showed that essential GATA2 and PITX1 REs are located between 116 and 198 bp upstream of the start codon. These REs are highly conserved in non-mammalian species. Additionally, NR5A1 (steroidogenic factor 1) suppressed both GATA2- and PITX1-induced promoter activity and may therefore play a role in restricting *CRHR2* expression in gonadotropes. We conclude that the expression of *CRHR2* in chicken thyrotropes is stimulated by GATA2 with interactions with POU1F1 and PITX1, in the absence of NR5A1.

1. Introduction

Thyroid-stimulating hormone (TSH) is secreted by the thyrotropic cells, or thyrotropes, of the anterior pituitary gland and controls the synthesis and secretion of thyroid hormones (Jackson, 1982). Typically, hypothalamic thyrotropin-releasing hormone is regarded as the main regulator of TSH secretion (Chen and Meites, 1975; Dussault, 1974). However, corticotropin-releasing hormone (CRH) is also able to induce TSH release in some non-mammalian vertebrates (reviewed by De Groef et al. (2006) and Watanabe et al. (2016)). At least in chickens (*Gallus gallus*), zebra finches (*Taeniopygia guttata*) and American bullfrogs (*Lithobates catesbeianus*), the TSH-releasing ability of CRH is mediated by type 2 CRH receptors (CRHR2) expressed by pituitary thyrotropes (De Groef et al., 2003; Okada et al., 2007; Watanabe et al., 2017). In these species, *CRHR2* mRNA expression within the anterior pituitary gland is largely concentrated in the thyrotropes. However, the transcription factors (TFs) and their corresponding response elements (REs) that drive expression of *CRHR2* in the thyrotropes of these non-mammalian species have not been investigated.

Thyrotrope-specific expression has been studied most extensively for the gene encoding the β-subunit of TSH, *TSHB*, in mammals (Gordon et al., 1997, 2002; Kashiwabara et al., 2009). In human and mouse, synergy between two TFs, namely GATA-binding protein 2 (GATA2) and POU class 1 homeobox 1 (POU1F1; also known as PIT1, pituitary-specific positive transcription factor 1), drives the expression of *TSHB* in thyrotropes specifically. Mammalian POU1F1 is expressed in somatotropes (growth hormone-producing cells), lactotropes (prolactin-producing cells) and thyrotropes, while GATA2 is expressed only in gonadotropes (gonadotropin-producing cells) and thyrotropes. Therefore, the only pituitary cell type in which both TFs are co-expressed is the

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thyrotrope (Simmons et al., 1990; Dasen et al., 1999). The synergy between POU1F1 with GATA2 determines the terminal differentiation of the thyrotropes and is important for their normal function (Anderson and Rosenfeld, 1994; Dasen et al., 1999; Scully and Rosenfeld, 2002; Charles et al., 2006). In gonadotropes, GATA2 is thought to interact with other TFs, including nuclear receptor subfamily 5, group A, member 1 (NR5A1; better known as SF1, steroidogenic factor 1), to stimulate expression of the β-subunit of luteinising hormone (LHB) (Lo et al., 2011). In addition, paired-like homeodomain 1 (PITX1) and 2 (PITX2) are expressed in the developing and adult anterior pituitary gland and are involved in the differentiation of nearly all pituitary cell types, including thyrotropes (Drolet et al., 1991; de Moraes et al., 2012). Knock-out of *Pitx1* in mice resulted in reduced numbers of gonadotropes and thyrotropes with lower hormone expression (Szeto et al., 1999).

The current study aimed to identify the REs that drive expression of *CRHR2* in the thyrotropes of chicken, a non-mammalian species in which CRH is known to induce TSH release and thyrotropes have been shown to express *CRHR2* mRNA (De Groef et al., 2003). Expression studies and *in vitro* reporter gene activation studies with the chicken *CRHR2* promoter (cp*CRHR2*) were performed with the primary aim to determine whether POU1F1 and GATA2 drive expression of *CRHR2* in thyrotropes as is the case for the mammalian *TSHB* gene. In addition, the effects of NR5A1 and PITX1 on the activity of cp*CRHR2* were investigated.

2. Materials and methods

This study was carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8th edition, 2013) of the National Health and Medical Research Council. All animal procedures were approved by the Animal Ethics Committee of La Trobe University.

2.1. RT-PCR

Total RNA was isolated from a pituitary gland of a one-day-old Cobb chicken using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's guidelines. The quantity and purity of the RNA sample were assessed using a Nanodrop Lite (Nanodrop, Wilmington, DE, USA). One μg of RNA was reverse transcribed in a total volume of 20 μl as described by Noy et al. (2017). The sample was incubated in a thermocycler at 25 °C for 10 min, 42 °C for 120 min, and 85 °C for 5 min. Using the resulting pituitary cDNA as template, partial sequences of chicken *GATA2, POU1F1* (α variant), *NR5A1* and *PITX1* were amplified. High-fidelity PCR was performed with 2 μl of pituitary cDNA, amplified in a 50-μl mixture, as described by Noy et al. (2017). Primers (Bioneer, Daejeon, Republic of Korea) were based on sequence data accessed through GenBank at the National Center for Biological Information (https://www.ncbi.nlm.nih.gov/genbank/) (Table S1). The samples were run in a thermocycler with initial denaturation at 94 °C for 4 min, followed by denaturation (94 °C, 30 s), annealing (30 s, see Table S1 for annealing temperatures), and extension (68 °C, 1 min/kb) for 35 cycles. The PCR products were then analysed on a 1% (w/v) agarose gel containing 0.05% (v/v) SYBR Safe DNA gel stain (Invitrogen). The resulting amplicons were visualised under UV light and imaged on a Chemidoc Gel Imaging System (Bio-Rad, Hercules, CA, USA).

2.2. In situ hybridisation and immunofluorescence

Pituitary glands from one-day-old Cobb chickens ($n = 3$) were fixed overnight in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4{\cdot}2H_2O$, 1.8 mM KH_2PO_4, pH 7.4) at 4 °C. The tissues were then cryoprotected in 4% paraformaldehyde and 30% (w/v) sucrose in PBS at 4 °C until tissues sank and stored at −80 °C until sectioned. Tissues were cut into 7-μm cryosections and transferred to Polysine slides (Thermo Fisher Scientific, Waltham, MA, USA). Sections were stored at −20 °C.

A partial sequence of chicken *GATA2*, *POU1F1* (α variant), *NR5A1* and *PITX1* were amplified with the primers listed in Table S1. These sequences were then inserted into a pCRII-TOPO vector (Invitrogen), and sense and antisense probes were transcribed from linearised plasmids using the DIG Labeling Kit (Roche Diagnostics, Risch-Rotkreuz, Switzerland). Pituitary sections underwent in situ hybridisation as per the protocol described by Watanabe et al. (2017). All sections were incubated in 200 μl of hybridisation buffer containing 100 ng/ml (*POU1F1, NR5A1*) or 400 ng/ml (*GATA2, PITX1*) of the labelled sense or antisense riboprobe 80 °C overnight under coverslips.

Adjacent slides were used for immunofluorescent staining of thyrotropes. Antigen retrieval was performed by soaking the slides in boiling 10 mM Tris.HCl with 1.3 mM EDTA and 0.05% (v/v) Tween in water (pH 9) for 10 min, then in boiling 1 mM Tris.HCl with 1.3 mM EDTA for 30 min, and finally washed in PBS for 15 min. Sections were incubated in a blocking solution containing 0.1 M lysine and 5% (v/v) normal goat serum (Antibodies Australia, Melbourne, Australia) in PBS with 4% (w/v) bovine serum albumin and 0.1% (v/v) Triton X-100 for 1 h at 4 °C. After being washed in PBS with 0.1% Triton X-100, the sections were incubated in anti-chicken TSHB (1.5 μg/μl diluted to 1:1000) overnight at 4 °C (Iwasawa et al., 2002). The following steps were then performed in the dark where the slides were washed 3 times in PBS containing 0.1% Triton X-100 before being incubated in 1:1000 of Alexa Fluor 594 donkey anti-rabbit (Invitrogen) for 1 h at room temperature. Six 5-min washes in PBS containing 0.1% Triton X-100 were then performed and NucBlue Fixed Cell Stain ReadyProbes reagent (Invitrogen) was applied according to the manufacturer's guidelines. The sections were finally washed in three 5-min washes of PBS before coverslips were mounted on the slides. The in situ hybridisation and immunofluorescence sections were imaged using an Olympus BX53 microscope (Olympus Scientific Solutions Americas Inc., Waltham, MA, USA).

