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Inducible cAMP Early Repressor Splice Variants ICER I and IIγ Both Repress Transcription of c-fos and Chromogranin A

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Abstract Inducible cAMP early repressor (ICER) splice variants are generated upon activation of an alternative, intronic promoter within the CREM gene. ICER is proposed to downregulate both its own expression, and the expression of other genes, containing cAMP-responsive promoter elements. To examine the biological function of the two ICER splice variants, I and IIγ, in comparable cellular systems, we generated HEK 293 cell variants with controllable overexpression of either ICER I or IIγ. These two splice variants contain two different variants of DNA binding domains. Overexpression of either ICER I or IIγ strongly represses CRE-driven reportergene transcription but not AP1- or NFκB-driven transcription. Thus, high specificity is maintained even at ICER overexpression. We here show that both ICER I and IIγ repress Pituitary adenylate cyclase-activating polypeptide (PACAP)-mediated c-fos mRNA induction with similar efficiency, indicating that both splice variants play an important role in modulating PACAP-mediated transcriptional activation of the c-fos gene. ICER I and IIγ also repress cAMP-mediated activation of chromogranin A (CgA), indicating that these splice variants may function as negative feedback regulators in CgA synthesis. The proliferation rate was not altered in cells overexpressing ICER I or IIγ. Thus, in the epithelial cells HEK 293, ICER I and IIγ splice variants seem to exert similar biological function. J. Cell. Biochem. 101: 1532–1544, 2007.

Key words: ICER; Gene regulation; alternative splicing; transcription factors; CREM; c-fos; CgA

The transcription of many genes is regulated by increased intracellular cAMP levels in response to extracellular stimuli. CREB/CREM/ATF [Rosenberg et al., 2002; Servillo et al., 2002] transcription factors mediate the nuclear response to the cAMP pathway [Mayr and Montminy, 2001]. The CREB/CREM/ATF family comprises homologous proteins which all contain a basic DNA binding domain (DBD) that recognizes a highly conserved sequence known as the cAMP response element (CRE) [Montminy et al., 1986; Short et al., 1986; Lewis et al., 1987; Bokar et al., 1988; Kim et al., 1993]. The proteins in this family share other characteristics as well: a leucine zipper domain for protein dimerization, a phosphorylation site for modulation of functional activity, and a transactivation domain that interacts with other components of the transcriptional machinery [Mayr and Montminy, 2001; Quinn, 2002; De Cesare et al., 2003]. The CREB and ATF-1 proteins are both expressed ubiquitously, whereas CREM gene products are expressed in a cell-, tissue-, and development-specific manner [De Cesare and Sassone-Corsi, 2000; Mayr and Montminy, 2001]. The CREM gene gives rise to several alternative splice products that encode polypeptides with distinct repressor or activator properties [Foulkes et al., 1991; Delmas et al., 1992; Laoide et al., 1993; Daniel et al., 2000]. Inducible cAMP early repressor (ICER) is transcribed from the intronic CREM gene promoter P2 (Fig. 1), which contains four CRE-like elements and directs the

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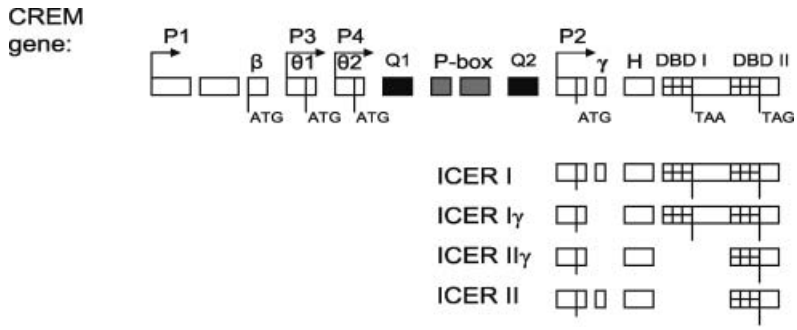

Fig. 1. Schematic representation of the CREM gene with ICER splice variants.

expression of a truncated protein, composed of the basic domain and leucine zipper motif only [Molina et al., 1993]. Thus, in contrast to the other CRE-binding proteins, the principal determinant of ICER activity is its intracellular concentration and not its degree of phosphorylation. Different members of the CREB/CREM/ATF family can form homo- and/or heterodimers and bind to similar DNA binding sites. ICER can form heterodimers with all other CREM proteins as well as with CREB proteins, although ICER homodimer formation is more favored [Molina et al., 1993; Stehle et al., 1993]. ICER containing dimers bind efficiently to CREs and can competitively block DNA binding of CREM activators or other members of the CREB/CREM/ATF family to promoters like, for example, the somatostatin promoter [Stehle et al., 1993] and the insulin promoter [Inada et al., 1999]. Occupation of CRE promoter elements by a dimer containing a repressor isoform results in repression of transcription, presumably due to impaired interaction with the coactivator protein CBP or TFIID or both [Sun and Maurer, 1995; Nakajima et al., 1997]. ICER is subject to an autoregulatory feedback loop since it downregulates its own expression via the CRE-elements in its own promoter [Molina et al., 1993; Foulkes et al., 1996].

Alternative splicing of the γ exon and the two different DNA binding domains DBD I and DBD II generates ICER splice variants I, Iγ, II, and IIγ (Fig. 1). The expression of ICER proteins with alternative DBDs raises the possibility that splice variants containing alternative DBDs may show different promoter element sequence specificity or otherwise differ in their modes of action as repressors. Also, the presence or absence of the γ exon may have an effect on ICER function.

The aim of the present study was to elucidate the biological function of ICER I and IIγ splice variants. We found that both ICER I and IIγ repress CRE-driven reportergene transcription as well as c-fos gene expression. This indicates that both ICER I and IIγ may act as anti-oncogenes inhibiting the expression of growth-related genes. Furthermore, both splice variants repress cAMP-mediated activation of chromogranin A (CgA), indicating that both ICER I and IIγ may function as modulators of synthesis of this protein known to be ubiquitous and functionally important in the neuroendocrine system [Taupenot et al., 2003].

