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Estrogen-dependent Transcription of the *NEL-like 2* (*NELL2*) Gene and Its Role in Protection from Cell Death*[S]

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NELL2 (neural tissue-specific epidermal growth factor-like repeat domain-containing protein) is a secreted glycoprotein that is predominantly expressed in neural tissues. We reported previously that *NELL2* mRNA abundance in brain is increased by estrogen (E2) treatment and that NELL2 is involved in the E2-dependent organization of a sexually dimorphic nucleus in the preoptic area. In this study we cloned the mouse *NELL2* promoter and found it to contain two half-E2 response elements. Electrophoretic mobility shift assays and promoter assays showed that E2 and its receptors (ERα and ERβ) stimulated *NELL2* transcription by binding to the two half-E2 response elements. Hippocampal neuroprogenitor HiB5 cells expressing recombinant NELL2 showed increased cell survival under cell death-inducing conditions. Blockade of endogenous synthesis of NELL2 in HiB5 cells abolished the cell survival effect of E2 and resulted in a decrease in phosphorylation of extracellular signal-regulated kinase 1 and 2 (ERK1/2). These data suggest that the *NELL2* gene is trans-activated by E2 and contributes to mediating the survival promoting effects of E2 via intracellular signaling pathway of ERK.

A gene encoding neural epidermal growth factor (EGF)[4]-like protein (NEL) was first identified in the chick embryo (1). A subsequent study reported two mammalian homologs of the chick *NEL* gene, *NEL-like 1* (*NELL1*) and *NEL-like 2* (*NELL2*), isolated from a human fetal brain cDNA library (2). NELL2 has a greater sequence similarity to chicken NEL than NELL1 and is strongly expressed in mammalian brain in an apparent neuron-specific manner (3–5). NELL2 is a secreted, *N*-glycosylated protein (3). A large fraction of NELL2 is secreted into culture medium from transfected COS7 and HiB5 cells (3, 6) and from *in ovo*-transfected chicken spinal cord cells (7). The released NELL2 promotes proliferation and differentiation of neural cells (7) and increases survival of *in vitro* cultured primary cortical and hippocampal neurons (8).

NELL2 is also involved in promoting the neuronal survival required for the formation of a sexually dimorphic nucleus of the preoptic area (SDN-POA) in male rats (6). The volume of the SDN-POA in male rats is much larger than females (for review, see Ref. 9). This has been known to result from the actions of estrogen (E2) on cells of the male SDN-POA (9). E2 exerts multiple effects on developmental processes taking place in the mammalian central nervous system, such as neurogenesis, survival, and differentiation of different neuronal populations (for review, see Ref. 10). A prominent function of E2 in the nervous system is to protect neurons from cell death (for review, see Ref. 11). E2 produced locally by aromatization of circulating testosterone promotes survival of the SDN-POA in neonatal male rats, whereas this effect is not observed in females because fetoneonatal E2 binding protein blocks E2 action in the female brain (12–14). Because blockade of NELL2 synthesis in the neonatal male rat brain resulted in a decreased size of SDN-POA (6), it has been suggested that NELL2 is involved in the E2-induced protection of SDN-POA in male rats. This notion was inferentially supported by the results of a differential display analysis of RNA samples from the hypothalamus of E2-injected female rats indicating that *NELL2* is an E2-responsive gene (15).

We now report that (*a*) E2-its receptor (ER) complexes bind and activate the *NELL2* promoter, and (*b*) E2-induced cell survival is blocked by silencing the *NELL2* gene via RNA interference (RNAi) and by overexpression of a mutant NELL2 lacking

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[4] The abbreviations used are: EGF, epidermal growth factor; NEL, EGF-like protein; Aβ, amyloid β-(25–35) peptide; AMPA, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate; ChIP, chromatin immunoprecipitation; dbESTs, expressed sequence tags database; E2, estrogen; E2-BSA, bovine serum albumin-conjugated E2; EMSA, electrophoretic mobility shift assay; ER, estrogen receptor; ERE, estrogen-response element; ERK, extracellular signal-regulated kinase; NELL2, neural epidermal growth factor-like 2; NELL2-P, NELL2 promoter; RT, reverse transcription; SDN-POA, sexually dimorphic nucleus of the preoptic area; siRNA, small interference RNA; SP1RE, SP1 response element; TSS, transcription start site; TUNEL, terminal deoxynucleotidyltransferase dUTP nick-end labeling; VMH, ventromedial nucleus; MAPK, mitogen-activated protein kinase; mNELL2, mouse NELL2; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; CS, complementary sequence.

EGF-like domains. These results suggest that NELL2 is a mediator of E2-induced neuronal survival in the central nervous system.

EXPERIMENTAL PROCEDURES

Cloning of the Mouse NELL2 (mNELL2) Gene 5′-Flanking Region—A mouse genomic DNA library (Invitrogen) was constructed in *Escherichia coli* LE392 after ligating partial Sau3AI DNA digests of mouse genomic DNA (strain 129/Sv) at the BamHI site of a phage vector lambda EMBL3 (Promega, Madison, WI). For screening clones containing the m*NELL2* gene, $\sim 1 \times 10^7$ phage plaques were transferred to Hybond N^+ membrane (Amersham Biosciences) and hybridized to a [α-^{32}P]dCTP (50 μCi/μl, Amersham Biosciences)-labeled m*NELL2* cDNA probe; the cDNA fragment (a 680-bp fragment spanning nucleotides 21–700 in m*NELL2* mRNA) was cloned by PCR amplification based on the deposited DNA sequence (NCBI GenBank™ accession number NM_016743). Hybridization was performed overnight at 50 °C in a hybridization buffer containing 45% formamide, 5× SSC (1 × SSC: 8765 g of sodium chloride, 4.41 g of sodium citrate/liter), 1× Denhardt solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll, and 0.02% bovine serum albumin), 1% SDS, 5% dextran sulfate, and 10 mg salmon sperm DNA (Sigma). After hybridization, the membranes were washed with 1× SSC and 0.1× SDS and exposed to x-ray films (AGFA CP-G plus). This process yielded positive clones.

The *NELL2* promoter region containing about 1.2 kb upstream from the translation initiation site was amplified by PCR using the above clone and a primer set based on the m*NELL2* genomic DNA sequence (NCBI GenBank™ accession number NM_016743): sense primer, 5′-CCA CCT ATC TAG GTA CCC CAT CC-3′; antisense primer, 5′-ATG GCT CGA CCG GGG AAC AT-3′. The amplified product was cloned into the pGL3 basic luciferase reporter vector (Promega). The sequence of the cloned DNA was verified by comparing it to that of the mouse genome data base.

Primer Extension—To identify the transcription start site (TSS) of the m*NELL2* gene, primer extension of mRNA samples from the mouse brain was employed. A 36-oligonucleotide long primer (5′-TGA GAA CGT TCT GCG TGA TCC TCG GGC TTG GAG CGG-3′) complementary to nucleotides downstream from the translation initiation site within exon 2 of the m*NELL2* gene was end-labeled with [γ-^{32}P]ATP (Amersham Biosciences) and 1 μl of T4 polynucleotide kinase (Invitrogen) at 37 °C for 60–90 min. Poly(A)$^+$ mRNA from mouse brain was hybridized with the radiolabeled primer in 30 μl of hybridization buffer (40 mM bis-Tris, pH 6.4, 0.4 M NaCl, 1 mM EDTA, and 50% formamide) at 65 °C for 90 min. After the mixture was slowly cooled down to room temperature, the hybridized mRNA was reverse-transcribed at 42 °C for 1 h using Superscript II Moloney murine leukemia virus reverse transcriptase (Promega). The extended cDNA products were extracted and separated on a 6% denaturing polyacrylamide gel, and the putative site of TSSs was determined by comparison with adjacent sequencing reaction that used the same oligonucleotide primer and a genomic clone containing this region as a template.

Reverse Transcription-PCR Confirmation of the TSS—Total RNA was isolated from mouse (C57/BL6) brain using TRI reagent (Sigma) and reverse-transcribed using oligo(dT) as primer. PCR amplification was performed using 30 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 45 s preceded by 95 °C for 5 min and followed by 72 °C for 7 min. The PCR primers (indicated in Fig. 1*B*) used in this experiment were: antisense primer (5′-GGT CAC CAA GAT TGT GAA CTC GTG-3′) designed from the DNA sequence located on exon 4; sense primer 1 (5′-GGT AGA ATC CCC TTG CCT TGC CCT TTG AC-3′) located upstream of exon 1; sense primer 2 (5′-CCT TCG GTC TCC CGC GCC TGT C-3′) spanning a region located upstream of exon 1 and the 5′ end of exon 1; sense primer 3 (5′-CGC CCG ATT TCG AGG GGG-3′) corresponding to the 3′ end of exon 1; sense primer 4 (5′-CCA TTG TCT CCG CCT TTC CAA C-3′) located on intron 1; sense primer 5 (5′-GAT TTC GAG GGG GGA GGG AGA CGA T-3′) spanning the 3′ end of exon 1 and 5′ end of exon 2; sense primer 6 (5′-GAC GAT GGA CTG AGA CGA TGC AC-3′) on exon 2; sense primer 7 (5′-CGT TCT GCG TGA TCC TCG GGC TTG-3′) located at the 3′ end of exon 2. The amplified products were separated by agarose gel electrophoresis, isolated, and cloned into pGEM-T easy vectors (Promega), and their DNA sequence was confirmed by sequencing.

