5-2009

Generation of an FSH Inducible Inh-[alpha]-FLAG-gg-ICER I[gamma] Ovarian Specific Gene Construct

Goce Bogdanoski

ABSTRACT

Avian animal models have led to many essential discoveries in molecular biology and biochemistry, including the discovery of vitamins, the chemistry behind vision and the development of transgenic hens as natural bioreactors for pharmaceutically relevant proteins. Hyperovulation in domesticated hens is considered a highly desirable genetic trait, a feature that has been explored in multiple studies and remains the focus of many biotech companies. Unlocking the molecular mechanism that controls ovulation may provide a basis for development in new reproductive technologies.

Previous research done on rodents provided data and identified a single transcription factor, the Inducible cAMP Early Repressor (ICER), found to directly mediate the nuclear response to gonadotropins and trigger hyperovulation. Transgenic mice carrying tissue-specific FSH-inducible ICER construct showed two-fold ovulation rate increase following hormonal activation of the cAMP second messenger relay.

In this study, I focus on generation of a transgenic construct derived and specific for the domesticated hen, *Gallus gallus*. For this purpose, I amplified the 3kb promoter sequence of the Inhibin-α subunit gene. I also subcloned the avian homolog of the ICER Iγ isoform to the pFLAG-CMV-2 plasmid and created the FLAG-gg-ICER Iγ transgene construct. The DNA sequencing analysis showed successful amplification of gg-ICER Iγ. Following transfection into 293T (Human Kidney Carcinoma cells), the Western Blot analysis identified gg-ICER Iγ expression under control of CMV promoter.

I am now focusing on subcloning the Inh-a-FLAG-gg-ICER Iγ transgenic construct onto an expression vector and perform functional analysis in cell culture. Last, my goal

would be to utilize a lenti-viral delivery vector to infect fertilized *Gallus gallus* eggs. Using a well established signaling pathway, my hypothesis is that the extracellular signals of the Follicle Stimulating Hormone (FSH) will trigger changes in gene expression that will induce hyperovulation. This molecular mechanism would provide evidence for the complex interaction between the reproductive tract and the central nervous system.

MONTCLAIR STATE UNIVERSITY

Generation of an FSH inducible Inh-α-Flag-gg-ICER Iγ ovarian-specific gene construct

by

Goce Bogdanoski

A Master's Thesis Submitted to the Facility of

Montclair State University

In Partial Fulfillment of the Requirements

For the Degree of

Master of Science

May 2009

College/School College of Science and Mathematics

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2009

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ACKNOWLEDGMENTS

I would like to thank the Biology and Molecular Biology department for giving me the opportunity to participate in this study. I would specifically include my thesis sponsor, Dr Carlos A. Molina and my two committee members, Dr Quinn Vega and Dr Kirsten Monsen for providing anything from technical assistance to guidance towards completing this work. I would like to give credit to students who directly or indirectly were involved and participated in this project.

I would finally like to dedicate this work to may parents, Ljubica and Vojo Bogdanoski, this thesis is my return for their trust and sacrifice.

INTRODUCTION

The domesticated hens aided the discovery of essential vitamins and provided specific clues to the differences between T and B cells. Used as natural therapeutic protein bioreactors, the avian remains one of the primary animal models used in research (Lillico *et al.*, 2007, Petltte and Mozdzlak, 2007). From a practical perspective, the domesticated hens contributed a great deal to the market solutions of the increase in food demand. Hyperovulation in domestic hens remains a highly desirable genetic trait and focus of biotech companies. Related but not limited to the global food crisis and production of cost-affordable therapeutic proteins, developing transgenic lines of domesticated hens may also provide the basis for development of new reproductive technologies (Lillico *et al.*, 2007).

Ovulation in mammals is controlled by the cyclical action of hormones, specifically gonadotropins FSH and LH (Molina *et al.* manuscript in preparation). Hormonal regulation of the cell cycle by gonadotropins FSH and LH is crucial for folliculogenesis, ovulation and luteinization (Muniz *et al.*, 2006). Changes in gene expression in response to FSH and LH directly control the progression of the ovarian follicular development (Burkart *et al.*, 2005). Maturation of the follicles requires proliferation and differentiation of the follicular cell compartment. Following hormonal activation of the cAMP relay, coordinated gene expression between these two processes must be tightly regulated (Molina *et al.*, manuscript in preparation).

cAMP is found to be one of the most prevalent signaling molecules that regulates gene expression (Burkart *et al.*, 2005). Many hormones use cAMP as a secondary messenger to control gene expression in target cells by activating cAMP responsive proteins (Mukherjee *et al.*, 1998). The intracellular cAMP relay mechanism plays a central

role in essential processes such as cell proliferation, cell differentiation and apoptosis (Sandrine *et al.*, 1997). Elevated levels of cytoplasmic cAMP initiate phosphorylation of nuclear factors used for transcriptional control, thus regulating specific gene expression (Sandrine *et al.,* 1997). The activation of genes by cAMP so far is explained by the presence of cAMP-response elements (CREs) that serve as binding sites for cAMP activated proteins. The CRE is constituted by an 8bp palindromic sequence (TGACGTCA) (Fazia *et al.*, 1997).

CRE-binding protein (CREB) and CRE-modulator protein (CREM) are members of the bZIP family of transcription factors that have been best characterized up to this date. Upon phosphorylation, the bZIP activated proteins bind to specific CREs located in the promoter of cAMP responsive genes and activate transcription (William *et al.*, 1998). Alternative RNA processing of CREB and CREM family of transcription factors creates protein isoforms that can act as activators and repressors (Burkart *et al.*, 2005). One of those downstream nuclear factors is the Inducible cAMP Early Repressor (ICER). ICER is found to play a crucial role in multiple processes including cell survival/death (Sandrine *et al.*, 1997), circadian control of transcription in the pineal gland, cyclin A expression in insulin producing b-cells (Inada *et al.*, 2005), cell-cycle control in fibroblast and FSH receptors expression in sertoli cells (Folco and Koren, 1997). Transcription of ICER is also regulated by cAMP signaling as an internal P2 promoter located in the 3' region of the CREM gene is activated and regulates ICER expression (Molina *et al.*, 1993, William *et al.*, 1998). The four ICER isoforms are the smallest CRE-binding nuclear factors described to date (Morales *et al.* 2003). ICER and the rest of CREM isoforms share the DNA binding and dimerization domains but lack the kinase and transactivation domains (Muniz *et al.*,

2006). ICER has been implicated in the transcriptional repression of FSH inducible genes during the process of folliculogenesis (Molina *et al.*, manuscript in preparation). The gonadotropins FSH/LH-cAMP-ICER cascade has been proposed to have dual mode, to activate or repress cell cycle progression in the ovaries, hence regulating folliculogenesis and ovulation. ICER plays a key role as a dominant negative transcriptional repressor binding as homodimer or heterodimer to CRE sequences located on the promoter region of target genes (Molina *et al.*, 1993, Muniz *et al.,* 2006).

LH mediated expression of ICER in granulosa cells is found to downregulate Inhibin-α subunit gene expression just before ovulation. Downregulated Inhibin-α subunit gene expression leads to prolonged FSH surge that is crucial for recruitment of additional ovarian follicles (Mukherjee *et al.,* 1998). EMSA's and transfection studies identified all ICER isoforms being capable of binding and repressing Inhibin-α subunit gene. In different cellular events, CREB and ICER show competitive action for binding Inhibin-α subunit CRE-like element as a result of FSH and LH stimulus, respectively. Replacement of CREB by ICER may be the final event in repression of Inhibin-α subunit gene (Burkart *et al.,* 2005). A basic pattern of FSH stimulation and LH repression during the estrous cycle was examined multiple times for a number of genes expressed in the ovaries (Mukherjee *et al.,* 1998). Gonadotropins induced regulation of gene expression in rat ovaries was examined by in situ hybridization using radiolabeled CREM antisense riboprobe and riboprobes for the shorter repressor forms like ICER (Mukherjee *et al.,* 1998). CREM and ICER expression in response to gonadotropins closely correspond. However, there were data showing selective activation of the P2 promoter that specifically generates the four ICER isoforms. In addition, the time sequence analysis showed that ICER regulation is directly

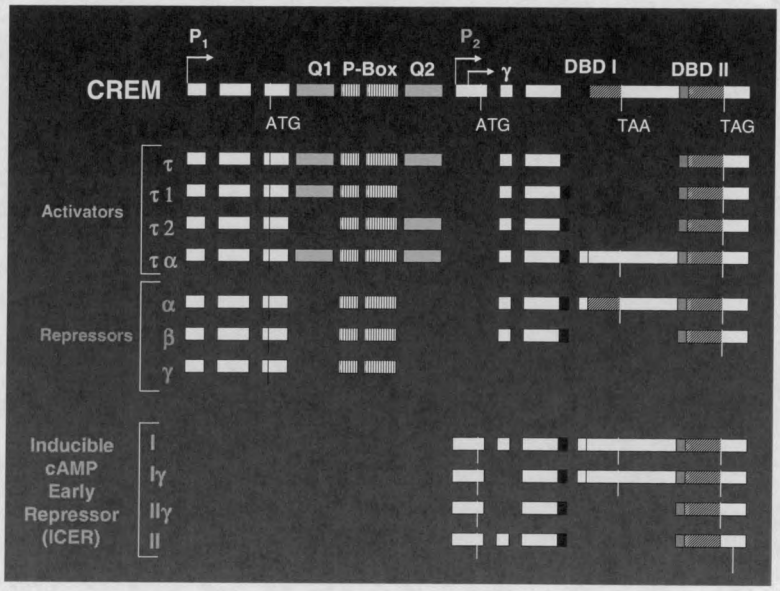

Figure 1: CREM and ICER isoforms – CREM gene contains two promoters, P1 and P2, regulating expression of 11isoforms, transcriptional activators and repressors. The P2 promoter regulates expression of 4 repressors, the only inducible isoforms of CREM. The graphical representation shows that CREM and ICER share the DNA binding and dimerization domains but lack the kinase and transactivation domains.

controlled by LH surges, upregulated and downregulated in specific time intervals. The actual relationship between ICER expression and Inhibin-α subunit downregulation in response to LH was still not confirmed. The Western Blot data identified a close relationship of ICER peak expression at 4h after hCG treatment, a time period when Inhibin- α subunit mRNA levels dramatically begin to decrease. Electrophoretic mobility shift assays identified ICER as a component of a larger complex attached to the Inhibin alpha-subunit CRE. Comparative analysis of ICER I sense and antisense construct were performed to test ICER ability to suppress Inhibin-α subunit gene expression. While the antisense construct had no effect, ICER I sense construct reduced the promoter activity thus downregulated Inhibin-α subunit gene expression (Mukherjee *et al.,* 1998). Hormonally activated cAMP mechanism of cell signaling is found in many tissues and cell types (Mukherjee *et al.,* 1998). The multiple genes involved, such as CREB and CREM and their many isoforms, along with the alternative RNA processing, phosporylation and the posttranslational modifications exponentially increase the overall effects of these nuclear factors. Such effects may be analyzed in a tissue or a cell specific manner.

