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Identification of a Novel Endoplasmic Reticulum Stress Response Element Regulated by XBP1*

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Michael Misiewicz[‡§], Marc-André Déry[§¶1], Bénédicte Foveau[§¶], Julie Jodoin[§], Derek Ruths[‖], and Andréa C. LeBlanc[‡§¶2]

From the [‡]Department of Anatomy and Cell Biology, McGill University, the [§]Lady Davis Institute for Medical Research, Sir Mortimer B. Davis Jewish General Hospital, the [‖]School of Computer Science, McGill University, and the [¶]Department of Neurology and Neurosurgery, McGill University, Montreal, Québec H3T 1E2, Canada

Background: Endoplasmic reticulum (ER) stress maintains cellular protein homeostasis.
Results: A novel ER stress-responsive element, ERSE-26, identified in 38 genes, is regulated by sXBP1 during ER stress.
Conclusion: ER stress increases levels of prion and other proteins not previously known to be involved in the ER stress response.
Significance: ERSE-26 implicates novel genes regulated by the ER stress response.

Understanding the regulatory mechanisms mediating *PRNP* gene expression is highly relevant to elucidating normal cellular prion protein (PrP) function(s) and the transmissibility of prion protein neurodegenerative diseases. Here, luciferase reporter assays showed that an endoplasmic reticulum stress element (ERSE)-like element, CCAAT-N26-CCACG in the human *PRNP* promoter, is regulated by ER stress and X-box-binding protein 1 (XBP1) but not by activating transcription factor 6 α (ATF6α). Bioinformatics identified the ERSE-26 motif in 37 other human genes in the absence of canonical ERSE sites except for three genes. Several of these genes are associated with a synaptic function or are involved in oxidative stress. Brefeldin A, tunicamycin, and thapsigargin ER stressors induced gene expression of *PRNP* and four randomly chosen ERSE-26-containing genes, *ERLEC1*, *GADD45B*, *SESN2*, and *SLC38A5*, in primary human neuron cultures or in the breast carcinoma MCF-7 cell line, although the level of the response depends on the gene analyzed, the genetic background of the cells, the cell type, and the ER stressor. Overexpression of XBP1 increased, whereas siRNA knockdown of XBP1 considerably reduced, *PRNP* and *ERLEC1* mRNA levels in MCF-7 cells. Taken together, these results identify a novel ER stress regulator, which implicates the ER stress response in previously unrecognized cellular functions.

The regulation of gene expression from the human *PRNP* gene encoding the cellular prion protein (PrP)[3] is not well characterized. Yet, the importance of the normal cellular PrP is undisputable. The levels of PrP influence prion disease transmission. The absence of PrP eliminates infection by transmissible prion diseases, and low levels of *PRNP* gene expression associated with promoter nucleotide polymorphisms decrease the risk of infection by bovine spongiform encephalopathy (1–3). In contrast, transgenic overexpression of *PRNP* accelerates transmissible prion disease progression (1, 4, 5). In addition, polymorphisms in the regulatory region of *PRNP* may present a risk for Creutzfeldt-Jakob disease (6, 7). Furthermore, accruing evidence implicates the normal cellular PrP in a number of essential cellular functions including neuroprotection against oxidative stress (8, 9), copper toxicity (10), Bax-mediated cell death (11), and ischemia (12–15). There is also evidence that PrP participates in normal synaptic function (16) and myelination (17, 18). Fully understanding the regulation of *PRNP* gene expression is essential to developing therapies against transmissible prion diseases and will also help elucidate the function of the normal cellular PrP.

PrP is developmentally and ubiquitously expressed in most organs and tissues, with a higher expression in brain, lung, heart, and muscle (19–25). The *PRNP* promoter contains GC-rich repeats, which are common to housekeeping genes. It does not contain the core promoter TATA box sequence but does contain Sp1, Ap-1, Ap-2, and CCAAT box transcriptional binding sites (26, 27). Regulatory elements include MyoD, heat shock elements (28), and metal regulatory element MTF-1 (29). Up-regulation of *PRNP* gene expression occurs via NGF (30) or hypoxic conditions in brain (15, 31), in HIV infection of astrocytes (32), during hematopoietic differentiation (24), and by cellular exposure to copper (29) and has been associated with a change in chromatin conformation (33).

We recently identified four endoplasmic reticulum stress response elements (ERSE) in the *PRNP* promoter and showed that ER stress transcriptionally up-regulates *PRNP* gene expression (34). The unfolded protein response is a well conserved process that cells use to restore ER homeostasis or enter apoptosis. In higher eukaryotes, three sensors of ER status exist (reviewed in Ref. 35). IRE1α (inositol-requiring enzyme 1) is a transmembrane ribonuclease that splices and activates X-box-

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[1] Recipient of the Frederick Banting and Charles Best doctoral scholarship from the Canadian Institutes of Health Research.
[2] To whom correspondence should be addressed: Bloomfield Ctr. for Research in Aging, Lady Davis Inst. for Medical Research, Sir Mortimer B. Davis Jewish General Hospital, 3755 Ch. de la Côte Sainte Catherine, Montréal, Québec H3T 1E2, Canada. Tel.: 514-340-8222, ext. 4976; Fax: 514-340-8295; E-mail: andrea.leblanc@mcgill.ca.
[3] The abbreviations used are: PrP, prion protein; ER, endoplasmic reticulum; ERSE, endoplasmic reticulum stress response element; sXBP1, spliced X-box-binding protein 1; BFA, brefeldin A; TM, tunicamycin; Thps, thapsigargin; AMV-RT, avian myeloblastosis reverse transcriptase; AEBSF, 4-(2-aminoethyl)benezenesulfonyl fluoride hydrochloride; ANOVA, analysis of variance.

binding protein (*XBP1*) mRNA when the foldase BiP (Grp78, encoded by *HSPA5*) dissociates from IRE1α and preferentially attaches to misfolded client proteins (35, 36). Concurrently, the transmembrane protein ATF6α (activating transcription factor 6), which is normally bound by BiP, is released and translocates to the Golgi complex, where it is cleaved by the transmembrane Site 1 and Site 2 proteases to form a highly active transcription factor (37, 38). The third branch of ER stress signal transduction is mediated by the transmembrane PKR-like ER localized kinase (PERK), which upon BiP dissociation phosphorylates eukaryotic translation initiation factor 2 (eIF2α). Phosphorylation of eIF2α arrests translation of most messages except for several with upstream activating open reading frames, such as activating transcription factor 4 (*ATF4*) mRNA (35, 39). Following ER signal transduction, spliced XBP1 (sXBP1), cleaved ATF6 (ΔATF6α), and ATF4 translocate to the nucleus where they are able to activate the expression of ER stress-responsive genes. ΔATF6α, sXBP1, and ATF4 interact with several promoter DNA motifs called ERSE, resulting in the transactivation of unfolded protein response target genes (35).

One of the *PRNP* ERSE, CCAAT, separated from CCACG by 26 nucleotides (CCAAT-N26-CCACG or ERSE-26), was of particular interest, as it resembled almost exactly the classical ERSE, CCAAT-N9-CCACG (ERSE-9), characterized by Yoshida *et al*. (37) in ER stress up-regulation of glucose-regulated proteins. ERSE-9 is transactivated by ΔATF6α (36, 37). Although XBP1 can bind to ERSE-9 and transactivate a luciferase reporter under the regulation of five tandem repeats of ERSE-9 (40), overexpression of XBP1 did not transactivate the glucose-regulated proteins (36). Separation of the CCACG from CCAAT with eight or 10–13 nucleotides instead of nine nucleotides abolished the ability of ΔATF6 to bind to the ERSE-9 motif (36, 37). Therefore, it would be expected that *PRNP* CCAAT-N26-CCACG might not be transactivated by ΔATF6α. Nevertheless, the homology of *PRNP* ERSE-26 with the classical ERSE-9 and the ability of ER stress to induce *PRNP* gene expression (34) prompted us to further investigate this motif. We found that ERSE-26 is transactivated by sXBP1 at the CCACG motif but is not transactivated by ΔATF6α. A bioinformatic analysis of the human genome revealed 37 other genes containing ERSE-26 within their promoter region. Several were confirmed to be up-regulated by ER stress in primary human neuron cultures or in the breast carcinoma MCF-7 cell line. These results identified a novel ER stress-regulated ERSE motif that is common to 38 human genes.