Mutant NELL2 Promoter Construct—Mutant *NELL2* promoters carrying deletion of the TATA-like sequence, intron 1, half-EREs, and SP1 response elements (SP1REs) were generated using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) and specific oligonucleotide primer sets; the intended mutations were confirmed by sequence analysis. The oligodeoxynucleotide primers used were (*a*) a primer set for deletion of intron 1 (sense primer, 5′-CCC CCT CGA AAC AGC TGT CGG GC-3′; antisense primer, 5′-GAG GGA GAC GAT GGC AGC TGA CTG AGA C-3′), (*b*) a primer set for deleting TATA-like sequence (5′-CCC AGC ACC TCT TGG TTT TTA TCT TAA AGA GGG-3′ and its complementary sequence (CS)), (*c*) a set for deleting half-ERE (5′-CCC CGC AGG TCC CCG AGA GCC CGG CTG CGG-3′ and its CS) located at the *NELL2* promoter (−223), (*d*) a primer set for deletion of the other half-ERE sequence (5′-CCT TGC CTT GCC CTT CTT GCT GCT GTG TAG-3′ and its CS) located at −1047 of the promoter, (*e*) the SP1RE sequence (5′-GGC TGC GGG CGT GCG GGA GCG ATG CGC GCA GAG-3′ and its CS) at −209, (*f*) the SP1RE sequence (5′-GGC TCG CGC CTC CCG GAG CGG TCT C-3′ and its CS) at −430, (*g*) SP1RE sequence (5′-GAA AAA GAG GCC GCC GCG CCC CGG CTC-3′ and the CS) at −453.

Cell Culture and Promoter Assays—The conditionally immortalized rat hippocampal cell line HiB5 was used for this study. Because HiB5 cells were conditionally immortalized by the stable expression of a temperature-sensitive mutant form of the SV40 virus large T antigen (16), they proliferate at the permissive temperature of 33 °C and stop growing at 37 °C. They were cultured in phenol-red free medium (Sigma) supplemented with 10% charcoal-stripped fetal bovine serum (Invitrogen) and antibiotics at 33 °C, 95% O_2, 5% CO_2.

To determine whether E2 regulates *NELL2* transcription, HiB5 cells were transiently transfected with m*NELL2* promoter

(NELL2-P)-luciferase reporter constructs (NELL2-pGL3) using Lipofectamine/PLUS (Invitrogen) and rat ERα and ERβ expression vectors (50 ng each) (kindly provided by Dr. Keesook Lee, Chonnam National University, Kwangju, South Korea). After 24 h the cells were treated with E2 or E2-conjugated with bovine serum albumin (E2-BSA, Sigma) that cannot penetrate through the membrane for 24 h and lysed with Cell Culture Lysis Reagent (Promega). Luciferase assays were performed using a luciferase reporter assay kit (Promega). Transfection efficiency of each assay was normalized by cotransfecting the plasmid pCMV-β-gal (Clontech, Palo Alto, CA) at 60 ng/ml.

Real-time PCR—To determine the effect of E2 on the endogenous *NELL2* expression, RNA (2 μg), isolated from HiB5 cells treated with E2 (10 nM) with or without ERs, was reverse-transcribed and amplified by a real-time PCR using two sets of primers: NELL2 sense primer, 5′-CGG TAG GTG GTT CCC TTC AT-3′, and antisense primer, 5′-CAG GAC TGC ATA ATG GGA CG-3′; glyceraldehyde-3-phosphate dehydrogenase sense primer, 5′-GTG ATG GCA TGG ACT GTG GT-3′, and antisense primer, 5′-TGG TGA AGG TCG GTG TGA AC-3′. Real-time PCR reactions (20-μl total volume containing 5 pmol of primer, 10 μl of SYBR Green dye (Qiagen, Valencia, CA), and 2 μl of cDNA) were carried out with a DNA Engine Opticon Continuous Fluorescence Detection System (MJ Research Inc., Waltham, MA) for ~40 cycles.

Western Blotting—Protein from HiB5 cells was homogenized in M-PER lysis buffer (Pierce). Extracted protein (15 μg) was separated by SDS-polyacrylamide gel electrophoresis and transferred to a membrane by electrophoretic transfer. The membrane was incubated with rabbit anti-rat NELL2 antibody (17), rabbit anti-extracellular signal-regulated kinase (ERK) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-phospho-p44/42 (p-ERK1/2) antibody (Cell Signaling Technology Inc., Beverly, MA), and rabbit anti-Bax antibody (Santa Cruz). Immunoreactivity was detected with an enhanced chemiluminescence (ECL) kit (Amersham Biosciences).

Electrophoretic Mobility Shift Assays (EMSAs)—HiB5 (5×10^6) cells were transfected with 1 μg each of ERα and ERβ expression vectors and cultured in the presence of 10 nM E2. After 24 h of incubation, nuclear protein extracts were prepared according to the method of Andrews and Faller (18) utilizing protease inhibitors (19). The double-stranded oligodeoxynucleotide probes used for the assays were end-labeled with [γ-^{32}P]ATP and purified over a NICK column (Amersham Biosciences). The binding assay was performed as described (20) with minor modifications using 10 μg of nuclear protein extracts, 20,000 cpm of probe, and 1 μg of poly(dI-dC). The reaction mixtures were separated by electrophoresis on a 6% non-denaturing polyacrylamide gel. The gels were then dried and exposed to film at −85 °C.

The oligodeoxynucleotide probes employed were (*a*) a positive control probe (5′-GGA TCT AGG TCA CTG TGA CCC CGG ATC-3′) containing the palindromic ERE and flanking sequence adopted from the promoter of the vitellogenin gene (21), (*b*) a negative control probe (5′-GGA TCT Agt aca CTG TGA CCC CGG ATC-3′) containing a mutation in one arm of the palindromic sequence of the positive control probe (21), (*c*) a probe containing the half-ERE sequence (5′-GAG AGC CTG ACC CGG CTG C-3′) located at the m*NELL2* promoter (−223), (*d*) a probe containing the other half-ERE sequence (5′-TGC CCT TTG ACC CTT GCT G-3′) located at −1047 of the promoter, (*e*) the SP1RE sequence (5′-CTG CGG CCC GCC GCG TGC G-3′) at −209, (*f*) the SP1RE sequence (5′-CTC GCG CCC CGC CCT CCC G-3′) at −430, and (*g*) the SP1RE sequence (5′-AGG CCG CCC CGC CCG CGC C-3′) at −453.

Chromatin Immunoprecipitation (ChIP) Assay—After lysis of the HiB5 cells transfected with expression vectors for ERs, nuclei were extracted and resuspended with nuclear lysis buffer (50 mM Tris, pH 8.1, 10 mM EDTA, 1% SDS, and protease inhibitors). Chromatin was sheared by sonication and diluted 5-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris, pH 8.1, 167 mM NaCl, and protease inhibitors). The reactions were incubated with 5 μg of antibodies against ERα (Affinity BioReagents, Golden, CO) and ERβ (Santa Cruz) at 4 °C overnight. Immune complexes were collected by reacting with 60 μl of the salmon sperm DNA/protein A-agarose for 1 h at 4 °C and then washed consecutively for 5 min each with buffers (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.1) containing different concentration of salts (150–500 mM) and 0.25 M LiCl. DNA from the protein-DNA cross-links was extracted by incubating the reactions with solution (1% SDS, 0.1 M $NaHCO_3$, 10 μg RNase, and 0.3 M NaCl) at 65 °C for 4 h and was further purified with phenol/chloroform. PCR amplification was performed using 35 cycles of 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s, preceded by 94 °C for 5 min, and followed by 72 °C for 10 min. Primer sets used for the PCR amplification were a primer set for half-ERE at −233 (sense primer, 5′-CGC CTT TCC AAC AGG TTC-3′; antisense primer, 3′-AAC TTG GAG CCG CCT CTG-5′) and a primer set for the other half-ERE at −1047 (sense 5′-ATG GGG TAG AAT CCC CTT GC-3′; antisense, 3′-AGC CCA GAC TGA GAG GCT TT-5′).

In Situ Hybridization and Immunohistochemistry—Localization of *NELL2* mRNA in the female rat hypothalamus was carried out after an *in situ* hybridization procedure (22) reported in detail elsewhere (23). In brief, the brains were transcardially perfused with 4% paraformaldehyde in borate buffer, pH 9.5. The sections (25 μm) were prepared using a freezing sliding microtome, mounted on glass slides, and dried overnight under vacuum. The next day the sections were prehybridized, hybridized with [^{35}S]UTP-labeled *NELL2* cRNA probe, and washed as reported (22, 23). After dehydration in graded alcohols, the slides were dipped in Ilford K5 emulsion and developed after 3 weeks of exposure. Control sections were hybridized with a *NELL2* sense RNA probe. The probes were prepared by SP6 RNA polymerase-directed *in vitro* transcription of a 333-bp rat *NELL2* cDNA template obtained by PCR amplification of a portion of the *NELL2* mRNA-coding region corresponding to nucleotides 548–880 in rat *NELL2* mRNA (23).

A combined immunohistochemistry/*in situ* hybridization procedure was used to determine whether NELL2 is expressed in the ERα-expressing cells in the rat brain. Floating 30-μm sections were incubated for 18–20 h at 4 °C with a mouse monoclonal ERα antibody (clone 1D5 diluted 1:100, LabVision

Neomarkers, Fremont, CA) and incubated with a horse anti-rabbit γ-globulin (1:250, Vector Laboratories, Burlingame, CA) for 1 h at room temperature and then with AB complex (Vector Laboratories) for 1 h. The immunohistochemistry reaction was then developed to a brown color with 3,3′-diaminobenzidine-HCl and 0.005% H_2O_2. After washing, the sections were mounted onto glass slides and dried overnight before hybridization with the radiolabeled *NELL2* cRNA probe. After an overnight hybridization at 55–56 °C, the slides were washed. After dehydration, the slides were dipped in Ilford K5 emulsion and exposed for 3 weeks at 4 °C. At this time the slides were developed, quickly dehydrated, dried, and coverslipped for microscopic examination.