MATERIALS AND METHODS

Cells and Reagents

Flp-In T-REX 293 (human embryo kidney cells, Invitrogen, Carlsbad, CA), were maintained in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, 0.1 mg/ml L-glutamine and 10 U/ml penicillin-streptomycin (GIBCO, Invitrogen) supplemented with 10% fetal calf serum (FCS; Euroclone Ltd, Devon, UK), 100 μg/ml Zeocin (Invitrogen) and 15 μg/ml Blasticidin (Invitrogen). Forskolin, CPT-cAMP, and phorbol-12-myristate-13-acetat (PMA), were purchased from Sigma Aldrich (St. Louis, MO), Pituitary adenylate cyclase-activating polypeptide (PACAP-38) was obtained from Bachem (Bobendorf, Switzerland) and Human rhTNFα was purchased from R&D Systems (Abingdon, UK). The peptides were dissolved and stored frozen at −20°C.

Reporter Assays

pCRELuc containing 4× CRE somatostatin consensus promoter elements (TGACGTCA) and pNFκBLuc, containing five copies of the

NFκB enhancer element (TGGGGACTTTCC-GC), were obtained from Stratagene (La Jolla, CA). pAP1-Luc containing four tandem copies of the AP1 consensus sequence (TGA(G/C)TCA) were obtained from Clontech (Palo Alto, CA). The plasmid pXp100Luc containing 100 bp of the proximal CgA promoter [Wu et al., 1995] was a generous gift from Dr. Daniel O'Connor (University of California, San Diego, CA). Cells were seeded in 96-well plates (4×10^4 cells/well) or 6-well plates (1.3×10^6 cells/well) and transfected after 24 h. *Transfection in 96-well plates*: 0.24 μg luciferase reporter plasmid DNA was transfected by use of 0.8 μl Lipofectamine 2000 transfection reagent (Invitrogen). After 4 h the medium was replaced with normal growth medium. Cotransfections of reporter plasmids and siRNA were performed with 0.15 μg luciferase reporter plasmid DNA and 0.08 μg siRNA by use of 0.8 μl XtremeGENE transfection reagent. *Cotransfections in 6-well plates* were performed with 1 μg siRNA and 2 μg plasmid DNA by use of 10μl XtremeGENE reagent. The next day cells were either harvested for protein isolation (6-well plates), or treated with stimuli (96-well plates) and after 6 h the medium was removed followed by lysis in 20 μl of lysis buffer (Promega, Madison, WI). Luciferase activity was measured by Wallac 1420 Victor ^{3TM} plate reader (Perkin-Elmer Life and Analytical Sciences, Boston, MA) by using the Luciferase Reporter Assay System (Promega).

PCR and Real-Time PCR

Total RNA was isolated using RNeasy mini kit (Qiagen, Germantown, MD) (6-well plates) or RNeasy Midi kit (Qiagen) (75 cm^2 bottle) according to the manufactures instruction. cDNA synthesis was performed with 500 ng total RNA in a 10 μl reaction containing 1× PCR buffer II, 5 mM $MgCl_2$, 500 μM each dNTP, 2.5 μM Oligo d(T) 16 primer, 0.4 U/μl RNase inhibitor and 2.5 U/μl MuLV Reverse transcriptase (Applied Biosystems). cDNA synthesis was performed at 10 min at 25°C followed by 1.5 h at 48°C and 5 min at 95°C. After synthesis, the cDNA was diluted 1:2 with RNase-free water. *Regular PCR* was performed with 1× PCR buffer, 0.05 U PLATINUM Taq DNA Polymerase or PLATINUM Taq DNA Polymerase High Fidelety (Invitrogen), 0.5 μM of each primer (forward and reverse), 7 mM $MgCl_2$, 0.8 mM of each dNTP (Applied Biosystems) in a 20 μl reaction. PCRs were performed in a GeneAmp PCR System 9700 (Applied Biosystems) using the following cycling parameters: activation for 10 min at 94°C followed by 35 cycles at 94°C s for 15 s, 61°C for 15 s, and 72°C for 30 s. *TaqMan Real Time PCR* was performed with 1× Quantitect Probe PCR Master Mix (Qiagen), 400 nM of each primer, 200 nM TaqMan Probe (Eurogentec, Belgium) and cDNA equivalent to 62.5 ng total RNA in a total reaction volume of 25 μl. The Real-Time PCR was performed in Stratagene's Mx3000P Real Time PCR system; 15 min at 95°C, 45 thermal cycles of 15 s at 95°C, 1 min at 56°C, and 30 s at 76°C. Fold induction of gene expression level was estimated by the ΔΔCt-method, where: Fold change $= 2^{-\Delta\Delta Ct}$ and $\Delta\Delta Ct = (Ct_{GOI} - Ct_{GAPD\text{-}CtGAPDH})_{untreated} - (Ct_{GOI} - Ct_{GAPDH})_{treated}$ [Livak and Schmittgen, 2001].

Primers and Probe Design

Regular PCR: the following PCR primers were used for ICER (Genbank^{TM}/EBI Data Bank accession number NM_182717): ICER-S: 5′-TGGAACACTTTATGTTGAACTGTGG-3′; I-CER-AS: 5′-CAGTTCATAGTTAAATATTTC-TAGTA-3′. *TaqMan Real Time PCR*: using computer software [GenScript Corp. Real-Time PCR Primer Design] (http://www.genscript.com/ssl-bin/app/primer) primers and probes were designed to recognize human CgA (Genbank^{TM}/EBI Data Bank accession number NM_001275), c-fos (Genbank^{TM}/EBI Data Bank accession number K00650), and GAPDH (Genbank^{TM}/EBI Data Bank accession number NM_002046) mRNA sequences. The sequences of primers and probes used for TaqMan Real Time PCR analyses are as follows: *CgA-S*: 5′-TACAAGGAGATCCGGAAAGG-3′; *CgA-AS*: 5′-CTCCTCTTTCTGCTGGGAGT-3′, *CgA-probe*: 5′-(FAM) TCCACAGCCAGAGCCTCC-GA (Dark Quencher)-3′, *c-fos-S*: 5′-GGGCA-AGG-TGGAACAGTTAT-3′; *c-fos-AS*: 5′-TCCT-TCTCCTTCAGCAGGTT-3′, *c-fos-probe*: 5′-(FAM) CAGCTCCCTCCTCCGGTTGC (Dark Quencher)-3′ and *GAPDH-S*: 5′-GAAGGT-GAAGGTCGGAGTC-3′, *GAPDH-AS*: 5′-GAA-GATGGTGATGGGATTTC-3′ and *GAPDH-probe*: 5′-(FAM) CAAGCTTCCCGTTCTCAGCC (Dark Quencher)-3′.