2.3. Construction of reporter and expression plasmids

Total RNA was isolated from a pituitary gland of a one-day-old Cobb chicken using TRIzol reagent and reverse transcribed as above. This pituitary cDNA was used to clone the entire coding sequences of chicken *GATA2*, *POU1F1*, *PITX1* and *NR5A1* by PCR, using the protocol described in Section 2.1 and the primer pairs specified in Table S1. Genomic DNA was isolated from a one-day-old Cobb chicken brain using the PureLink® Genomic DNA Mini Kit (Thermo Fisher Scientific) following the manufacturer's guidelines. Using this genomic DNA, putative promoter fragments of the *CRHR2* gene (cp*CRHR2*) were amplified with a fixed reverse primer containing a *Xho*I restriction site at the 5′ end, and a varying forward primer containing a *Mlu*I restriction site at the 5′ end, to generate amplicons of different lengths (Table S1).

High-fidelity PCR was performed with 100 ng of genomic DNA or 2 μl of cDNA, amplified in a 50-μl mixture as described in Noy et al. (2017). Primers (Bioneer) were designed based on sequence data accessed through GenBank and contained a restriction site at their 5′ end (Table S1). The samples were run in a thermocycler with initial denaturation at 94 °C for 4 min, followed by denaturation (94 °C, 30 s), annealing (30 s, see Table S1 for annealing temperatures) and extension (68 °C, 1 min/kb) for 35 cycles. The PCR product was then analysed on a 1% agarose gel containing 0.05% SYBR Safe DNA gel stain, excised, and purified using the Wizard SV Gel and PCR Clean-Up system (Promega, Madison, WI, USA). PCR products were subcloned into the pCRII-TOPO vector and used for transformation of Subcloning Efficiency DH5α Competent Cells (Invitrogen) or Chemically Competent *Escherichia coli* (TOP10) cells (Invitrogen). The cells were grown on a culture plate containing Luria-Bertani (LB) medium with 100 μg/ml ampicillin (Bioline, Alexandria, NSW, Australia) and 20 μl of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (50 mg/ml; Bioline). A white colony was

subsequently grown overnight in LB medium containing 100 μg/ml ampicillin, and plasmids were purified using the Purelink Quick Plasmid Midiprep Kit (Invitrogen) and sent for sequencing Australian Genome Research Facility (Melbourne, VIC, Australia). Insert sequences were confirmed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Restriction digestion was initially performed on 7.5 μg of pCRII-TOPO plasmids containing the *CRHR2* promoter regions with 5 μl 10× restriction buffer D (Promega), 0.5 μl acylated bovine serum albumin (BSA) (10 mg/ml), 2.5 μl of *Xho*I and *Mlu*I restriction enzyme (12 U/μl) (Promega) and nuclease-free water to a total volume of 50 μl. The solution was subsequently incubated at 37 °C for 4 h and subject to gel electrophoresis. Empty pGL3 basic vector (7.5 μg, Promega) was similarly digested with *Xho*I and *Mlu*I. After digestion, promoter sequences and vector were subjected to gel electrophoresis, excised and purified as above. Ligation was performed with 1 U T4 DNA ligase (Promega), 1 μl 10× ligase buffer (Promega), 100 ng digested vector and the appropriate amount of insert (3:1 M vector-to-insert ratio). The solution was incubated at 22 °C overnight. DH5α competent cells (Invitrogen) were transformed with the promoter constructs according to the manufacturer's guidelines. The Purelink HiPure Midiprep kit (Invitrogen) was then used to isolate the plasmid DNA in larger quantities from a 50-ml bacterial culture. The sequence and orientation of the inserts were confirmed by DNA sequencing (AGRF).

The promoter inserts were subsequently subcloned into the pGL4.10[*luc2*] vector (Promega), which has been upgraded to reduce anomalous transcription. Restriction digestion was performed on 7.5 μg of the promoter–pGL3 vector with 5 μl 10× restriction enzyme buffer J (Promega), 0.5 μl acylated BSA (10 mg/ml), 2.5 μl of *Kpn*I restriction enzyme (12 U/μl) (Promega) and nuclease-free water to a total volume of 50 μl. The mixture was subsequently incubated at 37 °C for 4 h and subjected to gel electrophoresis. After the digested plasmids were excised and purified, they were further digested with 2.5 μl *Xho*I restriction enzyme (12 U/μl) (Promega) in 5 μl 10x restriction enzyme buffer D, 0.5 μl acylated BSA (10 mg/ml). Empty pGL4.10[*luc2*] vector (7.5 μg) was digested with *Kpn*I and *Xho*I following the same procedure. The digested promoter sequences and vector were subjected to gel electrophoresis, excised and purified as described above.

Likewise, restriction digestion was then performed on 7.5 μg of the PCR-TOPO vector, and on the empty pCI Mammalian Expression Vector (Promega) vector with *Eco*RI and *Xba*I (for *PITX1*) or *Mlu*I and *Xho*I (*GATA2*, *POU1F1* and *NR5A1*) in a reaction containing 5 μl 10× restriction buffer H (Promega), 0.5 μl acylated bovine serum albumin (10 mg/ml), 2.5 μl of each *Eco*RI and *Xba*I or *Mlu*I and *Xho*I (12 U/μl) and nuclease-free water to a total of 50 μl. This mix was incubated at 37 °C for 4 h and subjected to agarose gel electrophoresis. The fragments were excised and purified as above.

A ligation reaction was performed using 1 U T4 DNA ligase (Promega), 1 μl 10× ligase buffer (Promega), 100 ng digested vector and the appropriate amount of insert (3:1 M vector-to-insert ratio). The mix was incubated overnight in a thermocycler at 22 °C. Transformation of DH5α competent cells with the ligation reaction product was performed following the manufacturer's protocol. The Purelink Quick Plasmid Midiprep Kit was used to purify the pCI and pGL4 vectors containing the coding sequence for the chicken TFs and cp*CRHR2* fragments, respectively, and the orientation and sequence of the inserts were confirmed by sequencing.

2.4. Site-directed mutagenesis

Substitution mutations were introduced in GATA2 and PITX1 REs using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Ipswitch, MA, USA) following the manufacturer's instructions. The template DNA used was 100 ng pGL4.10 plasmid containing a fragment of cp*CRHR2*, from bp –198 to +15 relative to the start codon. Mutations were created in two predicted GATA2 REs, located from bp −118 to −113 and from bp −192 to −178, and two of the predicted PITX1 REs, located from bp −197 to −192 and from bp −145 to −140, using the primers and annealing temperatures listed in Table S1 with an elongation time of 2 min (also see Fig. S3). The PCR products were analysed on an agarose gel as described above. PCR products were subjected to the 'kinase, ligase and *Dpn*I' treatment, incubated for 30 min at room temperature, and used for transformation of NEB 5α Competent *E. coli* cells (New England Biolabs). Five colonies from each reaction, grown overnight on LB agar with 100 μg/ml ampicillin (Bioline), were selected. These colonies were then grown for 8 h in 5 ml LB medium containing 100 μg/ml ampicillin and 1 ml of the resulting bacterial culture was grown overnight in 49 ml LB medium with 100 mg/ml ampicillin. The plasmids were purified with the Purelink Quick Plasmid Midiprep Kit. Mutations were confirmed by sequencing and sequence alignment using the default settings of Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Purified constructs containing the desired mutation were used for subsequent transfection experiments.