Mutant NELL2 Protein Constructs—NELL2 contains several different functional domains: a signal peptide, a thrombospodin-1-like module, five von Willebrand factor C domains, and six EGF-like domains, three of which are Ca^{2+}-binding EGF-like domains (3). Using overlapping PCR, we prepared two different mutant cDNAs encoding proteins lacking EGF-like domains: NELL2-ΔEGF-Ca^{2+} (lacking the three Ca^{2+}-binding EGF-like domains) and NELL2-ΔEGF (lacking all 6 EGF-like domains). Primer sets carrying a digestion site by NotI were used for generation of mutants: sense primer for NELL2-ΔEGF, 5′-CTC AGA ACA GCG GCC GCG AAG TCA TAA CC-3′; antisense primer for NELL2-ΔEGF, 5′-ATG GAA AGA ACT GCG CGG CCG CAC AG-3′; sense primer for the first round of PCR generation of NELL2-ΔEGF-Ca^{2+}, 5′-AAT GTC CCG GCC GCT TCA CAG TAG GCA TTG-3′; antisense primer for the first round of PCR for NELL2-ΔEGF-Ca^{2+}, 5′-TCG TGT GCG GCC GCA CGG AAC-3′; sense primer for the second round of PCR for NELL2-ΔEGF-Ca^{2+}, 5′-CAA TGT CGC GGC CGC TGT CTC AC-3′; antisense primer for the second round of PCR for NELL2-ΔEGF-Ca^{2+}, 5′-ATG GAA AGA ACT GCG CGG CCG CAC AG-3′. The PCR products were digested with NotI and finally cloned into the expression vector pcDNA 3.1-zeo (Invitrogen), and intended mutations were confirmed by DNA sequencing.

Stable Transfection of NELL2 and NELL2 Mutants—To determine the effect of NELL2 on the cell survival, we used HiB5 cells stably transfected with NELL2 or NELL2 mutant expression vectors. NELL2-transfected cells efficiently release NELL2 into the culture medium (6). As a control, other cells were transfected with the pcDNA 3.1-zeo vector. The cells constitutively expressing the transfected vectors were selected using Zeocin (400 μg/ml) (Invitrogen) and screened for NELL2 expression by RT-PCR.

Viability Assays—HiB5 cells (5000 cells/well) expressing NELL2 or NELL2 mutants were plated in 96-well plates containing 100 μl of phenol-red free Dulbecco's modified Eagle's medium supplemented with 10% charcoal stripped fetal bovine serum. The cells were exposed to 10 nM E2 for 24 h. Thereafter, the medium was changed to Dulbecco's modified Eagle's medium supplemented with 2% charcoal-stripped fetal bovine serum followed by an addition of the same amount of E2. Thirty minutes later, 10 μM amyloid β-(25–35) (Aβ) and 5 mM α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) (both reagents, Sigma) were added. After 20 h of incubation, cell viability was assessed by using a modified methylthiazolyltetrazolium (MTT) assay (24). After incubating 20 h with the appropriate reagents, 10 μl methylthiazolyltetrazolium (5 mg/ml stock) was added and incubated for 4–5 h. At this time, 100 μl solubilization solution (50% dimethylformamide, 20% SDS, pH 4.8) was added to each well. Absorption readings were performed at 570 nm using a spectrophotomer.

Terminal Deoxynucleotidyltransferase dUTP Nick-end Labeling (TUNEL) Assays—For detection of apoptotic cells, TUNEL assays were performed using the Fluorescent In Situ Cell Death kit, TMR red (Roche Diagnostics). After treatment with E2 and neurotoxins as indicated above, the HiB5 cells (5000 cells/well) were fixed in a 4% paraformaldehyde for 10 min at 4 °C and treated with 0.1% Triton X-100 in an ice-cold 0.1% sodium citrate for 5 min. The cells were treated with 50 μl of TUNEL reaction mixtures for 60 min at 37 °C in a humidified atmosphere. Samples were analyzed by confocal fluorescence microscopy.

Statistical Analysis—The results were analyzed with a one-way analysis of variance followed by the Student Neuman-Keuls multiple comparison test for unequal replications. Student's *t* test was used to compare two groups.

RESULTS

TSSs of NELL2 Gene—The TSS of the *NELL2* gene has not been experimentally determined. Although it is clear that the ATG site is located in exon 2 (NCBI GenBank™ accession number NM_016743.1), the existence of an exon 1 has also been reported (GenBank™ accession number NM_016743.2). To determine the location of the TSS, we first performed primer extension studies using mRNA extracted from the mouse brain and an antisense primer complementary to a sequence in exon 2. One strong band and several weak bands were detected (Fig. 1*A*). Sequence analysis determined that the last nucleotide of the strongest and longest product was a G (Fig. 1*A*), which corresponds to the first nucleotide of exon 2 (Fig. 1*D*). A blast search of the mouse dbESTs (Expressed Sequence Tags data base) was performed using the nucleotide sequence of exons 1 and 2 of m*NELL2* as a query to identify potential upstream TSSs. Although two clones correspond exactly to the 5′ end of exon 1 (BU612839, AU080563), 17 clones match with the 5′ end of exon 2, suggesting that the 5′ end of exon 2 is the major TSS of m*NELL2*.

To confirm the TSSs determined by the primer extension and blast search, RT-PCR was performed on RNA isolated from mouse brain by using combinations of sense primers corresponding to regions around 5′ end of exons 1 and 2 and antisense primer at exon 4 (Fig. 1*B*). The resulting products consisted of cDNA fragments generated by sense primers containing exon 1 (primers 2 and 3) and exon 2 (primers 5, 6, and 7) sequences but not by sense primers located upstream of exon 1 (primer 1) and within intron 1 (primer 4) (Fig. 1*C*). DNA sequencing revealed that the 5′ end of exons 1 and 2 exactly coincides with the 5′ end of cDNA products generated by primer 2 (spanning part of sequence upstream from exon 1 and the 5′ end of exon 1), and primer 5, spanning the 3′ end of exon 1 and the 5′ end of exon 2. Thus, m*NELL2* appears to have two alternative TSSs located at the 5′ end of exons 1 and 2. However, the paucity of ESTs containing exon 1 and the relatively

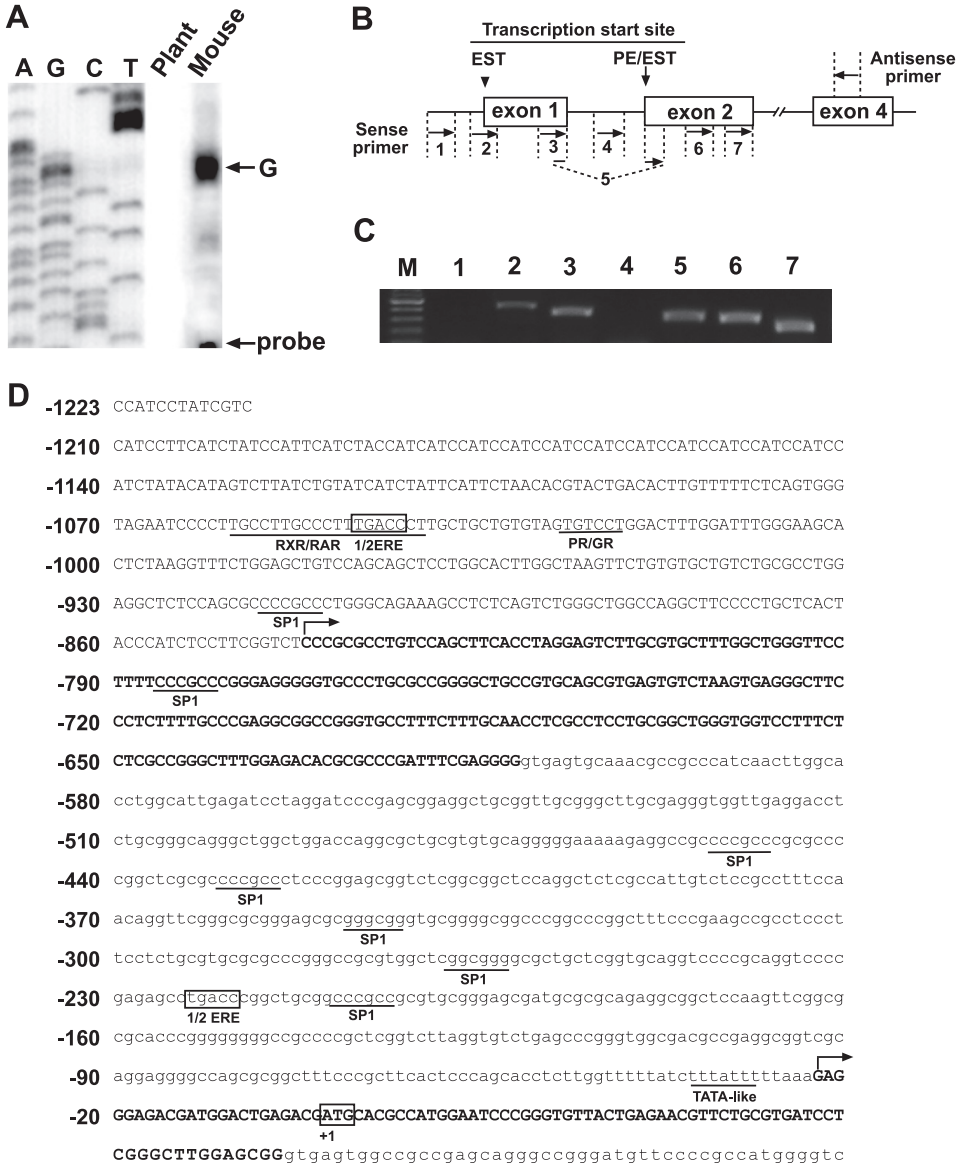

FIGURE 1. **Identification of TSSs and nucleotide sequence of 5′-flanking region in the m*NELL2* gene.** *A*, primer extension analysis of TSSs is shown. Poly(A)$^+$ mRNA was purified from mouse brain and analyzed by primer extension using an antisense primer complementary to a sequence contained in exon 2. *B*, shown is a schematic illustration of the position of primers for RT-PCR analysis of TSSs. A combination of an antisense and different sense primers indicated as numbers was used for PCR amplification of cDNA. *EST*, sequence found by dbEST analysis. *PE/EST*, sequence obtained by primer extension and dbEST analysis. *C*, verification of TSSs by RT-PCR is shown. PCR products were separated by agarose gel electrophoresis. *Numbers on top of gel* represent the sense primer numbers used. *D*, nucleotides are numbered by assigning position +1 to the ATG translational start site. The first and second exons are shown in *bold letters*, and the first intron is indicated with *lowercase letters*. The two TSSs are indicated by *arrows*. Based on sequence analysis (NCBI GenBank™ accession number GU290311), putative binding domains for transcription factors such as SP1, retinoid X receptor (RXR)/retinoic acid receptor (RAR), and progesterone receptor (PR)/glucocorticoid receptor (GR) are *underlined*, and the two half-EREs are *boxed*.

weaker PCR products produced by amplifications using primers 2 and 3 suggest that the TSS at exon 1 is much less frequently used than the one at exon 2.