EXPERIMENTAL PROCEDURES

Genome-wide Search for Genes Bearing an ERSE-26—To find genes in the human genome with an ERSE-26 in their promoter, a program was written in Python. The sequences of the complete human genome, version GRC37.1 from the Genome Reference Commission (41), and the yeast genome (*Saccharomyces cerevisiae*, S288c-NCBI) were scanned using regular expressions representing the plus and minus strand versions of ERSE-26. The promoters of all human genes were searched. The region from −2000 bp upstream of the transcription start site to +500 bp downstream of the transcription start site was considered to be the promoter region of the gene. The regular expression pattern used was "ccaat. 26ccacg", matching precisely (no deviations were permitted). Both ERSE-26 elements in the forward and reverse orientations were searched. Additionally, promoters in the positive and negative strands of the genome were examined. Source code is provided in a Git repository.

Cell Culture Conditions—Human primary neuron cultures, obtained from human fetal brains subject to McGill University ethical approval, were cultured as described previously (42). Briefly, brains were minced, trypsinized, plated on polylysine-coated flasks, and grown in the presence of 5-fluorodeoxyuridine, 1× penicillin-streptomycin, and minimum essential medium supplemented with 10% bovine calf serum in a 5% CO_2 environment at 37 °C. MCF-7 cells (from ATCC) were grown in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum (FBS) in a 5% CO_2 environment at 37 °C. HEK293 cells were grown in DMEM supplemented with 1.7 g/liter $NaHCO_3$ and 10% FBS in a 5% CO_2 incubator at 37 °C.

Pharmacological Induction of ER Stress and Other Cell Treatments—Neurons and MCF-7 cells were subjected to ER stress under conditions that were optimized experimentally to minimize cell death and maximize ER stress induction. In neurons, ER stress was induced by 5 μg/ml brefeldin A (BFA), 0.5 μg/ml tunicamycin (TM), and 3.25 μg/ml thapsigargin (Thps) from Biomol. Drugs were dissolved in DMSO, and cells were treated for 6 h using the equivalent concentration of DMSO as control followed by immediate total RNA extraction. MCF-7 cells were treated for 18 h, with all drugs at a concentration of 5 μg/ml.

Total RNA Extraction and Reverse Transcriptase-Polymerase Chain Reaction—Total RNA was extracted from neurons using TRIzol (Invitrogen) as described by the manufacturer's protocol. For MCF-7 cells, the Chomczynski guanidine thiocyanate method was used (43). RNA was quantitated on an H4 plate reader (Biotek, Winooski, VT) for neurons or a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). cDNA was prepared using avian myeloblastosis reverse transcriptase (AMV-RT) following the manufacturer's protocol (Roche Applied Science). Briefly, 1 μg of total RNA was used for cDNA synthesis by AMV-RT with poly(dT) primers, and cDNA used as a template for subsequent PCRs.

Primer Design and PCR of ERSE-26 Genes—For PCRs, the primers were designed using the NCBI PrimerBlast software available online (44). Primers were designed to have an annealing temperature of 60 °C. All oligonucleotides were ordered from a commercial supplier (Invitrogen) and optimized with a gradient of annealing temperatures. Table 1 contains all primer sequences. During all subsequent PCRs, the optimal annealing temperature of a primer pair of a gene was always used. cDNA was diluted to 1:200, and PCR was performed with *Taq* DNA polymerase (New England Biolabs) following the manufacturer's protocol. Three biologically independent neuron preparations were used, and two technical replicates per gene per preparation were tested. For MCF-7 cells, three independent experiments were conducted in triplicate for all ER stress drug treatments and sXBP1 transfections.

TABLE 1
Primers used for RT-PCR

Gene	For	Rev	Optimal TM
C16orf78	GGGAGGGAACCGCAGGGACA	TGGGTTTGGCCGCTGAGTGG	60
C1orf68	TTCGGGACTAGGGGCTGGGC	CGAAGGTCCGGGGAGCCAGA	50.8
C6orf118	TGAAGGAGGCCCTGGCCCAC	TCCTGCGCCATCCAGGAGGG	56.7
C6orf201	GGGGAGGACAACAAGCCTCAGGC	CCATGTCGCGGAGAGCTCAGC	60
CHST7	CCGCTCTGGAGGGTCGGTGA	CGCCAGGTGGCATGCACGTA	60
CRTAP	CTGGACAAGTACAGCGGCGAGCA	TATGCCCGCACTGCTCGGATG	54.1
ERLEC1	GCTCACTGTTGGGACAACCCACA	CCATGTCCCGACAACCACAGAGG	52.8
FOXO4	GGGCCCCTGTCAGCAGGAGA	CCAGACTGCTGGGGCCTGGA	60
GAD2 (v2)	CAGCTGCTCCAAAGTGGATG	ATCTTGCAGAAACGCCAAAG	60
GADD45B	GCCGAAGCGTCGGACTACCG	TGGGTCCACATTCATCAACTTGGCC	59.1
GJB2	TGCACGTGGCCTACCGGAGA	GTGGGCCGGGACACAAAGCA	52.8
GNAQ	GGAAGCCCGGCGGATCAACG	AGGGTCAGCTACGCGGTCCAA	60
HADH	AGACCGATTCGCTGGCCTCCA	AACCCGAGTTAGAATCACCACACGT	60
HYAL3 T1, T2, T3, T4	TGTTGTGACGCGCAAACCAGG	TCCGCGTCCTAGCTCCGCAC	60
HYAL3 T1, T2, T5	TGCAGGTTTCCATCCTTGGGGAAT	CGGCCCAGAGCCAATGCAGTT	59.5
KBTBD2	GCGGGTCTGCGTGGAGCTTT	TTAGATTGGCGGCGCCCCAC	55.4
LRFN4	TCAGCTCTCCCGCCTGGACC	GCTGGCCTTCCAGCACCCAG	60
LRRC55	CACAACCCCTGGCTGCGGAG	TCGACTTCAGGAGGGCCCCG	56.7
MYEOV2-T1	CAGTCCTCCATGGTTCCCGCAC	GGTGCCACACAGCGGATGTTC	60
MYEOV2-T2	GCACCGGGCTCTTGATGGACTT	GCTCTGTACCAAGGGCTTGGCCC	58
NAT6	CCTTAGAACGCCGTGGCGTGCC	GGCATGTGGAATCCAGGGGCAG	60
NOL10	TCGTTCGGCCGGACACCTGA	GGCGGGGCCAGCTTCAAAGT	59.5
NUDT9	AGGCTTCGGCGTCACGTGC	GATTTGGGCCCCATCGCCCC	58
PRM3	GGCCGGGGCCACGAATCCTC	GGAGGGTGTCTGCTTGGGCTC	60
PRNP	AGAGGCCCAGGTCACTCC	GAGCTTCTCCTCTCCTCACG	62
PTGER4	CAAACTCCAGTAGCCGCCCGTGC	GCGGACGAATTGACCCCGGGA	60
SERPIND1	CCGGGTGGTGGAGAGATGGC	GGACTTGGGTGGACAGCGGC	59.5
SESN2	CAGCTTCTGGCGCCTGCACT	TTTGCGCAGCTTCTCGGGGG	54.1
SLC38A5	GCCGTGGCCAACGTGTCCAT	TGGCCAGCTGAAGGCCTTGC	52.8
SMPD1	AACCCAATGTGGCTCGCGTGGG	CGGCTTCGGCACAGTAGGCAA	55.4
SPG7	CCATGTCATCGTCCTGGCGTCC	CAAGGCGTGGCCCGACTCATG	52.8
SPTBN5	TTCCCATCCTGCTGCAGCGC	CCTTCAGCTGCTGGGCCCAC	60
TGS1	GCAGCGTCCGGGCTAGTTCC	AACATTTCCGCCACGCGGCT	52.8
TIMM44	AAGACGGCCAAGCAGTCGGC	CCGGGATGCCCGGATGAACG	54.1
TMEM168	TGACCCGCCACAGCTAGGTGA	TCCAGTGCTTGGCCACATCGC	52.8
USP4	CTGCGGAGCGTGAAACACGG	GTGCATCCCACAGGTGCTAGTGC	54.1
YPEL3	GGCTCCCTCTGCTCCCCGTG	CAGCATGGAGGCCGGTCAGC	60
ZNF721	CCGAGAAGAAATCCGAGCAGCGA	TCCAGCCTAGCGTGTCCACGA	60
ZPBP2	GTGCTCTGGTGCCTCACAGG	GGCCCAATCCATAAGTAGGTGGGGT	60