Various ICER target genes have been reported in recent studies. Cyclin D2 (Ccnd2) is found to mediate folliculogenesis in response to FSH. Transgenic mice having Ccnd2 null mutation show impaired granulosa cell proliferation (Muniz *et al.,* 2006). Cyclins (D1, D2 and D3) belong to a family of cell cycle regulators involved in the G1/S phase transition, they activate genes involved in DNA synthesis and have been shown to be crucial for proper follicular development (Muniz *et al.,* 2006). Also, Cyclin D2 levels are found to be elevated in human granulosa cell tumors. The levels of Ccnd2 are reduced after LH/hCG surge. The proposed repressor of Ccnd2 is most likely to be the short isoform of CREM,

ICER. The Cyclin D2 mRNA levels have been shown to be induced by FSH via cAMP/PKA pathway and are rapidly decreased by LH (Muniz *et al.,* 2006). Expression of P450-arom in response to FSH is essential in granulosa cell differentiation (Morales *et al.,* 2003). P450-arom is a product of CYP19 gene and its expression can be detected in several tissues: human placenta, brain, adipose and gonads. The coding region of CYP19 is identical in every tissue. FSH activates CYP19 expression in ovaries due to the presence of CLS (CRE-like sequence) that puts in use the cAMP-dependent inducability of the ovarian promoter. The mechanism of downregulation of the P450-arom may be investigated by LH-induced ICER- suppression of the CYP19 promoter that controls P450-arom expression (Morales *et al.,* 2003). The importance of the cAMP signaling and the cAMP inducible genes has been broadly studied in context of hepatectomy, diabetes and epileptogenesis. Sassone-Corsi *et al.* have identified cAMP as an essential second messenger in liver as well as the role of CREM in liver regeneration (Sassone-Corsi *et al.,* 2002). In addition, ICER I-γ was found to be greatly increased in diabetes, directly repressing cyclin A gene expression. More importantly, the overall potential effect is the anti-proliferation in min6 cell line which has been identified as insulin producing cell line (Inada *et al.,* 2005). Studies on pineal gland identified ICER as negative regulator of cAMP-driven transcription of genes involved in the circadian FIX rhythm (Xiadong *et al.,* 1998). ICER may have a specific function in apoptosis of neurons presumably by downregulating expression of anti-apoptotic genes. Adenoviral vector delivering ICER overexpresion evokes programmed cells death in three different kinds of cultured neurons (Jacek *et al.,* 2003, Nicholas *et al.,* 1997). Following *status epilepticus*, the mRNA and protein levels of ICER in neurons are found to be increased (Porter *et al.,* 2008). Recent

advances in the field of neuroscience provided more sophisticated approaches for studying genes and proteins involved in neurosignaling, brain development and neuronal regeneration. Porter *et al.* studied CREM and ICER mode of action following *status epilepticus* (SE). Increased expression of CREM and ICER following pilocarpine induced *status epilepticus* may be one of the neuronal defense mechanism (Porter *et al.,* 2008).

Collectively, these reports include wide expression pattern of ICER related to multiple tissues and tissue specific processes. Transcriptional regulation of ICER with a use of a tissue specific and closely regulated promoter can thus be used to fight diabetes or epilepsy and provide additional information for the mechanism of mammalian reproduction at molecular level. In this study and based on data collected on rodents carrying transgenic ICER, I focused on generation of the Inh-α-FLAG-gg-ICER Iγ construct derived and specific for the domesticated hen, *Gallus gallus*. My perspective was derived from several key benefits from developing an avian model carrying ICER transgenic construct regulated by an ovarian-specific transgenic promoter. Using a well established signaling pathway, my hypothesis is that the extracellular signals of the Follicle Stimulating Hormone (FSH) will trigger changes in gene expression that will induce hyperovulation. To confirm the regulatory mechanism in addition to the cloning of the transgene I perform several Q-RT-PCR experiments to test the Inh- α subunit gene expression pattern using mouse-derived cDNA. The selected animals carried the ICER transgene specific for mice and were confirmed to have ICER overexpression. Comparing the expression levels of Inh- α subunit gene in transgenic and wild-type mice I may be able to confirm the molecular mechanism that regulates ovulation and provide evidence for the complex interaction between the reproductive tract and the central nervous system.

MATERIALS AND METHODS

RNA/DNA Extraction

Both RNA and DNA were extracted from frozen chicken ovaries using the TRIzol Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. Tissue from multiple ovaries was collected and a total of 39 DNA and 18 RNA samples were purified. Both RNA/DNA integrity was confirmed by agarose gel electrophoresis with ethidium bromide staining. The RNA/DNA concentration was measured before each experiment by using the NanoDrop (Thermo scientific) spectrophotometric analysis method.

PCR amplification of Inhibin-α subunit gene promoter

PCR was used to amplify the Inhibin-α subunit gene promoter sequence of 3049bp (Textbox 1). AccuPrime DNA Polymerase for GC-Rich templates (Invitrogen, Cat Nu. 12337-016) was used and all reactions were performed according to the manufacturer's protocol and cycling parameters. DNA concentration was adjusted for a range of 10ng-100ng final concentration with diH2O following a Nanodrop reading for each specific extract. The following oligos were used: Forward 5'- GCTCCGCCACCTCCACGTCC - 3' and Reverse 5'– GGCCGTGGGGCAGCTCAAGG - 3'. Re-amplification experiments were performed using two additional enzymes, the HotStart Tag-Plus DNA polymerase (Qiagen) and the HotStart Mastermix (Denville Scientific). Each enzyme was used for the PCR amplification according to the manufacturer's protocol, using the specified amounts of reagents and cycling parameters. All reactions were set for 25µl final volume.

RT-PCR amplification of gg-ICER Iγ

PCR with reverse transcription was used to generate chicken ICER (GenBank AF509471). AccessQuick RT-PCR system (Promega) components were prepared as indicated in the manufacturer's protocol for a total volume of 50μl per reaction. Primers we specifically designed the amplify the ICER mRNA based of the NCBI data (AF509471) : Forward 5'-ATGGCTGTTACAGGAGATGA-3' and Reverse 5'-TTATTCTGCTTTATGACAAT-3'. Reverse transcriptase was added to the reaction tubes followed by 45min incubation at 45°C. AccessQuick DNA polymerase was then added and PCR parameters were set as directed by the manufacturer. 2% agarose gel electrophoresis was performed to verify the size of the initial 327bp fragment.

Topo TA Cloning and Restriction Digest

The RT-PCR amplified ICER Iγ fragments were ligated onto a pCR 2.1 TOPO cloning vector. 6μl Topo cloning reactions (Invitrogen) were prepared using the volumes and components according to the manufacturer's instructions. Following 5min room temperature incubation, 2μl of each cloning reaction was added to a vial of OneShot Top10 chemically competent cells. Cells were incubated on ice for 30min and heat-shocked for 30sec at 42°C. 250μl of pre-warmed S.O.C. medium was added following 1hour incubation at 37°C. Cells were spread on LB w/ ampicillin selective plates and grown overnight. Growing colonies indicating successful integration of the ICER fragment onto the TOPO vector were picked and grown in 3ml LB medium at 200rpm overnight. 1.5ml aliquots were collected and centrifuged for 1min at 12000rpm. Plasmid DNA was purified using the QIAprep Miniprep Kit (Qiagen) and according to the manufacturer's protocol. Plasmid and

RT-PCR fragments were digested w/ EcoRI, XbaI/HindIII and XbaI/BglII supplemented with the appropriate buffers.

Sequencing of the pFLAG-CMV-2-gg-ICER Iγ construct

Following the restriction digest, ICER Iγ was cut off from the pCR 2.1 Topo and was excised from the gel piece. Gel extraction was performed using QIAquick Gel Extraction Kit (Qiagen) followed by an agarose gel electrophoresis confirmation and NanoDrop concentration analysis. The purified ICER fragment was then ligated onto pFLAG-CMV-2 expression plasmid. 10μl reactions were prepared using T4 ligase (Sigma). Ligation reactions were incubated overnight at 16°C. OneShot Top10 chemically competent cells were transformed on LB plates w/amp. Samples that developed viable colonies were sent to GeneWiz for sequencing (Sanger Method). CMV-Forward 21mer primer was used: 5'-CGCAAATGGGCGGTAGGCGTG-3'

Cell Transfection

Two plates (6 wells each) were prepared for DNA transfection. Each plate contained ICER Iγ, however, the pSV40-ICER Iγ had the mouse homolog of ICER and was used as control. The pFLAG-CMV-gg-ICER Iγ plasmid carried the *Gallus gallus* ICER Iγ. Human Kidney Carcinoma 293T cells were trasfected following 3 different protocols: lipofectamine (Invitrogen), Ca-phosphate (Sigma) and lipofectamine (Sigma). Cells were incubated for ~48 hours, w/ one time medium change in intervals indicated by each protocol respectively.

SDS-PAGE and Western Blot

Transfected cells were harvested approximately after 48hour incubation. Both dishes were washed w/ 1ml cold HBSS twice. Cells were then scraped in 1ml ice-cold PBS and transferred to a 1.5ml tubes, followed by a 1min spin at 14000rpm. 300µl 1X Lammli buffer (62.5mM Tris, pH 6.8, 2% SDS, 10% Glycerol, 5% mercaptoethanol and 0.001% bromophenolblue) was added to each sample. Samples were then heated for 5min at 100°C heating block and the centrifuged for 10min at 14000rpm. After quick vortex, samples were centrifuged for another 3min and supernatant was collected for SDS-PAGE. 6X loading dye was added to each sample. 1X running buffer was diluted from 10X stock (30.3g Tris base, 144g glycine, 10g SDS for a final volume of 1L). 30µl of each sample was loaded and the apparatus was set to run for 60min at 200V. Membranes were prepared according to the manufacturer's instruction (Western Breeze, Invitrogen) and soaked into transfer buffer (3.03g Tris, 14.4g glycine, and 200ml methanol for a total volume of 1L). System was run overnight at 30V. Anti-ICER (primary) and secondary AB's + stain were added to the nitrocellulose paper containing the transferred proteins.

Inhibin-α subunit gene promoter subcloning onto Topo II-Blunt and pFLAG plasmids

PCR amplified Inh-α sequence was ligated into TopoII-Blunt vector. Three separate reactions were prepared using 1, 2 and 3µl PCR-amplified DNA, 1µl salt solution and 1µl TopoII-Blunt plasmid. The reaction final volumes were adjusted with diH2O for a total of 6µl. Following a 30min room temperature incubation, 2µl of each reaction was pipeted into a 50µl vial of OneShot competent cells and incubated on ice for 15min. The cells were then heat-shocked in 42°C water bath for 30 sec, followed by addition of 250µl S.O.C. medium

(pre-warmed). Reaction tubes were then incubated in 37°C shaker for 1 hour at 200rpm. 200ul of each reaction was platted onto LB plates with kanamycin. Plates were incubated overnight at 37°C. Using the same DNA, another set of reactions was prepared using pFLAG-CMV-2 plasmid. An important change made to this plasmid was the excision of the CMV promoter sequence using HincII enzyme. It is now possible to use the plasmid to directly ligate Inh-α promoter sequence. The pFLAG-CMV-2 plasmid also contains the ICER Iγ gene in the multiple cloning site ligated between the two EcoRI sites. The reactions were prepared using 3μl plasmid DNA (ND concentration reading was 10ng/μl), 1μl insert DNA, 1μl 10X ligation buffer, 0.5μl 10mM ATP solution, 4.5μl PEG and 2μl T4 ligase. Reaction tubes were incubated overnight at 16°C. Collected colonies of each set were grown in 3ml LB medium at 37°C overnight at 180rpm. LB medium was prepared using ampicilin or kanamycin according to the plasmid selection gene.

Plasmid DNA Miniprep and Restriction digest

Plasmid DNA was later prepared using the QIAgen Miniprep kit according to the manufactures specification. 10μl of the final 50μl purified DNA were used for restriction digest using EcoRI restriction enzyme, in order to cut-off the insert from the plasmid. Reactions were set using 10μl plasmid DNA, 1μl EcoRI and 1ul 10X EcoRI-specific buffer. The digest reaction was incubated in water bath at 37°C overnight. The samples were then run on 1% agarose gel using 2μl 6X loading dye and 10μl (per gel box) EtBr.

