5-2011

Cloning and Characterization of the Promoter for the Ovarian-Specific Inhibin Alpha Gene Form Zebrafish (Danio rerio)

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ABSTRACT

Inducible cAMP Early Repressor (ICER) is an important regulator of folliculogenesis as it regulates the nuclear response to gonadotropins in ovarian tissue. Studies in mice demonstrated a 3.0 kb region of the alpha inhibin (INHα) promoter is a site of transcriptional activation in response to follicle-stimulating hormone (FSH). The purpose of this study was to isolate and construct an ICER transgene and characterize the putative 3.0 kb promoter region of the ovarian-specific INHα gene in *Danio rerio* (zebrafish).

The sequences for ICER and the INHα promoter in zebrafish were identified using BLAST searches with the known sequences from mouse and chicken. The putative sequences were subsequently isolated and amplified using RT-PCR and PCR. Successful isolation and amplification was confirmed with restriction enzyme mapping and DNA sequencing. ICER was cloned into pFLAG-CMV-2 plasmid to create the FLAG-dr-ICER Iγ transgene construct. Expression of FLAG-ICER protein was confirmed by Western blot after transfection into HEK293T human kidney cells. The INHα promoter was cloned into a pGL3-Basic luciferase expression vector for characterization studies. The transgene containing the INHα promoter was transfected into AB9 zebrafish tailfin cells and embryonic PAC2 zebrafish cells. Transfection experiments in AB9 and PAC2 cells demonstrated promoter induction by follicle-stimulating hormone (FSH) and repression of promoter activity by luteinizing hormone (LH).

MONTCLAIR STATE UNIVERSITY

CLONING AND CHARACTERIZATION OF THE PROMOTER FOR THE OVARIAN-SPECIFIC INHIBIN ALPHA GENE FORM ZEBRAFISH (*DANIO RERIO*)

by

Caitlin Marie Ament

A Master's Thesis Submitted to the Faculty of

Montclair State University

In Partial Fulfillment of the Requirements

For the Degree of

Masters of Science

May 2011

College/School College of Science and Mathematics

Department Biology and Molecular Biology

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Montclair, NJ

2011

ACKNOWLEDGEMENTS

A thesis is a journey of blood, sweat, and tears condensed into a few short pages. As such, many people deserve to be acknowledged for accompanying me on my path.

Thank you to the numerous students who have listened to my tales of progress and frustration during this process. I specifically would like to thank Ms. Diana Sanchez, Ms. Nadia R. Pasquale, and Ms. Dipika Patel for their guidance and patience.

Thank you to Dr. Kristin Monsen for her assistance and guidance during my college career.

My heartfelt appreciation belongs to my father, Mr. Edward A. Ament, my sister, Ms. Samantha L. Ament, and the rest of my family for supporting me and knowing when to leave well enough alone.

I am grateful to Dr. Carlos A. Molina for welcoming me into his lab and guiding me through this project.

Finally, I dedicate this work to my multiple "Moms": Dr. Ann Marie DiLorenzo for showing me how to find the story, Dr. Tina Pantano for giving me my foundation, Mrs. Nina Calpalbo for encouraging me, and Mrs. Sharon L. Ament, my real mother, for everything.

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INTRODUCTION

Oogenesis is the process in which primordial germ cells become ova. Early studies in *Oryzias latipes* (medaka), *Danio rerio* (zebrafish), and other teleosts yielded the presence of six distinctive stages. During the first stage, the primordial germ cells form and segregate to create the germline. The process of sex differentiation transforms these primordial germ cells into oogonia, which are later transformed into oocytes. Transformation of the oogonia into oocytes indicates the onset of meiosis. Following the first meiotic arrest, the oocyte matures until meiosis resumes and a mature ovum is produced. During ovulation, this ovum is expelled from the ovulatory follicle (Patino & Sullivan, 2002).

The ovarian follicle is one of the most studied structures involved in the ovulatory cycle. It develops when pre-follicle granulosa cells and an accompanying basement membrane envelop a developing oocyte in the late pachytene or diplotene stage of chromosome development. The granulosa cells form a monolayer around the oocyte while a heterogeneous layer of thecal cells forms externally over the basement membrane. Together these structures form the ovarian follicle (Patino & Sullivan, 2002).

Inducible cAMP Early Repressor (ICER) is a dominant negative transcriptional repressor necessary to regulate genes required for the granulosa to luteal cell transition in ovarian cells (Molina, Foulkes, Lalli, & Sassone-Corsi, 1993) (Muniz, Yehia, Memin, & Ratnakar, 2006). It is a member of the CRE Modulator (CREM) gene family (Muniz, Yehia, Memin, & Ratnakar, 2006) (Foulkes, Borrelli, & Sassone-Corsi, 1991). ICER often heterodimerizes with other proteins from the cAMP-Response Element (CRE-) Binding Protein (CREB) family in order to exert transcriptional control (Molina, Foulkes,

Lalli, & Sassone-Corsi, 1993) (Foulkes, Borrelli, & Sassone-Corsi, 1991) (Hoeffler, Meyer, Y., Jameson, & Habener, 1988) (Burkart, Mukherjee, & Mayo, 2006) (Mukherjee, Urban, Sassone-Corsi, & Mayo, 1998). Specifically, ICER has been shown to suppress the activity of follicle-stimulating hormone (FSH) inducible genes including inhibin alpha subunit (INHα), Cytochrome P450 Aromatase (*Cyp 19a*), and cyclin D2 (Molina, Foulkes, Lalli, & Sassone-Corsi, 1993) (Burkart, Mukherjee, & Mayo, 2006) (Mukherjee, Urban, Sassone-Corsi, & Mayo, 1998) (Morales M. , Gonzalez-Robayna, Santana, Hernandez, & Fanjul, 2006) (Morales V. , et al., 2003) (Perlman, et al., 2006). Under normal physiological conditions, ICER is expressed during the preovulatory luteinizing hormone (LH) surge in granulosa cells (Burkart, Mukherjee, & Mayo, 2006) (Mukherjee, Urban, Sassone-Corsi, & Mayo, 1998) (Morales V. , et al., 2003).

Gonadotropin cycling of FSH and LH in ovarian tissue controls the expression of genes responsible for follicular development (Burkart, Mukherjee, & Mayo, 2006) (Yehia, Schlotter, Razavi, Alessandrini, & Molina, 2001). Both FSH and LH have been shown to activate stimulatory G protein-coupled receptors located in the cell membrane of ovarian cells (Hunzicker-Dunn & Maizels, 2006). The binding of these ligands to receptors indirectly activates adenylyl cyclase and produces the secondary messenger cAMP (Mukherjee, Urban, Sassone-Corsi, & Mayo, 1998).

FSH is secreted from the pituitary and stimulates immature ovarian follicles through the expression of FSH-responsive genes, such as the INHα subunit, in granulosa cells (Mukherjee, Urban, Sassone-Corsi, & Mayo, 1998) (Hunzicker-Dunn & Maizels, 2006). INHα is a dimeric hormone that suppresses synthesis and secretion of pituitary FSH (Mukherjee, Urban, Sassone-Corsi, & Mayo, 1998). FSH is partially responsible for

positive regulation of INHα while LH is responsible for negative regulation of INHα (Burkart, Mukherjee, & Mayo, 2006). A feedback loop exists between FSH secretion and INHα expression in the ovarian cells and suppression of FSH production in the pituitary by INHα.

FSH also encodes LH receptors in immature ovarian follicles. Once the follicle matures, the immune receptors are activated to become functional LH receptors. During the preovulatory LH surge prior to proestrus, activity of the LH receptors trigger the morphological and biochemical changes necessary for oocyte ovulation and luteinization of remaining follicles. The LH surge also causes the downregulation of INHα. Suppression of INHα increases FSH secretion causing a secondary surge of FSH, which occurs at the start of estrus (Mukherjee, Urban, Sassone-Corsi, & Mayo, 1998). This second increase in FSH levels is also responsible for recruiting new ovarian follicles.

Expression of ICER is transient and immediately follows preovulatory surge of LH (Mukherjee, Urban, Sassone-Corsi, & Mayo, 1998) (Kameda, Mizutani, Minegishi, Ibuki, & Miyamoto, 1999). Binding of ICER to the CRE in the INHα promoter is required for downregulation of INHα (Burkart, Mukherjee, & Mayo, 2006). Previous studies have shown downregulation of INHα leads to a prolonged surge of FSH and thus the recruitment of additional ovarian follicles (Mukherjee, Urban, Sassone-Corsi, & Mayo, 1998). It has been hypothesized that FSH signals will trigger changes in gene expression leading to the induction of hyperovulation if ICER is expressed earlier during the folliculogenesis pathway. Previous studies implicated a 3.0 kb region of the INHα promoter as a site of ICER transcriptional activation in response to FSH (Muniz, Yehia,

Memin, & Ratnakar, 2006). Although well-characterized in mammalian systems, the activity of a putative INHα promoter in zebrafish remains largely uncharacterized.

The aim of this study was to isolate and clone ICER and the implicated region of the INHα promoter in zebrafish. The cloned ICER was tagged with an identifying p-FLAG sequence in order to differentiate it from endogenous ICER. Expression of p-FLAG-ICER-Iγ in HEK-293 was confirmed with a Western Blot. The 3.0 kb INHα promoter region was isolated and cloned into a luciferase reporter vector in order to assess promoter activity. Transfection experiments in AB9 (zebrafish tailfin cells) and PAC2 (zebrafish embryonic cells) showed promoter activity induction by FSH and repression of promoter activity by LH. Induction and repression of the 3.0 kb region of INHα promoter by FSH and LH, respectively, suggested this region of the promoter functions similarly as the same promoter in other animal models.

MATERIALS AND METHODS

In Silico Analysis

The sequences pertaining to ICER and the INHα promoter were identified using a BLAST search with the known sequences from mouse and chicken (http://blast.ncbi.nlm.nih.gov). The sequences were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2). Restriction enzyme sites were determined using NEBCutter (http://tools.neb.com/NEBcutter2/index.php).