Western Blotting of PrP, BiP, and β-Actin in ER Stress-treated Neurons—Cells were lysed for 20 min on ice in lysis buffer (150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100 (v/v), 0.5% sodium deoxycholate (w/v), and 50 mM Tris-HCl, pH 7.5) containing fresh protease and phosphatase inhibitors (38 μg/ml AEBSF, 0.5 μg/ml leupeptin, 0.1 μg/ml pepstatin, 0.1 μg/ml *N*-α-*p*-tosyl-L-lysine chloromethyl ketone hydrochloride, 4 mM sodium orthovanadate, and 20 mM sodium fluoride). After centrifugation of the lysate at 11,500 × *g* for 10 min at 4 °C, the supernatant was collected as the detergent-soluble fraction. The protein concentration was determined with the BCA protein assay reagents (Fisher). One hundred μg of protein was precipitated with 4 volumes of ice-cold methanol overnight at −20 °C and centrifuged at 11,500 × *g* for 15 min at 4 °C; the dried pellet was solubilized in Laemmli sample buffer (2% SDS (w/v), 5% β-mercaptoethanol, (v/v), 10% glycerol (v/v), 0.01% bromphenol blue (w/v), and 62.5 mM Tris-HCl, pH 6.8). The proteins were boiled for 3 min, separated in a 10 or 15% SDS-

PAGE, and transferred to PVDF membranes. PrP was detected with the polyclonal R155 antiserum (1/500, anti-PrP^{36-56}) or 3F4 (1/2000, anti-$PrP^{109-112}$). Other antibodies used were β-actin (1:5000, clone AC-15, Sigma) and BiP (1:250, clone H-129, Santa Cruz Biotechnology Inc.). Immunoreactivity was revealed with 1/5000 anti-rabbit IgG conjugated to horseradish peroxidase secondary antibodies (GE Healthcare) and chemiluminescence reagents from GE Healthcare or Millipore (Billerica, MA) and was detected with the Molecular Dynamics Storm 840 (GE Healthcare) or Kodak Biomax MR film (Carestream Health, Toronto, Ontario, Canada).

Cloning of ERSE-26 Fragment into pMetLuc2 Vector and Site-directed Mutagenesis—The pMetLuc2-Reporter construct (pML2, obtained from Clontech) is a plasmid that encodes a luciferase gene from the copepod *Metrida longa*. This gene contains a powerful endogenous signal peptide, causing the protein to be secreted into the growth medium. This allows for a no-lysis protocol for assaying luciferase activity in transfected cells by simply reading the luciferase activity (when a substrate is added) of the medium. To clone the *PRNP* promoter fragment into pML2, primers were designed to amplify the region from −391bp to −274bp, around the ERSE-26, which was obtained from the pGL3 *PRNP* promoter construct, kindly given to us by the laboratory of Dr. Collinge (26). The following primers were used (restriction sites in bold): forward, 5′-**GAG CTC** TCT CCA TTA TGT AAC GGG GA-3′; and reverse, 3′-G**CG AAT TC**T CAG TTG ATA CCG CCT GCG G-5′. Primers contained the restriction endonuclease sites for EcoRI and SacI. The PCR product and pMetLuc2-reporter vector were digested using EcoRI and SacI followed by ligation with T4 DNA ligase (Fermentas). DH5α *Escherichia coli* were transformed using standard protocols, and DNA was prepared for transfection using the alkaline lysis method (45). The resulting pML2-EL26 construct was sequenced. Following cloning of the *PRNP* promoter fragment into the pMetLuc2 vector, the ERSE-26 was mutated by PCR with mutagenic oligos. Primers were designed to target the second part of the ERSE-26, the putative XBP1/ATF6 binding site; CCACG was changed to ATCTA. Two additional base pairs after the XBP1/ATF6 site were also changed from TC to GA, yielding a final mutation of ATCTAGA. The following primers were used for mutagenesis, (with the mutation in bold and the former location of the CCACG site underlined): 5′-GAT TTT TAC AGT CAA TGA G**AT CTA GA**A GGG AGC GAT GGC ACC C-3′ and 5′-GGG TGC CAT CGC TCC CTT C**TA GAT CT**C ATT GAC TGT AAA AAT C-3′. In addition, two other mutants were generated by inserting 4 or 8 base pairs at the center of the ERSE-26 element. For the elongated ERSE-30 and ERSE-34, the following primers were used (with the inserted base pairs underlined): ERSE-30-For 5′-GGG CCG AAT TTC CAA TTA AAG ATG ATT TTA AAA TAC AGT CAA TGA GCC ACG TCA GGG AGC G-3′ and ERSE-30-Rev 5′-CGC TCC CTG ACG TGG CTC ATT GAC TGT ATT TTA AAA TCA TCT TTA ATT GGA AAT TCG GCC C-3′; and ERSE-34-For 5′-GGA GCT TTG GGC CGA ATT TCC AAT TAA AGA TGA TTT TAA AAA AAA TAC AGT CAA TGA GCC ACG TCA GGG AGC GAT GGC AC-3′ and ERSE-34-Rev 5′-GTG CCA TCG CTC CCT GAC GTG GCT CAT TGA CTG TAT TTT TTT TAA AAT CAT CTT TAA TTG GAA ATT CGG CCC AAA GCT CC-3′. DNA was synthesized using *Pfu* Turbo (Agilent Technologies) and subjected to DpnI digestion to remove non-mutated plasmid DNA. DNA was then transformed into DH5α *E. coli* using standard techniques and prepared using the alkaline lysis method. Transfection in HEK293 cells was done as described below.