Organization of the mNELL2 Gene and Binding Motifs for Transcription Factors in the mNELL2 Promoter—Organization of the m*NELL2* gene was determined based on information obtained from genomic DNA sequences. A blast search (www.ncbi.nlm.nih.gov) of the mouse genomic DNA (NCBI GenBank™ accession number NM_016743) was performed using a mouse cDNA complementary to the *NELL2* mRNA coding region as the query. We determined that the m*NELL2* gene comprises 21 exons, with sizes ranging from 75 to 707 bp in length and a total gene size of 307 kb in chromosome 15 (supplemental Data 1 and 2). Exon 1 in the m*NELL2* gene spans 231 bp and contains the 5′-untranslated region. Exon 2 (87 bp long) contains the ATG translation initiation site (Fig. 1*D*) followed by a signal sequence. The exon-intron junctions contain the consensus 5′ splice donor site, GT, and the 3′ splice acceptor, AG, except the intron 1 (supplemental Data 2). Sizes of the introns vary from 98 bp to 54,478 bp in length.

To find motifs for DNA-binding proteins in the 5′-flanking region of the m*NELL2* gene, the cloned sequence (NCBI GenBank™ accession number GU290311) was analyzed with the aid of a search program (available online at TESS (Transcription Element Search System)) and shown in Fig. 1*D*. The two identified TSSs are located at the 5′ end of exons 1 and 2, respectively. A TATA-like sequence is located immediately upstream from the TSS at exon 2. The 5′-flanking sequence of the m*NELL2* gene contains several consensus motifs for transcription factors: retinoic acid receptor (retinoid X receptor/retinoic acid receptor), progesterone receptor, and glucocorticoid receptor. The m*NELL2* promoter has no palindromic ERE. However, it contains 2 widely spaced half-EREs at position −223 and −1047. Both half-EREs have a well conserved 5′-TGACC-3′ sequence (25). In addition, the promoter region has seven well conserved SP1REs (5′-CCCGCC-3′ or 5′-GGGCGG-3′). Interestingly, intron 1 contains several important binding domains for transcription factors such as a half-ERE and SP1REs.

Effect of Deleting a TATA-like Sequence and Intron 1 on Basal Promoter Activity—The *NELL2* promoter sequence contains a TATA-like sequence near the TSS at exon 2. Interestingly, deletion of this sequence increased instead of decreased basal promoter activity (Fig. 2*A*), suggesting that the sequence is not a functional TATA box, but it may serve as a binding site for a transcriptional inhibitor(s). Because intron 1 is located upstream from the TSS of the exon 2, it may be involved in

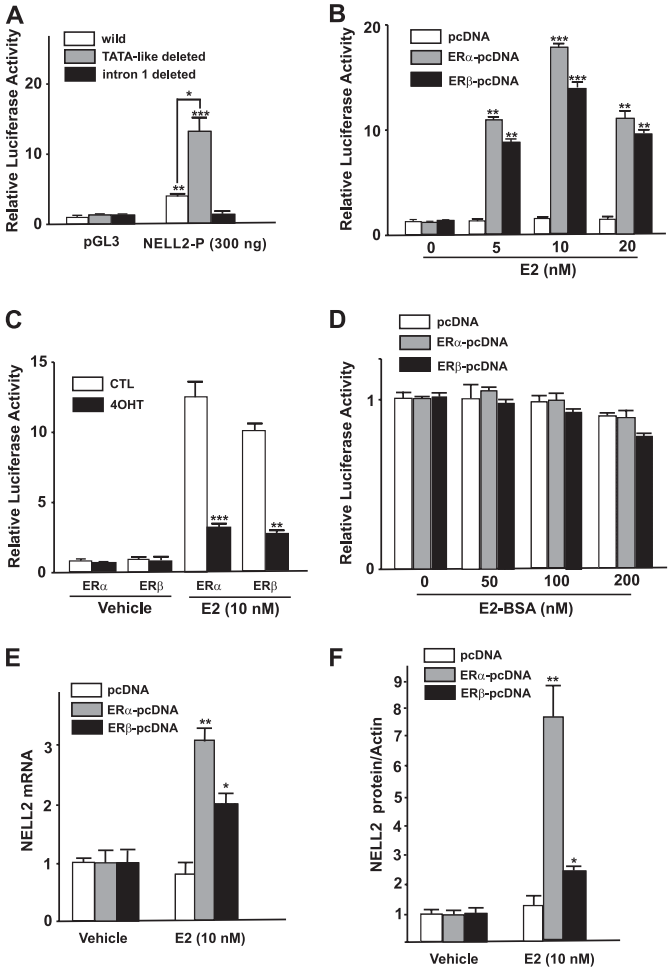

FIGURE 2. **Promoter assays assessing E2 action on *NELL2* transcription.** Luciferase reporter constructs (pGL3) containing the 5′-flanking region of the m*NELL2* gene (NELL2-P) were transfected into HiB5 cells with or without expression vectors carrying the rat ERα and ERβ-coding region and treated with E2 at the indicated concentrations. The cells were harvested for luciferase and β-galactosidase assays 24–30 h after transfection. *A,* shown is the effect of deleting the TATA-like sequence and intron 1, respectively, on the transcriptional activity of the *NELL2* promoter. *B,* transactivation of the *NELL2* promoter by different concentrations of E2 in the presence of expression vectors (50 ng each) encoding ERα (ERα-pcDNA) and ERβ (ERβ-pcDNA) is shown. *C,* shown is the effect of the ER antagonist, 4-hydroxytamoxifen (*4OHT*, 50 nM), on the E2-ER-induced transcriptional activation of the *NELL2* promoter. *CTL*, control. *D*, the absence of transactivation effect of E2-BSA on the NELL2 promoter is shown. *E* and *F*, shown is the effect of E2-ERs on the HiB5 cell endogenous expression of *NELL2* mRNA (*E*), determined by real-time PCR, and protein (*F*), analyzed by Western blotting, indicated as arbitrary units. β-Actin was used as an internal control for Western blotting. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *versus* control (pGL3 for *A*, 0 nM for *B*, CTL for *C*, and vehicle for *E* and *F*). Results are the means ± S.E. of at least six (*A–C*) and four (*D–F*) wells per group.

regulating transcription mediated by this site. Consistent with this notion, deletion of intron 1 abolished the basal transcriptional activity of the *NELL2* gene (Fig. 2*A*), suggesting that intron 1 is required for maintaining the m*NELL2* gene transcriptionally active. The effect of deleting TATA-like sequence and intron 1 was consistent at different concentrations of NELL2-P (data not shown).

Effect of Estrogen and Its Receptor on the NELL2 Transcription—We previously found that E2 increased the *NELL2* mRNA content in the hypothalamus of female rats (15). To determine whether E2 directly regulates transcription of the *NELL2* gene, we performed *NELL2* promoter assays in HiB5 cells. E2 strongly promoted *NELL2* transcription after transfection of the cells with ERα and ERβ expression vectors (Fig. 2*B*). The transcriptional activation effect of E2 was most prominent at the 10 nM concentration; at 20 nM, the effect decreased slightly. The specificity of this E2-ER effect on *NELL2* transcription was assessed using the ER antagonist, 4-hydroxytamoxifen. Treatment with 4-hydroxytamoxifen markedly reduced the effect of E2-ER on *NELL2* gene transcription (Fig. 2*C*), further suggesting that the trans-activational effect of E2 is specifically exerted via binding to ERs. To verify possible non-genomic action of E2, we performed *NELL2* promoter assays with E2-BSA. E2-BSA did not affect transcriptional activity of *NELL2* promoter (Fig. 2*D*), indicating that E2 exerts its effect on *NELL2* transcription through genomic action by binding with ERs.

To determine E2 action on the endogenous *NELL2* expression, we carried out real-time PCR and Western blot analysis of mRNA and protein extracts from the HiB5 cells treated with E2 together with or without ERs. E2 strongly increased endogenous mRNA (Fig. 2*E*) as well as protein (Fig. 2*F*) levels of NELL2 in the presence of ERα and ERβ.

ER Binds to Half-ERE Domains in the 5′-Flanking Region of NELL2 Gene—As stated above, the m*NELL2* promoter has no palindromic ERE. Instead, it contains two half-EREs. To determine whether ER is recognized by these domains in the *NELL2* promoter, we subjected the two sequences of the m*NELL2* promoter (located at −223 and −1047) containing the half-ERE 5′-TGACC-3′ core motif and flanking region to EMSA analysis. Both half-EREs showed shifted bands when exposed to nuclear extracts from the HiB5 cells transfected with either ERα (Fig. 3*A*) or ERβ (Fig. 3*B*) expression vectors and treated with E2. A positive control (labeled *C*) using the palindromic ERE showed shifted bands of an apparently similar size. In contrast, a mutated ERE (labeled *NC*) did not generate shifted bands.