Gateway Cloning

Oligonucleotide primers for the amplification of ICER DNA fragments were designed with

flanking attB1 or attB2 sites for insertion into the GATEWAY donor vector pDONR201 (Invitrogen). Primers with the following sequences were synthesized by Invitrogen (ICER specific sequence in bold): ICER-S, 5′-attB1- GGGGACAAGTTTGTACAAAAAAGCAGGCT**TGGAACACTTTATGTTGAACTGTGG**-3′; and ICER-AS, 5′-attB2-GGGG ACCACTTTGTACAAGAAAGCTGGGT**CAGTTCATAGTTAAATATTTCTAGTA**-3′. The PCR products were cloned directly into pDONR201, and the resulting plasmids were used to transfer the ICER sequences into pcDNA5/FRT/TO-cassetteA [T-Rex Flp-In compatible] via homologous recombination. *Preparation of Gateway compatible T-Rex plasmid*: The T-Rex Flp-In compatible plasmid pcDNA5/FRT/TO (Invitrogen) was digested with EcoRV and purified. The plasmid was ligated to the GATEWAY Reading Frame Cassette A (RfA), obtained from digestion with EcoRV from the RfA-containing plasmid pGEMA (generous gift from Dr. Ole Morten Seternes, University of Tromsø, Norway). The clones obtained represent GATEWAY-compatible "destination vectors" (pcDNA5/FRT/TO-cassetteA). The pDONR201 containing the ICER PCR-products were used to transfer the ICER sequences into the pcDNA5/FRT/TO- cassetteA [T-Rex Flp-In compatible] via homologous recombination. The pcDNA/FRT/TO-ICER plasmids were confirmed by sequencing.

Stable Cell Lines

Stable cell lines with tetracycline-induced ICER expression were established by transfecting the Flp-In T-Rex 293 cell line with pcDNA5/FRT/TO constructs with the recommended amount of pOG44. Two days post-transfection cells were split (1:8) into 24 well plates. After 6 h growth medium containing 150 μg/ml hygromycin and 15 μg/ml blasticidin was added. After 19 days individual colonies were picked, tested for Zeocin sensitivity, lack of β-gal activity and tetracycline (Invitrogen) inducible overexpression of the relevant construct by Western analysis.

The resulting variant cell lines were termed "HEK 293_{ind}-ICER I" and "HEK 293_{ind}-ICER IIγ" and represent model systems where a specific ICER splice variant can be induced without activating other genes. Flp-In T-Rex 293 cells transfected with empty vector, were termed "HEK 293_{ind}-Control."

siRNA Procedures

siRNA-ICER I, siRNA ICER IIγ, siRNA-CAT, and siRNA-EGFP (The Biotechnology Centre, University of Oslo [Amarzguioui et al., 2003; Amarzguioui and Prydz, 2004]), were annealed at 10 μM in 500 μl of 10 mM Tris-HCl, pH 7.4. siRNA-All-ICER (Qiagen), were annealed at 20 μM in 1,000 μl siRNA suspension buffer (Qiagen). siRNA species were designed targeting sites within human protein ICER: siRNA-ICER I: 5′-GAUGACACAGAUGAGGAAAC-T-3′, siRNA-ICER IIγ: 5′-GAGAUGACACAG-CUGCCACU-GG-3′ and siRNA-All-ICER: 5′-CAUUAUGGCUGUAACUGGATT-3′. Control siRNAs used: siRNA-CAT [Mousses et al., 2003]: 5′-GAGUGAAUACCACGACGAUU-3′ and siRNA-EGFP [Mousses et al., 2003]: 5′-GCAAGCUGACCCUGAAGUUC-3′.

Luciferase reporter plasmids, Luc-ICER, for control of ICER siRNA efficiency were generated as follows: pGL3-Control vector (Promega) was digested with XbaI, and subsequently filled of recessed 3' termini by Klenow fragment (New England Biolabs, Beverly, MA). The plasmid was ligated to the GATEWAY Reading Frame Cassette A (RfA) with the same procedure as above. The clones obtained represent GATEWAY- compatible "destination vectors" (pGL3-Control-cassetteA), with the cassette inserted following the Luc gene in the pGL3 plasmid. The ICER containing plasmid pDONR201, were used for cloning ICER into GATEWAY compatible pGL3-Control vector via LR reaction following the manufacturer's instructions. pGL3-Luc-ICER constructs were sequenced for control of correct reading frame and orientation.

Specificity of siRNAs were tested in reporter-gene studies using Luc-ICER plasmids, and the siRNAs had all potent and specific inhibitory effects (~80–90%) in the cotransfection assays. The inhibiting effect was confirmed on the protein level (results not shown).

Western Blotting

Cells were grown in 75 cm^2 flask (4×10^6 cells) or 6-well plates (1.3×10^6 cells) for two days, and then washed with PBS and harvested directly in 2,000 μl (500 μl for 6-wells) SDS sample-buffer, as described previously [Thommesen et al., 2000], except for 2 h incubation with primary

antibodies (diluted 1:100–1:1,000) and incubation with secondary antibody (1:1,000) for 1 h at room temperature. The following antibodies were used: Rabbit anti-human CREM-1 (X-12) (1:100) (Santa Cruz, CA), Rabbit α–CAT antiserum (1:5,000) (Invitrogen), rabbit anti-mouse Akt (1:1,000) (Cell Signaling Technology) and horseradish peroxidase conjugated goat anti-rabbit IgG (1:1,000) (Cell Signaling Technology).

