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The transcriptional repressor ICER binds to multiple loci throughout the genome

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ABSTRACT

The events culminating in ovulation are controlled by the cyclical actions of hormones such as Follical Stimulating Hormone (FSH) and Luteinizing Hormone (LH). The secondary messenger, cyclic AMP (cAMP) conveys the intracellular activity of these hormones. It is well established that a family of transcription factors facilitate cAMP mediated gene expression, yet it remains unknown how these factors directly affect ovulation. One of these factors, Inducible cAMP Early Repressor (ICER) has been implicated in the transcriptional regulation of cAMP inducible genes during folliculogenesis and ovulation. In order to better determine the role of ICER in ovarian function we have identified novel targets using a genome-wide approach. Using a modification of the chromatin immunoprecipitation (ChIP) assay we directly cloned and sequenced the immunoprecipitated ICER-associated DNAs from an immortalized mouse granulose cell line (GRMO2). The analysis of the immunoprecipitated DNA fragments has revealed that ICER's binding to DNA has the following distribution; 16% within the promoter region, 31% within an intron, 14% were not within a gene, 6% were within 20 kb of a promoter and 3% were within the 3′ end of genes.

1. Introduction

To date, there is no well-established, comprehensive model which characterizes the signaling events culminating in ovulation. Follicular development and subsequent ovulation is dictated in part by the follicle stimulating hormone (FSH) and luteinizing hormone (LH) and their affects on gene expression. A critical component of the intracellular activity of these two hormones is relayed by the second messenger cAMP [1]. Numerous genes expressed in the ovary are regulated by cAMP as a consequence of gonadotropin signaling [2]. The expression of cAMP-responsive genes is mediated by a large family of transcription factors of which CREM has been well characterized [3,4]. CREM is unique among the other CRE binding transcription factors in that an internal promoter exists and codes for an isoform that acts to regulate its expression [5]. This induced isoform, ICER (Inducible cAMP Early Repressor), serves as a dominant negative regulator of CREM expression. ICER is one of the smallest transcription factors to date, and represses the transcription of cAMP responsive genes by binding as a homodimer or heterodimer with other CRE-binding family members. The ICER protein possesses DNA-binding and dimerization domains while lacking the kinase-inducible and transactivation domains. ICER is characterized by a greater affinity to CRE-sequences than other transcription factors and thus makes ICER a powerful repressor. We and others have implicated ICER's role in regulating normal ovarian function [6] through its repression of inhibin alpha, aromatase Cyp19a1, and cyclin D2 [7–10]. An unbiased range of targets for ICER has yet to be explored. Since ICER negatively regulates the transcription of cAMP responsive genes by binding to a CRE, we performed a global analysis mapping the binding distribution of ICER along the entire mouse genome using a modification of the ChIP assay. This assay allowed us to clone and directly identify candidate genes that ICER could potentially regulate by physically binding to CREs present within their sequences. This analysis will lead to the identification of pathways involved in ovulation, potentially regulated by ICER pathways or by the cAMP pathway in general. In these report we identified a number of novel binding sites and our results support the role of ICER as an integral player in the signaling machinery responsible for normal ovarian function.

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2. Materials & methods

2.1. Reagents

AmpliTaq Gold DNA Polymerase with Buffer II and $MgCl_2$ solution for general PCR reactions were purchased from Applied Biosystems, Foster City, CA. Restriction enzymes and modification enzymes were purchased from New England BioLabs, Beverly, MA. TOPO TA Cloning Kit (with pCR2.1-TOPO vector pCRII-TOPO vector) with One Shot Chemically Competent *E. coli*, TOPO Shotgun Subcloning Kit and LipofectAMINE 2000 reagent were purchased from Invitrogen, Carlsbad, CA. Oligonucleotides for PCR based reactions were purchased from The Molecular Resource Facility UMDNJ, Newark, NJ. Cell culture media was purchased from Cellgro by Mediatech, Inc., Herndon, VA. Except Fetal bovine serum was purchased from Hyclone, UT. Insulin-Transferrin-Sodium Selenite media supplement was purchased from Sigma, St. Louis, MO.

2.2. Immortalized mouse granulosa cell line (GRMO2)

GRMO2 cells [11] (N.V. Innogenetics, Ghent, Belgium) were cultured in DMEM-F12, supplemented with Insulin-Transferrin-Sodium Selenite media supplement and 2% FBS in a humidified incubator at 37 °C and 5% CO_2. GRMO2 were transiently transfected using LipofectAMINE 2000 Reagent (Invitrogen, Carlsbad, CA).