Because SP1RE has also been reported to indirectly mediate E2 action on target genes (26–28), EMSAs using probes containing three SP1REs were also performed to identify indirect binding of E2-ERs to these sequences. SP1RE at −453 showed almost no shifted band, whereas SP1REs at −209 and −430 had shifted bands with ERβ (Fig. 3*B*), suggesting that E2-ERβ may indirectly interact with these SP1REs through binding to SP1 (26–28). Preincubation of the nuclear extracts with ERα antibody delayed migration of the protein-DNA complex generated with a probe containing half-EREs at −223 and −1047 (data not shown), indicating that ERα is indeed part of this complex.

To further determine *in vivo* interaction of ERs with NELL2 promoter containing the two half-ERE sequences, ChIP assays were performed using ERα and ERβ antibodies, and the precipitated DNA was amplified using PCR primer sets specific to the promoter regions containing the half-EREs (Fig. 3, *C* and *D*); one primer set amplified a 216-bp fragment of the m*NELL2* promoter region from −381 to −166 encompassing the half-ERE at −233; the other primer set amplified a 195-bp fragment corresponding to −1076 to −882 region of the *NELL2* promoter with the half-ERE at −1047. As shown in Fig. 3, *C* and *D*, *NELL2* promoter was immunoprecipitated with ERα and ERβ

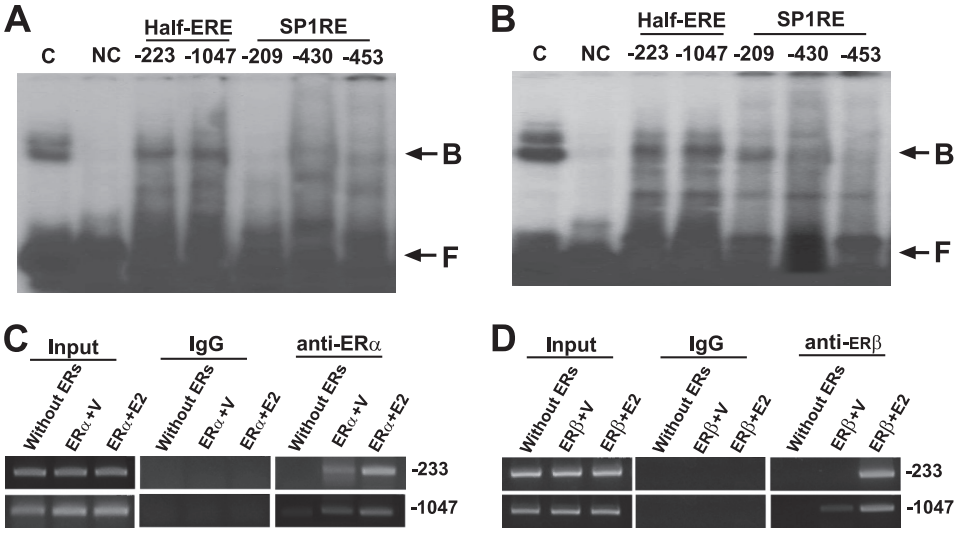

FIGURE 3. **EMSAs and ChIP assays.** EMSAs were performed using double-stranded oligomer probes containing the putative half-EREs and SP1REs found in the m*NELL2* gene 5′ flanking sequence (see "Experimental Procedures"). *A* and *B,* autoradiograms show binding of half-EREs and SP1REs derived from the m*NELL2* promoter to nuclear extracts (10 μg) prepared from HiB5 cells transfected with ERα (*A*) and ERβ (*B*) and treated with E2 (10 nM). *Numbers on top of the gel images* indicate the site in the *NELL2* promoter from where the oligomer probes were designed. *B*, protein-bound DNA; *F*, free DNA; *C*, control palindromic ERE; *NC*, negative control of mutant ERE. *C* and *D*, shown are ChIP assays of m*NELL2* promoter DNA using ERα (*C*) and ERβ (*D*) antibodies. HiB5 cells were transfected with expression vectors for the ERα (*C*) and ERβ (*D*), and the immunoprecipitated DNA was PCR-amplified with primers for two *NELL2* promoter fragments including the two half-ERE sequences (at −233 and −1047). *Input* represents the DNA extracted from the HiB5 cells before immunoprecipitation. Normal IgG from mouse (*C*) and rabbit (*D*) was included in the assay as negative controls. *V* = vehicle.

antibodies. The results revealed that PCR fragments containing the half-ERE sequences at −233 and −1047 were markedly increased in DNA samples from E2-treated HiB5 cells compared with DNA from vehicle-treated cells. No detectable band was observed in the control IgG preciptations, and very faint bands were found in the precipitations from cells without transfection of ERs.

E2-dependent Activation of NELL2 Transcription Decreases after Deletion of Half-EREs—To determine whether the two half-EREs found in *NELL2* promoter region are required for transactivation of the *NELL2* promoter by E2-ERα and E2-ERβ complexes, we deleted each site by the site-directed mutagenesis and examined the ability of E2-ERs to transactivate the mutated promoter in HiB5 cells. As shown in Fig. 4, deletion of each half-ERE resulted in a significant decrease of promoter activity induced by E2-ERα (Fig. 4*A*) and E2-ERβ (Fig. 4*B*). Deletion of the proximal half-ERE (at −223), located in intron 1, obliterated E2-ERβ-induced promoter activation (Fig. 4*B*), suggesting that this is a critical site mediating the effect of E2-ERβ on *NELL2* gene transcription. The −223 site also appears to be the most important for E2-ERα action (Fig. 4*A*). These results also suggest that intron 1 is critical not only for maintenance of basal transcription (see Fig. 2*A*) but also required for E2-ER-mediated transcriptional regulation of the m*NELL2* gene.

Because SP1REs are also involved in mediating E2-dependent transcriptional regulation via an interaction between E2-ER and SP1 bound to SP1RE (26–28), we determined if deletion of 3 SP1REs (at −209, −430, and −453) affects E2-ER-dependent *NELL2* gene transcription. Interestingly, single deletions of SP1RE at −209 and −430 strongly suppressed activation of *NELL2* transcription by E2-ERβ (Fig. 4*B*) but not that by E2-ERα (Fig. 4*A*). Thus, certain SP1REs could be indirectly involved in the regulation of *NELL2* transcription by E2-ER, but this action may be different between the two ERs.

ERα Protein and NELL2 mRNA Are Coexpressed in the Rat Hippocampus and Hypothalamus—Combined *in situ* hybridization/immunohistochemistry was performed to determine whether cells synthesizing ERα protein in the rat brain also express *NELL2* mRNA. The hippocampus and hypothalamus were selected as examples, because they are well known to contain ERα-expressing neurons. As shown in Fig. 5, *NELL2* mRNA was expressed at high levels in both the rat hippocampus and the ventromedial nucleus (VMH) of the hypothalamus. In the hippocampus, most NELL2-expressing cells (Fig. 5*A*) also showed ERα immunostaining (Fig. 5*C*), a colocalization best illustrated at a higher magnification (Fig. 5*E*). In the hypothalamus, cells of the VMH were rich in *NELL2* mRNA transcripts (Fig. 5*B*), and cells of the ventrolateral VMH region were also positive for ERα (Fig. 5*D*). A higher magnification view of this region shows that ERα-stained cells also contain silver grains, indicating the presence of *NELL2* mRNA transcripts (Fig. 5*F*). These results indicate that there are cells in the brain co-expressing both NELL2 and ERα, thus providing the morphological substrate for a functional interaction between these two molecules.

Effect of NELL2 on the Survival of Neuroprogenitor HiB5 Cells—An effect of NELL2 on cell survival was examined using methylthiazolyltetrazolium assays and HiB5 cells stably transfected with wild-type NELL2 or mutant NELL2 expression vectors (Fig. 6*A*) and treated with neural cell death-inducing agents in combination with E2. Wild-type NELL2 showed a strong neuroprotective effect on HiB5 cells treated with Aβ (Fig. 6*B*) and AMPA (Fig. 6*C*). However, mutant NELL2 lacking either the Ca^{2+} binding EGF domains (NELL2-ΔEGF-Ca^{2+}) or all six EGF-like domains (NELL2-ΔEGF) did not promote cell survival, indicating that these domains are essential for NELL2-dependent signaling to facilitate cell survival.

