Cold-Shock-Domain Protein A (CSDA) Contributes Posttranscriptionally to Gonadotropin-Releasing Hormone-Regulated Expression of *Egr1* and Indirectly to *Lhb*¹

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ABSTRACT

Gonadotropin-releasing hormone (GnRH), a hypothalamic neurohormone, regulates transcription of *Lhb* in gonadotrophs indirectly through transient induction and accumulation of EGR1, a zinc finger transcription factor. AlphaT3 and LbetaT2 cell lines model gonadotrophs at two distinct stages of development, prenatal and postnatal expression of *Lhb*. Although GnRH induces EGR1 in both cell lines, the levels of the DNA-binding protein are lower and disappear more quickly in alphaT3 than in LbetaT2 cells. Herein we show that overexpression of *Egr1* in alphaT3 cells rescues activity of a transfected *LHB* promoter-reporter, suggesting that its transcription is dependent on EGR1 crossing a critical concentration threshold. We also show that *Csda*, a gene that encodes an RNA-binding protein and is a member of the cold-shock-domain (CSD) family, is expressed at higher levels in LbetaT2 compared to alphaT3 cells. Transient expression studies indicate that at least one *Csd* element, residing in the 3' untranslated region of *Egr1* mRNA, increases activity of a chimeric pGL3 luciferase reporter vector in LbetaT2 cells. Additional experiments indicate that CSDA physically interacts with *Egr1* mRNA. Furthermore, siRNA-mediated reduction of endogenous *Csda* mRNA attenuates GnRH regulation of a transiently transfected *LHB* reporter vector. Taken together, these studies suggest that CSDA contributes posttranscriptionally to GnRH-regulated expression of *Egr1*, thereby enabling the transcription factor to cross a critical concentration threshold necessary for maximal accumulation of *Lhb* mRNA in response to the neurohormone.

cold-shock-domain protein A (CSDA), *Egr1*, GnRH, gonadotroph

INTRODUCTION

Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are pituitary gonadotropins essential for proper reproductive function in mammals. Both gonadotropins are heterodimeric glycoproteins composed of two noncovalently associated subunits: a common alpha subunit (CGA) and a unique beta subunit, either LHB or FSHB. The genes that encode these subunits are tightly regulated by gonadotropin-releasing hormone (GnRH), a neurohormone, and subject to feedback by gonadal steroids [1].

Upon release from hypothalamic neurons, GnRH binds GnRH receptor (GNRHR), a G-protein-coupled receptor, and signals through a cascade of intracellular pathways that culminates in increased transcription of at least 75 different genes [2, 3]. These genes can be classified as a hierarchy of primary, secondary, and tertiary responsive genes based on the kinetics of their induction and their functional relationship to one another [2–4]. The most notable of the GnRH-responsive genes are *Gnrhr*, *Cga*, *Lhb*, and *Fshb*, tertiary response genes that signify a functional gonadotroph [1].

GnRH regulates transcription of *Lhb* indirectly via regulated transcription of *Egr1*, a primary response gene that is a member of the immediate early gene family that encodes a zinc finger DNA-binding protein [5, 6]. EGR1 protein and its cognate mRNA have short half-lives; consequently, their levels rise and fall in concert with the pulsatile release of GnRH [7–9]. As such, it is the rise and fall of EGR1 that renders *Lhb* transcriptionally responsive to pulses of GnRH. Mice that harbor homozygous null alleles of *Egr1* further underscore its importance. Females fail to express *Lhb* and are infertile, whereas the phenotype of male mice varies from subfertile to completely infertile, depending on the strain and levels of LH [10, 11].

GnRH regulation of *Egr1* transcription requires two specific regions within the promoter that contain serum response elements (SREs) and Ets-binding sites [12]. The SREs bind serum response factor that in turn recruits the Ets protein ELK1 [7, 12, 13]. ELK1 is a downstream target of mitogen-activated protein kinase (MAPK) pathways, specifically MAPK1/3 and MAPK8/9 [7]. Both kinases are activated by GnRH and therefore link the MAPK signaling pathway with SRE-dependent transcription of *Egr1* [7]. The importance of MAPK signaling is further illustrated by pituitary-specific disruption of both *Mapk1/3*; the female mice are infertile and have reduced basal and GnRH-stimulated levels of *Egr1* [14].

Although EGR1 mediates GnRH-stimulated transcription of *Lhb*, two additional transcriptional factors are required for basal levels of *Lhb* expression. Mice that lack either PITX1 or SF1 have reduced levels of *Lhb* mRNA and secreted hormone and are infertile [15]. Levels of SF1 and PITX1 are refractory to pulses of GnRH, suggesting that they act permissively to render transcription of *Lhb* responsive to GnRH-induced changes of EGR1 [4].