2.5. Cell culture and transient transfection

For all transfection experiments, COS-7 cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with pyruvate (Invitrogen) containing 10% (v/v) foetal bovine serum (Thermo Fisher Scientific) and 1% (v/v) penicillin–streptomycin (10,000 U/ml; Invitrogen). Cells were seeded in 24-well plates coated with 0.1% (w/v) gelatine solution 24 h prior to transfection (Sigma-Aldrich, St. Louis, MO, USA) at a density of 5×10^4 cells per well in 500 μl of DMEM containing 10% foetal bovine serum. The cells were maintained in an incubator at 37 °C with 5% CO_2/95% air. The cell density on the day of transfection was 80–90% confluent. Cells were transfected with the relevant plasmids (as specified below) in 2 μl of Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions. Transfected cells remained in the CO_2 incubator for a total of 48 h at 37 °C. Media were refreshed 24 h post transfection. All transfections were performed in triplicate in three separate experiments. Cell lysates were collected by first removing all media and washing cells in 1× PBS. Passive Lysis Buffer (1×, Promega) was added and cells were removed with gentle rocking for 15 min. Firefly and *Renilla* luciferase activity was measured using a GloMax®-96 well Microplate Luminometer (Promega) using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's guidelines. Firefly activity was normalised to *Renilla* luciferase activity.

2.5.1. Deletion analysis to localise GATA2 binding sites in cpCRHR2

pGL4 plasmids containing a chicken *CRHR2* promoter (cp*CRHR2*) fragment of 500 bp, 997 bp, 1498 bp, 2000 bp, or 2500 bp upstream of the *CRHR2* start codon were co-transfected with pCI-GATA2. Each well of cells was transfected with 600 ng of promoter–luciferase plasmid and 120 ng of pCI-GATA2, as well as 5 ng of pRL-null vector (Promega) as an internal transfection control. The experiment was then repeated with pGL4 plasmids containing shorter cp*CRHR2* fragments of 500 bp, 416 bp, 359 bp, 198 bp, 116 bp or 62 bp, co-transfected with pCI-GATA2.

2.5.2. Effect of POU1F1 on GATA2-induced cpCRHR2 activity

pGL4 plasmids containing either cp*CRHR2* (bp −500/+15) or no promoter were transfected alongside pCI-POU1F1 and/or pCI-GATA2. Cells in treatment wells were transfected with 600 ng of pGL4 plasmid, 115 ng of pCI-GATA2 and/or 85 ng of pCI-POU1F1, and 5 ng of pRL-null. These vector amounts were based on previous studies (Gordon et al., 1997; Kashiwabara et al., 2009). The amount of expression vector DNA in all transfections in all experiments was equalised to 200 ng with empty pCI vector, and transfection with 200 ng of empty pCI vector was used as a negative control.

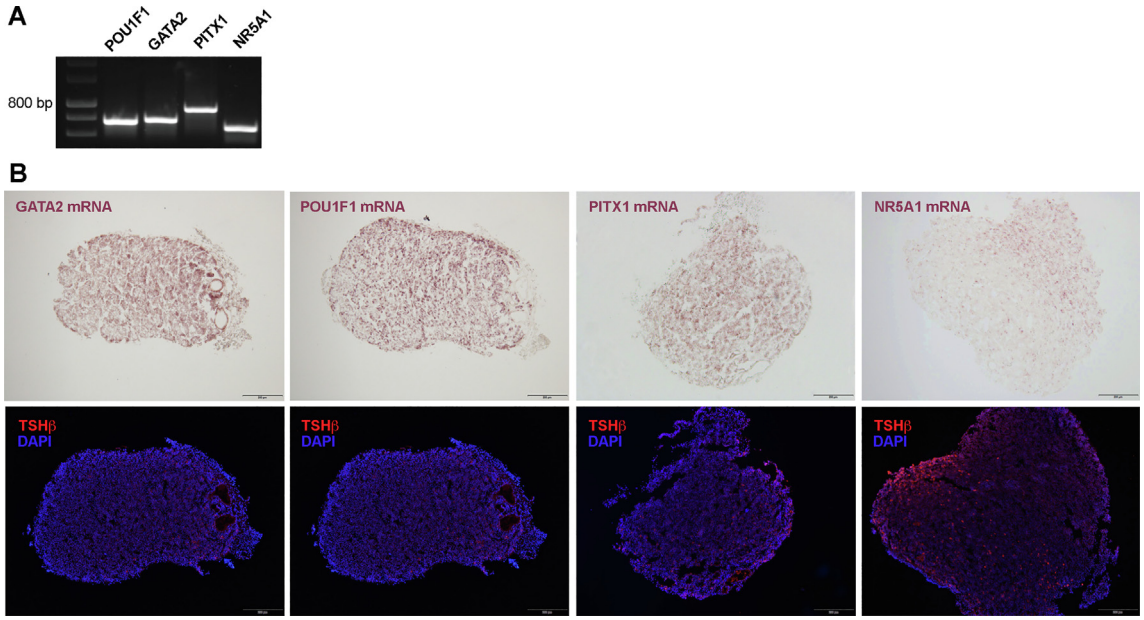

Fig. 1. mRNA expression of transcription factors in the pituitary gland of the chicken as shown by RT-PCR (A), and in situ hybridisation (B). A: Gel electrophoresis of partial sequences of transcription factors amplified by RT-PCR. The first lane shows a 1-kb DNA ladder. B: Top row: Purple staining represents transcription factor mRNA expression as indicated. Bottom row: Red staining represents TSHB protein expression (i.e. thyrotropes), while the blue staining is a nuclear counterstain. Pituitary glands were sectioned along different planes; parts with red staining represent the cephalic lobe and parts without red staining represent the caudal lobe of the anterior pituitary. Scale bars represent 200 μm.

2.5.3. Effect of NR5A1 on GATA2-induced cpCRHR2 activity

pGL4 plasmids containing either cp*CRHR2* (bp −500/+15) or no promoter were transfected alongside pCI-NR5A1 and/or pCI-GATA2. Treatment wells were transfected with 600 ng of pGL4 plasmid, 100 ng of pCI-GATA2, 100 ng of pCI-NR5A1 and 5 ng of pRL-null.

2.5.4. Effect of mutated GATA2 REs on GATA2-induced cpCRHR2 activity

pGL4 plasmids containing wild-type cp*CRHR2* (bp −198/+15) or cp*CRHR2* with a mutation in the first putative GATA2 RE (bp −192 to −178) or a mutation in the second putative GATA2 RE (bp −118 to −113) were transfected alongside pCI-GATA2. Treatment wells were transfected with 600 ng of pGL4 plasmid and 5 ng of pRL-null. Half of the wells in each condition were also transfected with 200 ng of pCI-GATA2 and the other half received 200 ng of empty pCI as negative controls.

2.5.5. Effect of PITX1 on GATA2-induced on cpCRHR2 activity

pGL4 plasmids containing cp*CRHR2* (bp −500/+15) or no promoter were transfected alongside pCI-PITX1 and pCI-GATA2. Each well of cells was transfected with 600 ng of pGL4 plasmid and 5 ng of pRL-null. Treatment wells also received 120 ng of pCI-GATA2 and/or 80 ng of pCI-PITX1, and transfection with 200 ng of empty pCI was used as negative control.

2.5.6. Effect of NR5A1 on PITX1-induced cpCRHR2 activity

pGL4 plasmids containing cp*CRHR2* (bp −500/+15) or no promoter were transfected with pCI-NR5A1 and/or pCI-PITX1. Treatment wells were transfected with 600 ng of pGL4 plasmid, 79 ng of pCI-PITX1 and/or 121 ng of pCI-NR5A1 and 5 ng of pRL-null. The amount of these vectors were changed to reflect the ratio of chicken PITX1 and chicken NR5A1, based on the size of the coding sequence. Transfection with 200 ng of empty pCI was used as negative control.