RNA/DNA Extraction

RNA and DNA were extracted from flash-frozen zebrafish using TRIzol® Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. RNA and DNA integrity was confirmed with a 1% agarose gel

electrophoresis stained with ethidium bromide. Prior to each experiment, the concentration of RNA or DNA was determined using a NanoDrop™ 1000 spectrophotometer (Thermo Scientific).

RT-PCR Amplification of ICER

ICER was amplified using the AccessQuick™ RT-PCR System (Promega) using the following primers: forward 5`-GCTCCGCCACCTCCACGTCC-3` and reverse 5'-GGCCGTGGGGCAGCTCAAGG-3`. Primers were designed using the sequences identified by the BLAST search and optimized for PCR. Restriction sites for XhoI and XbaI were created during subsequent rounds of PCR. All reactions were performed according to the manufacturer's protocol and cycling parameters. Successful amplification of ICER was demonstrated using a 2% agarose gel electrophoresis.

PCR Amplification of the INHα Promoter

Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen) was used to amplify the target sequence from the INHα promoter. The following primers were used to isolate the target sequence: forward 5`-GGTACATATTTTATTTTCCCCATT-3` and reverse 5`-CCTCAAAACTCAACAGAGGTTAAA-3`. Primers were designed using the sequences identified by the BLAST search and optimized for PCR. All reactions were performed according to the manufacturer's protocol and cycling parameters. A 1% agarose gel electrophoresis was used to demonstrate successful amplification of INHα.

TOPO® TA Cloning of ICER

The ICER Iγ fragments produced by RT-PCR were ligated into a pCR® 2.1 TOPO® cloning vector (Invitrogen) using the XhoI and XbaI restriction enzymes to open

the plasmid. Cloning reactions were prepared according to manufacturer's instructions. OneShot® TOPO10 chemically competent cells were incubated for five minutes at room temperature in preparation for transformation. After the incubation, 2µl of cloning reaction was added to the bacteria. The bacteria were subsequently incubated on ice for thirty minutes. Following a thirty-second heat-shock at 42°C, 250µl of pre-warmed S.O.C. medium was added to the bacteria. The bacteria were incubated for sixty minutes at 37°C before being spread on LB ampicillin selective plates and allowed to grow overnight. Colonies indicating successful integration of the TOPO-ICER Iγ plasmid were transferred to a 3 mL vial of LB ampicillin medium and allowed to grow overnight at 200 rpm. A 1.5 mL aliquot of the culture was centrifuged for one minute at 12,000 rpm to harvest the bacteria. The plasmid DNA was purified using the QIAprep® Miniprep Kit (Qiagen) according to the manufacturer's protocol. Restriction mapping with EcoRV and separation with a 1% agarose gel electrophoresis was used to determine the orientation of ICER Iγ in the plasmid. The fragment containing ICER Iγ was excised and purified using the QIAquick® Gel Extraction Kit (Qiagen).

Creation of the pFLAG-CMV-2-ICER Iγ Construct

Gel purified ICER Iγ fragments were ligated into the onto pFLAG-CMV-2 expression plasmid using XhoI and XbaI to open the vector. The ligation reactions were incubated overnight at 16°C. Transformation and incubation of OneShot® TOPO10 chemically competent cells was once again used to amplify the plasmid. Following isolation using the QIAprep® Miniprep Kit, the plasmid DNA was sent to GENEWIZ (South Plainfield, NJ) for Sanger sequencing.

SDS-PAGE and Western Blot

Hek293T cells were transfected with the FLAG-dr-ICER Iγ. The cells were harvested after forty-eight hours of incubation at 37°C in a 5% CO_2 environment. Cells were washed twice with 1 mL cold HBSS and scraped in 1 mL ice-cold PBS. Following a one-minute centrifugation at 14,000 rpm, a 300µl aliquot of 1X Laemmli buffer was added to each sample. A 100°C heating block was used to heat the samples for five minutes. The samples were then centrifuged for ten minutes at 14,000 rpm. Following a two-second vortex and a three-minute centrifugation at 14,000 rpm, the supernatant from each sample was collected for SDS-PAGE.

The addition of 6X loading dye was added to 30 µL to prepare the samples for SDS-PAGE. Samples were run using a 1X Tris base running buffer for sixty minutes at 200V. Nitrocellulose membranes were soaked in transfer buffer and run overnight at 30V. Primary anti-ICER antibodies and secondary rabbit antibodies were used to visualize ICER protein.

TOPO® TA Cloning of the INHα Promoter

The INHα promoter fragment produced by PCR was ligated into a pCR® 2.1 TOPO® cloning vector (Invitrogen) according to manufacturer's instructions. Plasmid DNA was amplified and collected by transforming OneShot® TOPO10 chemically competent cells and collected using the QIAprep® Miniprep Kit.

Cloning of INHα into the pGL3-Basic Vector

The QIAquick® Gel Extraction was used to extract the fragment containing the INHα promoter. The promoter was inserted into the pGL3-Basic plasmid using the KpnI and XhoI restriction enzymes to open the plasmid. OneShot® TOPO10 chemically

competent cells and the QIAprep® Miniprep Kit Plasmid DNA was used to amplify and collect plasmid DNA. Ligation efficiency and plasmid orientation were determined using a restriction digest with HindIII. Plasmid DNA was sent to GENEWIZ (South Plainfield, NJ) for Sanger sequencing.

Cell Culture Conditions

AB9 and PAC2 cells were cultured in L15 (Leibovitz) culture medium (Gibco), supplemented with 15% fetal bovine serum (FBS), 100 U/mL penicillin/100 mg/mL streptomycin, and 50 mg/mL gentamicin (Gibco). Cells were plated and allowed to grow for 72 hours in a 25°C incubator.

Cell transfections were prepared using a 4:1 ration of the FuGene® HD Transfection Reagent, according to the manufacturer's instructions (Roche). Where indicated, cells were transfected with the pGL3-Basic plasmid, pGL3-INHα plasmid, FSH receptor (FSHr) plasmid, and LH receptor (LHr) plasmid.

Luciferase Assay

A luciferase activity assay was performed using the Dual-Luciferase® Reporter Assay System (Promega). Cells were treated with 10 μg/μL of forskolin (FSK), human chorionic gonadotropin (hCG), or FSH, where indicated, for twenty-four hours. Prior to measuring luciferase activity, cells were washed with PBS and lysed using a passive lysis buffer (Promega). Luciferase and renilla activity were read using a TD 20-20 Luminometer (Turner Biosystems) programmed for a two-second delay and a ten-second measurement.

RESULTS

In Silico Analysis

The sequences for ICER and the INHα promoter in Danio rerio were identified via a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the known sequences from mouse and chicken. The sequence of ICER was determined to be 327 bp long (Figure 1) while the promoter region from INHα was found to be 3,049 bp in length (Figure 2). For both alignments, a high identity match suggested the isolated sequences were ICER and INHα.