Gonadotrophs in the anterior pituitary arise from the PROP1 lineage of precursor cells during the course of embryogenesis [16, 17]. Expression of *Cga* occurs on Embryonic Day 13 (e13) and is coincident with the expression of *Pitx1* and *Sf1* [18–20]. Expression of *Egr1* occurs around e15, with expression of *Lhb* and *Fshb* occurring much later at e17, near the end of gestation [18–20]. The temporal lag, however, between the appearance of three required transcription factors (PITX1, SF1, and EGR1) and expression of *Lhb* remains unclear. In this regard, studies with the gonadotroph-specific cell lines α T3 and L β T2 may

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provide an important clue. The α T3 and L β T2 cell lines model gonadotrophs at prenatal and postnatal stages of development, where *Lhb* expression is silent in the former and active in the latter [21–23]. Although GnRH induces *Egr1* in both cell lines, the expression levels of the mRNA encoding the DNA-binding protein are lower and disappear more quickly in α T3 than in L β T2 cells [6]. This suggests that expression of *Lhb* may require that the concentration of EGR1 reach a critical concentration threshold. Herein we provide data indicating that CSDA, a member of the cold-shock-domain (CSD) family of RNA- and DNA-binding proteins [24–27], may be one of the critical determinants that allows EGR1 to reach an effective concentration required for basal and hormonal regulation of *Lhb* transcription.

MATERIALS AND METHODS

Cell Culture

L β T2 and α T3 cells (provided by Dr. Pamela Mellon, University of California San Diego, La Jolla, CA) were maintained at 37°C with 5% CO₂ in high-glucose Dulbecco modified Eagle medium (DMEM; Invitrogen), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco) (complete medium). GnRH and PBS were purchased from Sigma. For actinomycin D (AMD; Sigma) treatments, the cells were rinsed after 30 min of GnRH treatment and AMD was added at a concentration of 10 μ M in complete medium.

Western Blot

The α T3 and L β T2 cells were grown to 75% confluence, then treated with 100 nM GnRH and harvested at the indicated time points by washing with cold PBS, then lysing with cold lysis buffer (50 mM Tris [pH 7.5], 200 mM NaCl, 0.5% Igepal, 0.1% SDS, 1 mM EDTA, Complete Mini Protease Inhibitor cocktail [Roche]) on ice for 30 min followed by centrifugation at 16000 $\times$ g for 10 min. Protein concentrations were determined using Coomassie Plus protein assay (Pierce). Equal protein amounts (20 μ g) were separated on a 12% polyacrylamide gel and transferred to polyvinylidene fluoride membrane (BioRad) followed by blocking in 5% nonfat milk/Tris-buffered saline with Tween 20 (TBST) for 1 h at room temperature. Primary antibody incubations followed either overnight at 4°C or for 1 h at room temperature using EGR1 antibody (1:5000, Egr1 C19, sc-189; Santa Cruz Biotechnology) or actin antibody (1:2000, Actin A2066; Sigma). Membranes were rinsed three times with TBST, then incubated with HRP-goat anti-rabbit antibody (Pierce) for 1 h at room temperature, followed by rinsing three times in TBST. Blots were then developed by chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate; Pierce). Quantitation was performed with Multi-Gauge V 3.1 after the Western blots were imaged on a Fuji LAS4000 Luminescent Image Analyzer (Fuji Film Corporations).

DNA Constructs and siRNA

The -779/+10-bovine LHB promoter has been previously described [28]. The *Egr1* expression vector (CMVNGF1A) was kindly provided by Dr. Jeffery Milbrandt (Washington University Medical School, St. Louis, MO) [29]. All *Egr1*-untranslated region (UTR) constructs were cloned using RT-PCR (Invitrogen), and the primers are listed in Supplemental Table S1 (available online at www.biolreprod.org). The resulting product was cloned into pCR-TOPO vector (Invitrogen). The sequence was removed from that vector using the restriction enzymes SpeI and XbaI (Invitrogen). These fragments were ligated using Takara ligase reaction mix (Takara) into a pGL3-Control vector (Promega) that was linearized with XbaI. These constructs placed the 3' UTR region downstream of the luciferase gene. Mutations of the 3' UTR *Csd* site were performed using the Stratagene quick change kit using primers listed in Supplemental Table S1. All plasmids were sequenced to ensure they were the correct sequence and in the proper orientation. Smart pools of small interfering RNA for *Csda* and nontargeting interfering RNA were obtained from Dharmacon.

RNA Extraction

RNA was isolated from α T3 and L β T2 cells using the TRIzol method according to the manufacturer's instructions (Invitrogen) and solubilized in RNase-free water (Invitrogen). RNA concentration and purity were obtained by spectrophotometry. Total RNA was resolved on a 1.2% denaturing agarose gel to check for RNA integrity.

Quantitative Real-Time PCR

Real-time PCR was used to quantify differences in expression of *Csda*. RT-PCR was used to generate cDNA from the total RNA isolated from α T3 or L β T2 cells. Total RNA (1 μ g) was reverse transcribed into cDNA by an oligo (dT)12–18 primer using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions, and used as the template for the real-time PCR. Primer Express 2.0 software (Applied Biosystems) was utilized to generate specific primers for genes that were being investigated (Supplemental Table S1). Real-time PCR was performed in a 96-well plate using a 7000 ABI prism sequence detection system (Applied Biosystems). The template cDNA for either α T3 or L β T2 cells were used in triplicate for each real-time PCR. The PCR contained between 5 and 10 ng of template cDNA, 1 X SYBR Green master mix (Applied Biosystems), and 600 nM of specific forward and reverse primers. The threshold cycle (C_T), which indicates the relative abundance of a particular transcript, was calculated for each reaction by the 7000 ABI prism sequence detection system. Primers for the housekeeping gene, cyclophilin B (*Ppib*), were used to normalize C_T values for each sample. The difference in expression between L β T2 and α T3 or the different siRNA knockdown experiments was calculated using the formula $2^{-\Delta\Delta C_T}$, as described in the SYBR Green protocol.