Q-RT-PCR for w.t. and transgenic gapdh and Inh-α subunit gene cDNA derived from w.t. and transgenic mice

The Q-RT-PCR amplification of two mouse-derived genes, the house-keeping gapdh and the Inh-α subunit gene, was carried out using the Sybr Green Master Mix. The reactions using primers specific for both genes were prepared using 12.5µl 2X Sybr Green MM, 2µl primer mix (0.8uM each primer), 0.5µl template cDNA and 10µl diH2O for a final volume of 25µl per reaction. The cycling parameters were set for a 10min initial denaturation at 95°C, followed by 40 cycles of 40sec at 95°C, 1min at 55°C and 40 sec at 72°C. Stratagene MX3000P instrument and analysis software was used. The following oligos were used: gapdh left 5'-ATGTTCCAGTATGACTCCACTCACG-3' and gapdh right 5'-GAAGACACCAGTAGACTCCACGACA-3', Inh-α left 5'-CTGTGCCTGTGTCCTTGGTA-3' and Inh-α right 5'-CCAGGAAAGGAGTGGTCTCA-3'. All primers were specific for the mouse homologs of the respective genes (Molina *et al.,* manuscript in preparation)

RESULTS

In Silico analysis data

The NCBI and the EBI GenBank were the main databases used to locate and analyze both, *Gallus gallus* Inhibin-α subunit gene promoter and ICER sequences. Both queries were based on known rodent sequences (Molina *et al.,* manuscript in preparation) and aligned for similarities for the chicken genome. The Inh-α promoter sequence was found on the European Bioinformatics website and was 3049bp long. 110bp from the 3' end of the sequence we were able to confirm the non-canonical CRE element (textbox 1,yellow hi-light). The chicken ICER Iγ sequence was available on the NCBI website. Accession number AF509471 query (*Gallus gallus* ICER Iγ mRNA, complete CDS) identified the mouse ICER Iγ sequence as the closest match with 94% (textbox 2).

In addition to the sequence analysis and alignment data from NCBI, we performed several analyses using the NebCutter V2.0 software from New England Biolabs (http://tools.neb.com/NEBcutter2/index.php) to determine restriction sites found on both, Inh-α gene promoter (fg.3) and gg-ICER Iγ sequences (fg.5). The collected data were used in the following restriction digest and restriction mapping experiments, mainly used to verify amplification and ligation efficiency. In addition, the specificity of restriction sites located on each sequence of interest, was used to verify orientation of the specific parts of the transgene construct on each plasmid. All these data were also incorporated and used to analyze restriction sites not present on the plasmid's Multiple Cloning Site (MCS) but found on the plasmid's circular DNA sequence. Performing sequence analyses (fg.2) I was able to confirm the high GC content of the Inh-α subunit gene promoter, a value of 66% which was our main consideration when setting the PCR reaction and cycling parameters.

5'GCTCCGCCACCTCCACGTCCTCCAGCGGCACCTGGAACCGCAGCCGGTGCTCTGCCACCGGGAGAGATGGGAGCTG
AGGGTGCTCAGCATCCTCCTGCTCAGCACCCTTCTGGTCCCCTGAACGCCCCCCCTGAGAGCACCCACGCATGCAGCG
CCCCCGCATTCCTCCCACATTGACAGTACGGCTGTGGGGAAGGAAGATGGCAGCCCCCCGTTCCCAGCAGCTATTTTG
GGATGTGGCAGCTGGGCGGGAGGCATTCGGTATGGGGCCTCAGGGCGCACAGCCCCCATGCAGGGGATCCAGCGTGG
CCCCGGGGCGCACACCTACCCTTCACCTGCAGCTGCACAGCACTCAGTGTCTGCCCGGCCGTGTTGGAGGCTGTGCAA
ACATACAGCCCCTGGTCCCGGGGCCGGCAGTACAGCACCTTCAGGATGAAGTAGCCCTCGCGGTCCTCGTAGATCAG
GTGCCGGCGGCCGGGGGCCACGGCCTTGCCATCCTTTTGCCAAATGATCTCTGGCTTGGGCTTGCCAGTGACATAGCA
GCGGAACTTGGCGTGCTTCCCCGCGCTGACGGCGAACGCCTTGGCTCTTGGCGTGCCGTTGGGTGCCGGCGCCCAGGT
GTCGGTGCATGCGTGCCGCAGTCTCCGCGGCTGGGTGCTGTCAGAAGGGGAGCACCGGGGCTCCACCAGGAGCACAG
CCGCAGCCAGGGCCTCCTTGGAGCCGCTGCGTGCCCGGCACACATAGACCCCCGCGTCTGGGGGCCGGACCCCAAAC
AGCTTAAGGTAATGCCAGTCCTCAGGCTGCCGGCCCACTGCAAAGTGGCTGCTCTCAAAGAGGTCACGCAGCCTGTG
GCCGTCCTTCTCCCACACGAGCAGCGGGCAGGGCTGCCCCAGCACTCGGCACGAGAAGGTCGAATCNNNNNNNNNN
GATGCTCCTCAGCCTCCACTCGCAGCGTGGCGGCAGCGTAGGTCTCGCCAACGCAGTTCTTGGCCTTGCAAACATAGA
GGCCGCCATCCTGCGGGGTGACGCAGGACACCCGTAGGCTGTACATGTCCCCCTCAGCCTCCATGCAGAAGCGGCCC
GAGGGCTGGATCGGGGCTGTGTCCTTCTCCCAGAGGACGCTGGGCCGGGGGTCACCTGCGATCTGGCACCTCAGCAT
GGCGTCGGTGCCGCTTTGTGCCGTGAAGGCGCGTGGGTAGGCCAGGAACCGAGGGGCTGCCCCATGGCTCTCCATCA
CCCTGGGATCGGGGCAGGAGGACGGCCAAGAGCTTCAGGCTGAGGACAGAGAGCGGCGTCAGGCTGTGGTGCGGGG
CATCCCACCCACAGCAATGCCACGTCAAGCCCAGGCTGGGAGGTGGGACCTGCCCGTGGGGCACGGGGCTCGCTGGG
TCCCTGCAGTGCTGGGGATGGGGTTGGCTGAGGAAGAACGGGCCCCAGCACGGCCCTTCCCCACCCGCTGGGGCATT
CCTGCAGTATCGGGTGGTGGCAGAGCCACATTCCTGCCGCATCTGGGCTGGCTCAGTGCACAGGTGCTGATAAGGGG
CTGTCATGGGGCTGGAGGCATCCGGATGCATCCGGGTGCTTCCTGCCCGGCTCAGCAGGGTGCATGGGAAGAGGACC
CCCGCCCGAGCGTGGTCCCAACTCCAGCCCCTGCGTGTCCAAGGCCAGGACTGGACATCACCGCAGGGTGCAGAGGG
CCAAACAGGGAACAAGTCCTGCTTTCGGAGGGTATCACCTCCACACCCAGTGCTGCAGGTACCCGTGCCCAGCACCA
CGGGCAGCAGAAGGGCTCTGTGCTTGAAGCCTTTCCTGGGCAAGCATAAACCTCAGCCCCTGCCCGTGCTGCACCCAG
GGGTGGACAGAGGCATCTATGCCTCCCTCAGGGAGAGCAGGGACAAAGAGAAACCCAAACATTCCCTGGCTGGGCTG
AAGGGGAATGTGGGACAGAGCTCATCCCCAGGGGCCAGGGCCAGGTTCCCCCCCCCCAAGCCCGCACTCCAGGAGCC
AGCACACACAGCCCCACAGCTCAGCCCCCCCCTCCTCTGATCACTCTGCCCAGAACCACCTCCTGCCTCAGGCACTCA
CCTTGTACCAACACCTCTGTCCTGCAGCTCCCGGTGCCGGCACCCCGCTCTGCTGCTCGCCCAGCTCTGCTCCCCAGCG
TCAGCATGCAGAGACCGACCGTTAGGAATGCCTGCAGCTGCCGTCCCCAAAAATACCCTCCGATGACATGCCGGGGT
GATAAGGCTGAGTCTGCTCCATGCAGCGCCTCGGCGGGGCAGGGCTGCCCTATGTATAGCACAGGCGGGCAGCACAG
CGGCAGCGGGGCGCAGCAGACCTGTTCCTGGTGGGTGCGGGTTCAGCCCATGGCACTGCACTGTGCACGGCACGCAG
CATAGGGATGGGATGCGCTGCAGCCAGCCAGGCCGAGTGGCACTGTGGGACCCTTCTCTTGTAACTGCCACAGTGTCC
CAAACTCTGTCCAATGCTGTCTCCAGCCATGGCCTGTCCCCAAGCAGAGGGTCCCTGTTCCCGCTTTTCCTCGTGTTCC
CAGTGCCCCACTTGTTGTGGCAATGCCACTGCCCTGAACCTTGCATCCTTGGGGCGTCCCATCCATACCCAGTAAGAG
GGCACCCAGCCTGCCACCCTCCTGTGCCTCTCACGCTGTTGCTCTGCCCGACGTGCACCCTTGGCCTCCTGCTGGCCCC
CAGACTCCAGCATGGAGGCACCCAGCTGCAGGCCCCCACGCCCCCTCCATCCCTGCCTACCCCCAGCCCCACGCACTC
ACCACTCCAGCAGCATGCACCCCACCCACCCCCCTGCCTGGGCGCCGTCCCCCCCCCCAGACCACACTGTCCTTGCAGT
CCTCACATTCCAGACGTCTCAGTGCTTTCACTGCCCAGAGATAATATTCACGGCGAGCAGACATTTGCGTCAGTGTCA
GATACGTCTCAATGCCACCCTGCCCCAGGGGGAGCGCAGCGGGCAGACGGGCCGGGCTGGCAAGTAGGAGCCCACG
GCAGAGCCCCTTGAGCTGCCCCACGGCC3'

Textbox 1: Inhibin-α subunit gene promoter sequence – 110bp from the 3' end of the sequence is the non-canonical CRE sequence (yellow hi-light) which is the proposed ICER protein binding site

ref|NT_039674.7|Mm18_39714_37 *Mus musculus* chromosome 18 genomic contig, strain C57BL/6J Length=73639148

Features in this part of subject sequence:
cAMP responsive element modulator

Score = 230 bits (124), Expect = 9e-58
Identities = 140/148 (94%), Gaps = 0/148 (0%)
Strand=Plus/Minus

```
CAGGGAAGCTGCCAGAGAATGTCGCAGGAAGAAGAAAGAATATGTCAAATGTCTTGAAAA  GG
|||||||||||||| | || ||||||||||||||||||||||||||||||||||||||
CAGGGAAGCTGCCCGGGAGTGTCGCAGGAAGAAGAAAGAATATGTCAAATGTCTTGAAAA  MM

TCGTGTGGCTGTGCTTGAAAACCAAAACAAGACTCTCATTGAGGAACTCAAGGCCCTCAA  GG
|||||||||||||||||||| |||||||||| |||||||||||||||||||||||||||
TCGTGTGGCTGTGCTTGAAAATCAAAACAAGACCCTCATTGAGGAACTCAAGGCCCTCAA  MM

AGATCTTTATTGTCATAAAGCAGAATAA                                  GG
||| |||||||| |||||||||| |||
AGACCTTTATTGCCATAAAGCAGAGTAA                                  MM
```

Textbox 2: *Gallus gallus* ICER Iγ mRNA alignment with *Mus musculus* ICER Iγ mRNA – results indicate 94% identity match b/w the mouse and chicken ICER mRNA

Display: - NEB single cutter restriction enzymes
- Main non-overlapping, min. 100 aa ORFs

GC=66%, AT=34%

Cleavage code	Enzyme name code
▼▲ | blunt end cut	Available from NEB
▼_ | 5′ extension	Has other supplier
_▲ | 3′ extension	Not commercially available
▼ | cuts 1 strand	*: cleavage affected by CpG meth.
	#: cleavage affected by other meth.
	(enz.name): ambiguous site

Figure 2: New England BioLabs NebCutter analysis data for Inh-α subunit gene promoter Sequence- the value of 66% GC content was my main consideration for setting the PCR parameters.