2.5.7. Effect of mutated PITX1 REs on PITX1-induced cpCRHR2 activity

pGL4 plasmids containing either wild-type cp*CRHR2* (bp −198/+15), cp*CRHR2* with a mutation in a first putative PITX1 RE (bp −197 to −192) or a mutation in a second putative PITX1 RE (bp −145 to −140), or no promoter were used for transfection. Each well was transfected with 600 ng of pGL4 plasmid and 5 ng of pRL-null. Half of the wells in each condition were also transfected with 200 ng of pCI-PITX1 and the other half received 200 ng of empty pCI as negative control.

2.5.8. Effect of mutated GATA2 REs on PITX1-induced cpCRHR2 activity

pGL4 plasmids containing either wild-type cp*CRHR2* (bp −198/+15), cp*CRHR2* with the first GATA2 RE (bp −192 to −178) mutated, or no promoter were used for transfection in the presence of either pCI-PITX1 alone or a combination of pCI-PITX1 and pCI-GATA2. Each well of cells was transfected with 600 ng of pGL4 plasmid and 5 ng of pRL-null. Those treatment wells that received only pCI-PITX1 received 200 ng of pCI-PITX1 and those that received both pCI-PITX1 and pCI-GATA2 received 120 and 80 ng, respectively. Transfection with 200 ng of empty pCI was used as negative control.

2.6. Bioinformatics

Putative REs within 500 bp upstream of the start codon of cp*CRHR2* were searched using the online transcription factor binding profile database JASPAR (http://jaspar.genereg.net; used to detect GATA2, POU1F1, PITX1 and PITX2 REs) and AliBaba 2.0 (http://gene-regulation.com/pub/programs/alibaba2/index.html; used to detect NR5A1 REs). NCBI (https://www.ncbi.nlm.nih.gov/) and Ensembl (http://asia.ensembl.org/index.html) genome assemblies were used to search for the 5′ promoter region of the CRHR2 in the following species: *Gallus gallus* (NC_006089.3), *Alligator sinensis* (NW_005842056.1), *Chrysemys picta* (NW_007359874.1), *Xenopus tropicalis* (NW_016683377.1), *Salmo salar* (NC_027302.1), *Oreochromis niloticus* (ENSONIG00000009454.1), *Meleagris gallopavo* (XM_003206922.3) and *Taeniopygia guttata* (XM_002196341.2).

2.7. Statistical analysis

Luciferase activity data were analysed using the statistical software package IBM SPSS statistics version 25. Normalised firefly luciferase

activity levels were analysed by one-way ANOVA, with post-hoc comparisons using Tukey's HSD test. Means were considered statistically significant if $P < 0.05$.

3. Results

3.1. Localisation of TF expression in the chicken pituitary gland

Using RT-PCR, *GATA2*, *POU1F1*, *PITX1* and *NR5A1* mRNA could be detected in the pituitary gland of chickens (Fig. 1A). We attempted to investigate the expression of these transcription factors in the thyrotropes specifically by a combination of in situ hybridisation and immunofluorescent staining of TSHB, but we were unable to combine both techniques on the same tissue section. Consecutive sections suggest that *GATA2*, *POU1F1* and *PITX1* mRNA are expressed in areas of the pituitary gland that also contain thyrotropes, but *NR5A1* mRNA was typically concentrated in areas without thyrotropes (Fig. 1B). Negative controls for in situ hybridisation using sense probes showed no signal (Fig. S1).

3.2. Effect of POU1F1 and GATA2 on cpCRHR2 activity

We generated pGL4.10[*luc2*] reporter constructs containing serially truncated segments of the 5′ flanking region of the chicken *CRHR2* (cp*CRHR2*). Fig. 2A shows the luciferase activity in COS-7 cells co-transfected with these deletion constructs and chicken GATA2- and POU1F1-expressing vectors. Transfection with the promoter construct containing 116 bp upstream of the start codon (−116/+15) did not result in an increase in luciferase activity compared to the promoterless vector (ANOVA, $P > 0.9999$). However, transfection with promoter constructs containing bp −198/+15, −416/+15, −997/+15 and −1536/+15 resulted in a significant ~5-fold increase in luciferase activity compared to the promoterless control ($P < 0.0001$). Transfection with constructs containing bp −359/+15 and −500/+15 resulted in a ~7-fold increase ($P < 0.0001$) and the bp −2000/+15 construct resulted in a 19-fold increase in luciferase activity compared to the promoterless control ($P < 0.0001$). However, the mean luciferase activity was not significantly different with construct −198/+15 compared with the −997/+15 ($P = 0.78$) and −1536/+15 constructs ($P = 0.60$). These results indicate that the combination of GATA2 and POU1F1 is able to stimulate cp*CRHR2* activity and that essential REs for these TFs are located between bp −198 and −116, although REs further upstream may enhance cp*CRHR2* activity further.

3.3. Localisation of GATA2 REs in cpCRHR2

To identify the location of the GATA2 RE within the promoter region involved in regulating chicken *CRHR2* expression, the luciferase reporter constructs containing serially truncated segments of the 5′ flanking region of chicken *CRHR2* were used. Fig. 2B shows luciferase activity in COS-7 cells co-transfected with these deletion constructs and chicken GATA2-expressing vectors. Transfection with promoter constructs containing 2500, 2000 or ~1500 bp upstream of the *CRHR2* start codon (−2500/+15, −2000/+15 and −1536/+15, respectively) did not result in a significant change in luciferase activity compared to the promoterless vector (ANOVA, $P > 0.05$). Deletion of the promoter fragment to bp −997 or −500 resulted in a significant ~15-fold increase in promoter activity compared to the promoterless vector ($P < 0.01$).

Further progressive deletion of the 5′-flanking sequence of chicken *CRHR2* led to a series of constructs containing sequences from bp −500 to −62 (Fig. 2C). Luciferase activity was not significantly different between constructs −500/+15, −416/+15 and −359/+15 (ANOVA, $P > 0.05$). The GATA2-induced activity of construct −198/+15 was significantly lower than that of −416/+15 ($P < 0.05$), whereas activities of −500/+15, −416/+15, −359/+15 and −198/+15 were all significantly higher than that of the promoterless vector ($P < 0.05$). Deletion to bp −116 resulted in a ~5-fold reduction of reporter activity ($P < 0.01$) compared to the longer constructs and lowered the activity to the level of the promoterless vector. Further deletion to bp −62 did not affect activity further. The results show, unexpectedly, that GATA2 is capable of activating cp*CRHR2* in the absence of POU1F1, and that the REs regulating this interaction are located in the same DNA fragment as that identified in the previous experiment, i.e. between bp −198 and −116.

3.4. Putative REs in cpCRHR2

The JASPAR tool was used to search for putative REs (Fig. S2) in the 500 bp upstream of the start codon of chicken *CRHR2*. Using the programme's default threshold of 80%, 15 GATA2 REs, 3 PITX1 REs, a TATA box and a GC box were found in either the forward or reverse direction, but no potential REs for POU1F1 were detected (not shown). When the threshold was lowered to 72%, 30 putative GATA2 REs, 2 POU1F1 REs and 26 PITX1 REs were detected; some of these REs are presented in Fig. S3. Two GATA2 REs, 4 PITX1 REs and no POU1F1 RE sites were found within the segment bp −198 to −116 of cp*CRHR2*; however, two PITX1 REs overlapped with the TATA box and GC box REs. To check the accuracy of the programme, we also analysed the promoter of mouse *Tshb*, known to contain a POU1F1 RE and GATA2 REs, with default settings (not shown). The programme detected the two functional GATA2 REs in the mouse *Tshb* promoter (Gordon et al., 1997, 2002; Kawasaki et al., 2003) at the threshold of 80%, but the POU1F1 RE detected was different to the reported RE (Steinfelder et al., 1992; Lin et al., 1994; Kawasaki et al., 2003). The latter was detected by the JASPAR programme at a threshold of 67%.