$$\Delta\Delta C_T = [(C_T \text{ of gene of interest} - C_T \text{ of Cyclophilin B})_A - (C_T \text{ of gene of interest} - C_T \text{ of Cyclophilin B})_B]$$

Transfections

Twenty-four hours prior to transfections, 2.5×10^5 – 5.0×10^5 L β T2 or α T3 cells were seeded onto a 24-well culture plate and maintained in complete medium. After 24 h, the cells were rinsed with PBS and a mixture of Lipofectamine (Invitrogen), DNA, and serum-free DMEM was added to each well for a period of 12–16 h. After this period, an equal volume of DMEM with 20% FBS was added, and the cells were treated with 100 nM GnRH or vehicle and incubated for another 24 h; they were subsequently assayed for luciferase activity using the Dual Luciferase Reporter assay kit (Promega).

A reverse-transfection paradigm was performed for the siRNA experiments with the *Egr1*-UTR-luciferase plasmid constructs. A suspension of 2.5×10^5 L β T2 cells per well was transfected with Lipofectamine (2 μ l/well), siRNA, and DNA in DMEM, and cells were allowed to attach to a 24-well plate. Twelve hours posttransfection, DMEM containing 20% FBS was added to each transfection well. Cells were assayed for luciferase activity 72 h posttransfection. For the AMD siRNA experiments, a suspension of 2.5×10^5 L β T2 cells was transfected with Dharmafect 1 (Dharmacon) according to the manufacturer's instructions. At 72 h posttransfection, cells were treated with 100 nM GnRH for 30 min, followed by a rinse with PBS and 10 μ M AMD treatment. Cells were then harvested in TRIzol and RNA was extracted.

Immunoprecipitation RT-PCR

To investigate if CSDA physically interacts with *Egr1* mRNA, an immunoprecipitation RT-PCR was performed similar to a previously published technique [24]. L β T2 cells were treated with 100 nM GnRH for 60 min; the cells were then rinsed two times with cold PBS, and scraped with 600 μ l of cell extract buffer (10 mM Hepes, pH 7.6, 3 mM MgCl₂, 14 mM KCl, 5% glycerol, 0.2% NP-40, 1 mM dithiothreitol, protease inhibitor [Roche Diagnostics], and RNaseOUT [Invitrogen] at 2 units/ μ l). The cell extract was incubated on ice for 30 min and the suspension was triturated every 5 min. The cell extract was then cleared by centrifugation at 8500 $\times$ g at 4°C. An equal volume of the dilution/binding buffer Tris-saline-azide [TSA] (10 mM Tris-HCl pH 7.5, 140 mM NaCl, 0.01% Na₂S₂O₃) was added, along with 1 mg of anti-CSDA (kindly provided by Dr. Robert Braun) or 1 mg of control rabbit IgG (Santa Cruz Biotechnology), and rotated overnight at 4°C with 30 μ l of Protein G Agarose (Upstate Biotechnology/Millipore). Protein G complexes were collected by centrifugation, and beads were rinsed seven times with TSA buffer. After the last centrifugation, 500 μ l of Tri-ZOL was added directly to the beads and RNA was purified as previously described. The RNA was then used for real-time RT-PCR as described above, using *Egr1* primers listed in Supplemental Table S1. PCR reactions were resolved on a 1% agarose gel and quantitated using GeneTools (Syngene) and the experiment was repeated three times.

Statistical Analysis

All statistical analysis was performed using GraphPad Prism (Version 5.0d) software using a one-way ANOVA analysis with a Tukey post hoc test. The variability associated with the siRNA studies reported in Figures 5 and 7

FIG. 1. The half-life of *Egr1* mRNA and the expression pattern of EGR1 protein differ between α T3 and L β T2 cell lines. **A)** The α T3 or L β T2 cells were treated with 100 nM GnRH for 15 min. After treatment they were washed twice with PBS and treated with 10 μ M AMD. RNA was harvested from the cells 15, 30, 45, 75, and 105 min post-AMD treatments and used for qRT-PCR, quantifying the relative expression of *Egr1*. The experiment was repeated three times and the data points were analyzed by nonlinear regression and fitted to a one-phase exponential decay curve. R^2 quantifies the goodness of fit to the one-phase decay curve, and ($r^2 = 0.92$ for L β T2 cells and $r^2 = 0.91$ for α T3) was used to estimate the half-life of the *Egr1* message. The curves were significantly different with a goodness-of-fit P value < 0.0008 . To confirm that the half-lives were significantly different, data points from each experiment were analyzed independently by nonlinear regression and refit to a one-phase decay curve. The three half-lives from either the α T3 or L β T2 curves were then averaged and analyzed by a one-tailed Student t -test and were significantly different ($P < 0.03$). Half-lives calculated by this latter approach were nearly identical to those calculated by the former approach (11 and 23 min versus 11 and 24 min). **B)** The α T3 or L β T2 cells were treated with 100 nM GnRH and cell lysates were harvested over a 2-h period and analyzed by Western blot using an antibody to EGR1 and an antibody to actin that served as a loading control. Shown is a representative blot. Quantitation was performed with Multi Gauge V3.1 software. Values represent the means $\pm$ SEM from three different experiments. * $P < 0.001$, two-way ANOVA.

required larger sample sizes ($n = 9$). These were generated from independent cultures of L β T2 cells sampled on several different days.