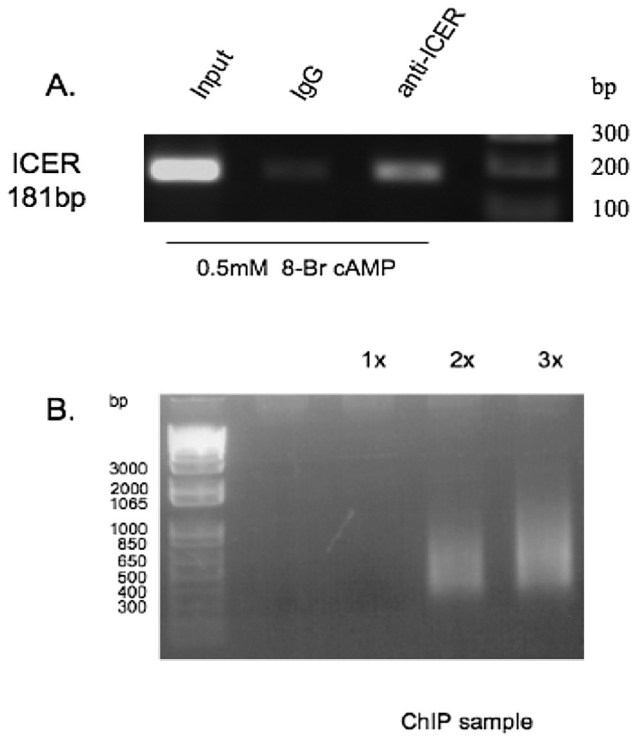


Fig. 1. Validation and amplification of immunoprecipitated chromatin fragments. GRMO2 cells were treated with 8-Br cAMP for 12 h to mimic the induced protein levels of ICER seen during the preovulatory LH Surge. A. Representative ChIP analysis of chromatin immunoprecipitated with either anti-ICER or pre-immune control rabbit IgG. Following DNA purification, samples were PCR amplified using a pair of primers covering the regions of Cyclin D2 and ICER gene promoter. B. Immunoprecipitated chromatin was first repaired to make the amplicons blunt ended. The blunted chromatin fragments were ligated with unidirectional double stranded oligonucleotide linkers. Chromatin amplicon were generated by 20 cycles of PCR and each reaction was purified using the Qiaquick PCR purification kit. The PCR protocol was repeated (1×, 2× and 3×) until enough amplicon was made. Pictured is an ethidium bromide stained gel with 5ul of the amplicon resolved on a 1% agarose gel, with fragments ranging between 300bp to 1 kb.

2.3. Antibodies

The anti-ICER polyclonal antibody was raised against bacterially purified ICER-IIγ and previously characterized [5,12]. This antibody has been shown to cross react with other CREM isoforms and ubiquitinated forms of ICER and does not cross react with CREB [5,12].

2.4. Chromatin immunoprecipitation (ChIP) assay and cloning

The ChIP assay for the purpose of cloning immunoprecipitated DNA fragments were performed as described recently using modification described elsewhere [13,14]. Briefly, GRMO2 cells were cultured to a confluency of 1×10^7 cells in 500 cm^2 dish and subjected to 8 h 0.5 mM 8-Br cAMP treatment. The cell pellets were resuspended in 2.0 ml lysis buffer Incubate on ice for 10 min. Cells were lysed (10 strokes) using an ice-cold dounce homogenizer. Nuclei were pelleted using a microfuge at 5000 rpm for 10 min at 40 °C. The nuclei pellet was resuspended in 2 ml nuclei lysis buffer plus the same protease inhibitors as the cell lysis buffer and incubated on ice for 10 min. Samples were processed a described in the ChIP assay. After elution from protein A-beads, samples were diluted using IP dilution buffer and subjected to another round of immunoprecipitation followed by washing and elution. After the second elution, the elutants were reverse cross-linked and the chromatin sample was purified using Qiaquick PCR purification kit (Qiagen), according to manufacture's protocol.

The Chromatin fragments were blunt-end repair and dephosphorylated using the TOPO Shotgun Subcloning Kit according to the manufacture's instructions. The DNA was subjected to ligation-mediated PCR (LMPCR), chromatin amplicon were generated by 20 cycles of PCR using the reported conditions [14], each reaction was purified using the Qiaquick PCR purification kit. The PCR protocol was repeated (1×, 2× or 3×) until enough amplicon was made. The resulting PCR products were cloned into PCR 2.1 TOPO vector. DNA plasmids from the recombinant colonies were screened by *EcoRI* digestion. *EcoRI* sites flank the PCR product insertion sites for excision of inserts. Colonies with inserts greater then 200 bps were sequenced using T7 primers. Obtained sequences were blasted against the mouse genome. Sequences were also subjected to a transcription factor binding site search [15,16].