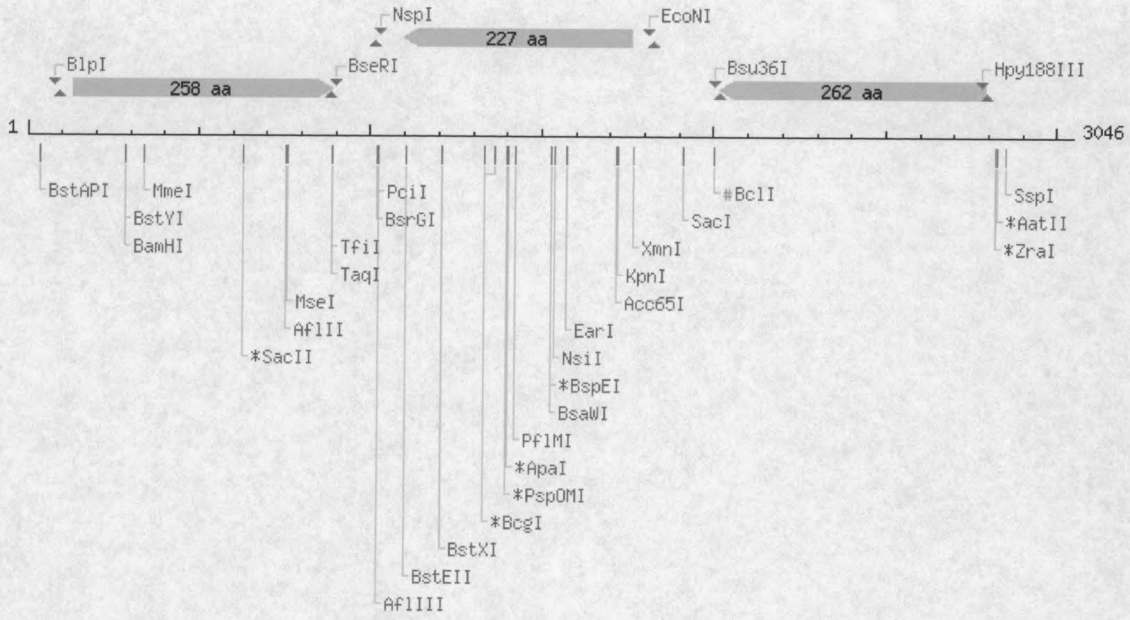

Figure 3: Restriction enzyme map for Inh-α subunit gene promoter sequence – restriction sites represent location for a single point cut for each respective enzyme listed. The indicated map was used to calculate the expected band size following restriction digest with the respective enzyme

Display: - NEB single cutter restriction enzymes
- Main non-overlapping, min. 100 aa ORFs

GC=47%, AT=53%

Cleavage code	Enzyme name code
blunt end cut	Available from NEB
5′ extension	Has other supplier
3′ extension	Not commercially available
cuts 1 strand	*: cleavage affected by CpG meth. #: cleavage affected by other meth. (enz.name): ambiguous site

Figure 4: New England BioLabs analysis data for *gg*-ICER Iγ gene sequence

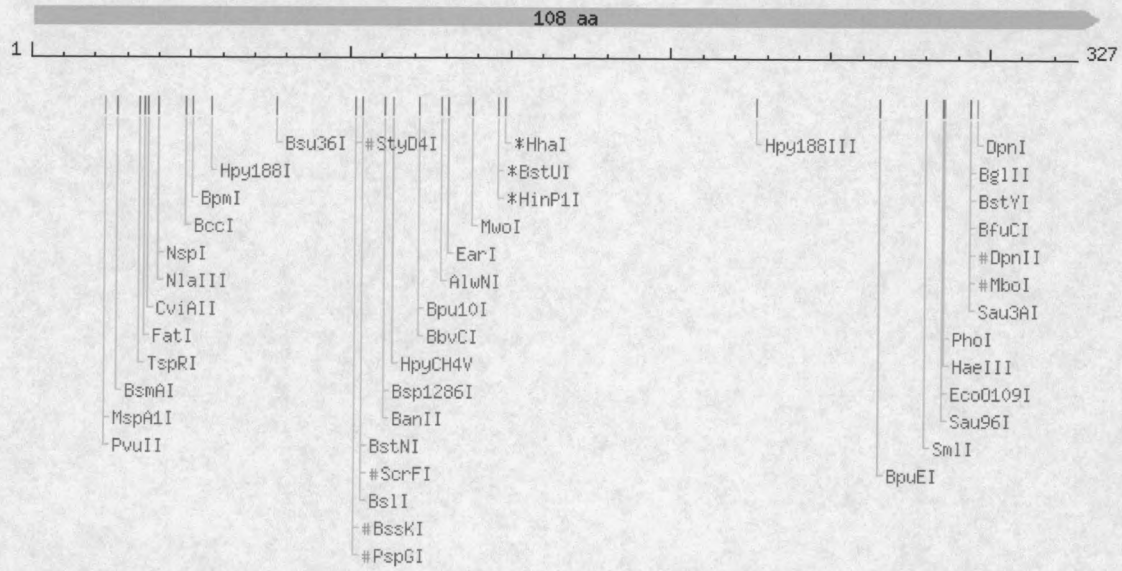

Figure 5: Restriction enzyme sites for gg-ICER Iγ sequence (single point cutters)

Test type: Nucleic Acid 3/11/2009 10:55

Max Buffer Size 200 Buffer Mode Save Report & Clear

User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
Default	3/11/2009	10:33 AM	140.09	2.802	1.911	1.47	1.55	50.00	230	1.802	0.016
Default	3/11/2009	10:34 AM	413.90	8.278	4.906	1.69	1.79	50.00	230	4.617	0.022
Default	3/11/2009	10:36 AM	84.67	1.693	1.194	1.42	1.30	50.00	230	1.306	0.027
Default	3/11/2009	10:37 AM	74.87	1.497	1.066	1.40	1.39	50.00	230	1.079	-0.706
Default	3/11/2009	10:38 AM	39.05	0.781	0.504	1.55	1.01	50.00	230	0.773	0.557
Default	3/11/2009	10:40 AM	29.77	0.595	0.343	1.74	1.12	50.00	230	0.531	0.009
Default	3/11/2009	10:41 AM	32.14	0.643	0.442	1.46	0.98	50.00	230	0.656	-0.134
Default	3/11/2009	10:42 AM	63.50	1.270	0.853	1.49	1.15	50.00	230	1.105	0.039
Default	3/11/2009	10:44 AM	443.46	8.869	5.063	1.75	2.04	50.00	230	4.349	0.057
Default	3/11/2009	10:45 AM	1652.30	33.046	21.425	1.54	1.85	50.00	230	17.871	0.286
Default	3/11/2009	10:46 AM	119.48	2.390	1.689	1.42	1.44	50.00	230	1.655	-0.001
Default	3/11/2009	10:47 AM	33.69	0.674	0.499	1.35	1.63	50.00	230	0.414	-0.011
Default	3/11/2009	10:48 AM	0.62	0.012	-0.001	-19.54	0.02	50.00	230	0.575	0.032
Default	3/11/2009	10:49 AM	3006.93	60.139	35.034	1.72	2.05	50.00	230	29.392	0.599
Default	3/11/2009	10:51 AM	1525.96	30.519	19.777	1.54	1.65	50.00	230	18.499	0.208
Default	3/11/2009	10:52 AM	66.89	1.338	0.914	1.46	1.18	50.00	230	1.134	0.001
Default	3/11/2009	10:53 AM	1084.89	21.698	14.746	1.47	1.36	50.00	230	15.987	0.189
Default	3/11/2009	10:54 AM	19.79	0.396	0.233	1.70	0.76	50.00	230	0.520	-0.107
Default	3/11/2009	10:54 AM	30.52	0.610	0.429	1.42	1.20	50.00	230	0.510	-0.103

Table 1: NanoDrop data for chicken genomic DNA – multiple samples listed indicate distinct extraction of chicken ovarian DNA (multiple ovaries used)

DNA/RNA Extraction

Following the TRIzol protocol for nucleic acids extraction, ~ 1ml of both DNA and RNA was extracted from chicken ovaries and purified for later experiments. The results obtained from the NanoDrop spectrophotometer for the purified genomic DNA are listed in Table 1. The purified RNA was run on 1% Agarose gel to verify the integrity of the purification procedure. Fg.6 clearly identifies two bands representing RNA derived from the 28S and 18S ribosomal subunits, confirming total RNA extraction.

RT-PCR

Amplification of the gg-ICER Iγ sequence starting from mRNA was successfully completed and the amplified DNA product was run on 2% agarose gel. The amplified sequence did fit the range of ~ 320bp, which was the expected size of the gg-ICER Iγ gene product (Supp. Fg.2). gg-ICER Iγ was then initially subcloned onto the pCR-2.1 Topo vector and restriction digested with EcoRI. As predicted by the restriction mapping analysis, this event produced two distinct bands, the gg-ICER Iγ with ~320bp and the larger band representing the remaining of the linearized plasmid sequence with ~4000bp (fg.7A). gg-ICER Iγ was then transferred onto the pFLAG-CMV-2 expression vector. Using EcoRI, once more we confirmed the restriction digest pattern and showed that we do have the gene sequence amplified (fg.7B). In addition, in a separate restriction digest experiment using EcoRI and BglII restriction enzymes, we were able to calculate the expected band pattern on the agarose gel and verify samples with correct orientation of the ICER gene insert on the plasmid. Only those samples indicating correct orientation of gg-ICER Iγ were later sent for sequencing (data not shown)

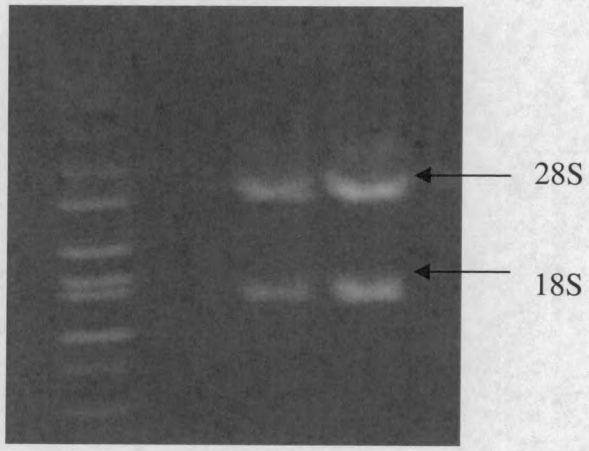

Figure 6: *Gallus gallus* RNA on 1% Agarose gel – following genomic RNA extraction