RESULTS

GnRH Differentially Regulates Accumulation of *Egr1* mRNA and EGR1 Protein in α T3 and L β T2 Cell Lines

A defining characteristic of α T3 and L β T2 cell lines is that GnRH stimulates accumulation of *Lhb* in the latter but not in the former [21–23]. This difference is consistent with the notion that α T3 cells model an early stage of embryonic cell specification whereas L β T2 cells model a later embryonic or postnatal stage [21–23]. Although α T3 cells fail to express appreciable levels of *Lhb* mRNA after treatment with GnRH, *Egr1* mRNA accumulates more rapidly as compared to L β T2 cells treated with GnRH [6]. This suggests that the half-life of *Egr1* mRNA may differ in the two cell lines. To test this possibility, α T3 and L β T2 cells were treated with 100 nM GnRH, and 15 min later treated with AMD to halt transcription. RNA was then harvested at different time points (0, 15, 30, 45, 60, 90, and 120 min) and analyzed by qRT-PCR. These data indicated that *Egr1* mRNA has a shorter half-life in

α T3 cells (11 min) when compared to L β T2 cells (24 min) (Fig. 1A; $P < 0.03$).

The difference in *Egr1* mRNA stability suggests that levels of EGR1 protein may not reach the same concentration after GnRH treatment in α T3 cells as they do in L β T2 cells. To address this possibility, α T3 and L β T2 cell lines were treated with 100 nM GnRH and cell lysates were harvested over a time course and analyzed by Western blot using an EGR1-specific antibody. Accumulation of EGR1 after GnRH treatment peaked at 1 h and then declined by 2 h in α T3 cells. In contrast, EGR1 continued to rise by 2 h in L β T2 cells by over 8-fold and was significantly higher in concentration when compared to the 2-h time point in α T3 cells ($P < 0.001$) (Fig. 1B). This is consistent with the possibility that although GnRH induces accumulation of EGR1 in α T3 cells the levels are too low to allow subsequent accumulation of *Lhb* mRNA.

Overexpression of EGR1 in α T3 Cells Rescues *Lhb*, Promoter Activity

If EGR1 fails to reach a minimally effective concentration in α T3 cells, then increasing its concentration via overexpression should lead to regulated expression of an *LHB* luciferase

A. α T3 cells **B. L β T2 cells**

FIG. 2. EGR1 is necessary for *LHB* promoter-reporter activity. The α T3 cells (A) and L β T2 cells (B) were cotransfected with the bovine *LHB* promoter-reporter luciferase construct (100 ng) and either an empty CMV expression vector (10 ng) or an expression vector for *Egr1* (10 ng). Luciferase was measured 24 h after treatment with 100 nM GnRH. Data shown are the means $\pm$ SEM from five independent experiments performed in triplicate. Different letters are significant at $P < 0.05$, one-way ANOVA with Tukey post hoc test.

reporter. To test this assumption, α T3 and L β T2 cells were transfected transiently with a bovine *LHB*-luciferase reporter [28] or cotransfected with the *LHB*-luciferase reporter along with an expression vector that encodes EGR1. Cells were then treated for 8 h with either vehicle or 100 nM GnRH.

Consistent with several previous studies [30], the *LHB*-luciferase reporter remains unresponsive to GnRH in α T3 cells but responds robustly in L β T2 cells by approximately 9-fold (Fig. 2, A and B). In contrast, overexpression of EGR1 dramatically up-regulates activity of the *LHB*-luciferase reporter by approximately 14-fold in α T3 cells. Overexpression of *Egr1* also enhances GnRH-stimulated activity of the *LHB*-luciferase reporter in L β T2 cells, increasing the fold change from approximately 9- to 14-fold. These data are consistent with the notion that levels of EGR1 in α T3 cells treated with GnRH fail to reach a minimally effective concentration required for activity of the *LHB* promoter.

FIG. 3. *Csda* is highly expressed in L β T2 cells. RNA from α T3 cells and L β T2 cells was isolated and quantified by qRT-PCR and plotted relative to the expression of the housekeeping gene *Ppib*. Data shown are the means $\pm$ SEM from three independent experiments performed in triplicate. * $P < 0.001$, one-tailed Student *t*-test.

Csda, a Member of the CSD Family of DNA- and RNA-Binding Proteins, Is Highly Expressed in L β T2 Cells

Although several previous studies indicate that the stability of EGR1 protein can be regulated posttranscriptionally and posttranslationally [31–35], factors that influence the stability of *Egr1* mRNA have been understudied. In this regard, the 3' UTR of *Egr1* is over 1000 nucleotides in length, nearly one third the total length of the mRNA (accession number NM_007913.5). Long 3' UTRs are a feature of posttranscriptionally regulated mRNAs [36]. Thus, we considered the possibility that differences in RNA-binding proteins in α T3 versus L β T2 cells might explain the difference in *Egr1* mRNA half-life.