Restriction maps of the sequences for ICER (Figure 3) and INHα (Figure 4) determined the location of restriction sites and the GC content. These maps and the restriction maps for the TOPO (Supplementary Figure 1), pFLAG-CMV (Supplementary Figure 2), and pGL3- basic vectors (Supplementary Figure 3) were used to determine the enzymes required to prepare the cloning sites.

```
CLUSTAL 2.1 multiple sequence alignment

Zebrafish       ATGGCTGTGACCGGAGATGAGACAGAATCAGCCACCACTGGAGGTATGTCAGGATATCAG 60
Mouse           ATGGCTGTAACTGGAGATGAAACT------GCTGCCACAGGTGACATGCCAACTTACCAG 54
                ******** ** ******** **        **  **** ** *  *** **   ** ***

Zebrafish       ATGACCTCTCCTGCATCTGGTCTTTCCCAGG------TCATGGACAGCTCACCCGACTCT 114
Mouse           ATCCGAGCTCCTACTACTGCTTTGCCACAAGGTGTCGTGATGGCTGCCTCACCAGGAAGC 114
                **     ***** *  *** * *  * ** *      * ****    ****** *

Zebrafish       CTGCCCAGCCCTCAGCTTCTGGCAGAGGAGGCGTCACGCAAAAGGGAACTCCGACTGATG 174
Mouse           CTGCACAGTCCCCAGCAACTAGCAGAAGAAGCAACTCGCAAGCGGGAGCTGAGGCTGATG 174
                **** *** ** ****  ** ***** ** **  * *****  **** **  * ******

Zebrafish       AAGAACAGGGAAGCTGCCCGAGAATGCCGGCGGAAGAAGAAAGAATATGTCAAATGTCTC 234
Mouse           AAAAACAGGGAAGCTGCTAAAGAATGTCGACGTCGAAAGAAAGAGTATGTGAAGTGTCTT 234
                ** ***************  ****** ** **    ******** ***** ** *****

Zebrafish       GAAAACCGAGTTGCCGTGCTTGAAAAACAAGACAAGACGCTCATCGAAGAACTCAAGGCT 294
Mouse           GAGAGTCGAGTCGCAGTGCTGGAAGTTCAGAACAAGAAGCTTATAGAGGAGCTTGAAACT 294
                ** *  ***** ** ***** ***   **  ****** *** ** ** ** ** **  *  **

Zebrafish       CTTAAAGACATATACTGTTGCAAAAATGAGTAA 327
Mouse           TTGAAAGACATTTGCTCTCCCAAAACAGATTAG 327
                 * ******** * ** *  *****  ** **
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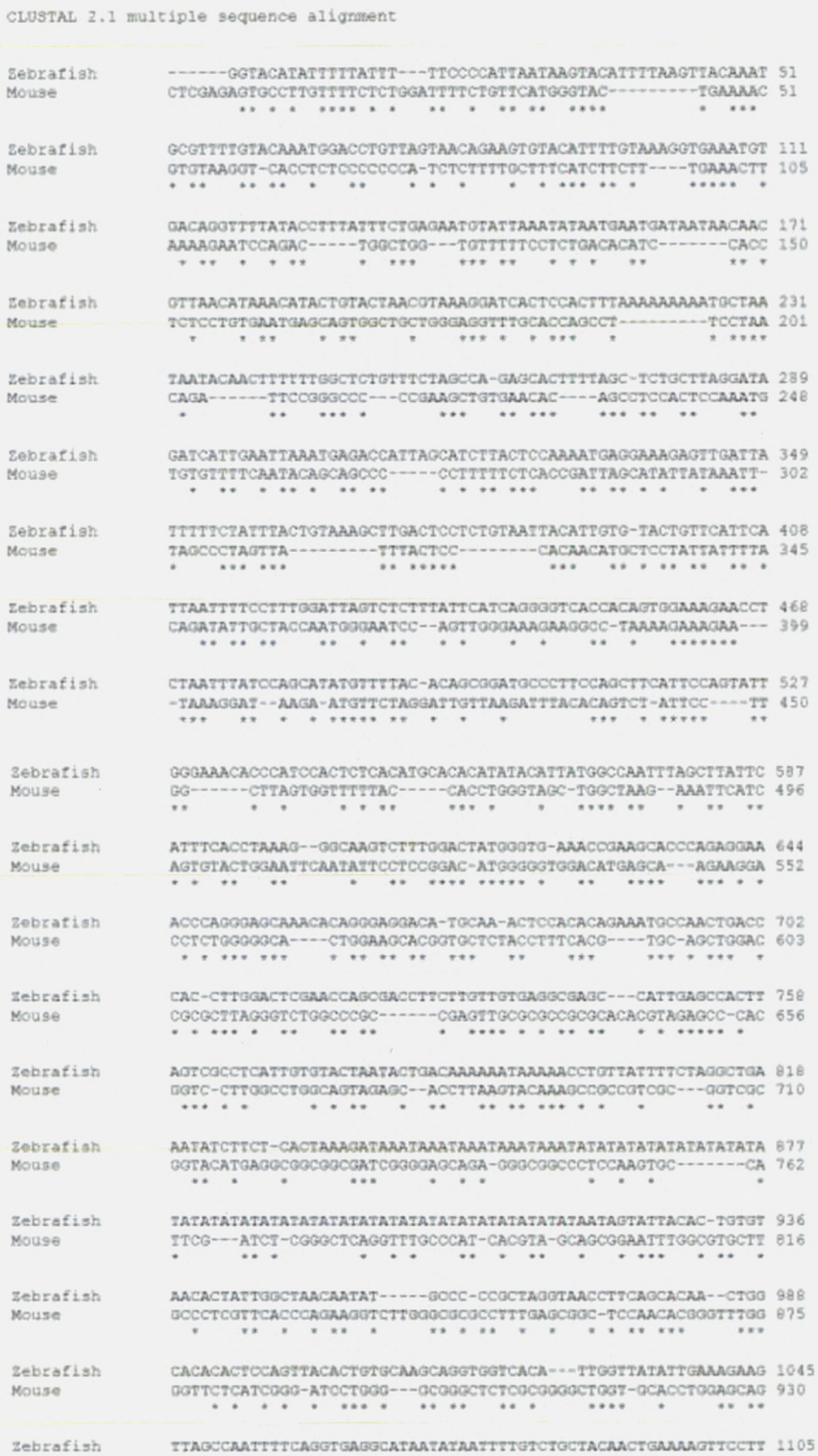
Figure 1: Alignment of zebrafish ICER Iγ mRNA and mouse ICER Iγ mRNA. The results indicate a 70% identity match between the 327 bp sequences from zebrafish and mouse.


CLUSTAL 2.1 multiple sequence alignment
Zebrafish ------GGTACATATTTTTATTT---TTCCCCATTAATAAGTACATTTTAAGTTACAAAT 51
Mouse CTCGAGAGTGCCTTGTTTTCTCTGGATTTTCTGTTCATGGGTAC---------TGAAAAC 51
Zebrafish GCGTTTTGTACAAATGGACCTGTTAGTAACAGAAGTGTACATTTTGTAAAGGTGAAATGT 111
Mouse GTGTAAGGT-CACCTCTCCCCCCCCA-TCTCTTTTGCTTTCATCTTCTT----TGAAACTT 105
Zebrafish GACAGGTTTTATACCTTTATTTCTGAGAATGTATTAAATATAATGAATGATAATAACAAC 171
Mouse AAAAGAATCCAGAC-----TGGCTGG---TGTTTTTCCTCTGACACATC-------CACC 150
Zebrafish GTTAACATAAACATACTGTACTAACGTAAAGGATCACTCCACTTTAAAAAAAAATGCTAA 231
Mouse TCTCCTGTGAATGAGCAGTGGCTGCTGGGAGGTTTGCACCAGCCT---------TCCTAA 201
Zebrafish TAATACAACTTTTTTGGCTCTGTTTCTAGCCA-GAGCACTTTTAGC-TCTGCTTAGGATA 289
Mouse CAGA------TTCCGGGCCC---CCGAAGCTGTGAACAC----AGCCTCCACTCCAAATG 248
Zebrafish GATCATTGAATTAAATGAGACCATTAGCATCTTACTCCAAAATGAGGAAAGAGTTGATTA 349
Mouse TGTGTTTTCAATACAGCAGCCC-----CCTTTTTCTCACCGATTAGCATATTATAAATT- 302
Zebrafish TTTTTCTATTTACTGTAAAGCTTGACTCCTCTGTAATTACATTGTG-TACTGTTCATTCA 408
Mouse TAGCCCTAGTTA---------TTTACTCC--------CACAACATGCTCCTATTATTTTA 345
Zebrafish TTAATTTTCCTTTGGATTAGTCTCTTTATTCATCAGGGGTCACCACAGTGGAAAGAACCT 468
Mouse CAGATATTGCTACCAATGGGAATCC--AGTTGGGAAAGAAGGCC-TAAAAGAAAGAA--- 399
Zebrafish CTAATTTATCCAGCATATGTTTTAC-ACAGCGGATGCCCTTCCAGCTTCATTCCAGTATT 527
Mouse -TAAAGGAT--AAGA-ATGTTCTAGGATTGTTAAGATTTACACAGTCT-ATTCC----TT 450
Zebrafish GGGAAACACCCATCCACTCTCACATGCACACATATACATTATGGCCAATTTAGCTTATTC 587
Mouse GG------CTTAGTGGTTTTTAC-----CACCTGGGTAGC-TGGCTAAG--AAATTCATC 496
Zebrafish ATTTCACCTAAAG--GGCAAGTCTTTGGACTATGGGTG-AAACCGAAGCACCCAGAGGAA 644
Mouse AGTGTACTGGAATTCAATATTCCTCCGGAC-ATGGGGGTGGACATGAGCA---AGAAGGA 552
Zebrafish ACCCAGGGAGCAAACACAGGGAGGACA-TGCAA-ACTCCACACAGAAATGCCAACTGACC 702
Mouse CCTCTGGGGGCA----CTGGAAGCACGGTGCTCTACCTTTCACG----TGC-AGCTGGAC 603
Zebrafish CAC-CTTGGACTCGAACCAGCGACCTTCTTGTTGTGAGGCGAGC---CATTGAGCCACTT 758
Mouse CGCGCTTAGGGTCTGGCCCGC------CGAGTTGCGCGCCGCGCACACGTAGAGCC-CAC 656
Zebrafish AGTCGCCTCATTGTGTACTAATACTGACAAAAAATAAAAACCTGTTATTTTCTAGGCTGA 818
Mouse GGTC-CTTGGCCTGGCAGTAGAGC--ACCTTAAGTACAAAGCCGCCGTCGC---GGTCGC 710
Zebrafish AATATCTTCT-CACTAAAGATAAATAAATAAATAAATAAATATATATATATATATATATA 877
Mouse GGTACATGAGGCGGCGGCGATCGGGGAGCAGA-GGGCGGCCCTCCAAGTGC-------CA 762
Zebrafish TATATATATATATATATATATATATATATATATATATATATATAATAGTATTACAC-TGTGT 936
Mouse TTCG---ATCT-CGGGCTCAGGTTTGCCCAT-CACGTA-GCAGCGGAATTTGGCGTGCTT 816
Zebrafish AACACTATTGGCTAACAATAT-----GCCC-CCGCTAGGTAACCTTCAGCACAA--CTGG 988