Transfection of HEK293 Cells and Luciferase Assay—HEK293 cells were plated in 6-well plates at ~500,000 cells/well. Twenty-four hours after plating, using the polyethyleneimine method (46), cells were transfected with pMetLuc2-Reporter-ERSE-26, pMetLuc2-Reporter-ERSE-30, or pMetLuc2-Reporter-ERSE-34 and plasmids pCGN-EGFP, pCGN-ATF6 encoding ΔATF6α (amino acids 1–373), or pCGN-sXBP1, kindly provided by Dr. Kaufman (40). Following 6 h of transfection, the medium was changed, and cells were allowed to grow for 20 h. After 20 h, the medium was collected. Luciferase assays were conducted on 50 μl of HEK293 growth medium. Substrate (Ready-to-Glow secreted luciferase substrate, Clontech) for the luciferase protein was prepared according to the manufacturer's protocols. Assays were conducted in a 96-well white, opaque plate (Costar), and total luminescence was determined using an H4 plate reader (Biotek, Winooski, VT). At least two reads were conducted per well in three biologically independent experiments. As a control, total RNA was extracted as described above to verify *sXBP1* and ATF6α transcripts in transfected cells.

Western Blotting of XBP1 and ATF6α in HEK293 Cells—HEK293 cells were extracted with Nonidet P-40 lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, and 5 mM EDTA, pH 8.0). Extracts were separated by 10% SDS-PAGE, transferred to PVDF membrane using a Bio-Rad Transblot Turbo apparatus, blocked for 1 h in 5% milk, and then incubated with 1:100 anti-XBP1 (sc7160, Santa Cruz Biotechnology) or 1:100 anti-ATF6 (IMG-273, Imgenex) to confirm expression of transgenes. Membranes were detected using anti-mouse/rabbit IgG horseradish peroxidase at 1:5000 in 5% milk (Jackson Immunoresearch) and visualized with ECL Prime (GE Healthcare) on Kodak BioMax MR film.

Transfection of MCF-7 Cells—MCF-7 cells from ATCC (Manassas, VA) were cultured as described above and transfected with a Nucleofector Kit V following the manufacturer's protocol (Amaxa™). Cells were transfected with either pCGN-EGFP-sXBP1 (pCGN-sXBP1) or vehicle (pCGN-EGFP), kindly given by the laboratory of Dr. Kaufman (40, 47). The siRNAs against XBP1 as well as scrambled siRNA controls were obtained from Santa Cruz Biotechnology (sc38627 and sc37007, respectively). Total RNA was extracted using Chomczynski's method, and cDNA was synthesized using AMV-RT as described above.

Quantitative PCR of ERSE-26 Genes—qPCR was conducted on cDNA from neurons and MCF-7 cells using SYBR Green *Taq* Master Mix (Quanta Biosciences). For several ERSE-26 genes, new primers had to be designed, and the additional qPCR-validated primers were obtained from the PrimerBank database (48), a repository of primer sequences synthesized by a commercial supplier (Invitrogen). These additional primers are as follows: *GADD45B*, For 5′-TACGAGTCGGCCAAGT-

TGATG-3′ and Rev 5′-GGATGAGCGTGAAGTGGATTT-3′; *XBP1*, For 5′-TGCTGAGTCCGCAGCAGGTG-3′ and Rev 5′-GCTGGCAGGCTCTGGGGAAG-3′; *GAD2*, For 5′-ATTGGGAATTGGCAGACCAAC-3′ and Rev 5′-TTGAAGTATCTAGGATGCCCTGT-3′; *SESN2*, For 5′-AAGGACTACCTGCGGTTCG-3′ and Rev 5′-CGCCCAGAGGACATCAGTG-3′; *GAPDH*, For 5′-TGCACCACCAACTGCTTAGC-3′ and Rev 5′-GGCATGGACTGTGGTCATGAG-3′; and *HPRT1*, For 5′-CCTGGCGTGGTGATTAGTGAT-3′ and Rev 5′-AGACGTTCAGTCCTGTCCATAA-3′. For neurons and MCF-7, three technical replicates for each of the three biologically distinct preparations were conducted. For MCF-7 cells, two technical replicates per biological replicate were done. Using ERSE-26-containing gene primers that produced a single unique amplicon, the standard curves and amplification constants were determined. An Applied Biosystems 7500Fast qPCR apparatus (Invitrogen) was used, and the manufacturer's default thermal cycle was used for all experiments. Primers were verified for specificity and performance for qPCR. All output was converted to -fold induction values compared with control treatments normalized with loading controls (*HPRT1*) using Pfaffl's method (49). In the siRNA experiments, we observed too much variability in the *HPRT1* mRNA levels with siRNA and drug treatments, and therefore the data were normalized to *GAPDH*.

Chromatin Immunoprecipitation—Chromatin immunoprecipitation (ChIP) assays were performed as described previously with some modifications (34). MCF-7 cells transfected with either pCGN-XBP1 or pCGN-ATF6 were incubated with 1% formaldehyde for 10 min at 37 °C. The cross-linking was stopped with glycine at a final concentration of 0.125 M. After 5 min at 37 °C, cells were lysed in swelling buffer (10 mM Tris-HCl, pH 8, 0.25 M sucrose, 0,5% Nonidet P-40, 2 mM DTT) containing fresh protease inhibitors (38 μg/ml AEBSF, 0.5 μg/ml leupeptin, 0.1 μg/ml pepstatin, and 0.1 μg/ml *N*-α-*p*-tosyl-L-lysine chloromethyl ketone hydrochloride) and incubated for 20 min on ice in sonication buffer (50 mM Hepes, pH 7.4, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1% SDS, and protease inhibitors). Nuclear lysates were sonicated to shear the genomic DNA into fragments of 200–1000 bp. The chromatin was precleared with protein G-Sepharose (Sigma) precoated with 1 μg/ml sonicated salmon sperm nuclei (S3126, Sigma) and 1 mg/ml BSA. Chromatin was then incubated overnight with precoated protein G-Sepharose and anti-ATF6 (IMG-273, Imgenex), anti-XBP1 (sc7160, Santa Cruz Biotechnology), control IgG antibodies (3 μg), or no antibody. After washing, the immunoprecipitates were eluted two times with 200 μl of elution buffer (50 mM $NaHCO_3$, 1% SDS, 1 mM EDTA, and 50 mM Tris-HCl, pH 8.0). Cross-linking was then reversed overnight at 65 °C. The immunoprecipitated and whole cell extract DNA were purified and used for PCR analyses using primers designed to amplify only the ERSE-26 of *PRNP*: *PRNP* sense, 5′-CTGAGCCTTTCATTTTCTCG-3′, and antisense, 5′-GAGATTCGCTTGAACACTTG-3′; *HSPA5* sense, 5′-GTGAACGTTAGAAACGAA TAGCAGCCA-3′, and antisense, 5′-GTCGAC CTCACCGTCGCCTA-3′; and *ACTB* sense, 5′-CTGGAACGGTGAAGGTGACA-3′, and antisense, 5′-AAGGGACTTCCTGTAACAATG CA-3′. The PCR products were visualized after electrophoresis on a 3% agarose gel containing ethidium bromide.

Data Mining of Existing Databases—Microarray databases in GeoDataset (NCBI) were explored for evidence of increased *HSPA5* mRNA levels as evidence of ER stress. The GeoDataSet named "*In vitro* generation of long-lived human plasma cells" (50) compared Illumina microarray profiles (Illumina human HT-12, V4.0, expression beadchip) of human adult peripheral B cells differentiated into plasma cells. A significant -fold change was taken when genes identified as containing ERSE-26 had an adjusted p value of $p < 0.05$.

Statistical Analysis—For the luciferase assays, a one-way analysis of variance (ANOVA) followed by Tukey's Honest Significant Difference post hoc test was used to determine whether luminescence values were statistically significant. For qPCR data, a treatment by gene ANOVA followed by Tukey's Honest Significant Difference post hoc analyses, pairwise one-way t test or as indicated in the figure legends on individual means (using SPSS version 17) was conducted on -fold increases in cDNA amplicons.