Preliminary studies with Affymetrix 430A microarrays and GeneSpring 7.3 software identified a transcript that encodes CSDA, a member of the CSD family of DNA- and RNA-binding proteins [25, 26], that was enriched significantly in L β T2 versus α T3 cells (data not shown). To confirm this predicted difference, we performed qRT-PCR using primers designed to *Csda* and *Ppib* (cyclophilin B); the cyclophilin B primers act as an internal control between the cell lines [37]. These data indicated that L β T2 cells harbor approximately 100-fold more *Csda* mRNA than α T3 cells (Fig. 3, $P < 0.05$), suggesting that this RNA-binding protein may contribute to the elevated levels of EGR1 in L β T2 versus α T3 cells.

The 3' UTR of *Egr1* mRNA Contains Elements with Posttranscriptional Activity

As CSDA was previously implicated in the stabilization of protamine mRNA in the testis [26, 27] and *Vegf* mRNA in vascular endothelial cells [24], we hypothesized that *Csda* may be linked to the difference in accumulation of *Egr1* mRNA and EGR1 protein noted between α T3 and L β T2 cells. If this is true, then *Egr1* mRNA should contain response elements that mediate the action of RNA-binding proteins such as CSDA. As mentioned earlier, the 3' UTR of *Egr1* mRNA is nearly one third the length of the mature mRNA (approximately 1000 nucleotides). More importantly, the 3' UTR contains several regions with striking homology to consensus sequences known to bind RNA-binding proteins. These include several regions with homology to *Csd* elements (Fig. 4A). Taken together,

FIG. 4. The 3' UTR of *Egr1* mRNA contains elements that act to modulate posttranscriptional activity of *Egr1* mRNA. **A**) Structure of *Egr1* mRNA indicating the UTRs (both 5' and 3'). The dark rectangles represent putative *Csd* consensus sites on the 3' UTR. Expression luciferase vectors that were used are also depicted, and are described in detail in *Materials and Methods*. **B**) L β T2 cells were transfected with the different luciferase constructs and luciferase values were measured. Relative fold change is based on the activity of the pGL3 empty vector construct. Values represent the means $\pm$ SEM of at least seven different experiments. Different letters are significant at $P < 0.05$, one-way ANOVA with Tukey post hoc test.

these regions of homology are consistent with the possibility that they may serve as *cis*-acting elements that modulate stability of *Egr1* mRNA independently of GnRH.

As an initial approach, we asked if the entire 3' UTR could act in *cis* to modulate activity of a pGL3-luciferase reporter. This entailed inserting the 3' UTR of *Egr1* immediately 3' to the luciferase coding sequence. Transient expression in L β T2 cells indicated that although the 3' UTR of *Egr1* increased activity of the chimeric pGL3 reporter by approximately 1.5-fold when compared to the parent pGL3 reporter, the difference was not statistically significant (Fig. 4B). Given the large size of the *Egr1* 3' UTR, we considered the possibility that RNA secondary structure constraints may dampen or even mask activity of response elements that mediate RNA stability, especially when the 3' UTR is appended to a heterologous mRNA. Thus, as a next step, we examined a 3' proximal region of the *Egr1* UTR that contains a single consensus site for *Csd*. Activity of this chimeric reporter was almost 3-fold greater than the parent construct ($P < 0.05$; Fig. 4B), suggesting that the *Csd* consensus site was responsible for the change in activity. Importantly, replacement of the *Csd* element with a

scrambled sequence significantly decreased activity of the chimera when compared to the reporter containing the 3' proximal UTR of *Egr1* ($P < 0.05$). This suggests that the proximal *Csd* element has the potential to contribute to the stability of *Egr1*.

Activity of the *Egr1* Proximal *Csd* Consensus Element Requires CSDA

To explore whether there is a functional connection between the *Egr1* proximal *Csd* consensus element and CSDA, we employed the use of transiently transfected siRNAs that either were specific for *Csda* or contained a nonspecific nucleotide sequence using a 72 h reverse transfection paradigm. Initial experiments indicated significant reduction of endogenous *Csda* mRNA 72 h posttransfection and even in cells that had been exposed to 100 nM GnRH for the last 30 min of the posttransfection period ($P < 0.05$; Fig. 5A). In subsequent experiments with the reverse transfection paradigm, the construct with the intact 3' proximal region of *Egr1* increased activity robustly in the presence of the scrambled control

FIG. 5. The 3' UTR proximal *Csd* consensus element and CSDA protein are functionally active in L β T2 cells. **A**) L β T2 cells were reverse transfected with either control RNAi or RNAi specific for *Csd*. Levels of *Csd* were quantified by qRT-PCR and plotted relative to levels of *Ppib*. In these experiments the cells were treated with 100 nM GnRH for the last 30 min of the 72-h reverse transfection. Actinomycin D (10 μ M) was added 15 min prior to termination to cease transcription. Data shown are the means $\pm$ SEM from three independent experiments performed in triplicate. * $P < 0.001$, one-tailed Student *t*-test. **B**) L β T2 cells were reverse cotransfected with either the pGL3, the *Egr1* proximal 3', or the *Egr1* scrambled *Csd* proximal 3' construct, along with either control RNAi or RNAi directed at *Csd*. Luciferase activity was recorded and relative fold change was based off the pGL3 vector that was cotransfected with control RNAi. Values represent the mean $\pm$ SEM of nine different experiments. Different letters are significant at $P < 0.05$, one-way ANOVA with Tukey post hoc test.