3. Results and discussion

3.1. ChIP analysis of genes potentially regulated by CREM/ICER

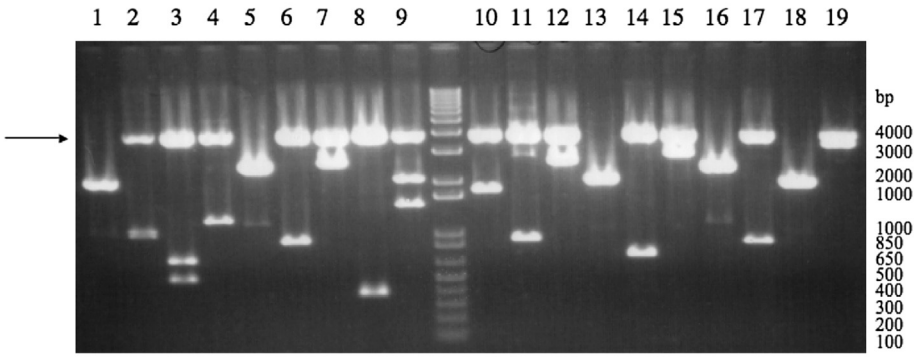
ICER has shown to be strongly induced in ovaries by exogenous gonadotropins in immature rats and is transiently expressed in the ovaries immediately after the preovulatory LH surge in adult cycling rats [7]. Our lab and others have suggested a role for ICER in ovarian function based on the observation that many FSH-responsive genes are transcriptionally repressed after the LH surge. We sought to perform a global analysis to map the binding distribution of CREM/ICER along the entire mouse genome.

We opted to use a modification on the ChIP assay to clone DNA associated with CREM/ICER as a means to directly identify candidate genes CREM/ICER could potentially regulate in the ovaries. This approach has been successfully utilized previously to clone novel E2F promoter targets [13]. Using a similar approach, we cloned and sequenced the immunoprecipitated CREM/ICER-associated DNAs from GRMO2 cells.

3.2. ChIP, target validation and amplification

The ChIP assay was performed with some modification as described [13]. Chromatin was extracted from GRMO2 cells

A.



B.

Chromosome	Annotated Region	Gene
11	intron	RIKEN cDNA 3300001P08 gene
4	20kb from gene	C Eph receptor A7
11	end of gene	E similar to hypothetical protein FLJ14166
15	promoter	tubulin, alpha 6
14	promoter	ribonuclease P 14 subunit (human)
4	promoter	membrane progestin receptor alpha
1	intron	MAP kinase-activated protein kinase 2
3	promoter	neuroblastoma ras oncogene
19	intron	C amyloid beta (A4) precursor protein-binding, family A, member 1
11	intron	C dopa decarboxylase
1	intron	MAP kinase-activated protein kinase 2
5	promoter	CDP-diacylglycerol synthase 1
5	intron	eukaryotic translation initiation factor 3, subunit 9 (eta)
8	intron	DDHD domain containing 2
17	intron	villin 2
5	intron	hypothetical protein B930074I24
5	intron	SH3-domain binding protein 2
13	intron	RIKEN cDNA 1810043M15 gene
1	intron	solute carrier family 26, member 9
12	intron	C ring finger protein 144
14	intron	C cDNA sequence BC065120
2	intron/exon	C RIKEN cDNA 1700048E23 gene

Fig. 2. Genes immunoprecipitated. A. PCR products were cloned directly into pCR II-TOPO vector and transformed into TOP10 competent cells. DNA mini-preps of the recombinant colonies were screened by *EcoRI* digestion. *EcoRI* sites flank the PCR product insertion sites for excision of inserts. Colonies with inserts greater then 200 bps were sequenced busing T7 primers. Obtained sequences were blasted against the mouse genome. Sequences were also subjected to a transcription factor binding site search. B. Representative list of the 70 sequenced clones. This list identifies the gene, location within the genomic DNA and chromosome number of the immunoprecipitated DNA fragment.