3.5. Effect of mutated GATA2 REs on GATA2-induced cpCRHR2 activity

Next, we determined which of the two putative GATA2 REs within bp −198 to −116 of cp*CRHR2* is responsible for GATA2-induced luciferase activity. Cells were transfected with either the wild-type −198/+15 promoter–reporter construct, or the same construct containing a mutation in the core sequence of the first putative GATA2 RE (bp −188 to −184) or in the second GATA2 RE (bp −118 to −114) (Fig. S4A), or a promoterless reporter plasmid, either with or without a GATA2-expressing vector. GATA2-induced activity was significantly reduced when the first GATA2 was mutated ($P = 0.02$) (Fig. 3). However, when a mutation was introduced in the second putative GATA2 RE, promoter activity remained the same as the wild-type vector in the presence of GATA2 ($P = 0.10$) (Fig. 3). This indicates that the first GATA2 RE (bp −188/−184), but not the second, is essential for GATA2-induced cp*CRHR2* activation.

3.6. Effect of POU1F1, NR5A1 and PITX1 on GATA2-induced cpCRHR2 activity

To determine whether *CRHR2* expression is regulated by synergy between GATA2 and POU1F1, as reported for human and mouse *TSHB*/*Tshb*, a vector construct containing bp −500 to +15 relative to the start codon of chicken *CRHR2* was transfected in the presence or absence of POU1F1 and/or GATA2 expression vectors into COS-7 cells. The presence of GATA2 alone caused a significant ~40-fold increase compared to the unstimulated promoter ($P < 0.0001$) (Fig. 4A). In the presence of POU1F1 alone, promoter activity was not significantly increased ($P = 0.78$). A significant increase in luciferase activity was detected in the presence of both POU1F1 and GATA2, compared to the unstimulated promoter ($P < 0.0001$), to the GATA2-stimulated promoter ($P < 0.0001$) and to the POU1F1-stimulated promoter ($P < 0.0001$) (Fig. 4A). These data reveal that while GATA2 alone is sufficient to activate cp*CRHR2*, POU1F1, by itself not capable of promoter activation, has a synergistic effect.

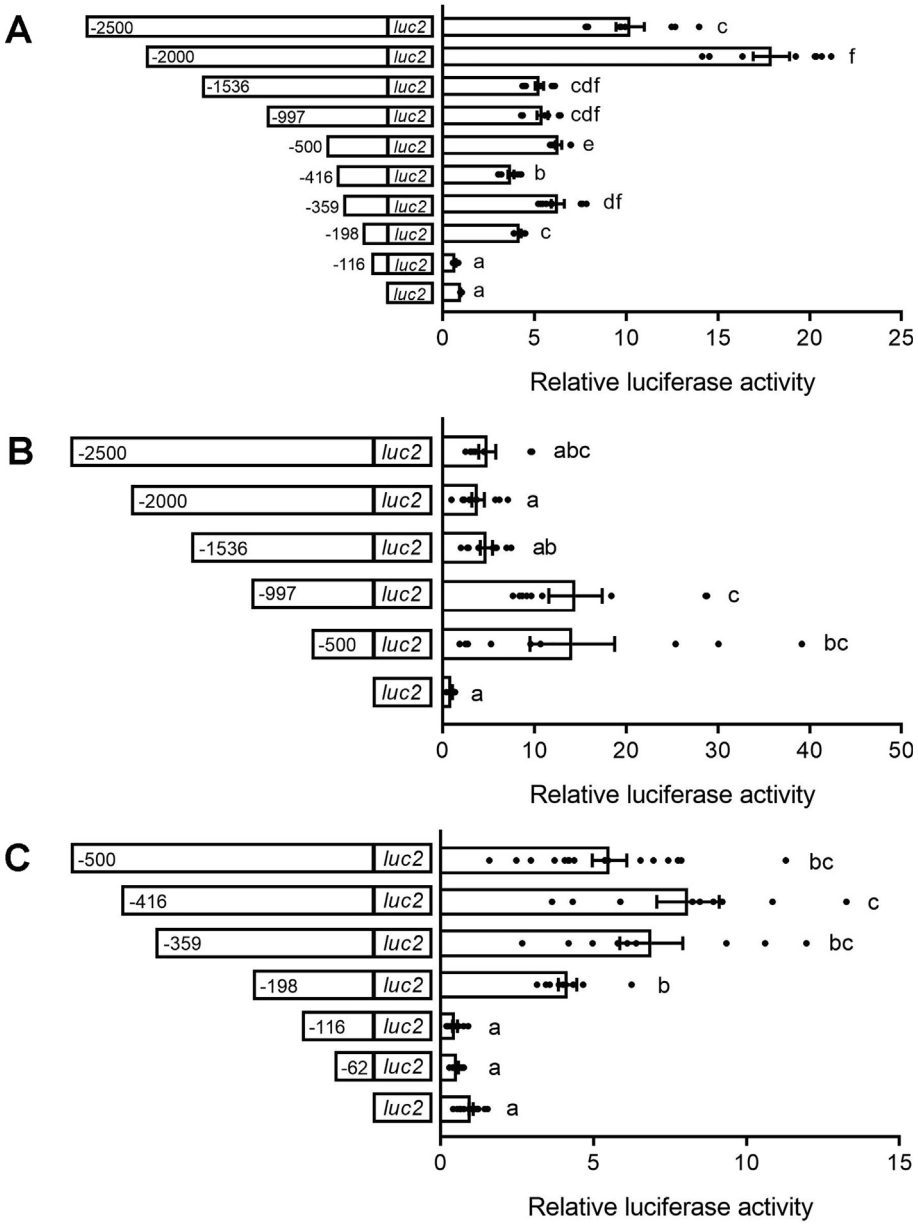

Fig. 2. Relative luciferase activity (right) in COS-7 cells transfected with pGL4.10[*luc2*] reporter constructs containing serially truncated segments of the 5′ flanking region of chicken *CRHR2* (left) along with chicken GATA2- and/or POU1F1-expressing plasmids. Values were normalised to *Renilla* luciferase activity, and are expressed as fold induction compared to empty pGL4. Each construct was tested three times in triplicate, and the bars represent means ± SEM. Data without a common letter are significantly different (ANOVA, $P < 0.05$). (A) Serially truncated segments from bp −2500 to −116, stimulated with both GATA2 and POU1F1. (B) Serially truncated segments from bp −2500 to −500, stimulated with GATA2 alone. (C) Serially truncated segments from bp −500 to −62, stimulated with GATA2 alone.

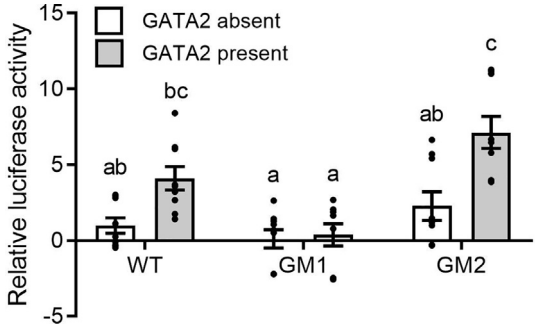

Fig. 3. Relative luciferase activity in COS-7 cells transfected with wild-type (−198/+15) cp*CRHR2* construct (WT), or (−198/+15) cp*CRHR2* with either the first (GM1) or the second (GM2) GATA2 response element mutated, in the presence or absence of GATA2. Each condition was tested three times in triplicate, and bars represent means ± SEM. Values were normalised to *Renilla* luciferase activity and activity of the promoterless vector, and are expressed as fold induction compared to the activity of the unstimulated WT promoter. Data without a common letter are significantly different (ANOVA, $P < 0.05$).

Next, we determined whether NR5A1 could suppress GATA2-induced cp*CRHR2* activity. Using the construct containing bp −500/+15 of cp*CRHR2* in the presence of GATA2 alone resulted in a significant ~30-fold increase in activity over the unstimulated promoter ($P < 0.0001$) (Fig. 4B). This activity was significantly reduced, but not completely abolished, in the presence of NR5A1 ($P < 0.0001$). The presence of NR5A1 alone did not change cp*CRHR2* activity to background levels ($P > 0.9999$) (Fig. 4B). Therefore, NR5A1 is capable of reducing GATA2-induced cp*CRHR2* activity.