siRNA when compared to the pGL3 control (approximately 20-fold, $P < 0.05$). We attribute the increased activity conferred by the proximal 3' UTR of *Egr1* to the use of the reverse transfection paradigm. In contrast to the control siRNA experiments, cotransfection with a *Csd*-specific siRNA reduced activity of the chimera to a value not significantly different than the pGL3 control. As expected, the chimera with the proximal 3' UTR containing a scrambled *Csd* sequence had reduced activity compared to the chimera with an intact proximal 3' UTR (approximately 7-fold versus 20-fold, $P < 0.05$) and was unaffected by transfection with the *Csd* siRNA. Together, these results establish a functional link between the *Csd* element and *Csd* mRNA.

CSDA Physically Interacts With *Egr1* mRNA

The previous experiments with the 3' UTR of *Egr1* mRNA were limited to transient expression assays in L β T2 cells. To further establish a link between CSDA and endogenous *Egr1* mRNA, we performed an immunoprecipitation RT-PCR. Here, L β T2 cells were treated with either vehicle or 100 nM GnRH for 60 min. The cells were lysed in the presence of RNase inhibitors and subject to immunoprecipitation using antibodies specific either for CSDA or normal IgG. RNA isolated from the immunoprecipitates was then analyzed by endpoint RT-PCR.

Input samples contained approximately 10% of the starting material. GnRH clearly induced expression of *Egr1* mRNA in the input samples (Fig. 6A) by approximately 10-fold when compared to the untreated input (Fig. 6B, $P < 0.05$, one-tailed Student *t*-test). The IgG lanes provide a reference point for

nonspecific interactions with the control antibody. Although GnRH increased the amount of EGR1 that associated with the IgG control, the increase was not significantly different ($P > 0.05$, one-way ANOVA with Tukey post hoc test). Similarly, immunoprecipitation of untreated extracts with the CSDA antibody yielded a signal that was statistically indistinguishable from the IgG control samples. In contrast, the amount of EGR1 that associated with the CSDA antibody in extracts from GnRH-treated cells was approximately 8-fold higher than detected in untreated extracts ($P < 0.05$), indicative of a specific interaction between CSDA and *Egr1* mRNA. In this regard, the large increase in *Egr1* pulled down after GnRH treatment supports the conclusion that transcript rather than DNA was immunoprecipitated with the CSDA antibody, because *Egr1* mRNA was greatly induced by GnRH treatment (see input lanes) whereas the amount of DNA present should have been unchanged. Together, these data are consistent with the notion of a physical association between CSDA and *Egr1* mRNA that presumably involves one or more of the *Csd* consensus sequences.

A Functional Correlation Exists Between *Csd* and GnRH-Induced Expression of *Lhb*

The *Lhb* promoter is a major downstream target of EGR1. Thus, changes in CSDA that impact EGR1 should also impact activity of the *Lhb* promoter. We examined this possible relationship in L β T2 cells with transient expression assays that included a luciferase reporter linked to the bovine *LHB* promoter along with control or *Csd*-specific siRNAs.

FIG. 6. CSDA physically interacts with *Egr1* mRNA. **A**) L β T2 cells were treated with 100 nM GnRH for 1 h. After treatment, cell extracts were prepared and immunoprecipitated with either normal IgG or an antibody to CSDA. The immunoprecipitate and 10% of the input was then subjected to RT-PCR with primers for *Egr1*. **B**) The RT-PCR was quantitated using GeneTools (SynGene) and values represent $\pm$ SEM of three different experiments. Input values were analyzed by a one-tailed Student *t*-test with $*P < 0.05$. Extracts from the immunoprecipitations were subject to analysis by one-way ANOVA and Tukey post hoc test. Different letters are significant at $P < 0.05$.

As noted earlier and reported in other studies [38, 39], activity of the *LHB*-luciferase reporter increases dramatically after treatment with GnRH, even in cells cotransfected with the control siRNA [39] (compare Figs. 2 and 7). In contrast, cotransfection with *Csda*-specific siRNA significantly attenuated the response of the *LHB*-luciferase reporter to GnRH ($P < 0.05$; Fig. 7). These results suggest a functional link between GnRH, CSDA, and activity of the *LHB* promoter.

When taken together, the collective data suggest a strong functional correlation between GnRH, EGR1, CSDA, and expression of *Lhb*. Since the levels of CSDA are unaffected by GnRH (data not shown), this RNA-binding protein appears to act permissively and posttranscriptionally in allowing GnRH to fully induce activity of the *Lhb* promoter.

DISCUSSION

GnRH-regulated transcription of *Lhb* involves a complex array of at least four major transcription factors: the immediate early gene product EGR1, the orphan nuclear receptor SF1, the

homeobox-binding protein PITX1, and a member of the canonical WNT signaling pathway β -catenin [1, 6, 38–42]. Of these, transcription of *Egr1* is regulated by GnRH, whereas transcription of the others occurs independently of GnRH. Yet targeted reduction of any of the four transcription factors or their respective binding sites attenuates GnRH-regulated transcription of *Lhb* [10, 11, 28, 38, 43]. This is consistent with a model where SF1, PITX1, and β -catenin act permissively to allow EGR1 to confer GnRH responsiveness to *Lhb* [39]. The work reported herein now expands this model to the CSD protein CSDA that acts posttranscriptionally and independently of GnRH to increase the stability of *Egr1* mRNA, further augmenting GnRH-regulated transcription of *Lhb*. As such, CSDA joins SF1, PITX1, and β -catenin as permissive factors that enable and maximize GnRH-regulated expression of *Lhb* through transcriptional and posttranscriptional mechanisms.