RESULTS

sXBP1, Not ΔATF6α, Transactivates ERSE-26—To determine whether ERSE-26 responds to ER stress-regulated transcription factors ATF6α and sXBP1, human embryonic kidney HEK293 cells were co-transfected with the pML2-ERSE-26 luciferase reporter construct and pCGN-sXBP1 or pCGN-ATF6-(1–373) encoding Δ*ATF6α*. Co-transfection of pML2-ERSE-26 and sXBP1 resulted in high luciferase activity (Fig. 1*A*), whereas non-transfected cells, mock transfected cells, or cells transfected with only pML2-ERSE-26, pCGN-sXBP1, or pCGN-ATF6-(1–373) showed little luciferase activity. Mutagenesis of the putative ATF6α/sXBP1 binding site, CCACG, to ATCTA in the ERSE-26 (pML2-ERSE26m) abolished transactivation by sXBP1 (Fig. 1*B*). RT-PCR (Fig. 1*C*) and Western blots (Fig. 1*D*) confirmed the expression of sXBP1 only in cells transfected with the pCGN-sXBP1 construct. In contrast to sXBP1, ΔATF6α did not activate ERSE-26-mediated transcription (Fig. 1, *A* and *B*). Again, RT-PCR (Fig. 1*C*) and Western blots (Fig. 1*D*) confirmed increased expression of ΔATF6α in the transfected cells. The length of the 26 interspersing nucleotides of ERSE-26 influence transcriptional regulation by sXBP1, as luciferase levels were decreased by almost 50% in ERSE-30 and ERSE-34 compared with ERSE-26 (Fig. 1*E*). Lastly, ChIP analyses confirmed the interaction of sXBP1 with ERSE-26 (Fig. 1*F*). In contrast, ATF6α did not co-immunoprecipitate the ERSE-26 (Fig. 1*G*). Together, these results indicate that sXBP1, not ΔATF6α, is the transactivating factor for the ERSE-26 motif.

ERSE-26 Motif Identified in the Promoters of 37 Additional Human Genes—To assess whether ERSE-26 is present in the promoter of genes other than *PRNP*, a Python program was written to search the Genome Reference Consortium human genome (Build GRC37.1). In total, 38 genes contained an ERSE-26 within −2000 bp and +500 bp of the transcription start site of the gene (Table 2). None of these genes contained canonical ERSEs except *C6orf20*, which contains an ERSE-II motif, and *GADD45B* and *LRRC55*, which contain XBP1 bind-

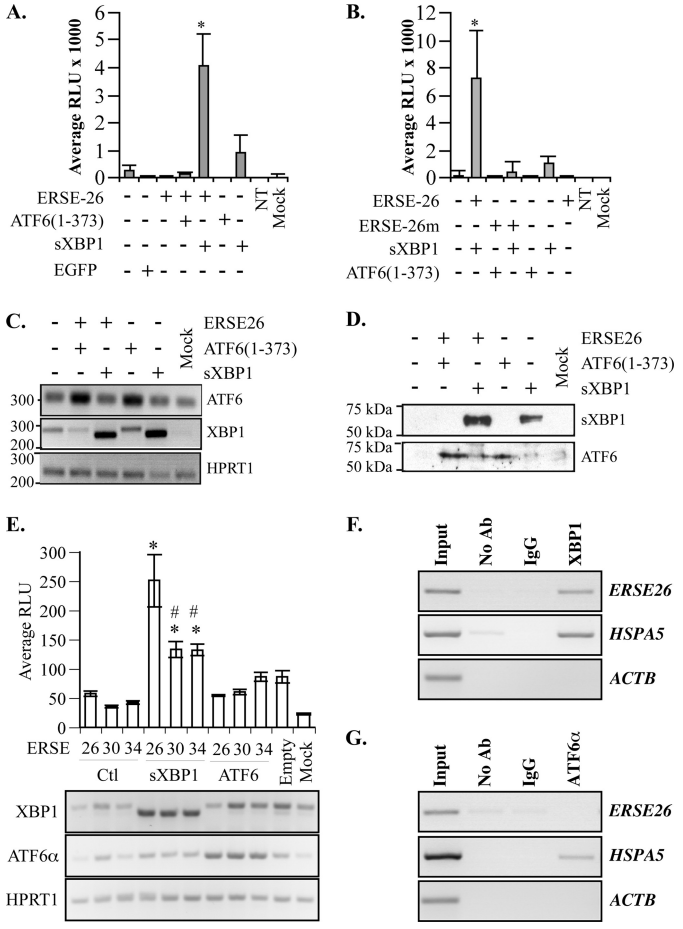

FIGURE 1. **ERSE-26 is transactivated by sXBP1.** *A*, relative luminescence units (*RLU*) from pML2 luciferase secreted from HEK293 cells transfected or co-transfected as indicated with the pML-2-ERSE-26 (ERSE-26), pCGN-ATF6α-(1–373), pCGN-sXBP1 (sXBP1), or the control pCGN-EGFP construct. The pML2 construct was used as a control (−, in all *panels*), and luminescence from non-transfected (*NT*) or mock transfected HEK293 cells is indicated in the *last two bars*. The results represent the mean ± S.E. of three independent experiments. *, $p < 0.001$ when compared with cells transfected with pML2-Empty and pCGN-sXBP1. *B*, relative luminescence units from pML2 luciferase secreted from transfected or co-transfected HEK293 cells, as indicated, to compare the transactivation of pML2-ERSE-26 *versus* the mutant pML2-ERSE-26m. The results represent the mean ± S.E. of three independent experiments. *, $p < 0.01$ when compared with cells transfected with pML2-EL26 and pCGN-sXBP1. *C*, ethidium bromide-agarose gel of sXBP1 and ATF6 amplicons obtained by RT-PCR of transfected cell mRNA. *D*, Western blot of sXBP1- and ΔATF6α-transfected HEK293 cells. *E*, relative luminescence units from HEK293 co-transfected with pML2-ERSE-26, pML2-ERSE-30, or pML2-ERSE-34 and pCGN-EGFP, pCGN-sXBP1 (sXBP1), or pCGN-ATF6α-(1–373). The results represent the mean ± S.E. of three independent experiments. *, $p < 0.001$ when comparing cells transfected with pCGN-sXBP1 to control (*Ctl*). #, $p < 0.01$ when comparing cells transfected with pML2-ERSE-30 or pML2-ERSE-34 with pML2-ERSE-26. *Lower panel*, ethidium bromide-agarose gel of *XBP1*, *ATF6α*, and *HPRT1* amplicons obtained by RT-PCR of transfected cellular mRNA. *F* and *G*, agarose gel showing RT-PCR of genes from the ChIP assay in MCF-7 cells transfected with either pCGN-XBP1 (*F*) or pCGN-ATF6 (*G*). Cross-linked chromatin immunoprecipitated with anti-XBP1 (*F*) or anti-ATF6α antibody, rabbit IgG, or no antibody (*G*) was used for PCR amplification of the *PRNP* ERSE-26 motif. The *HSPA5* promoter was used as a positive control, whereas PCR on the promoter of *ACTB* was used as an internal negative control.

ing sites in their promoters. Twenty-six ERSE-26 matches were found in the reverse (3′ to 5′) direction, and 12 were found in the forward direction. As a control for the search program, the search was repeated with the canonical ERSE-9 as a target pattern. Forty-four genes, including the previously known ERSE-containing genes *HSPA5* (BiP, Grp78), *XBP1*, and *PDIA6*, contained an ERSE-9 within −2000 bp to +500 bp of the transcription start site of the gene (Table 3). These results show that the ERSE-26 motif is not unique to the *PRNP* promoter and suggest that ER stress may regulate a wide variety of genes previously unsuspected to be involved in the ER stress response.