Mouse GCCCTCGTTCACCCAGAAGGTCTTGGGCGCGCCTTTGAGCGGC-TCCAACACGGGTTTGG 875
Zebrafish CACACACTCCAGTTACACTGTGCAAGCAGGTGGTCACA---TTGGTTATATTGAAAGAAG 1045
Mouse GGTTCTCATCGGG-ATCCTGGG---GCGGGCTCTCGCGGGGCTGGT-GCACCTGGAGCAG 930
Zebrafish TTAGCCAATTTTCAGGTGAGGCATAATATAATTTTGTCTGCTACAACTGAAAAGTTCCTT 1105
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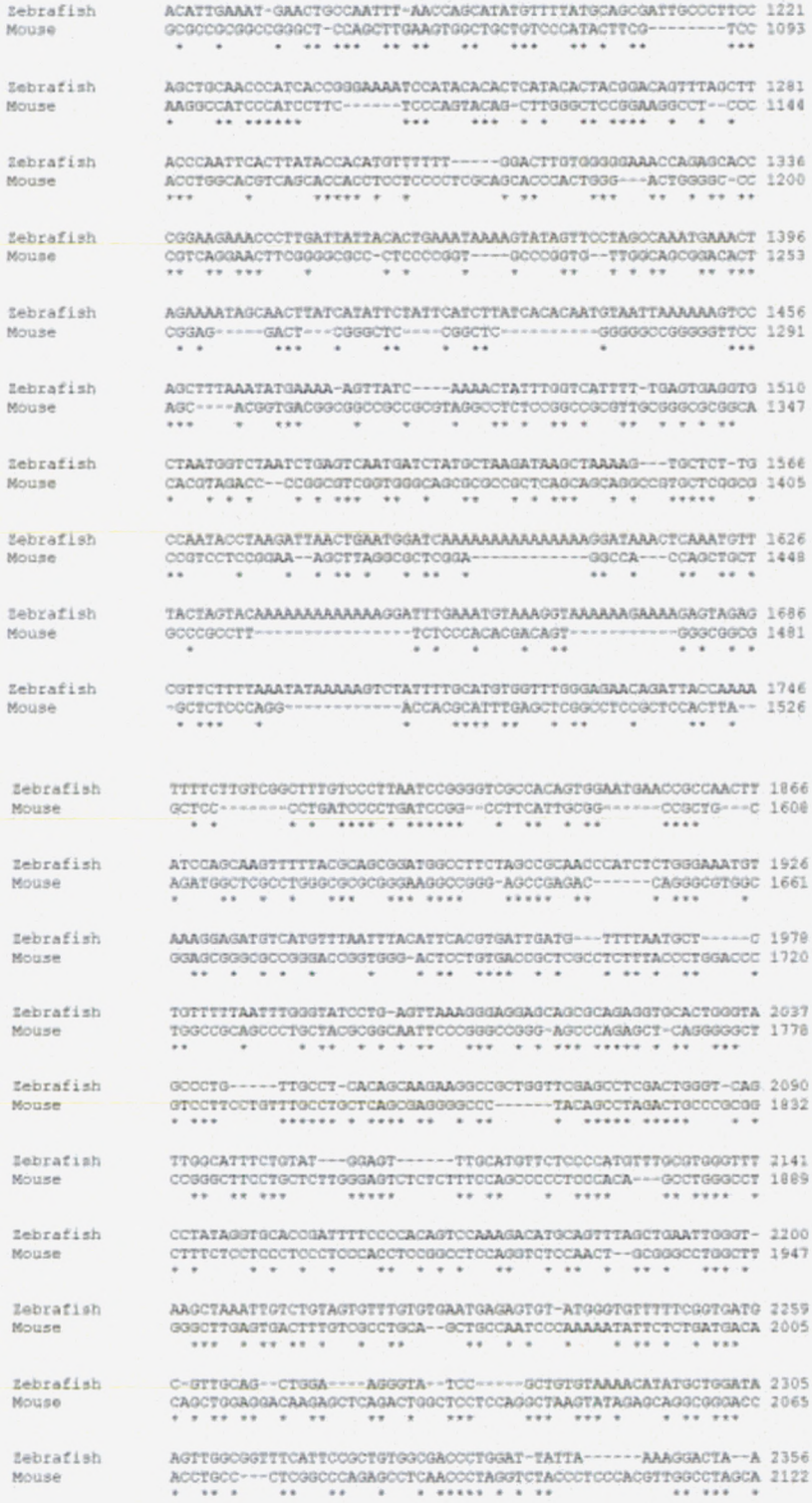
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Mouse GCGCCCGCGGCCGGGCT-CCAGCTTGAAGTGGCTGCTGTCCCATACTTCG-------TCC 1093
Zebrafish AGCTGCAACCCATCACCGGGAAAATCCATACACACTCATACACTACGGACAGTTTAGCTT 1281
Mouse AAGGCCATCCCATCCTTC------TCCCAGTACAG-CTTGGGCTCCGGAAGGCCT--CCC 1144
Zebrafish ACCCAATTCACTTATACCACATGTTTTTT-----GGACTTGTGGGGGAAACCAGAGCACC 1336
Mouse ACCTGGCACGTCAGCACCACCTCCTCCCCTCGCAGCACCCACTGGG---ACTGGGGC-CC 1200
Zebrafish CGGAAGAAACCCTTGATTATTACACTGAAATAAAAGTATAGTTCCTAGCCAAATGAAACT 1396
Mouse CGTCAGGAACTTCGGGGCGCC-CTCCCCGGT----GCCCGGTG--TTGGCAGCGGACACT 1253
Zebrafish AGAAAATAGCAACTTATCATATTCTATTCATCTTATCACACAATGTAATTAAAAAAGTCC 1456
Mouse CGGAG-----GACT---CGGGCTC----CGGCTC----------GGGGGCCGGGGGTTCC 1291
Zebrafish AGCTTTAAATATGAAAA-AGTTATC----AAAACTATTTGGTCATTTT-TGAGTGAGGTG 1510
Mouse AGC----ACGGTGACGGCGGCCGCCGCGTAGGCCTCTCCGGCCGCGTTGCGGGCGCGGCA 1347
Zebrafish CTAATGGTCTAATCTGAGTCAATGATCTATGCTAAGATAAGCTAAAAG---TGCTCT-TG 1566
Mouse CACGTAGACC--CCGGCGTCGGTGGGCAGCGCGCCGCTCAGCAGCAGGCCGTGCTCGGCG 1405
Zebrafish CCAATACCTAAGATTAACTGAATGGATCAAAAAAAAAAAAAAAAGGATAAACTCAAATGTT 1626
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Zebrafish TACTAGTACAAAAAAAAAAAAAGGATTTGAAATGTAAAGGTAAAAAAGAAAAGAGTAGAG 1686
Mouse GCCCGCCTT----------TCTCCCACACGACAGT----------GGGCGGCG 1481
Zebrafish CGTTCTTTTAAATATAAAAAGTCTATTTTGCATGTGGTTTGGGAGAACAGATTACCAAAA 1746
Mouse -GCTCTCCCAGG----------ACCACGCATTTGAGCTCGGCCTCCGCTCCACTTA-- 1526
Zebrafish TTTTCTTGTCGGCTTTGTCCCTTAATCCGGGGTCGCCACAGTGGAATGAACCGCCAACTT 1866
Mouse GCTCC-------CCTGATCCCCTGATCCGG--CCTTCATTGCGG------CCGCTG---C 1608
Zebrafish ATCCAGCAAGTTTTTACGCAGCGGATGGCCTTCTAGCCGCAACCCATCTCTGGGAAATGT 1926
Mouse AGATGGCTCGCCTGGGCGCGCGGGAAGGCCGGG-AGCCGAGAC------CAGGGCGTGGC 1661
Zebrafish AAAGGAGATGTCATGTTTAATTTACATTCACGTGATTGATG---TTTTAATGCT-----C 1979
Mouse GGAGCGGGCGCCGGGACCGGTGGG-ACTCCTGTGACCGCTCGCCTCTTTACCCTGGACCC 1720
Zebrafish TGTTTTTAATTTGGGTATCCTG-AGTTAAAGGGAGGAGCAGCGCAGAGGTGCACTGGGTA 2037
Mouse TGGCCGCAGCCCTGCTACGCGGCAATTCCCGGGCCGGG-AGCCCAGAGCT-CAGGGGGCT 1778
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Mouse GTCCTTCCTGTTTGCCTGCTCAGCGAGGGGCCC------TACAGCCTAGACTGCCCGCGG 1832
Zebrafish TTGGCATTTCTGTAT---GGAGT------TTGCATGTTCTCCCCATGTTTGCGTGGGTTT 2141
Mouse CCGGGCTTCCTGCTCTTGGGAGTCTCTCTTTCCAGCCCCCTCCCACA---GCCTGGGCCT 1889
Zebrafish CCTATAGGTGCACCGATTTTCCCCACAGTCCAAAGACATGCAGTTTAGCTGAATTGGGT- 2200
Mouse CTTTCTCCTCCCTCCCTCCCACCTCCGGCCTCCAGGTCTCCAACT--GCGGGCCTGGCTT 1947
Zebrafish AAGCTAAATTGTCTGTAGTGTTTGTGTGAATGAGAGTGT-ATGGGTGTTTTTCGGTGATG 2259
Mouse GGGCTTGAGTGACTTTGTCGCCTGCA--GCTGCCAATCCCAAAAATATTCTCTGATGACA 2005
Zebrafish C-GTTGCAG--CTGGA----AGGGTA--TCC-----GCTGTGTAAAACATATGCTGGATA 2305
Mouse CAGCTGGAGGACAAGAGCTCAGACTGGCTCCTCCAGGCTAAGTATAGAGCAGGCGGGACC 2065
Zebrafish AGTTGGCGGTTTCATTCCGCTGTGGCGACCCTGGAT-TATTA------AAAGGACTA--A 2356
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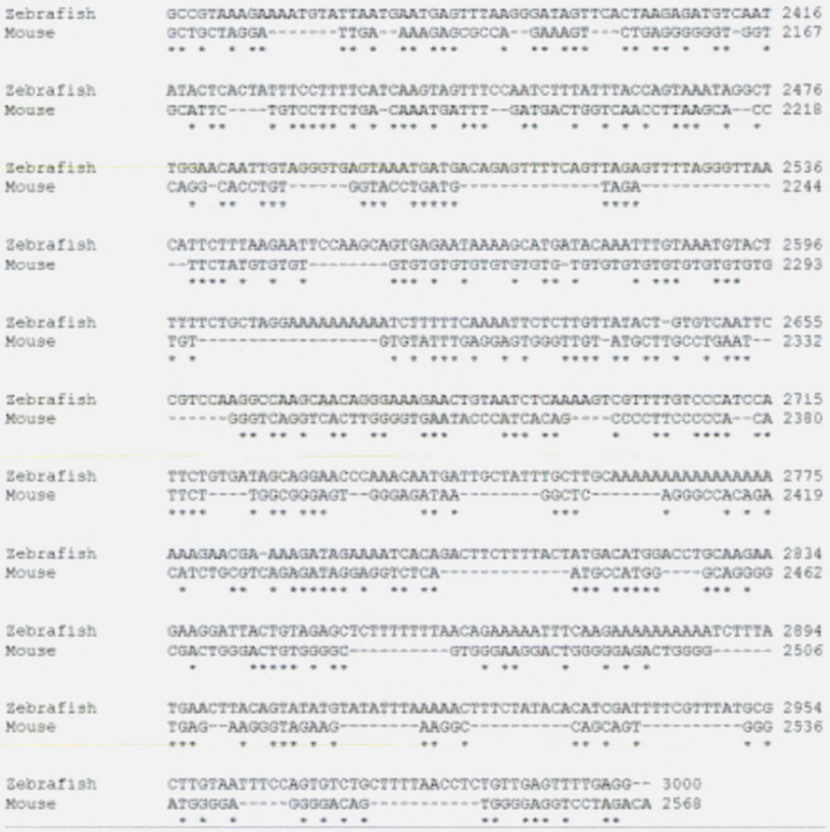

Figure 2: Alignment of zebrafish INHα mRNA and mouse INHα mRNA. The results indicate a 44% identity match between the sequences from zebrafish and mouse.

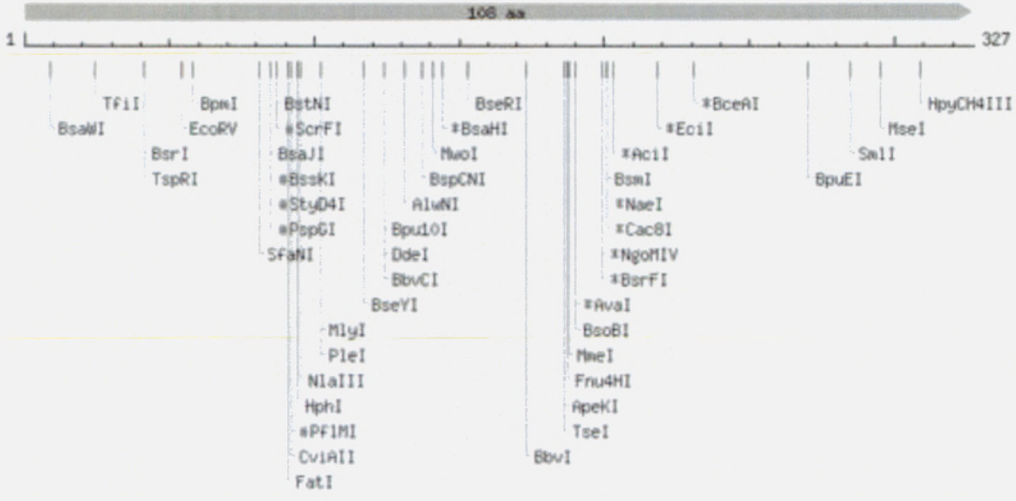

Figure 3: Restriction enzyme map of the zebrafish ICER. The GC content was found to be 50%.

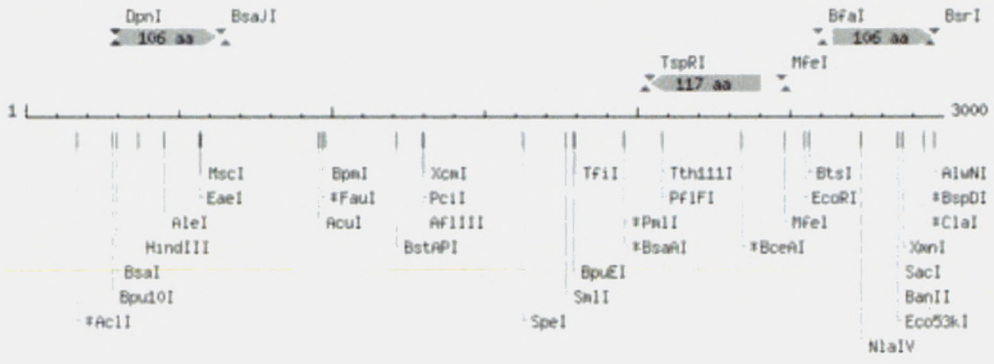

Figure 4: Restriction enzyme map of the zebrafish INHα mRNA. The GC content was found to be 35%.

RNA/DNA Extraction

Purified DNA and RNA were visualized using a 1% agarose gel electrophoresis. Isolation of RNA (Figure 5) was also achieved and evidence of genomic DNA contamination was not present.

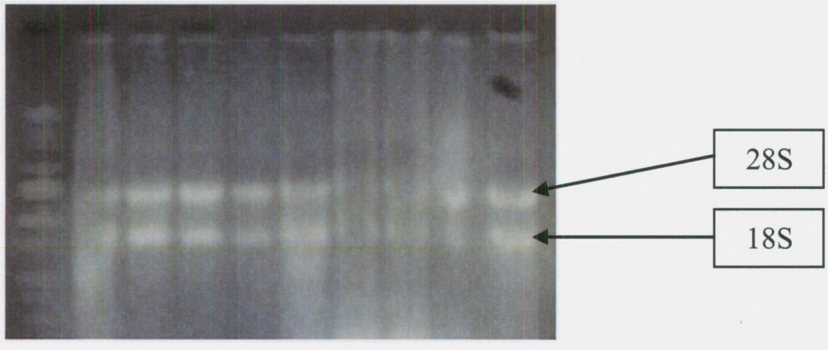

Figure 5: Isolation of RNA. RNA was successfully isolated using the TRIzol® reagent. Two bands are clearly visible indicating separation of the 28S and 18S ribosomal subunit.

Cloning of ICER into the pFLAG Vector

Amplification of the ICER Iγ sequence was confirmed with a 2% agarose gel electrophoresis (Figure 6a). The fragment was purified and subsequently cloned into the pCR® 2.1 TOPO® cloning vector. A restriction digest with EcoRV and gel