To determine whether the putative PITX1 REs located within 500 bp upstream of the chicken *CRHR2* start codon have an effect on the expression of *CRHR2*, the −500/+15 promoter construct was transfected with or without PITX1 and/or GATA2 expression plasmids. A significant ~16-fold increase in activity was observed in the presence of both PITX1 and GATA2 compared to the unstimulated promoter ($P < 0.0001$) (Fig. 4C). PITX1 alone also significantly stimulated the chicken *CRHR2* promoter ($P < 0.0001$), but this was not the case for GATA2 alone in this experiment ($P = 0.12$) (Fig. 4C). We conclude that PITX1 is also a stimulator of cp*CRHR2* activity.

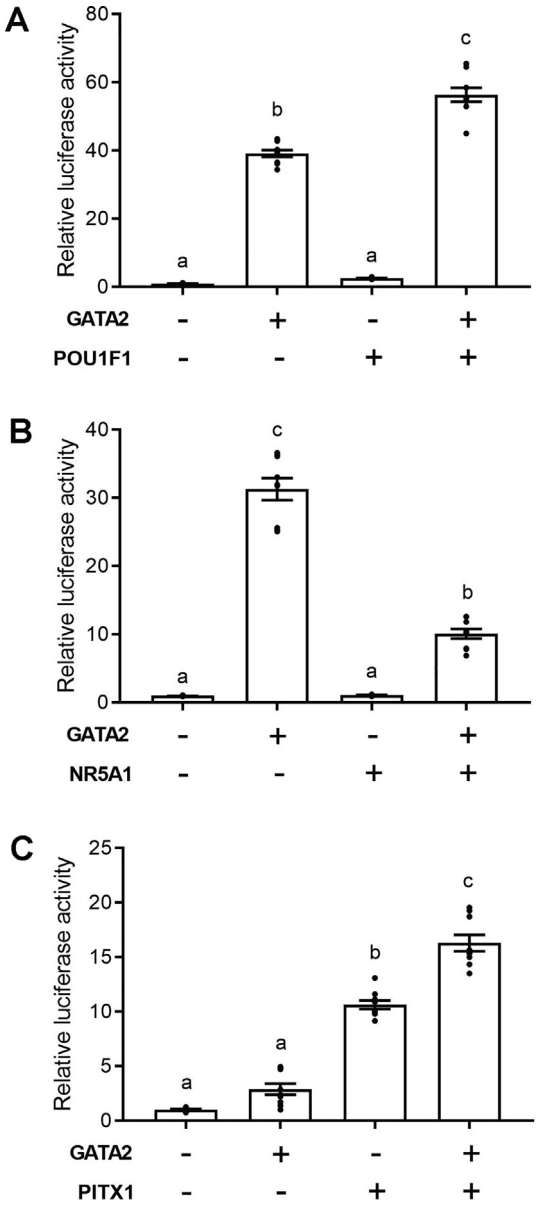

Fig. 4. Relative luciferase activity in COS-7 cells transfected with cp*CRHR2*-containing pGL4 vector in the presence or absence of chicken transcription factors, as indicated below the graphs. Each condition was tested three times in triplicate, and bars represent means ± SEM. Values were normalised to *Renilla* luciferase activity and activity of the promoterless vector, and are expressed as fold induction compared to the activity of the unstimulated promoter. Data without a common letter are significantly different (ANOVA, $P < 0.05$). (A) Relative luciferase activity of cp*CRHR2* (−500/+15) in the presence or absence of GATA2 and/or POU1F1. (B) Relative luciferase activity of cp*CRHR2* (−500/+15) in the presence or absence of GATA2 and/or NR5A1. (C) Relative luciferase activity of cp*CRHR2* (−198/+15) in the presence or absence of GATA2 and/or PITX1.

3.7. Effect of NR5A1 and mutated PITX1 or GATA2 REs on PITX1-induced cpCRHR2 activity

We then investigated whether NR5A1 can also suppress PITX1-induced cp*CRHR2* activity. Maximal activity was seen in the presence of PITX1 alone, a significant ~129-fold increase over the unstimulated promoter ($P < 0.0001$) (Fig. 5A). However, PITX1-induced activity was significantly decreased (~17-fold) by the addition of NR5A1 ($P < 0.0001$). The presence of NR5A1 alone did not change promoter activity ($P = 0.9999$) (Fig. 5A). These results show that NR5A1

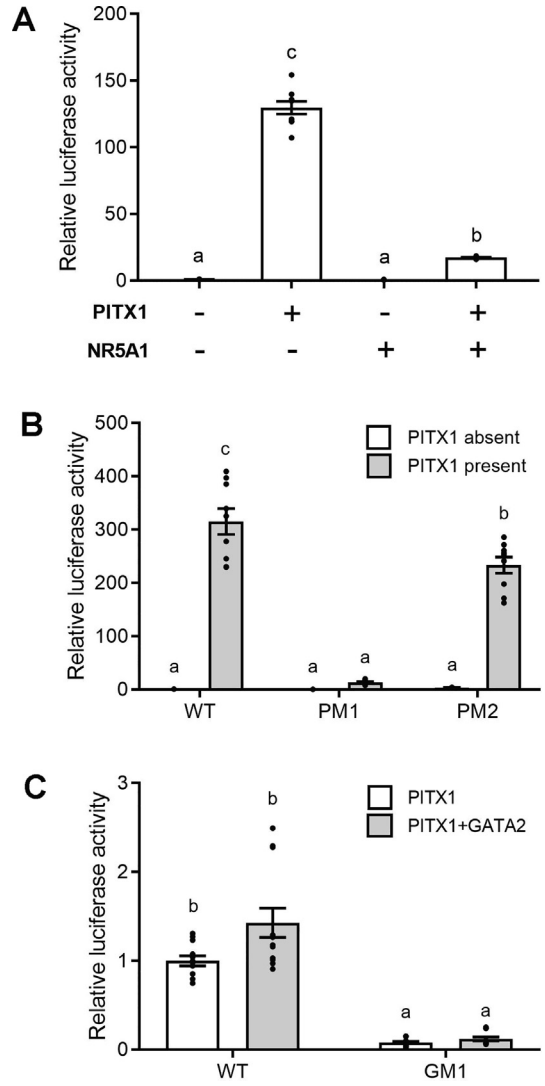

Fig. 5. (A) Relative luciferase activity in COS-7 cells transfected with (−198/+15) cp*CRHR2*-containing pGL4 vector in the presence or absence of PITX1 and/or NR5A1. B: Relative luciferase activity in COS-7 cells transfected with wild-type (−198/+15) cp*CRHR2* construct (WT), or (−198/+15) cp*CRHR2* with either the first (PM1) or the second (PM2) PITX1 response element mutated, in the presence or absence of PITX1. (C) Relative luciferase activity in COS-7 cells transfected with wild-type (−198/+15) cp*CRHR2* construct (WT), or (−198/+15) cp*CRHR2* with the first GATA2 response element mutated (GM1), in the presence of PITX1 and the presence or absence of GATA2. Each condition in A, B and C was tested three times in triplicate, and bars represent means ± SEM. Values were normalised to *Renilla* luciferase activity and activity of the promoterless vector, and are expressed as fold induction compared to the activity of the unstimulated promoter. Data without a common letter are significantly different (ANOVA, $P < 0.05$).

suppresses PITX1-induced cp*CRHR2* activity like it does with GATA2-induced activity.

Next, we determined which PITX1 REs within bp −198/+15 of cp*CRHR2* are responsible for PITX1-induced cp*CRHR2* activity. Therefore, two putative PITX1 sites identified by JASPAR, a first RE located at bp −197 to −192 and a second RE between bp −145 and −140, were mutated (Fig. S4B). In the presence of PITX1, the wild-type promoter showed the highest activity levels (Fig. 5B). However, when the first PITX1 RE was mutated, this resulted in a significant reduction in activity compared to the stimulated wild-type promoter ($P < 0.0001$). Mutation of the second PITX1 RE caused a significant reduction in PITX1-stimulated activity compared to the wild-type

promoter ($P < 0.0001$), but this activity level was still ~230-fold higher than unstimulated levels ($P < 0.0001$) (Fig. 5B). Thus, while the second PITX1 RE may be involved in PITX1-induced promoter activity, the first RE (bp −197/−192) seems to play a larger role.