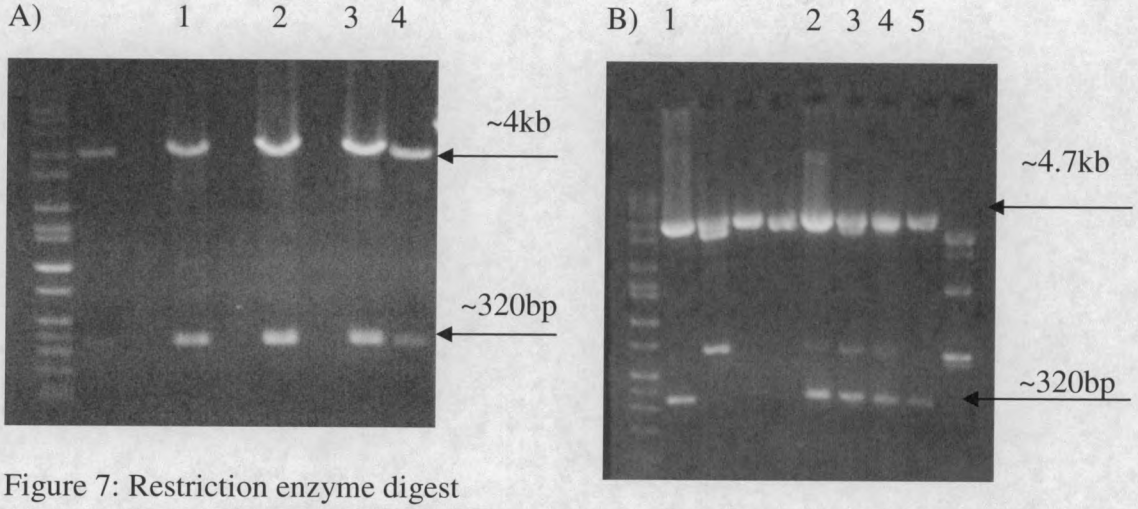

Figure 7: Restriction enzyme digest
A) pCR-2.1 Topo-gg-ICER Iγ EcoRI (lane 1-4 indicate successful subcloning)
B) pFLAG-CMV-2 gg-ICER Iγ EcoRI (lane 1-5 indicate ICER digest)

Sample ID: RVS 3hx CMV-Forward_C09.ab1

CGGGGGCNTAGCGAGCTGTTNGTGAACCGTCAGAATTGATCTACCATGGACTACAAAGACGATGACGACA
AGCTTGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATTCGCCCTTATGGCTGTTAC
AGGAGATGAAACAGCTGCCACTGGAGACATGCCATCTTACCAGCTTCGGACTCCCACTACTAACTTACCT
CAGGGAGTGGTAATGGCAGCCTCCCCAGGGGCTCTGCATAGTCCTCAGCAACTGGCAGAAGAGGCAACG
CGCAAGAGAGAGCTGCGACTTATGAAAAACAGGGAAGCTGCCAGAGAATGTCGCAGGAAGAAGAAAGA
ATATGTCAAATGTCTTGAAAATCGTGTGGCTGTGCTTGAAAACCAAAACAAGACTCTCATTGAGGAACTC
AAGGCCCTCAAAGATCTTTATTGTCATAAAGCAGAATAAAAGGGCGAATTCTGCAGATATCCATCACACT
GGCGGCCGCTCGAGCATGCATCTAGAGGATCCCGGGTGGCATCCCTGTGACCCCTCCCCAGTGCCTCTCCT
GGCCCTGGAAGTTGCCACTCCAGTGCCCACCAGCCTTGTCCTAATAAAATTAAGTTGCATCATTTTGTCTG
ACTAGGTGTCCTTCTATAATATTATGGGGTGGAGGGGGGGGTGGTATNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NN

Textbox 3: gg-ICER Iγ DNA sequencing data (GenWiz)

DNA Sequencing (gg-ICER Iγ)

To confirm the gg-ICER Iγ sequence, plasmid sample containing the gg-ICER Iγ sequence was sent for sequencing done by GeneWiz. The produced sequence was then aligned with the NCBI generated sequence in order to compare the efficiency of the amplification reaction. The resulted sequence had 100% match in nucleotide comparison with *Gallus gallus* mRNA for ICER Iγ (NCBI accession # AF509471,Suppl fg.1) The sequencing data provided additional evidence for orientation of the gg-ICER Iγ gene insert onto the plasmid. The collective data enabled continuation with the subcloning of the Inh-α subunit gene promoter sequence on the grounds of correct orientation of the gene sequence. In addition to the sequencing data, we performed another restriction digest reaction using three different restriction enzymes: EcoRI, XbaI and HincII. Based on the pFLAG-CMV-2 plasmid map and our collective restriction map for gg-ICER Iγ we were able to calculate the expected band sizes for each digest individually. Our focus was excision of the gene sequence using EcoRI, excision of the CMV promoter sequence using HincII and a control digest using XbaI that would only make a single cut on the MCS and linearize the plasmid. Following an agarose gel run, we were able to determine the experimental results matching our calculated data. Using three different restriction enzymes, EcoRI, XbaI and HincII, we were able to excise the gene insert, linearize the plasmid and excise the CMV promoter (fg.8). The last event, excision of the CMV promoter produced a plasmid with the gg-ICER Iγ gene incorporated into it and available to directly subclone the Inh-α promoter sequence. (fg.8, lane 4)

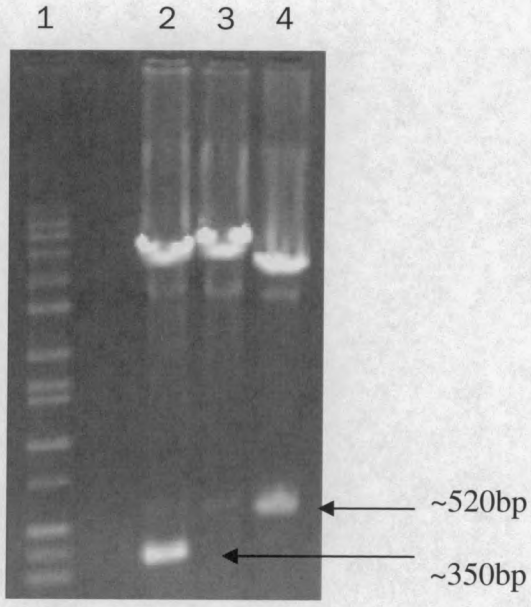

Figure 8: Agarose Gel analysis following EcoRI, XbaI and HincII digest
Lane 1: Molecular Marker
Lane 2:band: ~ 4700 and ~ 350 (EcoRI digest)
Lane 3: band: ~ 5060 (Xba digest)
Lane 4: band: ~ 4550 and ~ 520 (HincII digest)

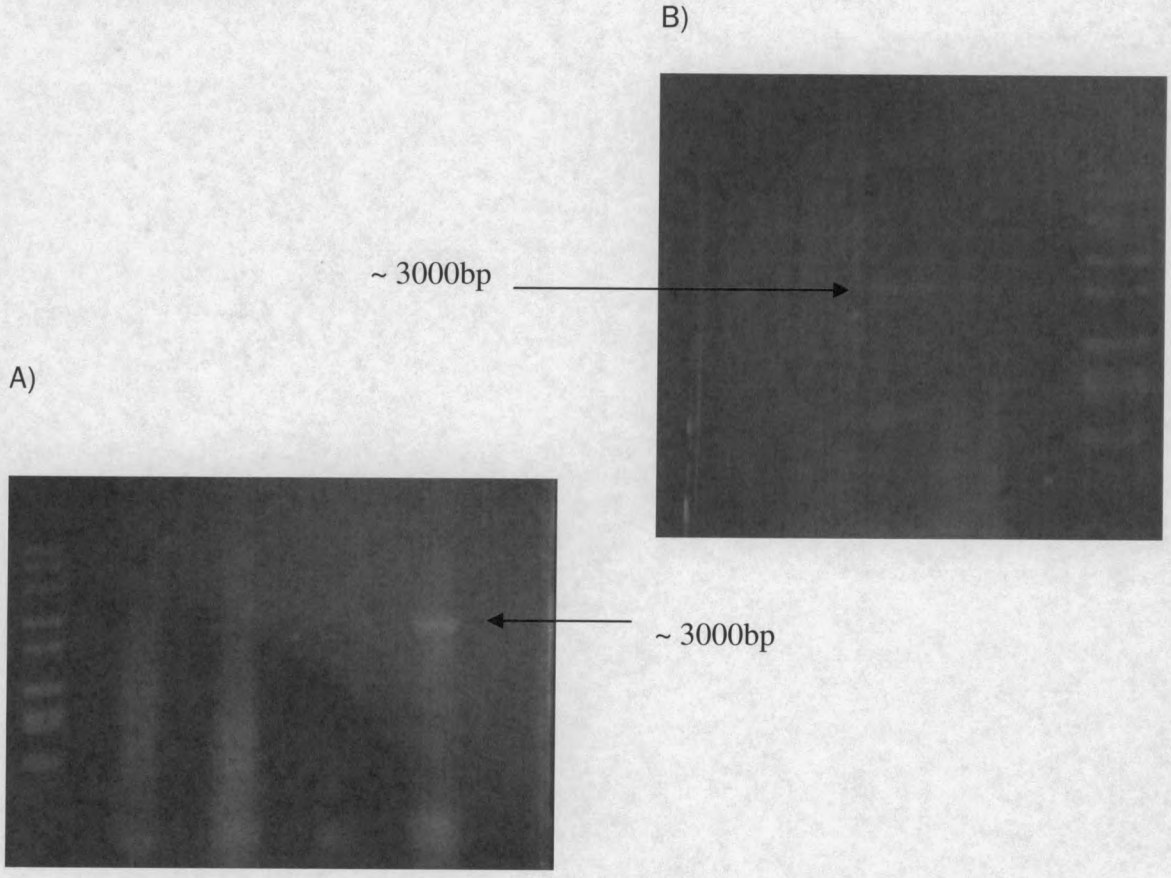

Figure 9: Inh-α Accu-Prime GC-Rich amplification on 1% agarose gel (w/ EtBr)
- fg.9A and fg.9B represent two separate amplification experiments

PCR amplification of the Inh-α subunit gene promoter

Using thermo-stable polymerase specific for GC-rich templates we successfully amplified the ~3046bp sequence of the Inh-α subunit gene promoter sequence. Two reactions carried under same conditions were concluded to contain this sequence following an agarose gel run. A distinct band at ~3kb, based on the molecular weight marker was identified, as expected based on the *in silico* analyzed sequence for the Inh- α subunit gene promoter (fg.9) Following the initial PCR amplification of the Inh-α subunit gene promoter sequence we performed several additional amplification reactions in order to produce sufficient amounts of the insert, to be used for subcloning and further analysis. However, at the time of the initial analysis, we were able to excise and collect the gel piece containing the DNA band of the expected size. Using the gel extraction/purification protocol specified in the materials and methods section, the isolated DNA was prepared and the integrity was again verified using agarose minigel (fg.10). As a result of the specific amplification method used to amplify the Inh-α subunit gene promoter sequence, namely the Accu-Prime GC-Rich polymerase, we then used pCR Blunt II-Topo plasmid to subclone the insert. The Topo cloning reactions yielded significant number of bacterial colonies that grew selectively on LB plates with kanamycin. Bacterial colonies were used to grow 44 minicultures overnight. In addition to the pCR Blunt II-Topo, we also used the previously generated pFLAG-CMV-2 plasmid and performed a T4 ligation using three different concentration of our PCR amplified insert. This time, however, we used the HincII digested pFLAG-CMV-2 without the CMV promoter sequence containing the gg-ICER Iγ in the MCS. LB plates and minicultures were amp selective.