E2 (10 nM) treatment alone markedly promoted survival of cells treated with Aβ (Fig. 6*B*) and AMPA (Fig. 6*C*). Cells expressing wild-type NELL2 responded to E2 treatment with a further increase in survival rate, but E2 failed to induce cell survival in the presence of either NELL2-ΔEGF-Ca^{2+} or NELL2-ΔEGF (Fig. 6, *B* and *C*). These results suggest that the survival promoting effect of E2 on HiB5 cells requires NELL2 intermediacy and that a defective NELL2 protein blocks the

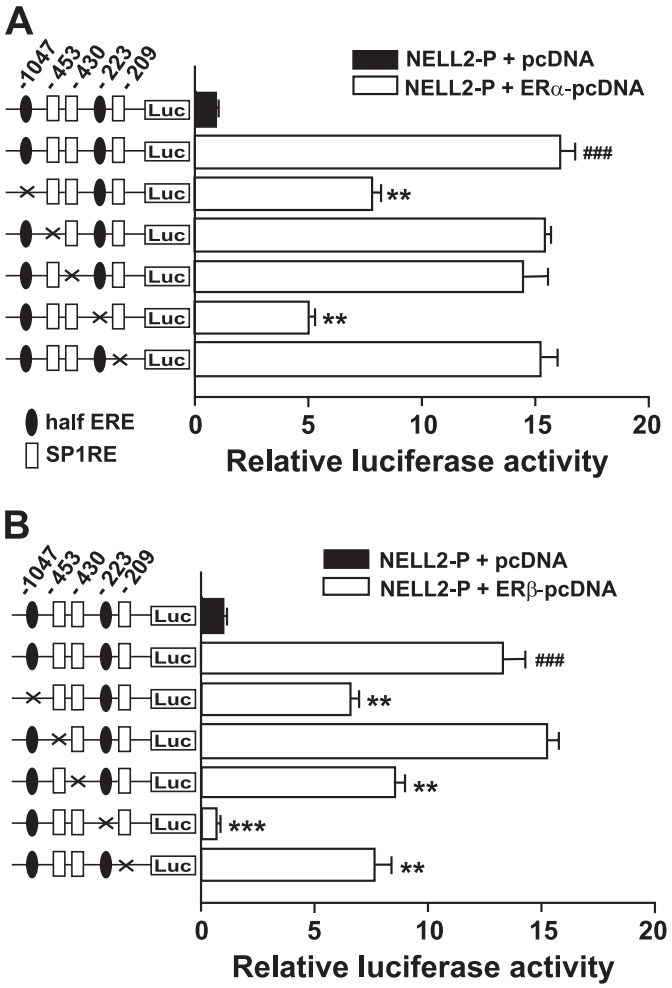

FIGURE 4. **Effect of site-specific deletion of half-EREs on the transactivation of the *NELL2* promoter by E2-ERs.** Single mutants carrying individual deletions of the half-EREs and SP1REs at −1047, −453, −430, −223, and −209 were cotransfected with 50 ng each of ERα (*ERα-pcDNA*, *A*) or ERβ (*ERβ-pcDNA*, *B*) expression vectors and treated with E2 (10 nM). The cells were harvested for luciferase and β-galactosidase assays 30 h after transfection. The positions of the deleted binding sites are indicated. The data are the means ± S.E. of nine wells per construct. ###, $p < 0.001$ *versus* NELL2-*p* + pcDNA; **, $p < 0.01$; ***, $p < 0.001$ *versus* wild NELL2-*p* + ERα-pcDNA or ERβ-pcDNA.

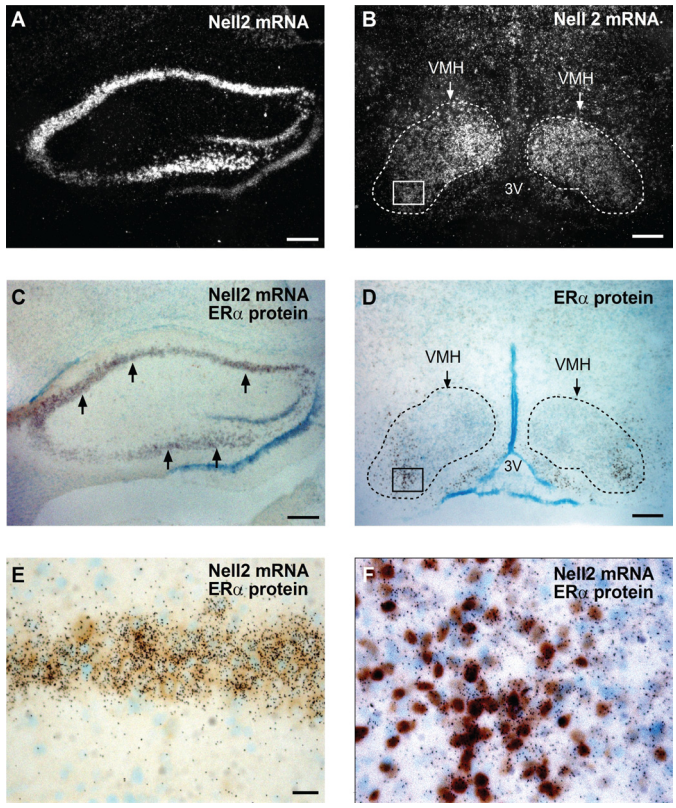

FIGURE 5. **Localization of *NELL2* mRNA and ERα protein in the rat hippocampus and hypothalamus.** Coronal sections of the female rat brain were first incubated with an ERα antibody, and the reaction was developed to a brown color. Thereafter, the sections were hybridized with a ^{35}S-labeled *NELL2* cRNA probe (white grains). *A*, the dark field image shows an abundance of *NELL2* mRNA-containing cells in the hippocampus including CA1 to CA3 layers and dentate gyrus. *B*, the dark field image shows that most cells of the VMH contain *NELL2* mRNA transcripts. *C*, shown is expression of *NELL2* mRNA (*black grains*) and ERα protein (*brown*) in the hippocampus. *Arrows* point to examples of colocalization in the hippocampal layers CA1, CA2, and CA3. *D*, localization of ERα protein in the ventrolateral portion of the VMH is shown. *E*, a higher magnification image highlights the colocalization of *NELL2* mRNA and ERα protein in the CA1 layer of the hippocampus. *F*, a high magnification image illustrates the colocalization of *NELL2* mRNA and ERα protein in the ventrolateral region of the VMH (*boxed region* in the *panel D*). *Bars*, 250 μm (*A–D*) and 20 μm (*E* and *F*). *3V*, third ventricle.

survival promoting action of E2, likely by antagonizing the effect of endogenous wild-type NELL2.

Effect of NELL2 on Aβ-induced Apoptosis—To determine whether the effect of NELL2 on the survival of HiB5 cells is due to an anti-apoptotic effect, we performed TUNEL assays. The number of TUNEL-positive cells showing a red coloration indicative of apoptotic nuclear condensation (supplemental Data 3) increased after Aβ treatment (Fig. 7*A*). This increase was reduced in cultures expressing wild-type NELL2 with or without E2 (Fig. 7*A*). Neither of the two mutant forms of NELL2 tested was able to rescue the cells from Aβ-induced apoptotic death. These results indicate that, as previously suggested (6), the survival-promoting effect of NELL2 is due to an anti-apoptotic effect. They also support the conclusion that NELL2 is required for E2 to protect cells from apoptosis.

To confirm the involvement of NELL2 in E2-induced cell survival, we performed one more set of TUNEL assays on HiB5 cells that was treated with E2 after knocking down endogenous synthesis of NELL2 with a *NELL2* siRNA. Transfecting the cells with *NELL2* siRNA resulted in a more than 90% loss of endogenous *NELL2* mRNA and significantly inhibited the anti-apoptotic effect of E2 while enhancing the apoptotic effect of Aβ (Fig. 7*B*). These results suggest that normal synthesis and action of NELL2 is critical for E2-induced cell survival.

To identify intracellular signaling molecules involved in the cell survival effect of NELL2, we performed Western blot analysis using the same experimental settings as Fig. 7*B*. Western blot analysis revealed that E2 increased phosphorylation of ERK1/2 in the Aβ-treated HiB5 cells (Fig. 7*C*), as previously reported in other systems (for a review, see Ref. 11 and 29), whereas it decreased pro-apoptotic protein Bax (Fig. 7*D*). Interestingly, *NELL2* siRNA exerted an effect exactly opposite to E2 action on the phosphorylation of ERK and Bax expression. The increase of Bax by *NELL2* siRNA further supports anti-apoptotic effect of NELL2. These results suggest that the cell survival effect of NELL2 is disclosed by its action on a signaling pathway of ERK and Bax.

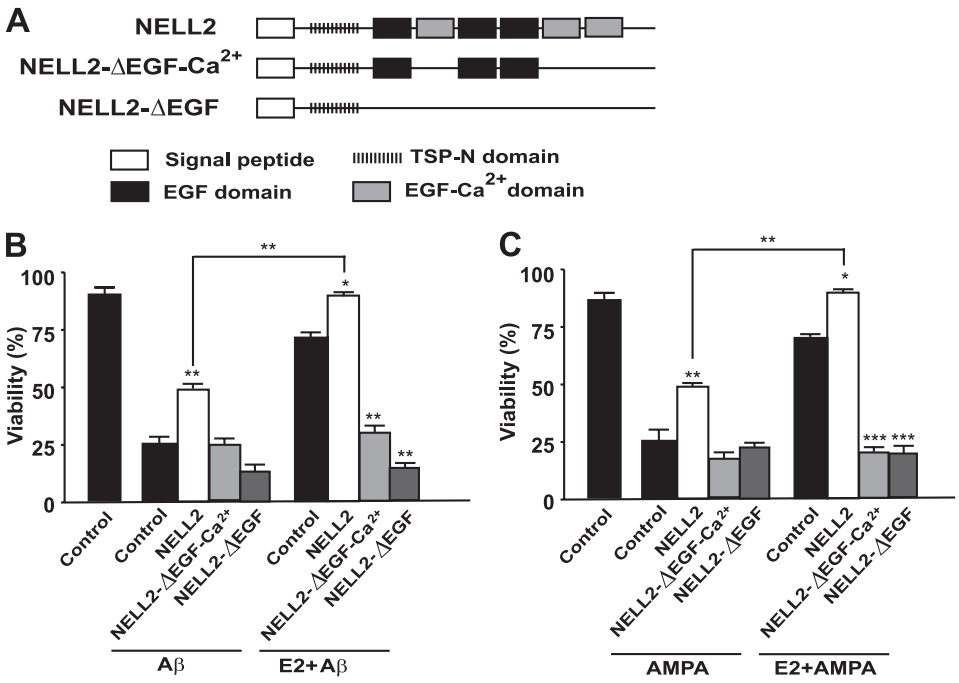

FIGURE 6. **Effect of NELL2 on the survival of HiB5 cells.** The cells stably expressing wild-type NELL2 or mutant NELL2 forms were plated at a density of 1×10^6 cells per well for 24 h and pretreated with E2 or vehicle for 24 h. The cells were then treated with neurotoxins for 20 h, and methylthiazolyltetrazolium (MTT) assays were performed. Results were calculated and presented as % of viable cells with regard to the total numbers of cells. *A,* schematic drawings show NELL2 and mutant NELL2 lacking three EGF-like Ca^{2+} binding domains (NELL2-ΔEGF-Ca^{2+}) and mutant NELL2 lacking all 6 EGF-like domains (NELL2-ΔEGF). *TSP-N,* N-teminal thrombospondin. *B,* shown is cell viability after treatment with Aβ peptide (10 μM) in the presence or absence of E2 pretreatment. *C,* viability of the cells treated with AMPA (5 mM) in the presence or absence of E2 pretreatment. *Bars* represent the means ± S.E. ($n = 6$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *versus* control group for each treatment.