Proteins that form the CSD family have been reported as the most evolutionary conserved family of nucleic acid-binding proteins found in all three major domains of life [44]. Features include a highly conserved 70-amino-acid CSD that in vertebrates exhibits more than 90% identity between any two family members [44]. They also contain a highly divergent amino terminus and a carboxyl terminal region consisting of characteristic four basic/aromatic islands of amino acids [44]. In mice, this family has three members: YBX1 (YB-1), YBX2 (MS2), and CSDA (MSY4) [27, 44, 45]. Although each of these three proteins is encoded by a different gene, they share a range of properties associated with CSD proteins that includes the ability to bind single-stranded and double-stranded DNA, and RNA [27, 46, 47]. Activities associated with the CSD family in mice include transcriptional regulation, mRNA splicing, and serving as a structural component of RNA-binding protein complexes [24, 25, 44, 45, 48]. Mouse CSD family members also bind selectively to a UCCAUCA consensus element [27]. Additionally, CSDA is highly expressed in mouse testis, where it binds specifically to protamine mRNA [26, 27] and has also been shown to mediate the stability of *Vegf* mRNA in vascular endothelial cells [24]. The studies reported herein are the first to define a role for CSDA outside of the testes, male and female germ cells, and vascular endothelium, namely the gonadotroph.

As mentioned earlier, SF1, PITX1, and EGR1 appear embryonically well before expression of *Lhb* occurs, which is near the end of gestation [11, 43, 49, 50]. The same temporal dissociation holds for β -catenin [51] as well. Previous studies have indicated that GnRH neurons project their axons to the median eminence by e16 [52, 53]. A more recent study has found that receptors for GnRH appear on gonadotrophs at e16.75 [54], suggesting that the fetal pituitary becomes responsive to GnRH as evidenced by detectable expression of *Lhb* in gonadotrophs. Since expression of *Lhb* requires GnRH, it is tempting to suggest that it is simply the arrival of GnRH that explains the temporal delay between the early appearance of the required transcription factors and the expression of *Lhb* much later. Yet, our studies with the α T3/L β T2 cell line model suggest that levels of EGR1 need to cross a critical concentration threshold before the transcription factor can effectively confer GnRH responsiveness to *Lhb*. Evidence includes the difference between amounts of EGR1 in the two cell lines (Fig. 1B), the differences in the half-lives of *Egr1* mRNA and EGR1 protein (Fig. 1A and B), the inability of GnRH to regulate a transfected *LHB*-luciferase reporter in α T3 cells (Fig. 2A), and the ability of overexpressed EGR1 to rescue the otherwise inactive *LHB*-luciferase reporter in α T3 cells (Fig. 2A). Thus, collectively these data suggest that

CSDA may play an important role by allowing EGR1 to reach a basal concentration that can be increased to an effective level by GnRH.

The proposed permissive role for CSDA is reminiscent of another transcription factor, *Nupr1* (also referred to as p8), with a concentration that is low in α T3 cells and high in L β T2 cells [55]. Knockdown of *Nupr1* mRNA in L β T2 cells abrogates GnRH-regulated expression of *Lhb*. Moreover, expression of *Nupr1* during cell specification of gonadotrophs occurs 1 day before expression of *Lhb*. Indeed, expression of *Lhb* near the end of fetal development is delayed by 1 day in mice with homozygous null alleles for *Nupr1*, suggesting its importance in defining the temporal pattern of *Lhb* expression in developing gonadotrophs [56]. Although yet to be studied, it is tempting to speculate that NUPR1 may contribute to GnRH regulation of *Egr1* transcription, providing yet another mechanism to ensure that EGR1 reaches maximally effective levels. Regardless of whether NUPR1 regulates transcription of *Egr1*, it can be added to the collection of transcriptional proteins like SF1, PITX1, and β -catenin that form a cadre of necessary and permissive factors that support GnRH-regulated transcription of *Lhb*.

In mature animals and humans, gonadotrophs in vivo are subjected to pulses of GnRH, and these pulses occur at different frequencies that serve to regulate different genes [57–61]. For example, *Fshb* is preferentially transcribed at slower GnRH pulse frequencies, whereas *Cga* and *Lhb* are transcribed at faster GnRH pulse frequencies [61]. These different pulse frequencies provide one avenue for controlling signaling pathways and transcriptional events in the gonadotroph. Thus, it is plausible that CSDA plays an important permissive role, maintaining sufficient amounts of *Egr1* transcript during the time period between pulses to ensure GnRH increases EGR1 to an effective concentration required for increased transcription of *Lhb*. Based on the studies presented, the most likely mechanism involves CSDA-mediated stabilization of *Egr1* mRNA. The functional importance of this event is underscored by the RNAi experiments indicating that reduction of *Csda* mRNA in L β T2 cells is accompanied by attenuated GnRH induction of an *LHB*-luciferase reporter (Fig. 7).