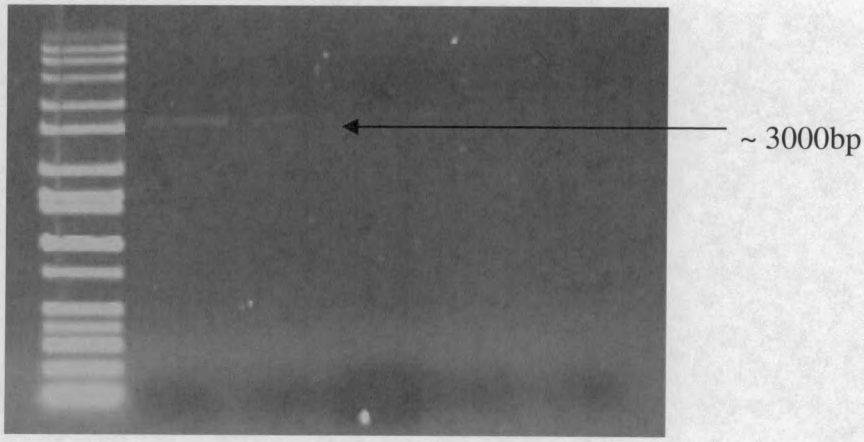

Figure 10: Inh-α subunit gene promoter sequence on 1% agarose minigel

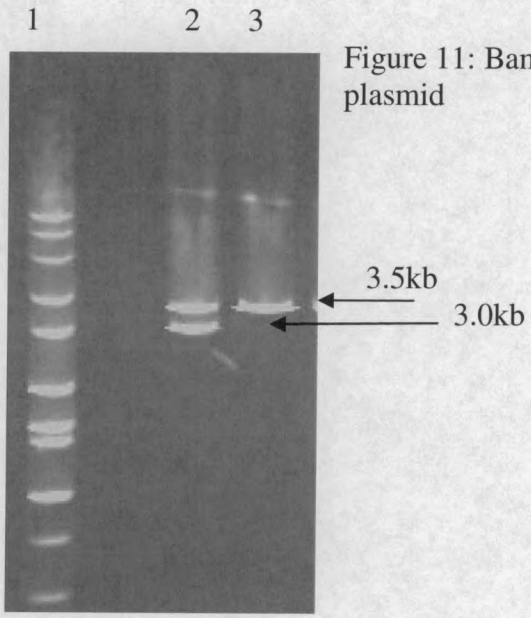

Figure 11: BamHI restriction digest of pCR-Blunt II-Topo plasmid

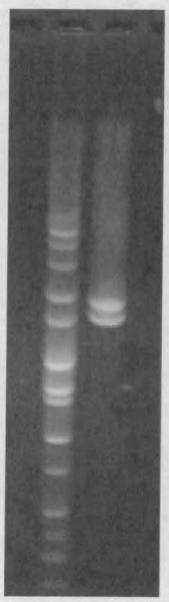

Figure 12: Confirmation digest of the pCR-Blunt II-Topo – BamHI restriction enzyme used

Following the BamHI restriction digest (fg.11) a single sample was observed to have two distinct bands with approximately 3.5 and 3kb, based on the molecular weight marker. As projected based on the restriction mapping analysis, the 3.5 band represents the linearized pCR-Blunt II-Topo plasmid and the lower band represents the ~ 3000bp sequence of the Inh-α subunit gene promoter. Collectively, these data indicated successful ligation of the Inh-α subunit gene promoter sequence to the pCR-Blunt II-Topo plasmid. A sample reaction thus was prepared and sent for sequencing. In addition to the DNA sequencing analysis results (textbox 4) and sequence alignment, along with our restriction mapping data, we were able to conclude that the observed two bands are actually a Topo-Topo self ligation where the pCR-Blunt II-Topo plasmid was ligated to itself. Additional analysis provided evidence that due to the presence of a specific restriction site (GGATCC) for BamHI (fg.12) the two bands were seen. In a rare coincidence situation the two bands of the unexpected self ligation reaction matched the expected size for the Inh-α subunit gene promoter sequence and the pCR-Blunt II-Topo plasmid.

Protein Extraction, SDS-PAGE and Western Blot

Parallel to the Inh- α amplification experiments I performed several cell transfection experiments (see Supp. fg.4). As a results of the induced 293T-cell protein expression, I was able to collect the protein extract, as specified in the materials and methods section. The purified protein extract was then used to perform an SDS-PAGE and Western Blot. The nitrocellulose membrane was incubated and stained with anti-ICER and anti-FLAG primary antibodies. Our results indicated that gg-ICER Iγ was transferred from the polyacrilamide gel and stained positively for anti-ICER Ab (fg.14). This confirmed expression of gg-ICER Iγ under the control of the strong CMV promoter located upstream

on the pFLAG-CMV-2 plasmid's MCS. Successful expression the of the gene construct was a requirement for continuation with the of subcloning the Inh-α subunit gene promoter as a replacement of the CMV promoter.

Q-RT-PCR of w.t. and transgenic mouse cDNA

Collective data of multiple Q-RT-PCR reactions were analyzed for amplification of initial mRNA levels. The analysis were based on the fluorescence readings accumulated by the MX3000P software (Stratagene) and calculated for each cycle of the Q-RT-PCR with total of 40 cycles. Our starting samples were prepared using cDNA derived from transgenic and wild type mice (Molina *et al.,* manuscript in preparation) Using these data inputs we then calculated the ratio between the house-keeping gapdh gene fluorescence levels and Inh-α gene fluorescence levels for each specific sample used. The ratio of gapdh/Inh-α of wild type mice were used to set reference mRNA translational levels. Using the wild-type calculated ratio of gapdh/Inh-α I was then able to compare the mRNA levels of transgenic animals and determine if Inh-α expression in animals carrying ICER transgene is downregulated, unaffected or induced (fg.14 and Supp. fg.5).

CNANATGCNTGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGCCCTTAGGCGA
ATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACCAAGCTTGATGCATA
GCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTG
TGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAA
GCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTC
CAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCG
GTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGC
TGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGA
TAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGC
CGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCT
CAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAG
CTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCT
TCGGGAAGCGTGGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTANGTCGT
TCGCTCCAAGCTGGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCC
GGTAACTATCGTCTTGAGTCCCACCCGGNAAGANACGACTTATNGCCNCTGGNAGCAGCCNC
TGNTAANAGGATTATCANANCGAGGNTTN .

Textbox 4: DNA sequencing output for Inh-α subunit gene promoter on pCR-Blunt II-Topo (BamHI restriction site in red)

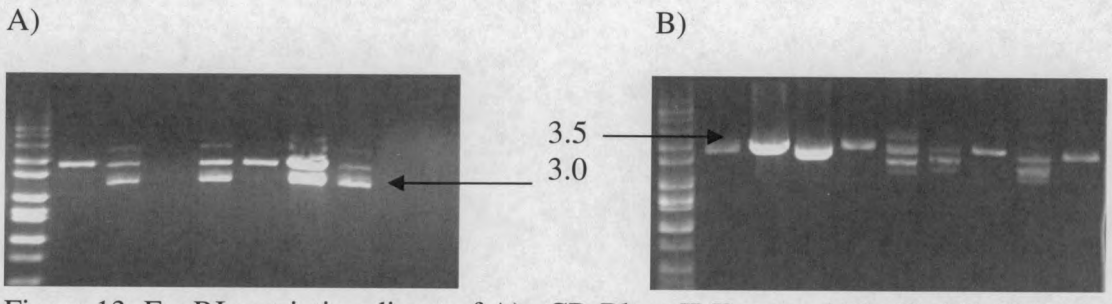

Figure 13: EcoRI restriction digest of A) pCR-Blunt II-Topo and B) pFLAG-CMV-2 gg-ICER γ plasmids carrying Inh-α promoter. Both pictures represent agarose gels following plasmid extraction and EcoRI restriction digest.

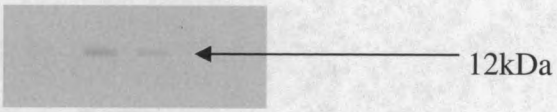

Figure 14: Western Blot for gg-ICER Iγ using anti-ICER primary antibody

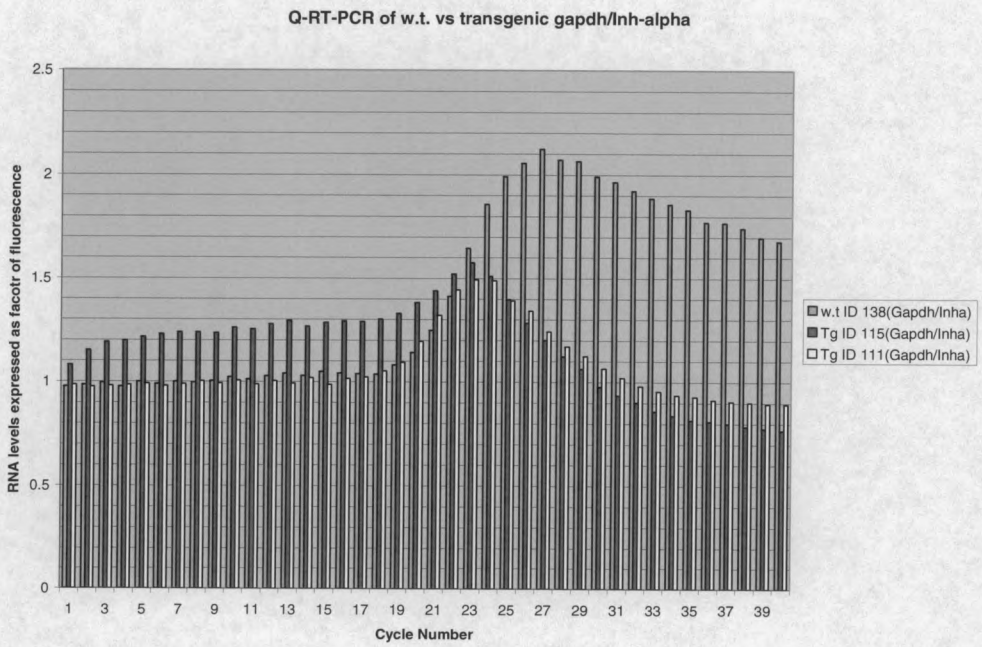

Figure 15: Q-RT-PCR output data for w.t. and transgenic cDNA amplification rates plotted as factor of fluorescence (mouse cDNA) – graph indicated that levels of Inh- α in transgenic animals is significantly downregulated, compared to the levels of expression in wild-type animals.

DISCUSSION

Transgenic animals including rodents and avian models became suitable systems for studies on gene function and gene interactions and have been used to unlock the mechanisms involved in specific developmental and reproductive processes. Comparative studies using nucleic acids and protein samples derived from wild type and transgenic animals represent a reliable method for validating specific genes and their involvement in specific processes. In addition, following the recent advances in the DNA microarray technology, studies of the expression patterns between wild type and transgenic animals became reliable tool for discovering specific genes that are induced or repressed as a result of the specific mutation introduced to the newly modified animal model.