Proliferation Assay

Proliferation rate was determined by measuring DNA synthesis using the Cell proliferation ELISA BrdU (5-bromo-2′-deoxyuridine) kit (Roche). Flp-In T-REX 293 cells (2×10^3) were seeded out in 96-well microtiter plates in 100 μl serum-containing medium, and after 30 h tetracycline was added to the cells. BrdU-labeling solution (10 μl per well) was added 17 h before incorporation of BrdU was measured, and the measurements were done as described previously [Thommesen et al., 2006]. Incorporation of BrdU was measured 2–7 days after cell seeding, and the number of cells was determined (five parallels).

Sequencing

Cycle sequencing was performed using the Big Dye sequencing kit (Applied Biosystems). Sequencing reactions were analyzed on an ABI377 Prism Sequencer (Perkin-Elmer Life and Analytical Sciences).

Statistical Analysis

P-values were estimated using a student two-tailed *t*-test, assuming unequal variance.

RESULTS

Previous studies examining the function of one specific ICER splice variant include studies of transgenic mice where increased β cell ICER Iγ expression leads to development of early severe diabetes [Inada et al., 2004, 2005], and studies using stably transfected ICER IIγ cells, showing that ICER IIγ inhibits growth of pituitary tumor cells and choriocarcinoma cells [Lamas et al., 1997; Razavi et al., 1998]. However, most studies of specific ICER variants up until now have been performed using transient transfection, like, for example, studies indicating that ICER IIγ evokes neuronal apoptosis in cell cultures [Jaworski et al., 2003; Tomita et al., 2003]. Use of transient transfection does not always guarantee a controlled increase in the protein of interest. We wanted to study the biological effects of specific ICER splice variants in a system where its levels could be determined, and where different ICER splice variants could be examined separately. The aim of this study was to compare the biological response of ICER I and IIγ, and examine their repressive effect on authentic CRE-containing promoters.

The stable cell lines for inducible ICER splice variant overexpression were termed "HEK 293_{ind}-ICER I" and "HEK 293_{ind}-ICER IIγ." They represent model systems where a specific ICER splice variant can be induced by tetracycline and where ICER levels are normal in the untreated condition.

To verify that ICER protein levels were increased in the tetracycline-induced state, HEK 293_{ind} cell variants were treated with tetracycline for different time intervals and ICER protein was analyzed by Western blot analysis. The result shows that both ICER I and IIγ protein levels are strongly upregulated upon 8 h tetracycline treatment, and that they are increased further upon treatment for 12 h. Furthermore, the high ICER protein levels still persist after 24 h treatment (Fig. 2).

Inducible ICER I and IIγ Proteins Repress c-fos mRNA Expression

The c-fos promoter contains, among multiple promoter elements, CRE elements important for c-fos gene expression [Berkowitz et al., 1989; Fisch et al., 1989]. HEK 293 cells were stimulated with the neuroendocrine peptide hormone Pituitary adenylate cyclase-activating polypeptide (PACAP), which induced a five- to eightfold increase in c-fos gene expression (Fig. 3). We here show that overexpression of either ICER I or IIγ splice variants repress PACAP-induced c-fos gene expression by 60% and 55%, respectively (Fig. 3A,B). Similarly, overexpression of either ICER I or IIγ repressed forskolin-induced c-fos gene expression by 75% and 60%, respectively (data not shown). Interestingly, basal c-fos expression (uninduced state) was also reduced (30–35%) in cells overexpressing either ICER I or IIγ (Fig. 3A,B).

Our results indicate that both ICER I and IIγ repress transcription from an endogenous, CRE-dependent promoter. Furthermore, our results show that both splice variants can interfere both with the forskolin-response,

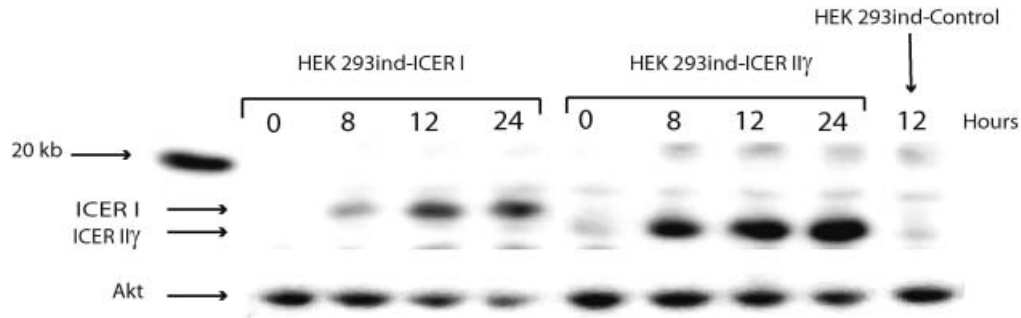

Fig. 2. Western blot verifying ICER overexpression in tetracycline-treated HEK 293_{ind}-ICER cells. Whole cell lysates from HEK 293_{ind}-ICER I and HEK 293_{ind}-ICER IIγ cells treated with tetracycline (1 μg/ml) for 8, 12, and 24 h were analyzed on Western blot using antiserum towards CREM and Akt. HEK 293_{ind}-Control (HEK 293 cells transfected with empty vector) treated with tetracycline for 12 h.

which is mediated via the protein kinase A (PKA) signaling pathway, as well as with the transcriptional response to the hormone PACAP, which has been reported to include both PKA [Kienlen Campard et al., 1997; Vaudry et al., 1998] as well as MAP kinases [Schafer et al., 1996], in its activation of c-fos gene expression.