electrophoresis demonstrated the sequence was ligated into the vector (Figure 6b). The fragment containing ICER was excised and gel purified prior to transfer into the pFLAG-CMV-2 vector. A restriction digest with EcoRV confirmed ligation and the orientation of the ICER sequence in the plasmid (Figure 6c). The sequencing results for the plasmid supported the evidence suggesting ICER was successfully cloned and orientated properly within the vector (data not shown).

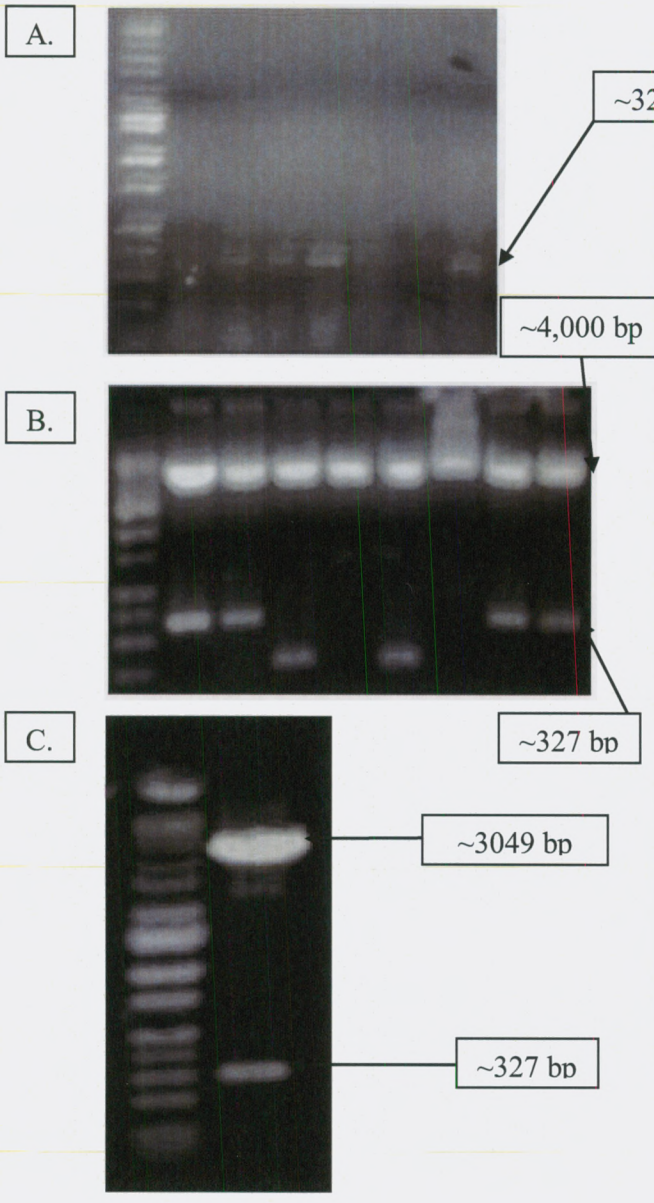

Figure 6: Cloning of ICER into the pFLAG-CMV vector. A. ICER was isolated using RT-PCR. The approximate 327 bp fragment corresponded to the length of the DNA sequence for ICER. B. Restriction mapping with EcoRV showed ICER was cloned into the TOPO vector. The approximate 4.0 kb fragment corresponded to the TOPO vector while the 327 bp fragment was ICER. C. Restriction mapping of the FLAG-dr-ICER Iγ construct with EcoRV produced the expected two fragments. The approximate 3.0 kb fragment corresponded to the pFLAG-CMV vector. The second fragment, which was approximately 300 bp in length, confirmed the insertion of ICER into the vector.

Western Blot

A SDS-PAGE and Western Blot were used to verify the CMV promoter was capable of driving ICER expression (Figure 7). Successful blotting with an anti-ICER antibody confirmed the presence of ICER.

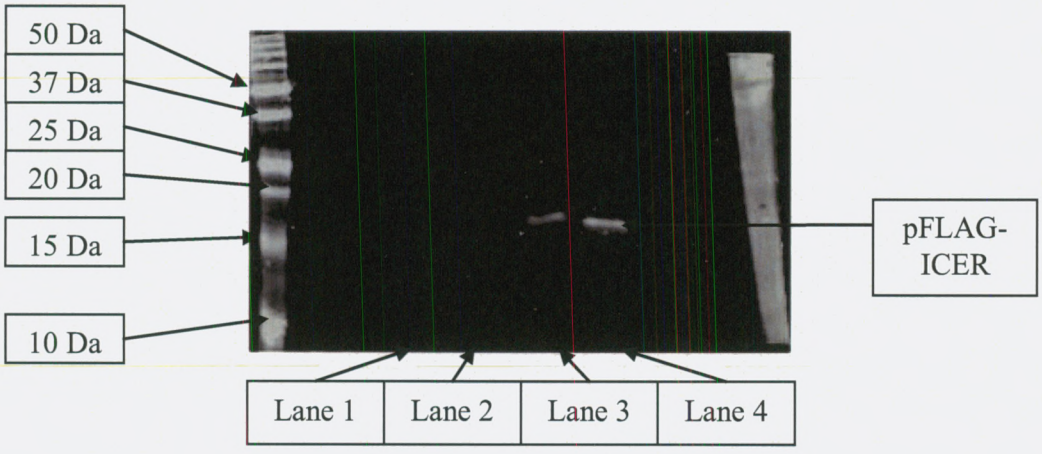

Figure 7: SDS-PAGE and Western Blot of ICER Iγ. Expression of the transgene ICER Iγ was confirmed by the appearance of a band in lanes three and four after blotting with anti-ICER antibodies. The negative control in lanes one and two demonstrated the specificity of the antibodies for ICER.

Cloning of the 3.0 kb INHα into the pGL3 Basic Vector

A 1% agarose gel electrophoresis was used to verify amplification of INHα (Figure 8a). A restriction digest with EcoRV was used to confirm cloning of the sequence into the pCR® 2.1 TOPO® cloning vector (Figure 8b). INHα was then cloned into the pGL3 vector. A restriction digest using HIND III performed to verify ligation efficiency (Figure 8c). Plasmid sequencing corroborated the restriction enzyme maps indicating INHα was cloned into the vector.

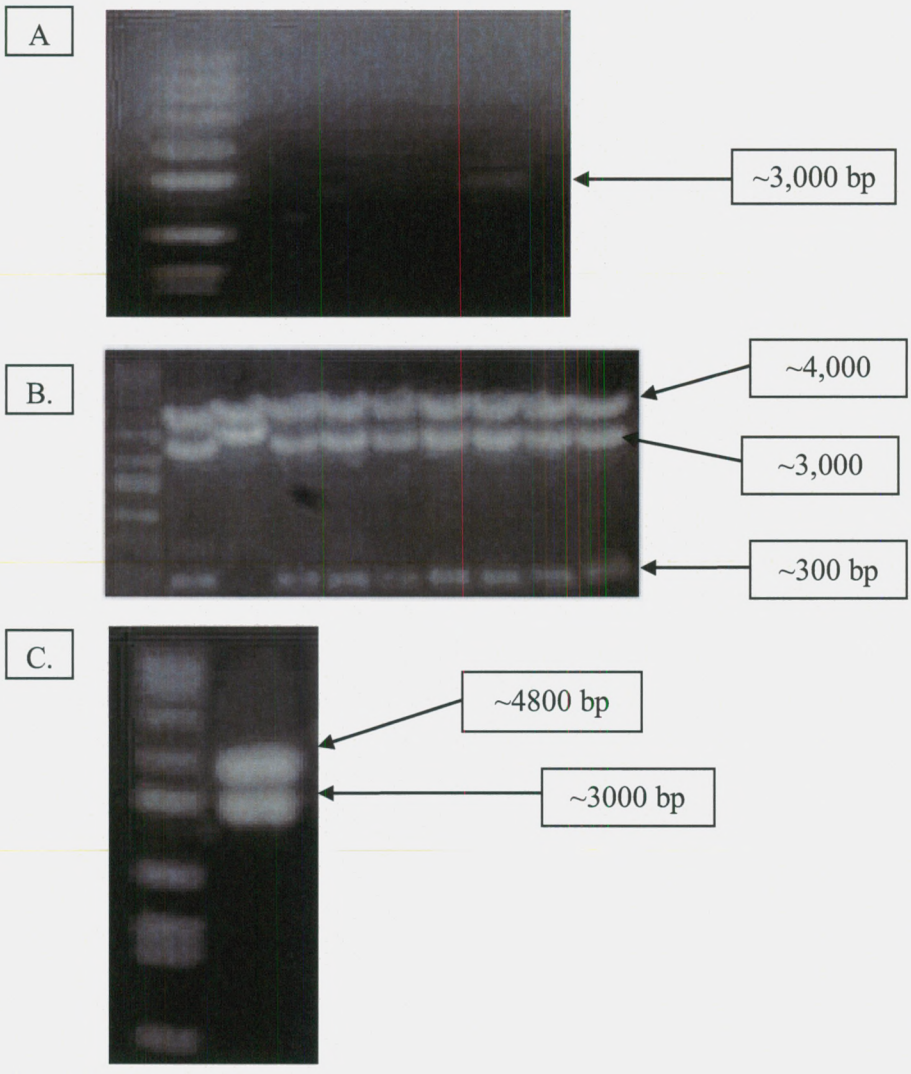

Figure 8: Cloning of INHα into the pGL3-Basic vector. A. INHα was isolated and amplified from genomic DNA using PCR. The resulting 3.0 kb fragment corresponded to the predicted size of the INHα promoter region. B. Restriction mapping with EcoRI showed INHα was cloned into the TOPO vector. The approximate 4.0 kb fragment corresponded to the TOPO vector while the 3.0 kb fragment was mapped to INHα. The restriction map also predicted a third fragment, which was also seen in the gel. C. Restriction mapping of the pGL3-INHα construct with HindIII produced the expected 4.8 kb fragment, which corresponded to the pGL3 vector, and 3.0 kb fragment, which was INHα.

Characterization of the pGL3-INHα Construct

A luciferase assay was used to determine if the putative INHα promoter could drive expression of a reporter gene. Basal expression of the luciferase reporter gene was

first measured to verify the functionality of the INHα promoter. A five-fold increase was seen in luciferase activity for the tailfin AB9 cells while a three-fold increase was seen in the embryonic PAC2 cells thus indicating this putative 3.0 kb region was indeed a promoter (Figure 9). The results suggested the regulatory sequences of INHα were not lost during the cloning process. Expression of the reporter gene also established the AB9 and PAC2 cell lines were sufficient for testing promoter activity despite the fact that neither of these cell lines originated from ovarian tissues.

Previous studies have shown gonadotropin signaling leads to increases in cellular cAMP (Mukherjee, Urban, Sassone-Corsi, & Mayo, 1998). Cells were treated with forskolin to determine if the INHα would respond to increased intracellular levels of cAMP. Luciferase activity in the AB9 cells increased almost two-fold indicating INHα was responsive to the increased concentration of cAMP. The increased activity also suggested the INHα promoter activity could be modulated using FSH and LH. Promoter activity in the PAC2 cell line was almost completely repressed in forskolin treated cells. This repression of luciferase activity might have been the result of competition for cAMP by other biochemical pathways in the embryonic cells.