CREM and ICER isoforms of activators and repressors in multiple studies have been implicated to regulate processes related to the reproductive system as well as the neuroendocrine and the nervous system (Mukherjee *et al.,* 2003). ICER has been reported to be the smallest nuclear transcription factor known with only 108 amino acid in its sequence. ICER has also been reported to be the only inducible isoform of CREM exerting its function as dominant negative transcriptional repressor. Representing the last signals of the well studied cAMP relay, CREM and ICER constructs have been developed for different animal models. The role of ICER in folliculogenesis however has not been fully validated since there is no ICER-null rodent model yet developed (Molina *et al.,* manuscript in preparation). Developing a mouse model where ICER expression was disrupted and ICER role was examined based on the newly introduced expression pattern was indeed a very useful tool for implicating this CREM isoform in the folliculogenesis, spermatogenesis and in the reproductive system in general (Molina *et al.,* manuscript in

preparation, William and Habener, 1996). In thus current study my focus was to generate a FLAG-ICER transgenic construct specific for the domesticated hen, *Gallus gallus*. My perspective was derived from several key benefits from developing an avian model carrying ICER transgenic construct regulated by an ovarian-specific promoter. From an economic stand point, genetically modified avian model with inducible ovulation rates may directly translate into increased food production. In chicken, hyperovulation would mean increased egg production per animal unit, a factor that is a great interest for the biotech sector and the poultry industry in general. Increased egg production would directly translate into significant increase of monetary gain. The hyperovulation rate had two-fold increase in rodents (Molina *et al.,* manuscript in preparation). From scientific perspective, a plausible effect would be the molecular mechanism involved in the reproductive system and the clues that may be unlocked by generating a hyperovulating system under hormonal (FSH or LH) or cAMP-inducible control. On the other hand, recent studies are focusing on generating avian transgenic animals with the ability to serve as natural bio-reactors for production of therapeutically relevant proteins that in other cases require costly manufacturing processes. The idea for developing avians as protein bio-reactors may simply come out from the fact that a single egg contains ~3.6g of albumin protein (Lillico *et al.,* 2007). Shifting from albumin production to a protein of interest can thus be made possible by designing specific transgenic constructs that will utilize the natural avian mechanism for protein production. For these specific goals, I focused on the domesticated hen, *Gallus gallus*. Among the reasons for using avian animal model are the availability and research-cost efficiency, previous work already reporting significant advances in gene

delivery as well as the availability of the completed chicken genome from the NCBI database (http:// www.ncbi.nlm.nih.gov/projects/ genome/ guide/chicken).

For the purpose of generating ovarian-specific construct, I amplified the 3kb Inhibin-α subunit gene promoter which has been implicated as site for transcriptional activation by CREB in response to FSH (Molina *et al.,* manuscript in preparation). The Inh-α subunit gene has tissue-limited expression thus making it suitable promoter sequence for transgene construct targeted for ovarian expression only. Inhibin-α subunit gene expression is limited to the ovaries, gonads, the pituitary gland and placenta, however, only by using the entire 3kb promoter sequence of the *Gallus gallus* Inh-α I hypothesize that the transgene expression will be limited to the ovaries only. My hypothesis is derived from the data acquired from study done on rodents (Molina *et al.,* manuscript in preparation). The 3kb promoter sequence of the Inh-α gene has not been amplified up to this date. Our hypothesis extends that under the control of the FSH inducible Inh-α promoter we can over-express FLAG-ICER transgene and induce hyperovulation. Using a sequence-homolog construct, specific for mice, two-fold rate increase in ovulation was observed (Molina *et al.,* manuscript in preparation). Analyzing the sequences for Inh-α and ICER between rodents and avian, I found 94% identical nucleotide arrangement thus I expect to see similar expression patter in *Gallus gallus.*

Inh-α subunit gene promoter was found to be ~3kb in size and 66% of GC content. Using the method indicated in the material and methods section, I was able to amplify this sequence and our agarose gel results showed the expected band size. Presumably, due to the size of the promoter and the high GC content, I was not able to neither repeat the amplification by performing direct PCR of genomic DNA nor re-amplify the PCR product.

For this reason I re-designed and used multiple primers sets specific for the Inh-α promoter sequence (suppl. fg.3) Every primer set used was re-designed and optimized for different annealing temperature, a factor that I gave priority when adjusting the PCR parameters. In addition, in multiple reactions I changed the primer concentration and the cycling parameters. The results of the new PCR conditions did not show any particular effect. Studies reporting amplification of high GC templates suggested that this lack of amplification efficiency may be due to the shorter readable sequences as a result of formation of distinct secondary structure (Choi *et al.,* 1999). For that reason I validated several key factors including changes in the DNA concentration and concentration of buffers and additives (DMSO, MgSO4). A 5% v/v DMSO was reported to increase denaturation of the dsDNA and increase the length of the segment amplified at any given time. No significant changes were observed following multiple adjustments made with DMSO and MgSO4, using one alone or together (Choi *et al.,* 1999). Next, I focused on testing different thermostable enzymes. These enzymes include the adjustments made to the initial Accu-Prime protocol (Invitrogen) the HotStart Tag Plus DNA Polymerase (Qiagen) and the HotStart Tag Mastermix (Denville scientific). I observed relatively small changes in amplification efficiency between different enzymes used, however all results lacked re-amplification of the expected sequence.

Following the extraction of DNA and RNA from chicken ovaries we successfully amplified both gg-ICER Iγ and Inh-α. I then used multiple cloning and expression plasmids to produce larger amount of the gene and the promoter sequences. gg-ICER Iγ was initially subcloned onto pCR Topo and later transferred onto pFLAG-CMV-2 plasmid. Under the control of the strong CMV promoter I was able to express the ICER gene following 293T

(Human Kidney Carcinoma) cell transfection. Western blot analysis provided evidence for gg-ICER Iγ expression, staining positive with anti-ICER primary antibody. At the same time, DNA sequencing analysis provided additional confirmation for the integrity of the amplified sequence. The BLAST and alignment data showed 100% nucleotide match with the known chicken (*Gallus gallus*) sequence, initially found on the NCBI database (AF509471). In addition, I was able to construct a variation of the original pFLAG-CMV-2 plasmid by removing the CMV promoter. Using the HincII restriction enzyme we observed band pattern characteristic for linearized plasmid without the promoter sequence. This variation of the pFLAG-CMV-2 plasmid was later used for subcloning the Inh-α subunit gene promoter sequences upstream of the gg-ICER Iγ located on the multiple cloning site. My initial data for the Inh-α promoter provided evidence for successful amplification of the 3kb sequence. I was able to surpass the main obstacle for amplifying a large construct with over 66% GC content using specific thermo stable DNA polymerase. Due to the specific amplification mechanism of the Accu-Prime polymerase enzyme, which generates sequences with blunt ends, I used pCR-Blunt II-Topo subcloning plasmid designed to incorporate blunt end sequences. To further increase the ligation rates, I de-phosphorylated the PCR product, which resulted with high yield bacterial colonies growth on amp-selective plates. I am next focusing on subcloning our final construct, Inh-α-FLAG-gg-ICER Iγ and test for expression in cell culture. The final stage would include the transfer onto lentiviral vector that would be used as vehicle for delivering the transgene construct into fertilized chicken eggs, as a first step towards creating a transgenic chicken.

In addition to the molecular cloning of the Inh-α-FLAG-gg-ICER Iγ transgene construct, I also focused on further analysis of mouse-derived cDNA. Namely, I performed

Q-RT-PCR and analyzed the levels of gapdh and Inh-α genes taken from wild type and transgenic animals. Inhibins have been shown to have limited expression in several tissues through the organism including the granulosa cells in females and sertoli cells in males (Shao-Yao *et al.,* 1995, Safi *et al.,* 2002, Chen *et al.,* 2007). The levels of Inh-α have been reported to increase at the pre-antral stage of follicular development reaching its peak at the antral stage (Johnson *et al.,* 2005). In the process of folliculogenesis, the antral or the tertiary phase represents the survival gate, when most of the follicles get arrested and sent for follicular atresia. The mechanism behind the follicular atresia and the molecular cues that initiate termination of development remain unknown. Several studies however have reported that following the increase expression of Inh-α in granulosa cells, the levels of FSH produced in pituitary gland are decreased (Safi *et al.,* 2002, Bernard *et al.,* Chen et al., 2007). The proposed mechanism suggests that Inh-α closes the negative signal loop that shuts down FSH production. Consequently, as a result of the FSH deficiency, found also at the antral stage, the follicular development is terminated for most of the developing follicles, followed by the initiation of the follicular atresia. My results support this hypothesis. In wild-type mice, following the initial increase in expression of Inh-α, the levels reach a peak expression after which a stabilization is observed. The levels of Inh-α were calculated setting the levels of the house-keeping gapdh gene as a reference line (Molina *et al.,* manuscript in preparation). In transgenic mice, however, the results were dramatically different. Following the initial increase in expression, Inh-α levels were found to be down-regulated, almost bringing it as low as the initial starting point levels. My hypothesis is that ICER Iγ overexpression, in transgenic mice, is repressing Inh-α gene expression by binding to the CRE sequence located on the Inh-α promoter. By doing so,

ICER is prolonging the FSH surge coming from the pituitary gland. Prolonged FSH production is then increasing the number of the developing follicles rescued from follicular atresia. The same observation I found repeating the Q-RT-PCR experiment twice, using a total of 2 wild-type derived cDNA and 4 transgenic cDNA samples. These result, however, were not observed when a DNA microarray analysis was performed (Molina *et al.,* manuscript in preparation). This effect may be due to the fact that at different stages of the mice estrous cycle, different expression patterns are regulating the processes of folliculogenesis, ovulation and follicular atresia, as well as the coordination between them all.

In this study I have shown data for successful cloning of the *Gallus gallus* Inducible cAMP Early Repressor (ICER Iγ). Following the RT-PCR amplification of the chicken RNA ICER Iγ, I performed several restriction digest reaction and verified the orientation of the gene, now ligated onto the pFLAG-CMV-2 plasmid. Placed under the control of the strong CMV promoter, I was able to induce gg-ICER Iγ expression in 293T (Human Kidney Carcinoma) cells and detect the gene expression using an antibody specifically designed for ICER Iγ. Western Blot analysis indicated expression of the target gene with the expected ~12kDa in size. Finally, our sequencing data indicated complete nucleotide sequence match of the amplified gg-ICER Iγ. The sequence alignment data (NCBI Blast) showed a 100% match with published sequences of *Gallus gallus* ICER Iγ. I can partially confirm amplification of the Inh-α subunit gene promoter sequence. The initial observation on the agarose gel indicated a 3kb band, as expected for the generated promoter sequence (European Bioinformatics web page). Following the subcloning reaction, I was able to confirm the right band patterns, using restriction enzymes specific

for the used pCR Blunt II-topo plasmid and the Inh-α sequence. I wasn't able to confirm the correct sequence following the sequencing experiment. My focus is now the re-amplification of the Inh-α sequence and ligating it to the pFLAG-CMV-2-gg-ICER Iγ plasmid. Important feature of this plasmid is that the CMV promoter has been excised out by a specific HincII digest. A complete construct of Inh-α-FLAG-gg-ICER Iγ can then be tested in cell culture and prepared for lentiviral gene delivery into the host avian animal. Developing a transgenic colony carrying our construct can then be analyzed for several developmental and reproductive features, including the FSH regulated hyperovulation as part of my main hypothesis.