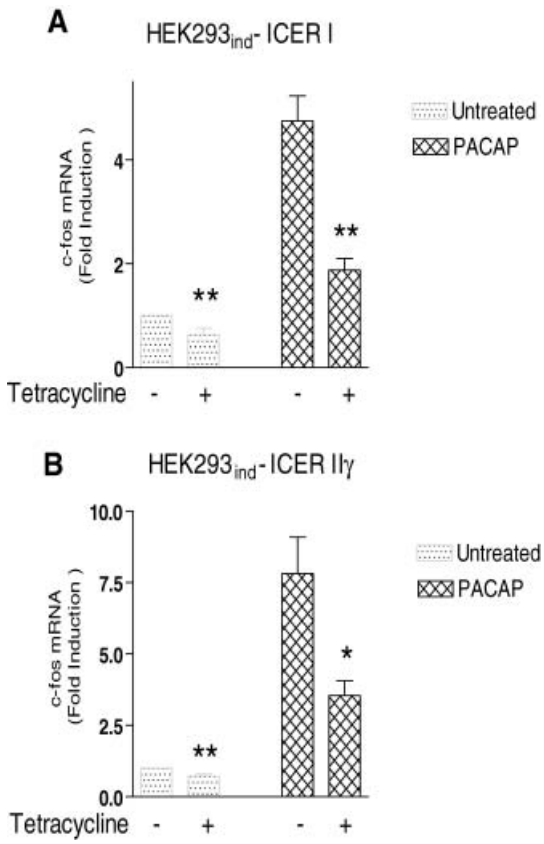

Fig. 3. Effect of ICER I and IIγ overexpression on c-fos gene expression. **A**: HEK 293_{ind}-ICER I and (**B**) HEK 293_{ind}-ICER IIγ cells were pre-treated with tetracycline (1 μg/ml) for 11 h, and treated with PACAP (0.2 μM) for 30 min before harvesting. c-fos mRNA expression were determined by quantitative TaqMan real time PCR. Mean ± SEM of at least three separate measurements are shown. $^{**}P < 0.05$, $^{*}P < 0.06$; significant difference from tetracycline untreated cells.

ICER Represses Transcription from the Chromogranin A (CgA) Promoter

We further studied another CRE-containing promoter, the CgA promoter, where the CRE-element is shown to play an indispensable role in expression of the gene [Wu et al., 1995]. Overexpression of ICER I repressed forskolin-activated CgA promoter-driven transcription by approximately 50% in a reportergene assay (Fig. 4A). ICER I also repressed activation of the CgA promoter by the non-hydrolysable cAMP analogue in CPT-cAMP to a similar manner (data not shown). Similarly, overexpression of ICER IIγ resulted in about 50% repression of CgA reportergene activity (Fig. 4B). In order to examine whether expression of the endogenous CgA gene was also repressed, we measured CgA mRNA levels in forskolin-induced cells with and without ICER overexpression. We found that endogenous CgA-gene is expressed at low levels in untreated HEK 293 cells and that overexpression of ICER I completely blocked forskolin-induced expression of the endogenous CgA gene (Fig. 4C). Our results strongly suggest that activation of the endogenous CgA gene is repressed by ICER, which has not been previously reported.

Overexpression of ICER Specifically Represses CRE-Driven Gene Expression

To examine whether the ICER splice variants target CRE promoter elements specifically, we tested the effect of ICER I and IIγ overexpression in reportergene assays with

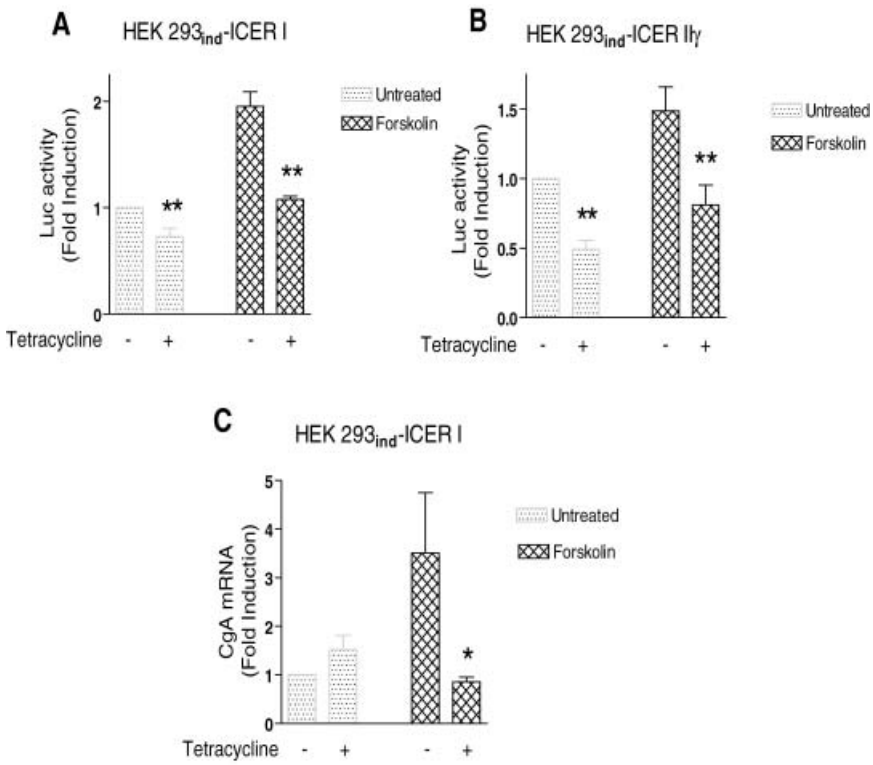

Fig. 4. Effect of ICER I overexpression on CgA reportergene and CgA gene expression. **A**: HEK 293_{ind}-ICER I and (**B**) HEK 293_{ind}-ICER IIγ cells, were transfected with CgA-reporter gene, pretreated with tetracycline (1 μg/ml) for 16 h, and treated with forskolin (10 μM) for 6 h. **C**: HEK 293_{ind}-ICER I cells pre-treated with tetracycline (1 μg/ml) for 11 h, and treated with forskolin (10 μM) for 1 h and CgA expression measured by quantitative TaqMan real time PCR. Mean ± SEM of at least three measurements are shown for the transfections (A,B), while two biological experiments (with three technical replicas) are shown for the real-time PCR data. $^{**}P < 0.05$, $^{*}P < 0.09$; significant difference from tetracycline untreated cells.

reportergenes driven by artificial promoters of the isolated promoter elements CRE, AP-1, and NFκB. Experiments with the CRE reporter plasmid pCRELuc showed that overexpression of either ICER I or IIγ resulted in strong reduction in forskolin-mediated CRE-driven transcription (Fig. 5A,B) Also PACAP-mediated CRE-driven transcription was repressed by overexpression of either ICER I or IIγ (Fig. 5A,B). The two splice variants inhibited reportergene activation to a similar extent, and both ICER I and IIγ repressed basal CRE activity (e.g., reportergene activity in the absence of stimuli) (Fig. 5A,B).