To assess whether ERSE-26 is evolutionarily conserved, we searched the yeast genome. The search identified eight yeast genes containing ERSE-26 (Table 4), but none of these were the same as the mammalian ERSE-26 genes. However, three of the eight genes may be relevant to ER function. Fun12p functions as a translation initiation factor (51), Chs6p is involved in Golgi to plasma membrane protein trafficking (Saccharomyces Genome Database), and Hap1p is a zinc finger transcriptional factor responding to oxygen and heme levels (52). These data indicate that ERSE-26 exists in lower organisms but not necessarily in genes that are orthologous to the mammalian ERSE-26 genes.

ERSE-26 Genes Are Up-regulated in Primary Human Neuron Cultures Treated with BFA, TM, or Thps—To confirm that the ERSE-26-containing promoters are transactivated during ER stress, primary human neurons were treated with the ER stressors BFA, TM, and Thps (Fig. 2). RT-PCR identified transcripts from 20 of the 38 ERSE-26 genes in primary human neuron cultures (Fig. 2*A*). ER stress induction under these conditions was confirmed by the presence of the *sXBP1* amplicon (Fig. 2, *A* and *B*) or increased *HSPA5* (BiP) mRNA (Fig. 2*B*) or protein (Fig. 2*C*) levels. The levels of ER stress-induced sXBP1 varied with the type of ER stress and also showed variable levels of induction in independent neuron cultures. Nevertheless, relative to the DMSO control, each of the pharmacological inducers of ER stress (BFA, TM, and Thps) induced expression of many of the ERSE-26 genes in at least one human neuron culture (Fig. 2*A*). To assess the levels of mRNA in a more quantitative manner, qPCR was conducted on a few ERSE-26-containing genes (Fig. 2*B*). Compared with the DMSO control, the three ER stressors significantly increased the levels of *HSPA5* and *XBP1*. Although the levels of *HSPA5* mRNA were similarly increased by each of three ER stressors, the -fold increase in *XBP1* levels varied considerably (20–60-fold). *PRNP* mRNA levels increased by 3–8-fold with the three different ER stressors. *ERLEC1* mRNA levels increased 4–6-fold with BFA and TM but not Thps. *GADD45B* mRNA increased 2-fold with Thps only. *SESN2* levels increased significantly (8–12-fold) with TM and Thps, whereas *SCL38A5* levels were not significantly increased with any of the three ER stressors. These variable results probably reflect inherent differences in mRNA stability for different genes or the presence of other transactivating or silencing factors in the promoter of these genes. Furthermore, the variability in response in different primary human neuron preparations indicates a potential influence of the genetic background on the ER stress response. As a negative control, neurons were submitted to serum deprivation as another stress. Compared with either the DMSO control or the non-treated cells, the mRNA levels of the ERSE-26-containing genes did not increase and neither did those of the ER stress-regulated genes *HSPA5* and *XBP1*. Together, these results indicate that ER stress can modulate the expression of many ERSE-26-containing genes.

TABLE 2
Genes containing an ERSE-26 in their promoters

Entrez GeneID	Gene Symbol	Match Direction	Product	Entrez GeneID	Gene Symbol	Match Direction	Product
123970	C16orf78	R	chromosome 16 open reading frame 78	79954	NOL10	R	nucleolar protein 10
1E+08	C1orf68	R	chromosome 1 open reading frame 68	53343	NUDT9	R	nudix-type motif 9
168090	C6orf118	R	chromosome 6 open reading frame 118	58531	PRM3	R	protamine 3
404220	C6orf201	R	chromosome 6 open reading frame 201	5621	PRNP	F	prion protein
56548	CHST7	R	carbohydrate (N-acetylglucosamine 6-O) sulfotransferase	5734	PTGER4	F	prostaglandin E receptor 4
10491	CRTAP	F	cartilage associated protein	3053	SERPIND1	R	serpin peptidase inhibitor, clade D
27248	ERLEC1	R	endoplasmic reticulum lectin 1	83667	SESN2	R	sestrin 2
4303	FOXO4	R	forkhead box O4	92745	SLC38A5	F	solute carrier family 38, member 5
2572	GAD2	F	glutamate decarboxylase 2	6609	SMPD1	F	sphingomyelin phosphodiesterase 1, acid lysosomal
4616	GADD45B	F	growth arrest and DNA-damage-inducible, beta	6687	SPG7	R	spastic paraplegia 7
2706	GJB2	R	gap junction protein, beta 2, 26kDa	51332	SPTBN5	F	spectrin, beta, non-erythrocytic 5
2776	GNAQ	R	guanine nucleotide binding protein, q polypeptide	96764	TGS1	F	trimethylguanosine synthase
3033	HADH	R	hydroxyacyl-Coenzyme A dehydrogenase	10469	TIMM44	F	translocase of inner mitochondrial membrane 44
8372	HYAL3	R	hyaluronoglucosaminidase 3	64418	TMEM168	F	transmembrane protein 168
25948	KBTBD2	R	kelch repeat and BTB (POZ) domain containing 2	7375	USP4	R	ubiquitin specific peptidase 4
1E+08	LOC100131680	R	similar to protein-kinase, interferon-inducible dsRNA dependent inhibitor	83719	YPEL3	R	yippee-like 3 (Drosophila)
78999	LRFN4	R	leucine rich repeat and fibronectin type III domain containing 4	170960	ZNF721	F	zinc finger protein 721
219527	LRRC55	R	leucine rich repeat containing 55	124626	ZPBP2	R	zona pellucida binding protein 2
24142	NAT6	R	N-acetyltransferase 6 (GCN5-related)	150678	MYEOV2	R	myeloma overexpressed 2

ERSE-26-containing Genes Are Up-regulated in ER-stressed Breast Carcinoma MCF-7 Cells—To determine whether the variability observed in different human neuron preparations is dependent on the genetic background of the cells, we conducted the same experiments on the breast carcinoma MCF-7 cell line. qPCR results showed a more consistent induction of the various genes studied in independent experiments (Fig. 3*A*). However, some of the genes responded differently to the three ER stressors. *HSPA5* levels increased 35-fold with BFA but only 10–12-fold with TM and Thps treatments. Similar results were