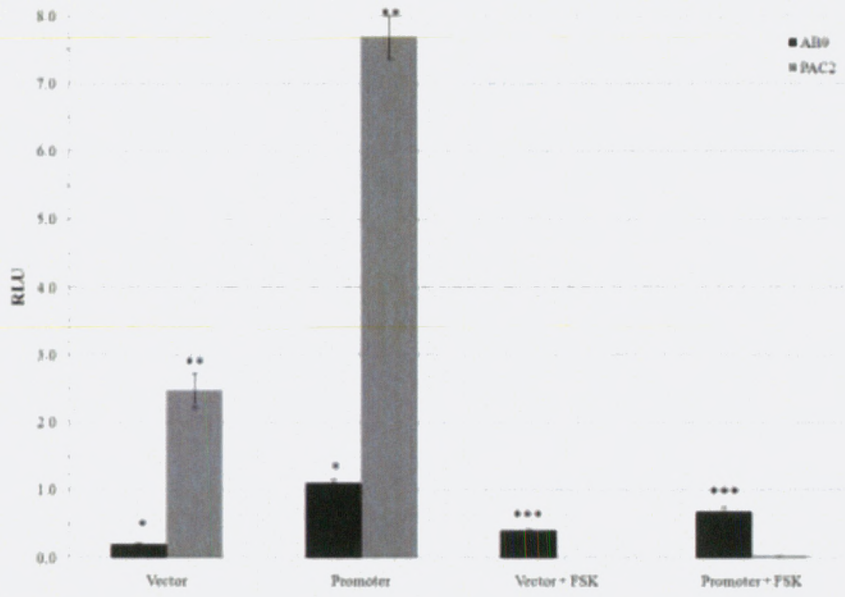

Figure 9: INHα promoter activity in AB9 and PAC2 cells in response to forskolin treatment. Data are presented as the mean ± SEM of three independent experiments. Bars (*, **, ***) differ significantly from controls, $P < 0.05$.

Previous studies in rats demonstrated LH was capable of suppressing INHα activity (Mukherjee, Urban, Sassone-Corsi, & Mayo, 1998). It was hypothesized LH suppression would be maintained due to the importance of LH in the recruitment of ovarian follicles in zebrafish. AB9 and PAC2 cells were co-transfected with FSH and LH receptors and treated with hCG. Treatment with hCG repressed INHα promoter activity in AB9 and PAC2 cells indicating the promoter was responsive to LH mediated suppression (Figure 10). Suppression of the INHα promoter in zebrafish supported the supposition zebrafish INHα functions similarly to the same promoter in mammals.

Induction of INHα activity was due to the presence of trace amounts of FSH innate to the fetal bovine serum in the culture media and the hCG hormone. Complete removal of this trace FSH is not possible. Evidence of the FSH hormone was seen when cells were co-transfected with the INHα promoter and FSH receptor. Treatment with

hCG was able to overcome some of the FSH induction in order to suppress INHα promoter activity.

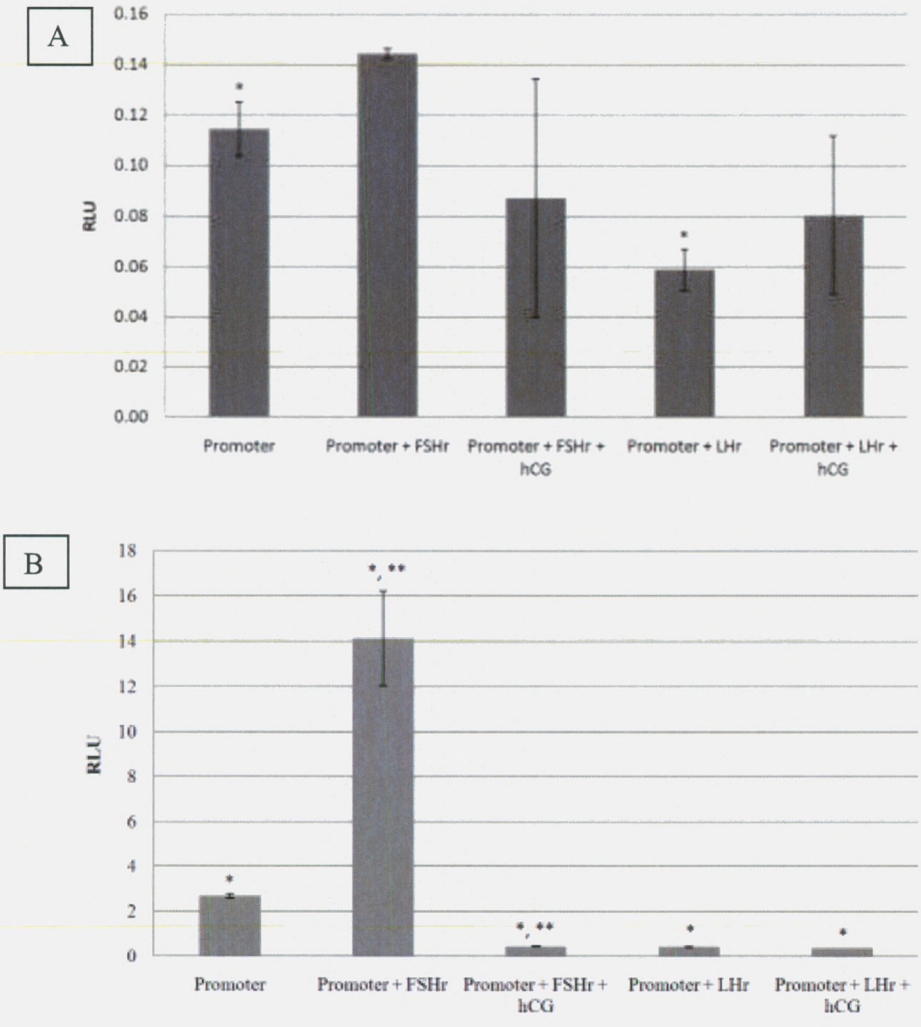

Figure 10: INHα promoter activity in AB9 and PAC2 in response to LH. Data are presented as the mean ± SEM of three independent experiments. A. AB9 cells were co-transfected with the pGL3- INHα construct and either FSH receptor or LH receptor as indicated. Bars (*) differ significantly from the control, $P < 0.05$. B. PAC2 cells were co-transfected with the pGL3- INHα construct and either FSH receptor or LH receptor as indicated. Bars (* and **) differ significantly from the controls, $P < 0.05$.

As already seen, INHα is a FSH-inducible promoter so luciferase activity should increase if cells are treated with FSH hormone. Once again, AB9 and PAC2 cells were co-transfected with FSH and LH receptors, however, the cells were treated with FSH

hormone to measure its effect. Increased levels of promoter activity after FSH treatment provided additional evidence indicating the promoter was responsive to FSH (Figure 11). Induction of the INHα promoter in zebrafish supported the hypothesis zebrafish INHα functions similarly to the same promoter in mammals.

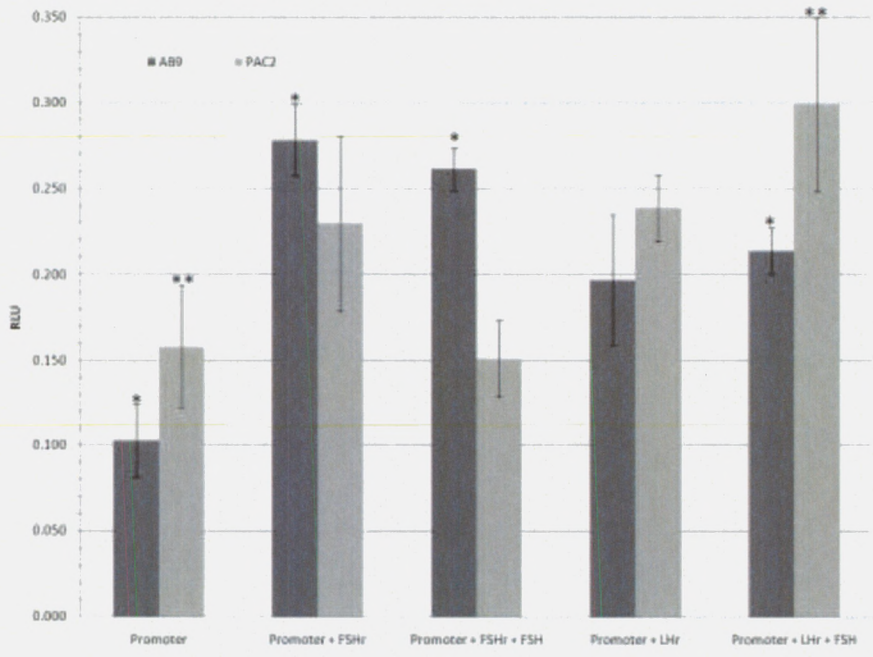

Figure 11: INHα promoter activity in AB9 and PAC2 in response to FSH. Data are presented as the mean ± SEM of three independent experiments. Bars (* and **) differ significantly from the controls, $P < 0.05$.

DISCUSSION

FSH and LH are responsible for folliculogenesis, ovulation, and luteinization in ovarian cells (Muniz, Yehia, Memin, & Ratnakar, 2006) (Burkart, Mukherjee, & Mayo, 2006). The cyclical actions of these two gonadotropins mediate changes in gene expression in a primordial follicle through cAMP signaling cascades (Molina, Foulkes, Lalli, & Sassone-Corsi, 1993). This signaling cascade leads to transcriptional activation of ICER during the preovulatory LH surge in granulosa cells (Mukherjee, Urban,

Sassone-Corsi, & Mayo, 1998). ICER subsequently heterodimerizes with CREB family members and suppresses activity of INHα and other FSH inducible genes (Morales V. , et al., 2003). Previous studies have shown downregulation of INHα leads to a prolonged surge of FSH and thus the recruitment of additional ovarian follicles (Mukherjee, Urban, Sassone-Corsi, & Mayo, 1998). Studies have implicated a 3.0 kb region of the INHα promoter as a site of ICER transcriptional activation in response to FSH (Muniz, Yehia, Memin, & Ratnakar, 2006). In this study, ICER and the INHα promoter was isolated and cloned into expression vectors to test the effectiveness of the INHα promoter.

Zebrafish systems are a good model for reproductive and ovulatory studies due to their short ovulatory cycles and readily accessible reproductive tracts. Previous studies demonstrated FSH and LH signaling fluctuates during ovulation so that levels increase prior to ovulation and decrease during egg release from the follicle (Muniz, Yehia, Memin, & Ratnakar, 2006). Moreover, LH was shown to induce final oocyte maturation and ovulation (Foulkes, Borrelli, & Sassone-Corsi, 1991). BLAST searches and CLUSTALW2 alignments between the murine sequences for ICER and INHα revealed high homology, which validated the use of zebrafish for a model in this study.