An important question is whether the approximate 2-fold effect of CSDA-mediated stability of *Egr1* mRNA is sufficient to have a significant impact on GnRH-regulated expression of *Lhb*. Here it is important to underscore that the interaction between SF1, PITX1, and EGR1 is synergistic [6]. The biological significance of synergistic interactions is that a small change in concentration in one of the interacting components can have a multiplicative effect on the overall activity of the interacting partners. Consequently, it makes intuitive sense that a modest change in stability of *Egr1* mRNA that leads to a corresponding change in EGR1 protein may have a greater than additive impact on its subsequent interaction with SF1 and PITX1.

The physiological significance of the mouse CSD family of proteins has been approached through generation of mice with homologous null alleles. Since the testes and male and female germ cells are major sites of expression, it is not surprising that infertility is common but not necessarily a universal phenotype. For example, the *Ybx2* male and female knockout mouse are infertile [62, 63], whereas the *Ybx1* knockout mouse displays embryonic lethality [45]. Mice with null alleles for *Csda* develop normally, with females appearing fertile, but with the caveat that only three females were examined [44]. In contrast, three of six males with null alleles for CSDA were infertile because of increased spermatocyte apoptosis and seminiferous tubule degeneration [63]. In transgenic mice that have CSDA

FIG. 7. There is a functional correlation between reduction of *Csda* and a reduction in *LHB* activity. L β T2 cells were cotransfected with the bovine *LHB* promoter-reporter and either control siRNA or siRNA directed at *Csda*. After transfection, the cells were treated with 100 nM GnRH for 24 h and luciferase activity was recorded, which is represented as fold change. Values represent means $\pm$ SEM of nine different experiments. Different letters are significant at $P < 0.05$, one-way ANOVA with Tukey post hoc test.

overexpressed in the testis, a loss of fertility occurs, which is postulated to be due to excessive translational repression of protamine, preventing spermatid differentiation to fully occur [64]. Unfortunately, because CSDA is expressed in the germ cells of testes and ovary and in many embryonic tissues during the course of development, it is not possible to link the phenotypes observed in these studies to effects that may have occurred through altered gonadotropin production in gonadotrophs. Making this determination will require gonadotroph-specific targeted ablation of *Csda*.

Although the study with mice lacking CSDA points to a limited physiological role in male testis, it is important to consider the possibility that other CSD family members may compensate for the lack of CSDA. For example, both CSDA and YBX2 have been demonstrated to bind the same specific *Csd* consensus sequence (5'-UCCAUCA-3') associated with the mediation of mRNA stability [27], and it has been hypothesized that this binding is important for repressing translation of genes, specifically protamine [26, 27]. With regard to the current study, even though siRNA-mediated reduction of CSDA approached 90% (Fig. 5A), we have observed only a marginal impact on endogenous *Lhb* mRNA after GnRH treatment (data not shown). This difference could reflect partial compensation provided by other RNA-binding proteins that are expressed in L β T2 cells in comparison to α T3 cells. In addition, the 3' UTR of *Egr1* mRNA harbors three *Csd* consensus sequences (Fig. 4A). One of these, located in the 3' proximal region, contributes to the increased activity of a chimeric pGL3 reporter as assessed by loss of activity upon deletion (Fig. 4B). Thus, more studies will be required to determine whether other RNA-binding proteins that are expressed in L β T2 cells can act through the 3' proximal *Csd* element and whether reduction of these proteins and CSDA attenuates GnRH regulation of *Lhb* mRNA.

Although we focused on only one specific *Csd* element, we predict that the array of elements with homology to *Csd*, AU-rich, or polypyrimidine consensus sequences in the 3' UTR of

Egr1 mRNA should provide a partially redundant combinatorial code that determines the stability of the mRNA. Defining this combinatorial code will require assay of individual and discrete combinations of elements to determine their level of autonomous as well as context-dependent activity.

Although we have emphasized that the striking difference in *Csda* in α T3 versus L β T2 cells may allow EGR1 to cross a critical concentration threshold required for activity of the *LHB* promoter, it is important to recognize that α T3 cells are missing other factors that contribute to the threshold level of EGR1. In this regard, although overexpression of EGR1 rescues activity of the *LHB* promoter in α T3 cells, overexpression of *Csda* fails to do the same (data not shown). Thus, we predict that CSDA is required but not sufficient for allowing EGR1 to cross a concentration threshold required for activity of the *LHB* promoter.

In summary, this is the first report indicating that CSDA interacts functionally with EGR1 and is one of the factors that renders the *LHB* promoter fully responsive to GnRH. Together, the data presented in this study and in our previous studies [28, 38, 39, 43] suggest that maximal GnRH regulation of *Lhb* expression requires factors that are required but function independently of GnRH. These include DNA-binding proteins like SF1 and PITX1, coactivators like β -catenin, and at least one RNA-binding protein, namely CSDA. Together these proteins act permissively to allow GnRH-dependent increases in EGR1 to up-regulate activity of the *Lhb* promoter. This complex set of protein partners provides the exquisite control necessary to ensure that levels of LH, the ultimate end product, remain in a physiological rather than pathophysiological range.

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