In order to verify that the observed repressing effect was caused by ICER, the activity of the CRE-driven reporterplasmid was measured in the presence of siRNA towards ICER. We found that siRNA towards ICER clearly reversed the repressing effect of overexpressed ICER on forskolin-induced CRE-driven transcription (Fig. 6). In ICER I overexpressing cells both ICER I specific siRNA as well as siRNA targeting all ICER splice variants increased CRE-driven transcription (55% and 65%, respectively; Fig. 6A). Similarly, in ICER IIγ overexpressing cells, both ICER IIγ specific siRNA as well as all-ICER siRNA increased CRE-driven transcription (60% and 85%, respectively; Fig. 6B). No effect of all-ICER siRNA was observed on forskolin-induced reportergene activity in the HEK 293_{ind}-Control cells (Fig. 6C). These cells were stably transfected with a control DNA-construct that did not contain ICER sequences. These results clearly indicate that the repression of forskolin-induced reportergene activity is caused by the overexpressed ICER splice variant.

Overexpression of either ICER I or IIγ had no repressing effect upon either forskolin or PMA-induced AP1 promoter activity (Fig. 7A). Likewise, no effect of either ICER I or IIγ overexpression was observed on tumor necrosis factor (TNF)-induced activity of the NFκB reporterplasmid (Fig. 7B). These results show that ICER overexpression does not lead to

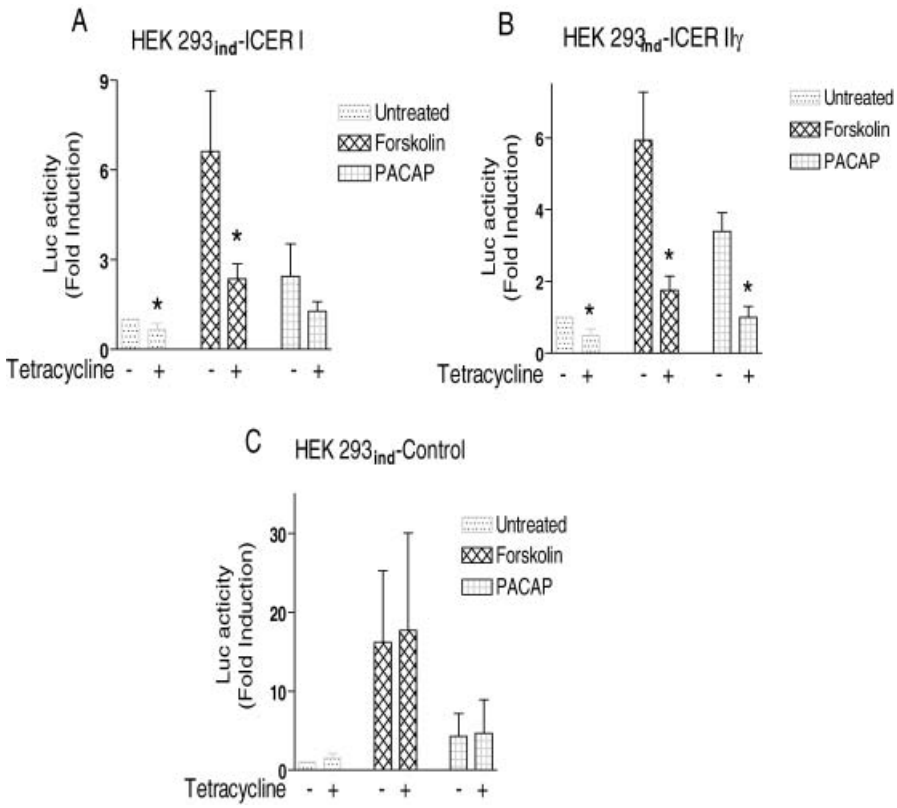

Fig. 5. Effect of ICER overexpression on pCRELuc activity. **A**: HEK 293_{ind}-ICER I cells (**B**) HEK 293_{ind}-ICER IIγ cells, and (**C**) HEK 293_{ind}-Control cells were transfected with pCRELuc, pretreated with tetracycline (1 μg/ml) for 24 h, and treated with forskolin (10 μM) for 6 h. Mean ± SEM of quintuple measurements are shown. $^*P < 0.05$; significant difference from tetracycline untreated cells.

general transcriptional repression and that the consensus AP1 and NFκB promoter elements are not affected by ICER I or IIγ. The fact that CRE-elements (*TGACGTCA*) are repressed but not AP1-elements (*TGA*(*G*/*C*)*TCA*), indicates that high specificity is maintained in the cell system used in the present study even though ICER protein levels are high in the tetracycline-induced state.Overexpression of either ICER I or IIγ represses the uninduced level of gene expression from all CRE containing promoters examined in the present study. This indicates that endogenous ICER levels in the uninduced state are not sufficiently high to completely silence the basal expression from these promoters.

Overexpression of ICER I and IIγ Proteins did not Effect Cell Growth

Razavi et al. [1998] have shown that ICER IIγ dramatically inhibits the growth and DNA synthesis of mouse pituitary tumor cells and human choriocarcinoma cells, while Memin et al. [2002] found that overexpression of ICER IIγ in prostate tumor AT6.3 cells did not affect cell growth. We examined the effect of ICER I and IIγ overexpression on the proliferation of HEK 293 cells, by comparing BrdU-incorporation in cells cultivated in the absence of tetracycline with BrdU-incorporation in cells cultivated in the presence of tetracycline. We found no significant differences in cell proliferation in the presence or absence of high levels of either ICER I or IIγ, indicating that neither of these splice variants affects normal growth in HEK 293 cells (Fig. 8A–C). Taken together with the results reported by others [Razavi et al., 1998; Memin et al., 2002], our results indicate that the influence of ICER on growth is cell type specific.