(1×10^7 cells) treated with 8-Br cAMP for 8 h. After the chromatin was fixed and sheared, the samples were divided and either immunoprecipitated with an antibody recognizing ICER or subjected to pre-immune rabbit IgG as a negative control. To minimize the occurrence of non-specific DNA carry-over, the immunoprecipated chromatin was eluted from the protein A beads and subjected to another round of immunoprecipitation. The assay was validated by PCR using primers flanking the four CREs within the *Crem* internal promoter [5], as a positive control for ICER binding (Fig. 1A). Since the PCR on the chromatin immunoprecipitated against ICER resulted, as expected, in the amplification of the targeted promoter regions, we next blunt-end repaired the sheared chromatin and added to its ends unidirectional double stranded oligonucleotide linkers. Chromatin amplicons were generated by 20 cycles of PCR and each reaction was purified using the Qiaquick PCR purification kit. The PCR protocol was repeated (1×, 2× and 3×) until enough amplicon was made, producing a smear between 400bp to 3 kb (Fig. 1B).

3.3. Sequencing and identity determination of immunoprecipitated DNA

The resulting amplified chromatin was subcloned into a pCR II - Topo vector. DNA extracted from the recombinant colonies were screened by *EcoRI* digestion and plasmids with inserts greater then 200bp were sequenced using T7 primers (Fig. 2A). The different DNA sequences obtained were screened against the mouse genome using the NCBI-BLAST program for mouse sequences (Fig. 2B). The DNA sequences were also subjected to a transcription factor binding site search using transcription factor search programs: TFSEARCH [15], TESS [16] and the CREB Target Gene Database [17].

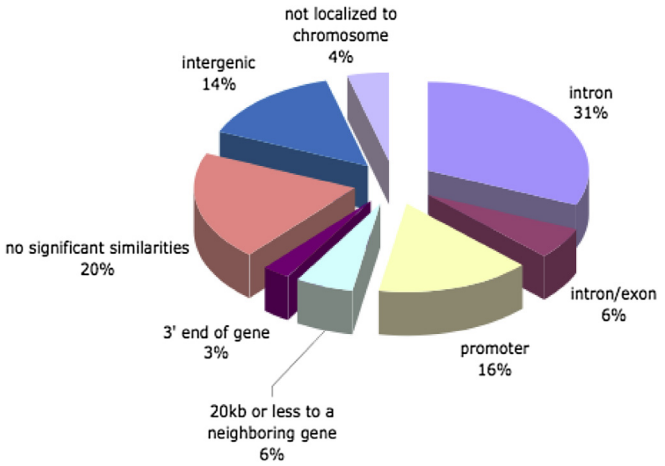


Fig. 3. Relative distribution of the immunoprecipitated chromatin fragments throughout the mouse genome.

We next examined the positions of the CREM/ICER binding fragments relative to annotated exons and introns. As summarized from the multiple categories shown in Fig. 3, 6% of the immunoprecipitated regions (4 fragments) lay within 20 kb of the 5′ end of the gene, and 31% (22 fragments) of the CREM/ICER binding regions lay within an intron. The 6% CREM/ICER binding fragments (4 fragments) intersected an annotated exon/intron region. Fig. 2B reports a representative list of the 70 sequenced clones. Only 16% of the CREM/ICER binding regions lay within a promoter relatively adjacent to a TATA sequence. Our data is consistent with those previously published, demonstrating that only a modest percentage of CREB sites were found near or within genes along human chromosome 22 [18].

The use of ChIP has served as a valuable tool in identifying *in vivo* binding of trans-acting factors binding to cis-elements on chromatin. Recently, researchers have begun using ChIP as an unbiased approach to globally identify a target gene. For example, modification of the ChIP assay has led to the discovery of novel E2F binding sites by cloning of immunoprecipitated fragments [13]. We employed this technique in our studies to globally identify CREM/ICER binding sites throughout the mouse genome. Our data supports previous findings demonstrating that CREB binds to multiple loci on human chromosome 22 [18]. However in that report, the immunoprecipitated chromatin was hybridized to a genomic DNA microarray containing all of the nonrepetitive DNA sequences of human chromosome 22. Their data demonstrated that only a small fraction of CREB binding sites lay near the well-defined 5′ ends of genes. Instead, the majority of sites were found within introns and unannotated regions. Similar results were obtained in our studies where we directly cloned and sequenced CREM/ICER targets. Their data also demonstrated that few CREB targets were found near full-length cyclic AMP response element sites, the majority of the CREB targets contained shorter versions or close matches to this sequence. Although the expression of CREB and CREM is believed to be ubiquitous, the dogma of constitutive occupancy of a CRE has been challenged. Several studies have demonstrated that CREB binding is highly tissue-specific and such binding were apparent at genes that were transcriptionally active but not on promoters of genes that were not expressed [19].

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