Identification of the ICER sequence in zebrafish showed the sequence to be 327 bp in length. The sequence was isolated using RT-PCR and cloned into the pFLAG-CMV2 expression vector. Insertion into the vector tagged ICER an identifying FLAG sequence so that exogenous ICER production was differentiable from endogenous expression. Restriction enzyme mapping and DNA sequencing provided evidence ICER was inserted successfully into the plasmid in the correct orientation.

ICER insertion into the pFLAG-CMV2 vector also placed ICER expression under the control of the CMV2 promoter. Western Blots with the FLAG-dr-ICER Iγ construct showed expression of FLAG-ICER was induced using the CMV2 promoter in HEK293T cells. Successful blotting with anti-ICER primary antibodies yielded additional evidence the isolated sequence was ICER.

Although identification and isolation of ICER in zebrafish was important, it was not the main purpose of this investigation. Instead, this study sought to isolate and characterize a 3.0 kb region of the INHα promoter. The area of interest in the INHα promoter was found to be 3,049 and shared a 44% identity match with the previously studied murine INHα promoter (Muniz, Yehia, Memin, & Ratnakar, 2006) (Mukherjee, Urban, Sassone-Corsi, & Mayo, 1998). It was surprising to find such high homology between the promoter regions considering the evolutionary distance between zebrafish and mice. The similarity between the nucleotide sequences suggested the zebrafish promoter would respond to gonadotropin signaling such that FSH would induce promoter activity and LH would suppress it.

Using PCR, the 3.0kb INHα promoter region was isolated from genomic DNA and cloned into the pGL3-Basic luciferase vector in order to assess promoter activity. Ligation efficacy into the vector was confirmed with restriction enzyme mapping and DNA sequencing.

Cloning of the promoter into this expression vector allowed its activity to be assessed using cells derived from sources other than ovarian tissues. Two cell lines, AB9 and PAC2, were used to test promoter activity. AB9 cells are derived from the tailfin tissue of adult zebrafish while PAC2 cells are collected from post-fertilization embryonic

zebrafish. After transfection with the pGL3-INHα vector, both cell lines were capable of supporting expression of the vector. The ability to characterize the INHα promoter in non-ovarian cells helped to ensure all reporter gene activity was in response to treatment rather than biochemical pathways inherent to ovarian cells.

FSH and LH signaling regulates ICER and INHα activity through activation of stimulatory G protein-coupled receptors (Hunzicker-Dunn & Maizels, 2006). Binding of FSH or LH to their respective receptor indirectly activates adenylyl cyclase, which in turn produces the secondary messenger cAMP (Mukherjee, Urban, Sassone-Corsi, & Mayo, 1998). Induction of the INHα promoter was seen in forskolin treated AB9 cells suggesting this zebrafish promoter was responsive to cAMP signaling. Very little promoter activity was seen in forskolin treated PAC2 cells. This lack of induction did not imply the promoter was not responsive to the increased levels of cAMP; other biochemical pathways in the embryonic cells may have been in competition with the promoter for cAMP. An increase in activity of these unknown biochemical pathways could have prevented expression of the luciferase reporter.

After establishing zebrafish INHα promoter activity can be induced by increased intracellular levels of cAMP, the next step was to determine if LH could repress promoter activity. Zebrafish cells were co-transfected with FSH receptors or LH receptors and the pGL3-INHα construct. Transfection with LH and FSH receptors was necessary to ensure the non-ovarian AB9 and PAC2 cells could respond to gonadotropin signaling. Introduction of FSH receptors and LH receptors into the cells revealed background FSH and LH present in culture serum. Increased promoter activity in cells containing FSH

receptors and decreased promoter activity in LH receptor containing cells implied the INHα promoter was responsive to gonadotropin signaling.