DISCUSSION

We have previously reported that hypothalamic *NELL2* mRNA abundance is increased by E2 treatment (15) and that NELL2 is involved in the E2-dependent formation of the SDN-POA in male rats (6). In the present study we show that E2 and its receptors, ERα and ERβ, trans-activate the *NELL2* gene and that NELL2 mediates the protective effect of E2 on cultured neuroprogenitor cells against cell death-inducing treatments.

A stimulatory effect of E2 on *NELL2* transcription appears to be at odds with the decrease in *NELL2* mRNA that occurs at the time of female puberty (23). NELL2 mRNA abundance increases in the hypothalamus during the juvenile period, when circulating E2 levels are low and decreases, instead of increasing, at the time of puberty, when circulating E2 levels are elevated. It is possible that the juvenile increase in NELL2 synthesis is independent from changes in E2 secretion and that the stimulatory effect of high (pubertal) E2 levels is counteracted by a repressive E2-independent mechanism set in motion at the time of puberty. Further investigation is obviously required to clarify this phenomenon.

Sequence analysis revealed that the m*NELL2* promoter does not have classical palindromic ERE(s). Instead, it contains two half-EREs and seven SP1REs. Both half-EREs strongly bind ERα and ERβ in EMSA, suggesting that these domains physically interact with ERα and ERβ. This interaction was further confirmed by supershift assays and ChIP assays. Deletion of each of the two half-EREs resulted in a significant decrease of E2-ER-dependent promoter activation. Thus, both the half-EREs are required for full E2-ER-dependent transactivation of *NELL2*. Earlier studies have demonstrated that E2-ER complexes regulate target gene promoters containing half-EREs (30, 31). Gel shift assays showed that ER specifically bound two conserved half-EREs in the prothymosin α gene (30) and several half-EREs in the Na^+/H^+ exchanger regulatory factor gene (31). The half-EREs in these genes were critical for the ERα- and ERβ-dependent transcriptional regulation, although binding affinities of these half-EREs to ER are much less than that of the canonical palindromic ERE. EMSA and ChIP assays and promoter analyses in our study revealed that E2-ERs regulate transcription of the *NELL2* gene also by directly binding to half-EREs.

Interestingly, deletion of some SP1REs differentially affected the ERα and ERβ dependence of *NELL2* transcription; deletion of SP1RE at −209 and −430 decreased ERβ-dependent-, but not the ERα-dependent activation of *NELL2* transcription. The mechanism underlying this difference remains to be ascertained. The decrease of ERβ-dependent activation of *NELL2* transcription by deletion of SP1RE at −209 and −430 suggests that the ERβ likely interacts with SP1 bound to these SP1REs. E2-ER action through interaction with SP1-SP1RE complexes has been reported to occur in several different genes and shown not to involve direct ER binding to SP1RE (26–28). Instead, ER appears to bind to SP1 and enhance SP1 binding to SP1REs.

Sequence analysis and activity assays of the m*NELL2* promoter revealed that the promoter contains neither a functional TATA box nor a CAAT box. Instead, the *NELL2* promoter appears to be GC-rich, a feature shared with other GC-rich promoters that contain several SP1REs and multiple TSSs spread over a large region (32). The majority of the genes lacking a TATA box exhibit multiple TSSs, which is a typical feature of housekeeping genes (33) that also contain SP1REs upstream from the TSSs (34). However, some members of this class do not have housekeeping functions but instead appear to be involved in regulation of growth and differentiation, such as the genes encoding c-Ki-ras (35), the EGF receptor (36), and nerve growth factor receptor (34). NELL2 is also believed to be involved in regulating proliferation and differentiation processes (5, 7), and thus, it may share similar mechanisms of transcriptional control with the aforementioned genes (34). It has been suggested that SP1 may control the accuracy of transcription initiation in TATA-less genes (37–39). Seven conserved

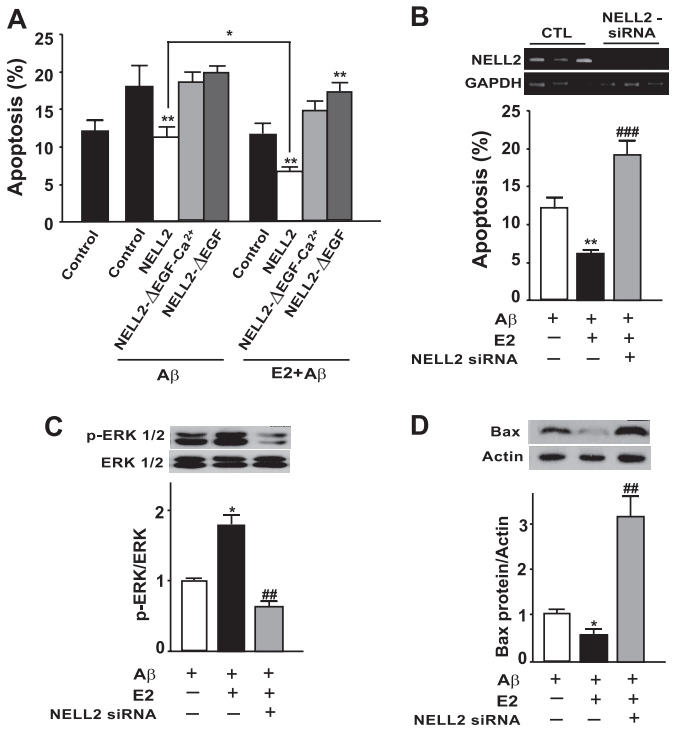

FIGURE 7. **NELL2 decreases apoptosis of HiB5 cells.** HiB5 cells stably expressing wild-type NELL2 or the NELL2 mutants indicated in the figure were treated with E2 at a concentration of 10 nM for 24 followed by exposure to 10 μM Aβ for 20 h. Cells were stained using the TUNEL method. *A*, quantitative analysis of the results showing differences in the number of TUNEL-positive cells between treatment groups ($n = 6$). **, $p < 0.01$ *versus* control for each treatment group. *B*, blockade of endogenous NELL2 synthesis via RNAi prevents E2-dependent protection from Aβ-induced apoptosis. The cells were transfected with *NELL2* siRNA and then treated with E2 for 24 h followed by treatment with Aβ for 20 h. The *gel on top of the graph* illustrates the results of a representative experiment assessing the content of *NELL2* mRNA by RT-PCR. Results are shown as the percentage (%) of apoptotic cells ($n = 8$/group). *CTL*, control. *GAPDH*, glyceraldehyde phosphate dehydrogenase. **, $p < 0.01$ *versus* Aβ; ###, $p < 0.001$ *versus* Aβ + E2. *C* and *D*, shown is the effect of *NELL2* siRNA on the phosphorylation of ERK1/2 (*C*) and content of Bax (*D*) in the HiB5 cells treated with Aβ and E2. Proteins extracted from the cells were subjected to Western blotting. *Autoradiograms on top* show representative results of repeated experiments ($n = 4$). Data were calculated in comparison with bands generated by total ERK proteins (*C*) and β-actin (*D*) and are indicated as arbitrary units. *, $p < 0.05$ *versus* Aβ; ##, $p < 0.01$ *versus* Aβ + E2.

SP1REs are found upstream from the TSS located at the 5′ end of exon 2 in m*NELL2* gene. Despite these features, the m*NELL2* promoter also contains putative binding sites for transcriptional regulators such as retinoic acid receptor/retinoid X receptor and progesterone receptor/glucocorticoid receptor, suggesting that it may be subjected to inducible control by factors binding to these sites.

Our transcription assays demonstrated that deletion of intron 1 of *NELL2* gene resulted in loss of basal promoter activity, suggesting that intron 1 is essential for normal *NELL2* transcription. In addition to the promoter region, the intron 1 contains a half-ERE at −223 and several SP1REs. Deletion of these sequences resulted in a decrease of E2-ER-dependent stimulation of *NELL2* transcription, supporting the notion that intron 1 is also important for the transcriptional regulation of *NELL2* gene by E2. Such an involvement of intronic regions in transcriptional regulation has also been reported for several other genes, including those encoding apolipoprotein B (40), renin (41), and vasopressin V_{1b} receptor (42).

E2 is an important steroid hormone that exhibits multiple effects on various tissues including the nervous system. E2 exerts neuroprotective action in various model systems where it reduces neuronal damage caused by serum deprivation (43, 44), β-amyloid treatment (45, 46), and exposure to glutamate (46, 47). In the present study we found that E2 protects HiB5 cells against toxicity induced by AMPA and Aβ peptide. Earlier studies suggested that the neuroprotection exerted by E2 may be mediated by rapid intracellular signaling events in addition to ERE-mediated transcriptional regulation (for review, see Ref. 11 and 29). For example, activation of the MAPK pathway is necessary for E2 to exert its neuroprotective effects, because blockade of this pathway with a mitogen/extracellular-signal regulated kinase kinase-1 (MEK-1) inhibitor prevented E2-dependent neuroprotection (48). MAPK activation was induced by E2 even in cells transfected with a mutated ERα lacking the ability to mediate ERE-dependent transcription, suggesting that MAPK-mediated neuroprotective action of E2 does not require E2-ER-dependent transcription (48).