DISCUSSION

The majority of all human genes are alternatively spliced [Black, 2003], and the biological activities of different splice variant products of

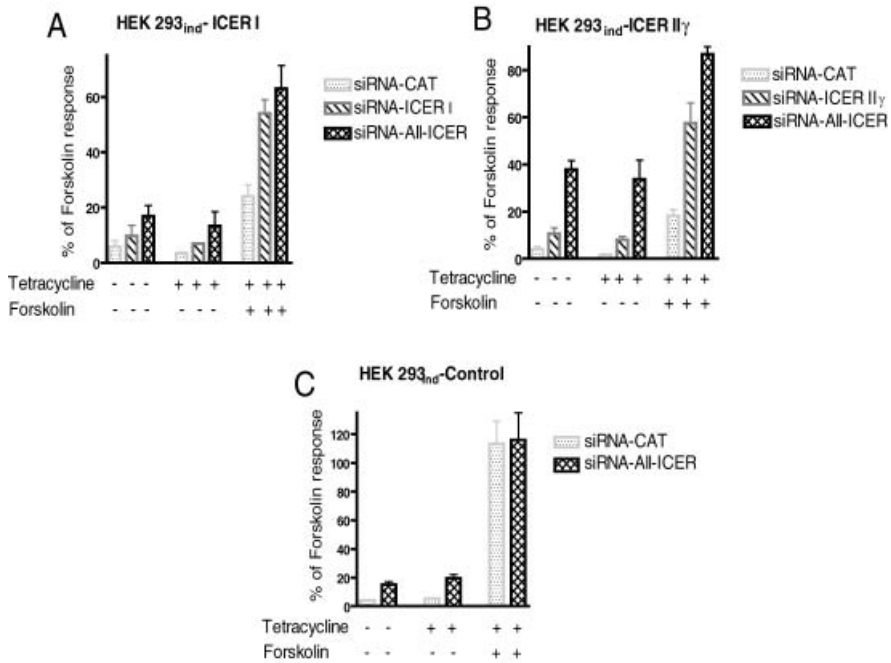

Fig. 6. Effect of ICER siRNAs on CRE-driven gene activity. Results are shown as % of forskolin response in absence of tetracycline. **A**: HEK 293_{ind}-ICER I cells (**B**) HEK 293_{ind}-ICER IIγ cells, and (**C**) HEK 293_{ind}-Control cells were transfected with pCRELuc and siRNA-ICER I (specifically targeting ICER I), siRNA-ICER IIγ (specifically targeting ICER IIγ), siRNA-All-ICER (targeting all ICER splice variants) or siRNA-CAT, pretreated with tetracycline (1 μg/ml) for 24 h, and treated with forskolin (10 μM) for 6 h. Results are shown as mean ± SD (n = 6), and the graph is representative of two separated experiments.

the same gene are often dissimilar as a result of particular domains being present in the protein or not. Splice events can transform membrane-bound proteins into soluble proteins, as is the case for the fibroblast growth factor receptors (FGFR) [Jang, 2002]. Changes in splicing of genes like CD44, the Wilms' tumor gene WT1, variants of BRCA1 and BRCA2, MDM2, FGFR, kallikrein family members, and a large number of other genes, may occur during tumor progression and this has been suggested to play an important role in carcinogenesis [Brinkman, 2004]. For the vast majority of alternative splice events their biological significance is still unclear.In order to understand why several ICER splice variants exist, it is of interest to characterize the role of each splice variant, and thus, each variant has to be studied explicitly. We have used cell systems where the level of overexpression of each of the splice variants ICER I and IIγ is controlled and separated from other cellular events. These cell systems thus enable us to study each of the two splice variants separately, with respect to their roles in transcription regulation and proliferation. Our results show that both splice variants repress CRE-driven transcription in a similar manner and that none of them seem to interfere with HEK 293 cell growth.So far there have been few reports characterizing biological differences between ICER splice variants, and studies that involve more than one splice variant have not detected differences in biological functions [Molina et al., 1993; Barabitskaja and Foulke, 2006]. Inada et al. [1999] shows that ICER I as well as ICER Iγ splice variants, both of which contain the DNA binding domain I (DBD I) (Fig. 1) act as repressors of the insulin promoter. Another study has reported that all four ICER splice variants bind to the inhibin α subunit CRE-element with similar affinities, and demonstrated that ICER splice variants are all capable of significantly downregulating both basal and forskolin-induced activity of the inhibin α subunit promoter, independent on whether they contain the alternative DNA binding domains DBD I or DBD II (Fig. 1) [Burkart et al., 2006]. Our findings indicate that the two ICER splice variants comprising either DBD I (ICER I) or DBD II

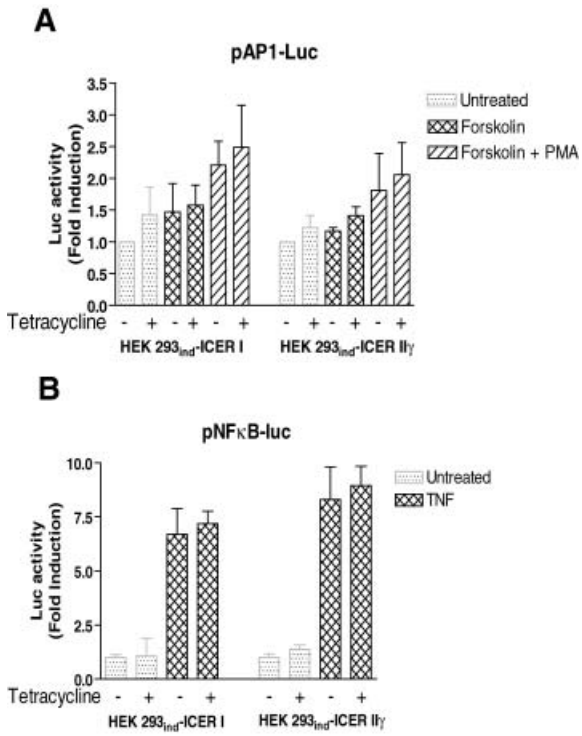

Fig. 7. Effect of ICER overexpression on AP1 and NFκB reportergene. **A**: HEK 293_{ind}-ICER I and HEK 293_{ind}-ICER IIγ cells were transfected with pAP1-Luc, and 16 h before lysis pretreated with tetracycline (1 μg/ml), and treated with Forskolin (10 M) and/or PMA (50 ng/μl) for 6 h. Mean ± SEM of triple measurements are shown. **B**: HEK 293_{ind}-ICER I and HEK 293_{ind}-ICER IIγ were transfected with NFκB-Luc, and 16 h before lysis pretreated with tetracycline (1 μg/ml), and treated with rhTNFα (20 ng/ml) for 6 h. Results are shown as mean ± SD (n = 3), and are representative of three independent experiments.