References:

1. Anna D. Burkart, Abir Mukherjee, and Kelly E. Mayo 2005, *Mechanism of Repression of the Inhibin -Subunit Gene by Inducible 3',5'-Cyclic Adenosine Monophosphate Early Repressor*, Molecular Endocrinology

2. Abir Mukherjee, Janice Urban, Paolo Sassone-Corsi, and Kelly E. Mayo 1998, *Gonadotropins Regulate Inducible Cyclic Adenosine 3',5'-Monophosphate Early Repressor in the Rat Ovary: Implications for Inhibin alpha Subunit Gene Expression*, Molecular Endocrinology

3. V Morales, I Gonzalez-Robayna, I Hernandez, J Quintana, P Santana, C M Ruiz de Galarreta and L F Fanjul 2003, *The inducible isoform of CREM (inducible cAMP early repressor,ICER) is a repressor of CYP19 rat ovarian promoter*, Journal of Endocrinology

4. Luis C. Muniz, Ghassan Yehia, Elisabeth Memin, Pillarisetty V.A.L. Ratnakar, and Carlos A. Molina 2006, *Transcriptional Regulation of Cyclin D2 by the PKA Pathway and Inducible cAMP Early Repressor in Granulosa Cells*, Biology of reproduction

5. Paolo Sassone-Corsi 1998, *Coupling gene expression to cAMP signaling: role of CREB and CREM*, The International Journal of Biochemistry & Cell Biology

6. Giuseppe Servillo, Maria Agnese Della Fazia and Paolo Sassone-Corsi 2002, *Coupling cAMP Signaling to Transcription in the Liver: Pivotal Role of CREB and CREM*, Experimental Cell Research

7. B. E. Porter, I. V. Lund, F. P. Varodayan, R. W. Wallace and J. A. Blendin 2008, *The role of transcription factors cyclic AMP responsive element modulator (CREM) and inducible cyclic AMP early repressor (ICER) in epileptogenesis*, Neuroscience

8. Nicholas S. Foulkes, Jimo Borjigin, Solomon H. Snyder and Paolo Sassone-Corsi 1997, *Rhythmic transcription: the molecular basis of circadian melatonin synthesis*, Trends Neurosci. (1997)

9. Akari Inada, Gordon C. Weir, Susan Bonner-Weir 2005, *Induced ICER Ic down-regulates cyclin A expression and cell proliferation in insulin-producing b cells*, Biochemical and Biophysical Research Communications

10. Jacek Jaworski, Barbara Mioduszewska, Amelia Sanchez-Capelo, Izabela Figiel, Agata Habas, Agata Gozdz, Tomasz Proszynski, Michal Hetman, Jacques Mallet, and Leszek Kaczmarek1 2003, *Inducible cAMP Early Repressor, an Endogenous Antagonist of cAMP Responsive Element-Binding Protein, Evokes Neuronal Apoptosis In Vitro,* The Journal of Neuroscience

11. Sandrine Ruchaud, Paule Seite, Nicholas S Foulkes, Paolo Sassone-Corsi and Michel Lanotte 1997, *The transcriptional repressor ICER and cAMP-induced programmed cell death*, Oncogene

12. Eduardo J Folco and Gideon Koren 1997, *Degradation of the inducible cAMP early repressor (ICER) by the ubiquitin-proteasome pathway*, Biochem J

13. William H Walker, Philip B Daniel and Joel F Habener 1998, *Inducible cAMP Early Repressor ICER down-regulation of CREB gene expression in Sertoli cells,* Molecular and Cellular Endocrinilogy

14. J.E. Fortune 1994, *Ovarian follicular growth and development in mammals*, Biology of Reproduction

15. Josef Bodor, Anna-Lena Spetz, Jack L Strominger, and Joel F Habener 1996, *cAMP inducibility of transcriptional repressor ICER in developing and mature human T lymphocytes,* Proc. Natl. Acad. Sci USA

16. Nicholas S Foulkes, Jimo Borjigin, Solomon H Snyder and Paolo Sassone-Corsi 1997, *Rhythmic transcription: the molecular basis of circadian melatonin synthesis* TINS

17. Akari Inada, Gordon C Weir, Susan Bonner-Weir 2005, *Induced ICER Iy down-regulates cyclin A expression and cell proliferation in insulin-producing b-cells*, Biochemical and Biophysical research communications

18 Maria Agnese Della Fazia, Giuseppe Servillo, Paolo Sassone-Corsi 1997, *Cyclic AMP signaling and cellular proliferation: regulation of CREB and CREM*, FEBS letters

19 Luis C Muniz, Ghassan Yehia, Pillarisetty V.A.L. Ratnakar and Carlos A. Molina: *Hyperovulation in an ovarian-specific transgenic mouse model*, Manuscript in preparation

20 Gian Maria Fimia and Paolo Sassone-Corsi: *Cyclic AMP signaling*, Journal of Cell Science,

21 Mark A. Sussman 2003, *ICER-Capades Putting Cardiac Cyclic AMP signaling "On Ice"* Circ Res

22. William H Walker and Joel F Habener 1996, Role of Transcription factors CREB and CREM and cAMP-Regulated transcription during spermatogenesis, Trends Endocrinology Metab

23 S.G. Lillico, A. Sherman, M.J. McGrew, C.D. Robertson, J. Smith, C. Haslam, P. Bernard, P.A. Radcliffe, K.A. Mitrophanous, E. A. Elliot and H.M. Sang, 2007: Oviduct-specific expression of two therapeutic proteins in transgenic hens, PNAS

24. Shao-Yao Ying, Si-Qing Li and Zhong Zhang 1995: *Expression and localization of Inhibin alpha subunit in rat retinal photoreceptor cells*, Life Sciences

25. M. Safi, O.M. Onagbesan, V. Briggeman, B. Vleugels, G. Volckaert and E. Decuypere 2002: *Regulation of Inhibin alpha and beta A subunit messenger ribonucleic acid levels by gonadotropins and IGF-I in cultured chicken granulosa cells*, General and comperative Endocrinology

26. Daniel Bernard, Stacey Chapman and Teresa Wooddruff: Mechanism of Inhibin Signal Transduction

27. P.A. Johnson, C.F. Brooks and A.J. Davis 2005: *Pattern of secretion of immunoreactive inhibin/activin subunits by avian granulosa cells*, General and comperative Endocrinology

28. Fengjian Chen, Xunping Jiang, Xiuping Chen, Guiqiong Liu and Jiating Ding 2007: *Effects of downregulation of Inhibin alpha gene expression on apoptosis and proliferation of Goose Granulosa Cells*, Journal of Genetics and Genomics

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gb|AF509471.1| UG Gallus gallus ICER mRNA, complete cds
Length=327

GENE ID: 378903 ICER | ICER protein [Gallus gallus]

 Score =  566 bits (306),  Expect = 5e-158
 Identities = 306/306 (100%), Gaps = 0/306 (0%)
 Strand=Plus/Plus

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Query  61   CAGGGAGTGGTAATGGCAGCCTCCCCAGGGGCTCTGCATAGTCCTCAGCAACTGGCAGAA
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Query  301
GAATAA  306
||||||
GAATAA  327
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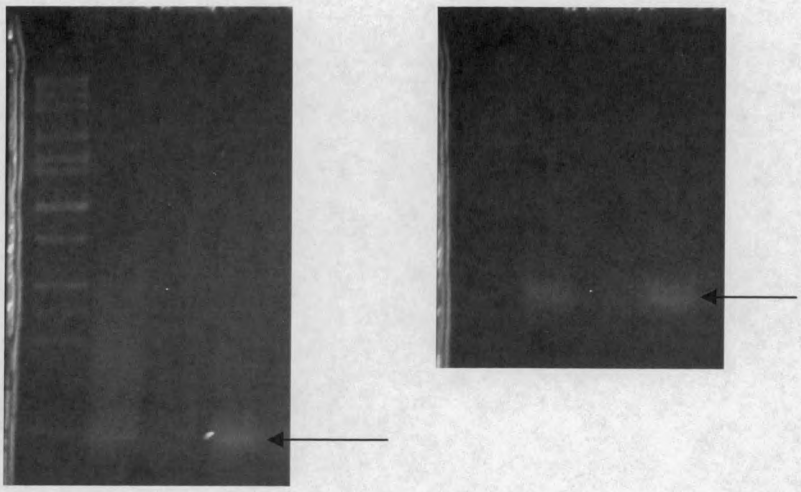
Supplement figure 1: Mega-BLAST analysis using the nucleotide sequence generated from the DNA sequencing experiment of the pFLAG-CMV-2-gg-ICER Iγ. Results indicate 100% match b/w the amplified ICER and the NCBI published sequence for ICER gene

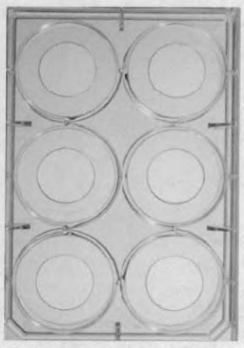


Supplement figure 2 RT-PCR Agarose gel of ICER Iγ (arrows indicate band for ~320bp ICER Iγ

Forward primer: 5' GCTCCGCCACCTCCACGTCC 3'
Reverse primer: 5' GGCCGTGGGGCAGCTCAAGG 3'

Supplement figure 3 Inh-α amplification primers (additional set)

Plate 1 pFLAG-ICER Iγ

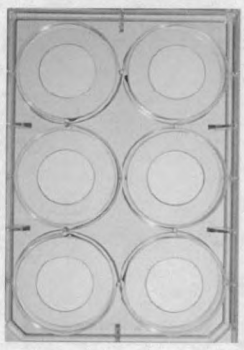


------Lipofectamine (Invitrogen)----

------Ca-Phospate (Sigma)----------

------Lipofectamine (Sigma)---------

Plate 2 pSV-ICER Iγ



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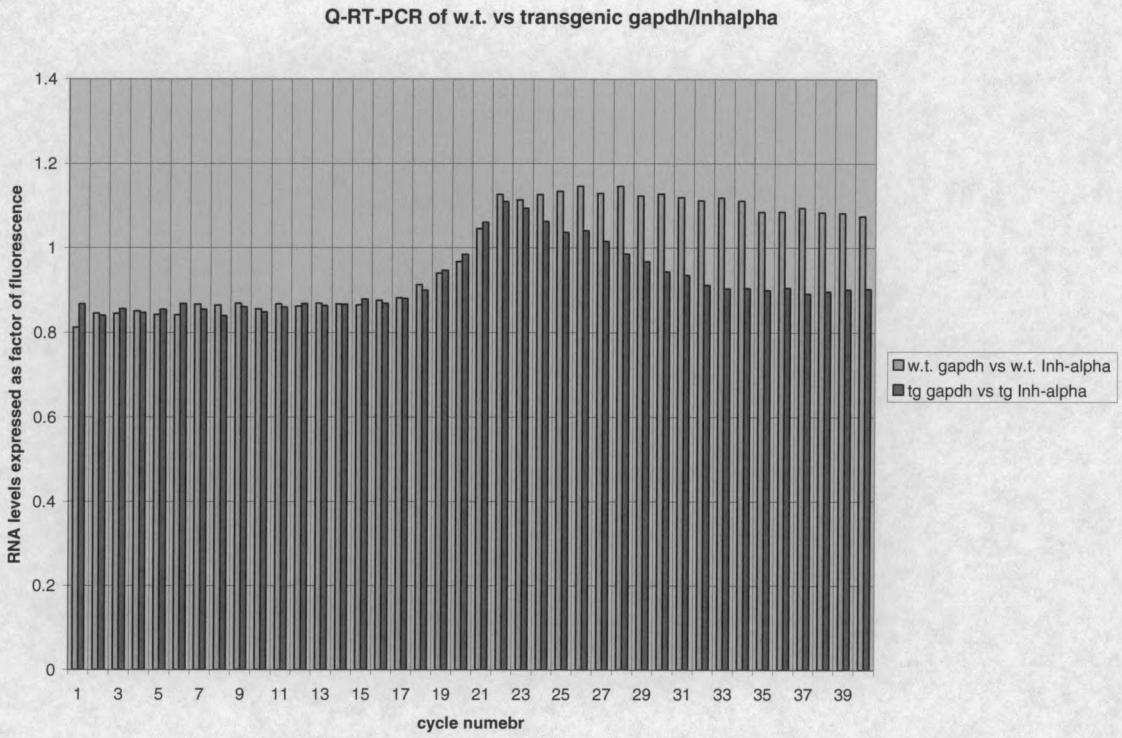
Supplement figure 4 Cell Transfection of gg-ICER Iγ:
Plate 1 pFLAG-CMV-2 gg-ICER Iγ
Plate 2 pSV-mm-ICER Iγ control



Supplement figure 5 Q-RT-PCR of w.t. vs transgenic gapdh/Inh-α (mouse cDNA)