TABLE 3
Genes containing an ERSE-9 in their promoters

Entrez GeneID	Gene Symbol	Match Direction	Product	Entrez GeneID	Gene Symbol	Match Direction	Product
79087	ALG12	F	alpha-1,6-mannosyltransferase	83746	L3MBTL2	R	l(3)mbt-like 2 (Drosophila)
242	ALOX12B	F	arachidonate 12-lipoxygenase	147945	NLRP4	F	NLR family, pyrin domain containing 4
342371	ATXN1L	R	ataxin 1-like	56104	PCDHGB1	F	protocadherin gamma subfamily B, 1
130162	C2orf63	R	chromosome 2 open reading frame 63	10130	PDIA6	F	protein disulfide isomerase
222166	C7orf41	R	chromosome 7 open reading frame 41	646780	PHKA1P1	R	Pseudo
821	CANX	F	calnexin	5260	PHKG1	R	phosphorylase kinase, gamma 1 (muscle)
858	CAV2	R	caveolin 2	648791	PPP1R3G	F	protein phosphatase 1, regulatory (inhibitor)
1160	CKMT2	F	creatine kinase, mitochondrial 2 (sarcomeric)	7905	REEP5	R	receptor accessory protein 5
79009	DDX50	F	DEAD (Asp-Glu-Ala-Asp) box polypeptide	1E+08	RPS16P6	R	microRNA:hsa-mir-192
83939	EIF2A	F	eukaryotic translation initiation factor 2A	26135	SERBP1	R	SERPINE1 mRNA binding protein 1
2009	EML1	R	echinoderm microtubule associated protein like 1	126669	SHE	F	Src homology 2 domain containing E
57579	FAM135A	F	family with sequence similarity 135	9197	SLC33A1	F	solute carrier family 33, member 1
113828	FAM83F	R	family with sequence similarity 83, member F	26776	SNORA71B	R	small nucleolar RNA, H/ACA box 71B
11153	FICD	F	FIC domain containing	84447	SYVN1	F	synovial apoptosis inhibitor 1, synoviolin
448831	FRG2	F	FSHD region gene 2	54499	TMCO1	F	transmembrane and coiled-coil domains 1
441581	FRG2B	F	FSHD region gene 2 family, member B	84286	TMEM175	R	transmembrane protein 175
9815	GIT2	R	ArfGAP 2	25963	TMEM87A	R	transmembrane protein 87A
2742	GLRA2	F	glycine receptor, alpha 2	7325	UBE2E2	R	ubiquitin-conjugating enzyme E2E2
7184	HSP90B1	F	HSP90 (Grp94)	9736	USP34	R	ubiquitin specific peptidase 34
3309	HSPA5	F	GRP 78 (BiP)	79674	VEPH1	F	ventricular zone expressed PH domain
3617	IMPG1	F	interphotoreceptor matrix proteoglycan 1	7494	XBP1	F	X-box binding protein 1
9776	KIAA0652	F	KIAA0652	7565	ZNF17	F	zinc finger protein 17

observed with *PRNP* and *ERLEC1*. In contrast, *XBP1*, *GADD45B*, *SESN2*, and *SLC38A5* levels increased similarly with the three different ER stressors. Except for TM on *SESN2*, all ERSE-26 genes had a significant increase in gene expression with the three different ER stressors when compared with control DMSO-treated cells. Although the MCF-7 cells responded more consistently to ER stress than primary cultures of human neurons, there was also a differential response of these genes to different ER stressors. To determine whether this differential response was dependent on XBP1 levels, MCF-7 cells were transfected with pCGN-EGFP-XBP1 or control vector pCGN-EGFP. As expected, *sXBP1* mRNA levels were increased 25-fold over pCGN-EGFP-transfected cells (Fig. 3*B*). *HSPA5*, *PRNP*, and *ERLEC1* mRNA levels increased 2–3-fold with XBP1 expression, but neither the *GADD45B* nor the *SESN2* mRNA level was modulated by XBP1 overexpression. Overexpression

TABLE 4
Yeast genes containing an ERSE-26 in their promoters

Match pattern		Distance from start	Match direction[a]	Gene name	Gene product	Function
		bp				
gcacc	taacc	−609, −573	F	*FUN12*	Fun12p	Translation initiation Factor IF2
ccaat	ccacg	−858, −822	F	*BUD9*	Bud9p	Controls yeast polarity and development.
ggtta	ggtgc	−1291, −1255	R	*MTC1*	Mtc1p	Maintenance of telomere-capping protein 1
gcacc	taacc	−479, −443	F	*CHS6*	Chs6p	Export of cargo from Golgi to plasma membrane
gcacc	taacc	−1293, −1257	F	*SMX2*	Smx2p	Part of spiceosome
gcacc	taacc	−550, −514	F	*HAP1*	Hap1p	Zinc finger transcriptional factor responding to O2 and heme
gcacc	taacc	378, 414	F	*RKM5*	Rkm5p	Protein-lysine methyltransferase
gcacc	taacc	−1053, −1017	F	*NHA1*	Nha1p	$Na^+/H+$ antiporter

[a] R, reverse; F, forward.

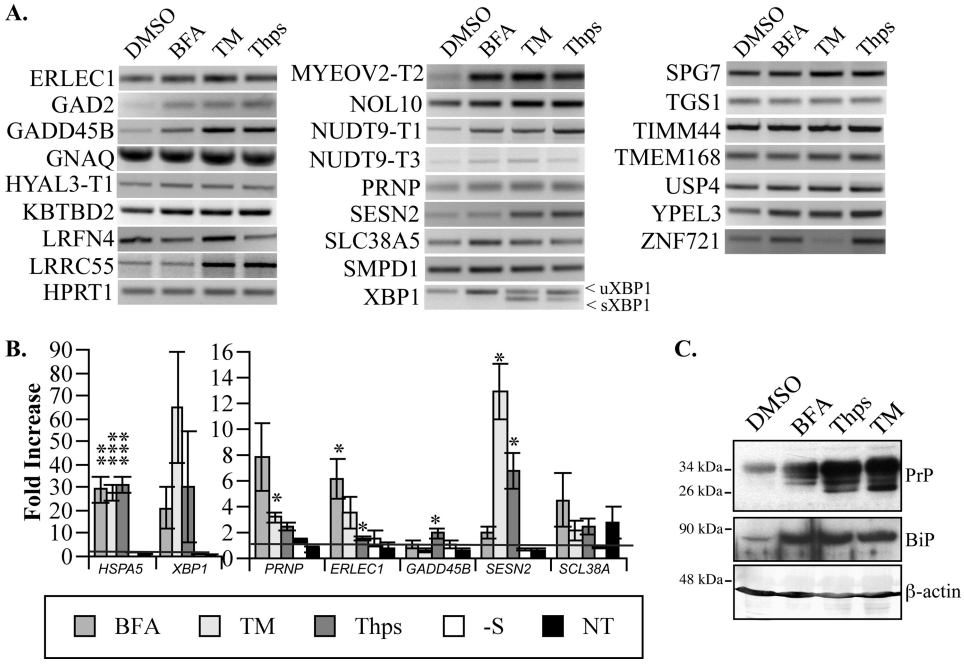

FIGURE 2. **Induction of ER stress increases ERSE-26 gene expression in cultured primary human neurons.** *A*, ethidium bromide-stained agarose gel of ERSE-26-containing gene amplicons. *Each panel* is a representative example from each gene, respectively. *B*, -fold increase of the indicated gene expression by qRT-PCR over the level obtained in the DMSO-treated control cells and arbitrarily set at 1. In addition to treatment with BFA, TM, and Thps, serum deprivation (−*S*) and non-treated cells (*NT*) were analyzed. The data represent duplicates of three independent neuron cultures; mean ± S.E. *C*, Western blot analyses of MCF-7 cell protein extracts for PrP, BiP, and β-actin levels.

of XBP1 protein in transfected MCF-7 cells was confirmed by Western blotting (Fig. 3*C*).We then tested the regulation of these genes by XBP1 in MCF-7 cells treated with BFA in the absence or presence of scrambled siRNA (siCtl) or siRNA against XBP1 (siXBP1). BFA increased the levels of *sXBP1* mRNA in MCF-7 cells and thus provided a model to determine whether sXBP1 can up-regulate ERSE-26-containing genes in live cells (Fig. 3*D*). Unexpectedly, the *HPRT1* mRNA levels varied with the different treatments, and thus we had to use *GAPDH* mRNA levels to normalize instead of *HPRT1*. *XBP1* mRNA level increased 2-fold in BFA-treated MCF-7 cells compared with the DMSO control-treated cells (Fig. 3*E*). The siXBP1 decreased these levels by 80%. *PRNP*, *HSPA5*, *ERLEC1*, and *SESN2* mRNA levels increased with BFA treatment. In the presence of siXBP1, *PRNP* and *ERLEC1* mRNA levels decreased by 50%, but *HSPA5* was only slightly decreased. This was not unexpected, because *HSPA5* is regulated also by ATF6α and ATF4 (36, 53). Furthermore, siXBP1 did not prevent BFA-induced *SESN2* mRNA levels, consistent with the inability of the sXBP1 to up-regulate this gene in MCF-7 cells (Fig. 3*B*). *GADD45B* was not up-regulated by BFA when normalized to *GAPDH*, and basal levels were unaffected by the siXBP1. Lastly, we confirmed the down-regulation of XBP1 protein in siXBP1-treated cells (Fig. 3*E*). Together, these results show that ER stress-induced or overexpressed sXBP1 can transactivate ERSE-26-containing genes but that the ERSE-26 motif is not always sufficient for sXBP1 transactivation.