(ICER IIγ) display similar repressive effects on transcription, which is in accordance with the earlier findings.

The c-fos proto-oncogene is known to play a key role in the control of intracellular events leading to cell proliferation, cell differentiation or apoptosis [Holt et al., 1986; Nishikura and Murray, 1987; Hu et al., 1996; Preston et al., 1996]. We have recently found that endogenous ICER suppresses gastrin-mediated transcriptional activation of c-fos in the rat neuroendocrine cell line AR42J [Steigedal et al., 2006]. In the present study we show that both ICER I and IIγ repress PACAP- and forskolin-induced c-fos gene expression in HEK 293 cells. In accordance with our findings, c-fos is reported to be repressed in ICER IIγ overexpressing AtT20 cells [Razavi et al., 1998]. ICER IIγ has previously been described as a tumor suppressor [Razavi et al., 1998]. Since we found

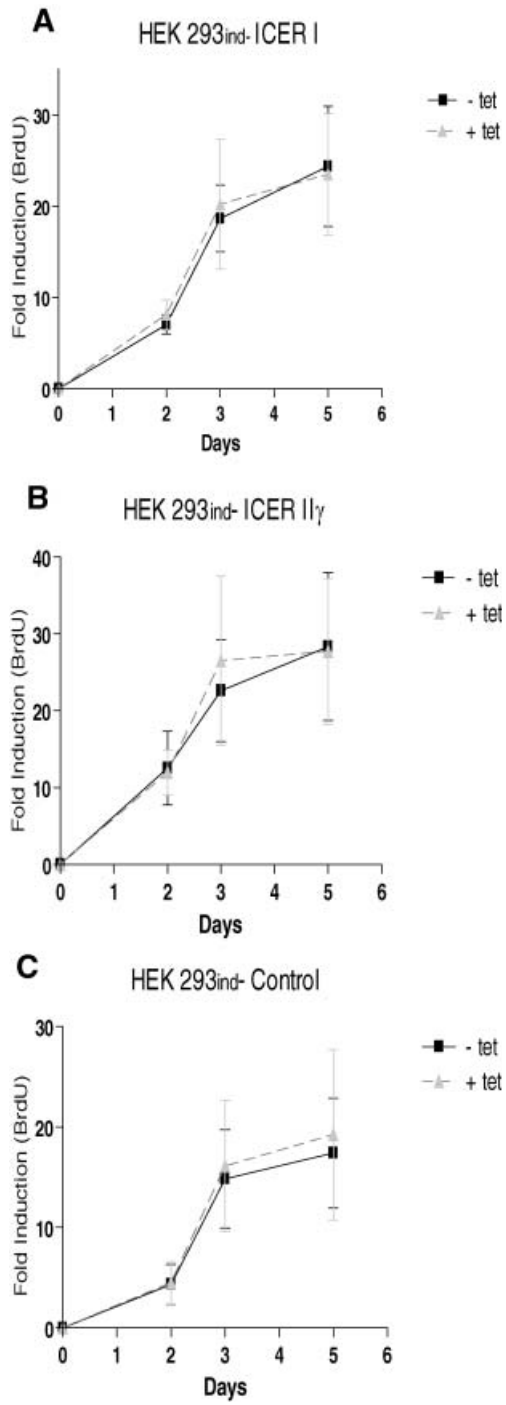

Fig. 8. Effect of acute ICER overexpression on proliferation in HEK 293_{ind} cells. **A**: HEK 293_{ind}-ICER I cells (**B**) HEK 293_{ind}-ICER IIγ cells, and (**C**) HEK 293_{ind}-Control cells were treated or not treated with tetracycline for 5 days, and proliferation measured by BrdU incorporation. Values shown are mean ± SD (n = 5). Similar results have been obtained in two independent experiments.

that the repressive effect of ICER I on c-fos is comparable to that of ICER IIγ, and since we show that both ICER I and IIγ specifically target CRE which is crucial for transcriptional activation of the c-fos promoter [Thommesen et al., 2001; Tenbrock et al., 2006], our study indicates that also ICER I may act as an anti-oncogene inhibiting the expression of growth-related genes like c-fos, and that both ICER I and IIγ might thereby function to inhibit the proliferate effect of mediators like PACAP.

Throughout the neuroendocrine system, CgA is a ubiquitous component of secretory vesicles wherein it may be stored and released together with a number of polypeptide hormones [Taupenot et al., 2003]. The CRE domain in the CgA promoter responds to cAMP [Wu et al., 1995], and plays a crucial role in neuroendocrine cell type-specific expression of this protein in response to, for example, PACAP which activates CgA gene transcription via PKA [Taupenot et al., 1998]. Our results showing that both ICER I and IIγ can modulate cAMP-activated CgA gene expression indicate that ICER may function as a negative feedback inhibitor in CgA synthesis induced by mediators like PACAP.The fact that no biological differences were observed between the splice variants ICER I and IIγ in their repression of CRE-driven transcription may indicate that putative different biological functions of the splice variants are not manifested as differences in protein function, but may rather be related to differences in their expression levels in given biological situations, and in specific cell types. ICER is known to form heterodimers with other CREM, CREB, or ATF1 proteins [Molina et al., 1993; Gellersen et al., 1997]. Thus, the splice variants may differ with respect to which transcription factor they interact with. Alternatively, heterodimers with the same non-ICER partner but with different ICER splice variants may exhibit dissimilar biological activities. Moreover, ICER splice variants may differ in their repressing effect depending on the context in which the CRE promoter element is located. It has been shown that cAMP-induced gene expression mediated by CREB changes when sequence and location of the CRE in the promoter is varied [Mao et al., 1998; Mayr and Montminy, 2001]. This might be the case for ICER as well, that is, the localization and context of the CRE element may be important for the ICER effect. Further studies should address questions whether different ICERs have different repressing activity depending on the promoter sequence, that is, whether it contains a consensus CRE or a CRE-like sequence [Bodor and Habener, 1998; Wang and Murphy, 2000], in order to increase our knowledge of biological functions of ICER splice variants.

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