To determine whether PITX1 and GATA2 act synergistically, and whether PITX1 requires bound GATA2 to induce a response, we co-transfected either wild-type cp*CRHR2* (−198/+15), cp*CRHR2* with the first GATA2 RE mutated or a promoterless vector, with either a PITX1 expression vector alone or a combination of PITX1- and GATA2-expressing vectors. The activity of the promoter with the mutated GATA2 RE was significantly lower when stimulated with either PITX1 alone or with the combination of PITX1 and GATA2 ($P < 0.0001$) (Fig. 5C). These results show that PITX1 is no longer capable of stimulating cp*CRHR2* activity when the first GATA2 RE is non-functional, suggesting that PITX1 requires GATA2 bound to the this RE in order to activate the promoter.

3.8. Effect of NR5A1 on GATA2- and PITX1-induced cpCRHR2 activity

Lastly, we investigated the activity of cp*CRHR2* (−500/+15) stimulated with both PITX1 and GATA2, in the presence or absence of NR5A1. The highest activity was observed in the presence of PITX1 and GATA2 without NR5A1, a significant ~160-fold increase over the wild-type control in the absence of all TFs ($P < 0.0001$) (Fig. 6). The addition of NR5A1 significantly suppressed the stimulatory effect of PITX1 and GATA2 ($P < 0.0001$), but activity was still significantly higher than that of the unstimulated promoter ($P < 0.0001$) (Fig. 6). Therefore, in the presence of NR5A1, GATA2- and PITX1-induced cp*CRHR2* activation is suppressed.

3.9. Evolutionary conservation of GATA2 and PITX1 REs in the crhr2 gene promoter

Genome mining showed that the promoter of *crhr2/CRHR2* contains a putative PITX1 RE in various selected non-mammalian vertebrate species, including birds, reptiles, amphibians and fishes (Fig. 7). All putative PITX1 RE sites were recognised by the JASPAR programme at a 78% or higher threshold. The GATA2 REs were recognised by JASPAR at a 70% or higher threshold. In zebra finch, chicken, turkey (*Meleagris gallopavo*), western clawed frog (*Xenopus tropicalis*), Nile tilapia (*Oreochromis niloticus*) and Atlantic salmon (*Salmo salar*, CRHR2-like isoform 2), the putative PITX1 RE is located within 20 bp upstream of a putative GATA2 RE. In other species, i.e. the painted turtle (*Chrysemys picta*) and the Chinese alligator (*Alligator sinensis*), the putative PITX1 and GATA2 REs are spaced further apart (Fig. 7). The location of the GATA2 and PITX1 REs is roughly within the first 200 bp upstream of the start codon in all species investigated.

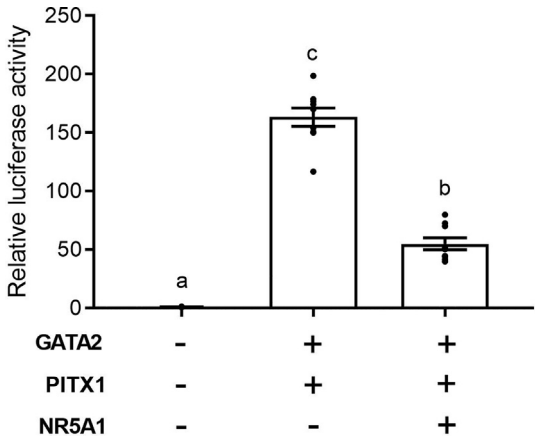

Fig. 6. Relative luciferase activity in COS-7 cells transfected with (−500/+15) cp*CRHR2*-containing pGL4 vector in the presence or absence of chicken transcription factors, as indicated below the graph. Each condition was tested three times in triplicate, and bars represent means ± SEM. Values were normalised to *Renilla* luciferase activity and activity of the promoterless vector, and are expressed as fold induction compared to the activity of the unstimulated promoter. Data without a common letter are significantly different (ANOVA, $P < 0.05$).

4. Discussion

The effect of the chicken TFs GATA2, POU1F1, PITX1 and NR5A1 on cp*CRHR2* activity was investigated, as to our knowledge, there are no studies that have been performed to elucidate the mechanisms that drive *CRHR2* expression in the thyrotropes, which allows CRH to act as a thyrotropic factor in certain species, like chicken.

In mouse, the *Tshb* gene, almost exclusively expressed in thyrotropes, is transcribed by synergy of GATA2 with POU1F1, whereby POU1F1 is required to block a suppressor region in the *Tshb* promoter that, in the absence of POU1F1, inhibits GATA2-induced *Tshb* expression (Kashiwabara et al., 2009). This promoter region of the mouse *Tshb* gene is located within 128 bp upstream of the start codon of the gene, and the suppressor region is located between bp −82 and −52 (Kashiwabara et al., 2009). This explains why TSHB is only expressed in thyrotropes, which contain both GATA2 and POU1F1, but not in gonadotropes, which express GATA2 but not POU1F1, nor in somatotropes that express POU1F1 but not GATA2. Induced expression of POU1F1 can convert gonadotropes into thyrotropes (Dasen et al., 1999). We speculated that a similar mechanism is responsible for the thyrotrope-specific expression of CRHR2 in non-mammalian species, like chicken, in which CRH is a TSH-releasing factor, an interaction mediated by CRHR2 on the thyrotropes (De Groef et al., 2003; Watanabe et al., 2016). Chicken POU1F1 is known to have four isoforms: α, β1, β2 and γ (the latter also referred to as PIT1W to distinguish it from mammalian PIT1γ). Only three of these (α, β1 and β2) are known to activate the promoter of the growth hormone gene (Mukherjee and Porter, 2012; Van As et al., 2000). In the current study, the α isoform of POU1F1 could only activate cp*CRHR2* if GATA2 was present, even though chicken POU1F1 was effectively expressed in the COS-7 cells after transfection, as verified by RT-PCR (Fig. S5). POU1F1α did synergistically increase the effect of GATA2 on cp*CRHR2* activation, possibly through binding one or more of the putative REs detected by JASPAR in the −500/+15 cp*CRHR2* fragment. Further analysis is required to determine which of these REs are functional. We also tested the ability of the γ isoform to stimulate cp*CRHR2* activity with or without GATA2, but similar to the growth hormone gene, the γ isoform was unable to activate cp*CRHR2* by itself nor did interact synergistically with GATA2 (Fig. S6).

Interestingly, in contrast to the mammalian *Tshb/TSHB* gene, GATA2 alone was sufficient to induce activity of the bp −198/+15 cp*CRHR2* fragment. A GATA2 RE, located between bp −178 and −192, was suggested by site-directed mutagenesis to mediate this interaction. It should be noted that we still saw an increase in luciferase activity of the promoterless vector in the presence of GATA2, despite the fact that the upgrade of the pGL3 basic vector to pGL4.10 was reported by the manufacturer to have resulted in a reduction of background luciferase activity and deletion of a GATA binding site (Promega, pGL4 Luciferase Reporter Vectors Technical Manual TM259), and the deletion of two additional putative GATA2 binding sites (Dougherty and Sanders, 2005). Analysis of the entire pGL4.10 sequence with JASPAR at an 80% threshold resulted in the detection of ten putative GATA2 binding sites, including one within the coding sequence of the *luc2* luciferase gene (data not shown). This necessitated the use of additional negative controls and correction for the activity of the promoterless vector in all experiments. pGL vectors have commonly been used to study GATA2 reporter activity (e.g. Kim et al., 2009; Simon et al., 2008), but we recommend the use of a different reporter vector to study GATA2 in future experiments to avoid these