Aihara *et al.* (8) reported that recombinant rat NELL2 promoted survival of primary cultured hippocampal and cortical neurons via the MAPK-dependent pathway. These authors found that NELL2-induced neuronal survival is partly due to inactivation of ERK and activation of c-Jun N-terminal kinase (JNK). In the present study, however, a decreased availability of NELL2 by siRNA induced a decrease of ERK phosphorylation and increase of Bax expression and, in turn, resulted in an increase of apoptosis. Discrepancy between the two studies may be due to differences of cells and treatments.

In this study we showed that E2-ERs activate *NELL2* transcription and that NELL2 promotes neuroprogenitor HiB5 cell survival. Therefore, E2-induced survival of HiB5 cells may be at least in part mediated by ER-mediated trans-activation of *NELL2*. E2 is involved in phosphorylation of ERK through membrane receptors (11, 29), and ERK signaling induces a decrease of Bax expression (for review, see Ref. 49). Thus, our results, showing that *NELL2* siRNA induced changes in ERK phosphorylation and Bax expression in the presence of E2 suggest that NELL2 may partly be also responsible to the non-genomic E2 action on ERK and Bax signaling for cell survival.

Because NELL2 is a released protein (3) and a larger portion of NELL2 was found in the culture medium than in cellular extracts of HiB5 cells overexpressing NELL2 (6), it seems plausible that the survival promoting effect of transfected NELL2 in HiB5 cells might require a paracrine effect of NELL2 released to the culture medium. Whether NELL2 is recognized by a specific receptor is not known.

The survival effect of E2 was inhibited not only by siRNA-mediated blockade of NELL2 synthesis but also by NELL2 mutants lacking EGF-like domains, suggesting that these domains are required for the survival effect of E2. It also appears that NELL2 mutants lacking EGF-like domains act as antagonists of NELL2, although the mechanism underlying this effect is not known.

In summary, our results indicate that the *NELL2* gene is trans-activated by E2 and that NELL2 mediates the survival promoting effect of E2 on HiB5 neuroprogenitor cells. The

detailed cellular mechanism underlying this effect remains to be further identified.

REFERENCES

1. Matsuhashi, S., Noji, S., Koyama, E., Myokai, F., Ohuchi, H., Taniguchi, S., and Hori, K. (1995) *Dev. Dyn.* **203,** 212–222
2. Watanabe, T. K., Katagiri, T., Suzuki, M., Shimizu, F., Fujiwara, T., Kanemoto, N., Nakamura, Y., Hirai, Y., Maekawa, H., and Takahashi, E. (1996) *Genomics* **38,** 273–276
3. Kuroda, S., and Tanizawa, K. (1999) *Biochem. Biophys. Res. Commun.* **265,** 752–757
4. Oyasu, M., Kuroda, S., Nakashita, M., Fujimiya, M., Kikkawa, U., and Saito, N. (2000) *Brain Res. Mol. Brain Res.* **76,** 151–160
5. Kim, H., Ha, C. M., Choi, J., Choi, E. J., Jeon, J., Kim, C., Park, S. K., Kang, S. S., Kim, K., and Lee, B. J. (2002) *J. Neurochem.* **83,** 1389–1400
6. Jeong, J. K., Ryu, B. J., Choi, J., Kim, D. H., Choi, E. J., Park, J. W., Park, J. J., and Lee, B. J. (2008) *J. Neurochem.* **106,** 1604–1613
7. Nelson, B. R., Claes, K., Todd, V., Chaverra, M., and Lefcort, F. (2004) *Dev. Biol.* **270,** 322–335
8. Aihara, K., Kuroda, S., Kanayama, N., Matsuyama, S., Tanizawa, K., and Horie, M. (2003) *Brain Res. Mol. Brain Res.* **116,** 86–93
9. Gorski, R. A. (1985) *J. Anim. Sci.* **61,** 38–61
10. McCarthy, M. M. (2008) *Physiol. Rev.* **88,** 91–124
11. Garcia-Segura, L. M., Azcoitia, I., and DonCarlos, L. L. (2001) *Prog. Neurobiol.* **63,** 29–60
12. Rhees, R. W., Shryne, J. E., and Gorski, R. A. (1990) *Brain Res. Dev. Brain Res.* **52,** 17–23
13. Arai, Y., Murakami, S., and Nishizuka, M. (1994) *Horm. Behav.* **28,** 313–319
14. Davis, E. C., Popper, P., and Gorski, R. A. (1996) *Brain Res.* **734,** 10–18
15. Choi, E. J., Ha, C. M., Choi, J., Kang, S. S., Choi, W. S., Park, S. K., Kim, K., and Lee, B. J. (2001) *Brain Res. Mol. Brain Res.* **97,** 115–128
16. Renfranz, P. J., Cunningham, M. G., and McKay, R. D. G. (1991) *Cell* **66,** 713–729
17. Jeong, J. K., Kim, H. R., Hwang, S. M., Park, J. W., and Lee, B. J. (2008) *Mol. Cells* **26,** 186–192
18. Andrews, N. C., and Faller, D. V. (1991) *Nucleic Acids Res.* **19,** 2499
19. Kuhn, R., Monuki, E. S., and Lemke, G. (1991) *Mol. Cell. Biol.* **11,** 4642–4650
20. Kim, M. S., Hur, M. K., Son, Y. J., Park, J. I., Chun, S. Y., D'Elia, A. V., Damante, G., Cho, S., Kim, K., and Lee, B. J. (2002) *J. Biol. Chem.* **277,** 36863–36871
21. Klinge, C. M., Peale, F. V., Jr., Hilf, R., Bambara, R. A., and Zain, S. (1992) *Cancer Res.* **52,** 1073–1081
22. Simmons, D. M., Arriza, J. L., and Swanson, L. W. (1989) *J. Histotechnol.* **12,** 169–181
23. Ha, C. M., Choi, J., Choi, E. J., Costa, M. E., Lee, B. J., and Ojeda, S. R. (2008) *Neuroendocrinology* **88,** 199–211
24. Behl, C., Davis, J., Cole, G. M., and Schubert, D. (1992) *Biochem. Biophys. Res. Commun.* **186,** 944–950
25. Klinge, C. M. (2001) *Nucleic Acids Res.* **29,** 2905–2919
26. Rishi, A. K., Shao, Z. M., Baumann, R. G., Li, X. S., Sheikh, M. S., Kimura, S., Bashirelahi, N., and Fontana, J. A. (1995) *Cancer Res.* **55,** 4999–5006
27. Qin, C., Singh, P., and Safe, S. (1999) *Endocrinology* **140,** 2501–2508
28. Li, C., Briggs, M. R., Ahlborn, T. E., Kraemer, F. B., and Liu, J. (2001) *Endocrinology* **142,** 1546–1553
29. Toran-Allerand, C. D., Singh, M., and Sétáló, G., Jr. (1999) *Front. Neuroendocrinol.* **20,** 97–121
30. Martini, P. G., and Katzenellenbogen, B. S. (2001) *Endocrinology* **142,** 3493–3501
31. Ediger, T. R., Park, S. E., and Katzenellenbogen, B. S. (2002) *Mol. Endocrinol.* **16,** 1828–1839
32. Dynan, W. S., and Tjian, R. (1983) *Cell* **35,** 79–87
33. Dynan, W. S. (1986) *Trends Genet.* **2,** 196–197
34. Sehgal, A., Patil, N., and Chao, M. (1988) *Mol. Cell. Biol.* **8,** 3160–3167
35. Hoffman, E. K., Trusko, S. P., Freeman, N., and George, D. L. (1987) *Mol. Cell. Biol.* **7,** 2592–2596
36. Ishii, S., Xu, Y. H., Stratton, R. H., Roe, B. A., Merlino, G. T., and Pastan, I. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82,** 4920–4924
37. Smale, S. T., and Baltimore, D. (1989) *Cell* **57,** 103–113
38. Lu, J., Lee, W., Jiang, C., and Keller, E. B. (1994) *J. Biol. Chem.* **269,** 5391–5402
39. Javahery, R., Khachi, A., Lo, K., Zenzie-Gregory, B., and Smale, S. T. (1994) *Mol. Cell. Biol.* **14,** 116–127
40. Brooks, A. R., Blackhart, B. D., Haubold, K., and Levy-Wilson, B. (1991) *J. Biol. Chem.* **266,** 7848–7859
41. Voigtländer, T., Ganten, D., and Bader, M. (1999) *Hypertension* **33,** 303–311
42. Rabadan-Diehl, C., Lolait, S., and Aguilera, G. (2000) *J. Neuroendocrinol.* **12,** 437–444
43. Bishop, J., and Simpkins, J. W. (1994) *Mol. Cell. Neurosci.* **5,** 303–308
44. Green, P. S., Bishop, J., and Simpkins, J. W. (1997) *J. Neurosci.* **17,** 511–515
45. Fitzpatrick, J. L., Mize, A. L., Wade, C. B., Harris, J. A., Shapiro, R. A., and Dorsa, D. M. (2002) *J. Neurochem.* **82,** 674–682
46. Behl, C., Widmann, M., Trapp, T., and Holsboer, F. (1995) *Biochem. Biophys. Res. Commun.* **216,** 473–482
47. Singer, C. A., Rogers, K. L., and Dorsa, D. M. (1998) *Neuroreport* **9,** 2565–2568
48. Mize, A. L., Shapiro, R. A., and Dorsa, D. M. (2003) *Endocrinology* **144,** 306–312
49. McCubrey, J. A., Steelman, L. S., Chappell, W. H., Abrams, S. L., Wong, E. W., Chang, F., Lehmann, B., Terrian, D. M., Milella, M., Tafuri, A., Stivala, F., Libra, M., Basecke, J., Evangelisti, C., Martelli, A. M., and Franklin, R. A. (2007) *Biochim. Biophys. Acta* **1773,** 1263–1284