Downregulation of INHα activity in response to LH signaling was required to verify promoter function in zebrafish. Treatment with hCG suppressed promoter activity in both AB9 and PAC2 cells. Although hCG also contains a FSH domain, background FSH and this domain did not interfere with LH-mediated suppression of the promoter. The results suggested the zebrafish INHα was downregulated in response to LH, which supported the hypothesis this promoter functions similarly to the INHα promoter in mammals.

Upon establishing INHα could be downregulated with LH, the next task was to demonstrate the promoter could be upregulated with FSH. Promoter induction by FSH had already been suggested by background FSH from the culture serum and activity resulting from hCG treatment in cells transfected with FSH receptors. Treatment with FSH hormone significantly ($P < 0.05$) increased luciferase activity in AB9 cells. Increased promoter activity was also seen in the PAC2 cells after FSH treatment. Evidence for INHα upregulation in response to FSH treatment further supported the hypothesis the zebrafish INHα is partially responsible for mediating the cellular response to gonadotropin signaling.

The purpose of this study was to isolate zebrafish ICER and a 3.0 kb region of the INHα promoter. Moreover, characterization of the INHα promoter was required since this promoter had never been characterized in zebrafish. The sequence for ICER was found to be 327 bp in length and maintained a 70% nucleotide identity match with murine ICER. The 3,049 bp region of interest for the INHα promoter shared a 44%

nucleotide identity match with the murine version of this promoter region. Restriction enzyme mapping and DNA sequencing demonstrated the sequences for ICER and INHα promoter were independently isolated and cloned into the pFLAG-CMV and pGL3-Basic expression vectors, respectively. The FLAG-dr-ICER Iγ plasmid was transfected into Hek293T cells. ICER protein was identified following SDS-PAGE and Western Blot analysis. Transfection experiments and luciferase assays using the pGL3-INHα provided evidence indicating the isolated region could support promoter activity. Moreover, this region of the promoter was upregulated by FSH treatments and downregulated by LH/hCG. This pattern of upregulation and downregulation has previously been documented in rats and mice (Burkart, Mukherjee, & Mayo, 2006) (Yehia, Schlotter, Razavi, Alessandrini, & Molina, 2001).

This study sought to characterize ICER and the INHα promoter's roles in the regulatory pathways controlling ovulation in zebrafish. It was hypothesized FSH signals could trigger changes in gene expression leading to the induction of hyperovulation if ICER could be expressed earlier during the folliculogenesis pathway. This study demonstrated zebrafish INHα promoter was inducible by FSH signaling and repressed by LH in non-ovarian zebrafish cells. The use of AB9 and PAC2 cells to study INHα promoter activity allowed for direct manipulation of FSH and LH levels and prevented intercellular competition for the gonadotropins.

Characterization of the INHα promoter was an important step in understanding ICER regulation in ovulation. Further work is required to establish if the INHα promoter can drive ICER expression in zebrafish. ICER and the INHα promoter are already isolated in separate expression vectors so the next task would be to place FLAG-ICER

under control of the INHα promoter. Once it is determined INHα can induce ICER expression, the INHα-FLAG-ICER Iγ construct should be prepared for gene delivery using a lentiviral vector for the creation of transgenic zebrafish. The development of transgenic individuals would assist in understanding FSH regulation of ICER and INHα in ovulation.

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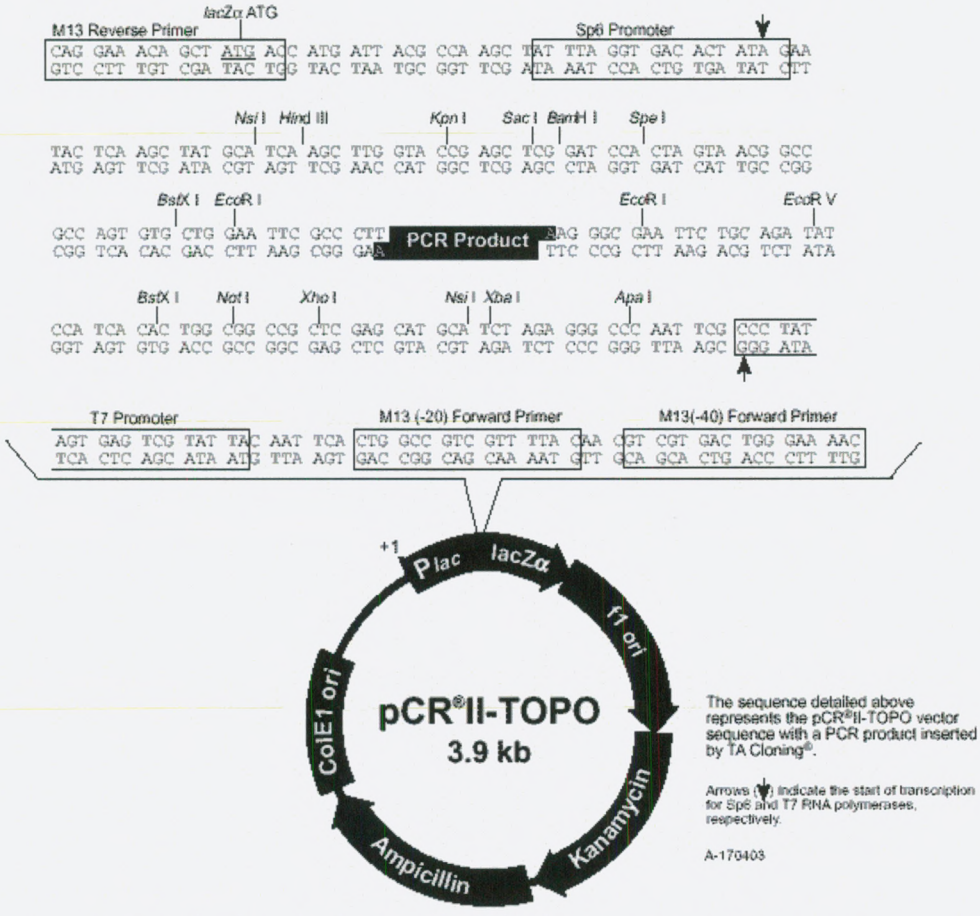
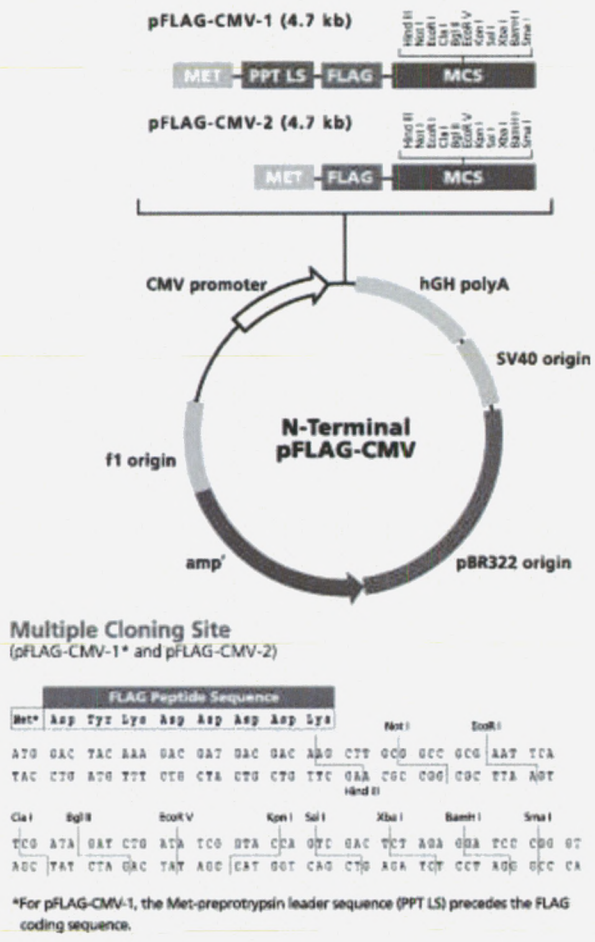
Ubiquitin-mediated Destruction. *Journal of Biological Chemistry , 276*, 35272-35279.

Comments for pCR®II-TOPO
3950 nucleotides

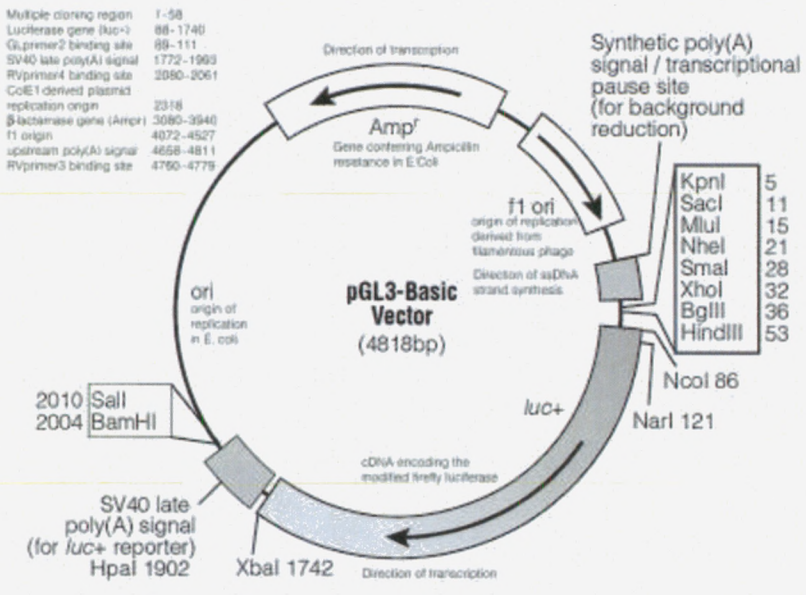
LacZα gene: bases 1-588
M13 Reverse priming site: bases 205-221
Sp6 promoter: bases 239-256
Multiple Cloning Site: bases 269-399
T7 promoter: bases 406-425
M13 (-20) Forward priming site: bases 433-448
M13 (-40) Forward priming site: bases 453-468
f1 origin: bases 590-1004
Kanamycin resistance ORF: bases 1338-2132
Ampicillin resistance ORF: bases 2150-3010
ColE1 origin: bases 3155-3828



Supplementary Figure 1: Restriction Map of the TOPO vector.



Supplementary Figure 2: Restriction Map of the pFLAG-CMV vector.



Supplementary Figure 3: Restriction Map of the pGL3-Basic vector.