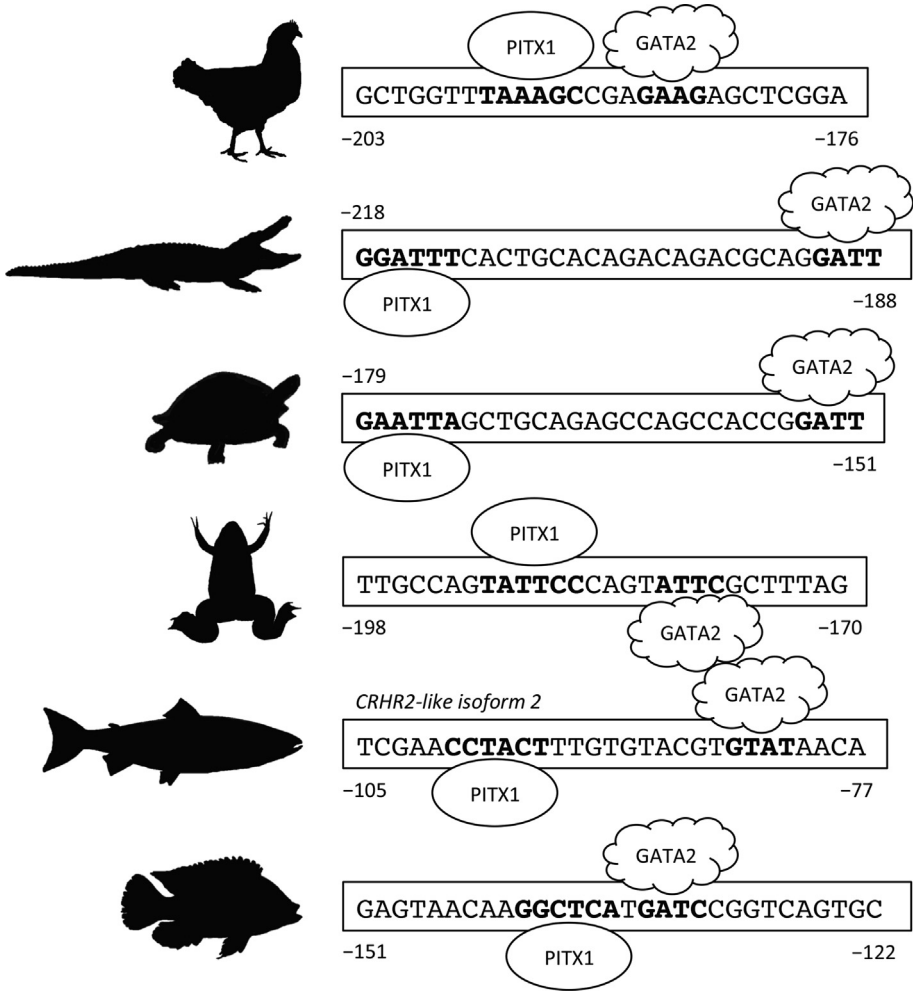

Fig. 7. Conservation of the GATA2 and PITX1 response elements responsible for transcriptional activation of *CRHR2/crhr2* in selected non-mammalian vertebrate species as detected by JASPAR. Threshold settings were as follows: *Gallus gallus*: PITX1 79%, GATA2 72%; *Alligator sinensis*: PITX1 82%, GATA2 70%; *Chrysemys picta*: PITX1 87%, GATA2 89%; *Xenopus tropicalis*: PITX1 78%, GATA2 72%; *Salmo salar*: PITX1 87%, GATA2 80%; *Oreochromis niloticus*: PITX1 78%, GATA2 85%.

complications.

The stimulatory effect of GATA2 was further enhanced by PITX1, a TF that is expressed in most pituitary cell types in mouse, including the thyrotropes. Using bioinformatics, we showed that there are putative REs for GATA2 and PITX1 within the first 220 bp upstream of the start codon of *crhr2/CRHR2* in several non-mammalian vertebrate species, including species in which CRH is a thyrotropic factor and/or CRHR2 is expressed on the thyrotropes – chicken, zebra finch, *Xenopus* and salmon (De Groef et al., 2003; Larsen et al., 1998; Okada et al., 2007; Watanabe et al., 2017). This may indicate that the thyrotropes of other non-mammalian vertebrate species express CRHR2 as well, or in other words, that CRH is a thyrotropic factor in all these species. We also showed that PITX1 exerts its effect by binding a RE close to the GATA2 RE, but only when this GATA2 RE is functional (i.e. not mutated). This suggests that a protein–protein interaction between PITX1 and GATA2 is enabling activation of cp*CRHR2*. PITX1 is known to activate the promoters of many hormone-coding genes within the pituitary gland, seemingly in co-operation with cell-specific TFs (Tremblay et al., 1998). PITX1 is known to physically interact with other TFs, namely basic helix–loop–helix TFs, to control the transcription of the pro-opiomelanocortin gene in rodents (Poulin et al., 2000). The requirement of a functional GATA2 RE for PITX1 to stimulate cp*CRHR2* seems contradictory with the observation that PITX1 is able to activate cp*CRHR2* in the absence of exogenous GATA2, but this activation could be explained by interaction of PITX1 with endogenous GATA2 within the COS-7 cells. COS-7 cells are derived from green monkey kidney cells, known to express GATA2 (Yu et al., 2014). It is therefore likely that COS-7 cells express GATA2 as well, as indicated by western blotting of COS-7 cell protein extracts with an anti-GATA2 antibody (Aviva Systems Biology, Sand Diego, CA, USA; product protocol sheet OAAF00202). Further experiments, like chromatin immunoprecipitation and electrophoretic mobility shift assays, are required to unequivocally demonstrate binding of GATA2 and PITX1 to the REs identified here through promoter activity assays and mutagenesis.

If GATA2 alone, or in conjunction with PITX1, is able to stimulate the expression of *CRHR2*, this raises the question why *CRHR2* is not more strongly expressed by gonadotropes, which also express GATA2 and PITX1. In the chicken pituitary gland, *CRHR2* mRNA is largely confined to the thyrotropes (De Groef et al., 2003). We therefore hypothesised that in the gonadotropes, *CRHR2* expression is suppressed by NR5A1. NR5A1 is expressed in the mammalian pituitary gland as early as embryonic day 13.5 and is known to not only be involved in gonadotrope-specific *Lhb* gene expression, but also the β-subunit of follicle-stimulating hormone (Baratta et al., 2003; Brown and McNeilly, 1997; Tremblay et al., 1998; Zhu et al., 2005). In the current study, we showed that NR5A1 suppressed GATA2 + PITX1-induced activity of cp*CRHR2*, suggesting that this mechanism limits *CRHR2* expression in the gonadotropes *in vivo*. JASPAR does not allow detection of NR5A1 REs and a different programme, AliBaba 2.0, did not yield any hits for cp*CRHR2*. It therefore remains to be investigated whether NR5A1 REs exist in cp*CRHR2*. However, NR5A1 is known to physically interact with GATA proteins and PITX1 as part of its transcriptional control of

other genes, like *Lhb* (Dasen and Rosenfeld, 1999; Tremblay et al., 1998; Tremblay and Viger, 1999). It is possible that a similar mechanism of protein–protein interaction between NR5A1 and the GATA2 and/or PITX1 proteins is involved in downregulating the expression the *CRHR2* gene in gonadotropes.

In summary, we found that GATA2 can strongly activate cp*CRHR2* on its own, as well as through interaction with POU1F1 and PITX1, suggesting that GATA2 is the main driver of cp*CRHR2* activity in chicken thyrotropes. However, in the presence of NR5A1, the GATA2-, PITX1- and combined GATA2-and-PITX1-induced activity of cp*CRHR2* was significantly reduced, suggesting a possible mechanism reducing or abolishing *CRHR2* expression in the gonadotropes. Given the conservation of essential GATA2 and PITX1 REs in various non-mammalian species, this mechanism may be conserved across non-mammalian vertebrates.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.113263.

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