Evidence for ERSE-26 Physiological Function—To determine whether the ERSE-26 motif is potentially transcribed by sXBP1 in a physiological condition, we examined available microarray data sets comparing the mRNA profiles of human XBP1-dependent B cell differentiation into plasma cells (Table 5) (54). In this array, *XBP1* was up-regulated 4.3-fold (Log2). All ERSE-26 genes identified by our bioinformatics analysis were present in the data set, and 13 of 38 showed a significant (adjusted $p <$ 0.05) Log2 -fold change in mRNA levels when activated B cells were differentiated into plasma cells. Of the 13 genes, *ERLEC1* (2.4-fold), *FOXO4* (1.29-fold), and *YPEL3* (2.1-fold) were up-regulated, and *PTGER4* was down-regulated (−2.3-fold). Foxo4 negatively regulates cell cycle and thus could be up-reg-

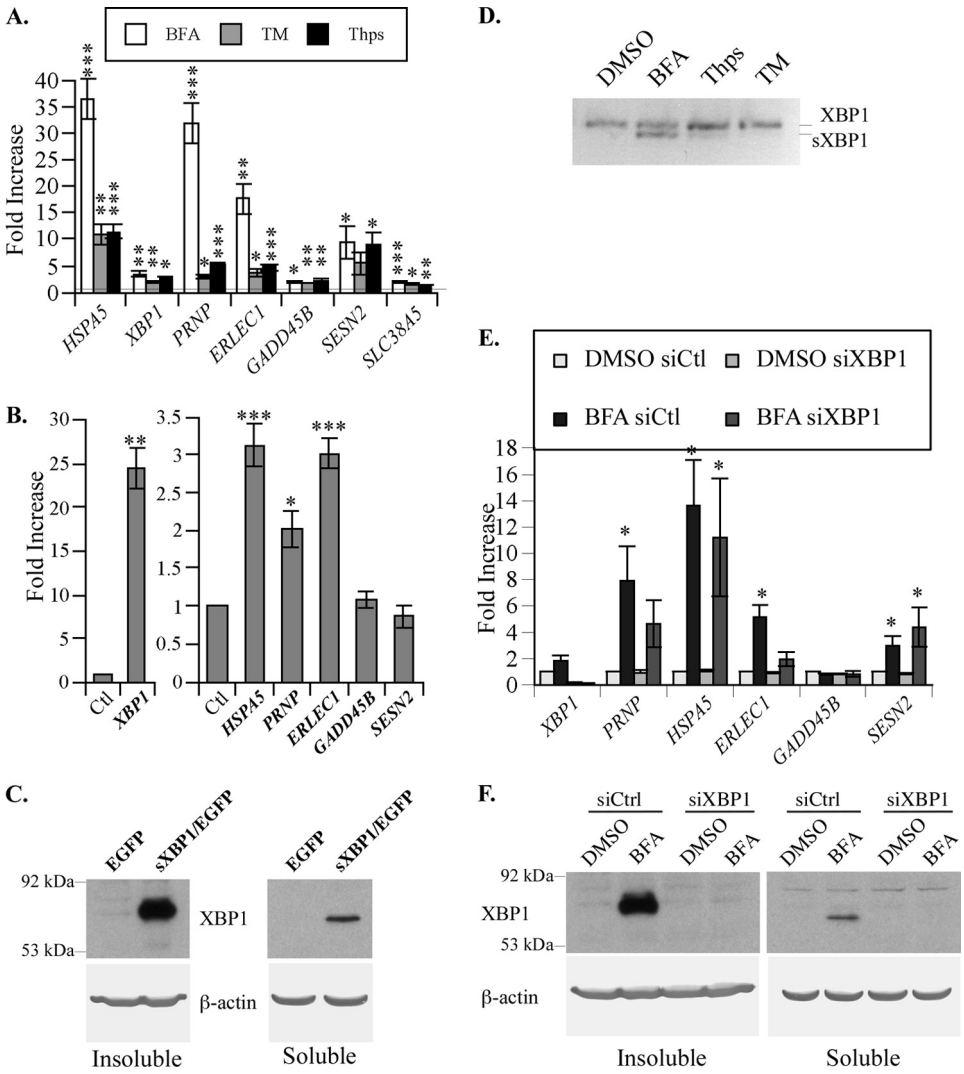

FIGURE 3. **MCF-7 cells show induced ERSE-26 gene expression following ER stress treatment or sXBP1 transfection.** *A*, -fold induction of ERSE-26 genes in MCF-7 cells treated with BFA, TM, or Thps for 18 h as determined by qRT-PCR. ANOVA, $p < 0.001$ followed by planned paired *t* test using a Bonferroni correction between DMSO and BFA, DMSO, and TM and between DMSO and Thps. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *B*, ERSE-26 gene expression levels in pCGN-EGFP-sXBP1-transfected MCF-7 cells compared with pCGN-EGFP-transfected cells (control (*Ctl*)). The results represent an average ± S.E. of three experiments done in triplicate. A paired *t* test was used to assess XBP1 levels (*left panel*), and ANOVA ($p < 0.0001$) and Tukey-Kramer multiple comparisons were used to assess the levels of gene expression in the *right panel*. ***, $p < 0.001$; *, $p < 0.05$. *C*, Western blot with anti-XBP1 or anti-β-actin antibodies in Nonidet P-40-soluble or -insoluble protein extracts from MCF-7 cells transfected with pCGN-EGFP (*EGFP*) or pCGN-sXBP1-IRES-EGFP (*sXBP1/EGFP*). *D*, ethidium bromide-stained agarose gel of XBP1 RT-PCR from MCF-7 cells treated with BFA, Thps, and TM. *E*, ERSE-26 gene expression levels in siRNA scrambled control (*siCtl*) or siRNA against XBP1 (*siXBP1*)-transfected MCF-7 cells submitted to BFA. The results represent the mean ± S.E. of four independent experiments. *, $p < 0.05$ comparing ±BFA. *F*, Western blot with anti-XBP1 or β-actin antibodies in Nonidet P-40-soluble and -insoluble protein extracts from MCF-7 cells treated with DMSO or BFA (5 μg/ml, 18 h) 24 h after transfection with control scrambled or XBP1-targeting siRNAs.

ulated to stop proliferation and allow differentiation of the B cells into plasma cells (55). *ERLEC1* is an ER-resident protein that has been implicated in metastasis and the survival of lung cancer cells, in the regulation of the unfolded protein response, and in trafficking of glycoprotein in the ER quality control system (56). Ypel3 is associated with cellular senescence and is repressed in colon and mammary tumors, suggesting a novel role as a tumor suppressor (57). Ptger4 promotes proliferation in endometrial cancers (58). Therefore, these ERSE-26-containing genes could be implicated in differentiated plasma cells via XBP1.

DISCUSSION

The role of ER stress in disease is broad, and many conditions that disturb ER homeostasis by affecting protein folding can have a large impact on the physiological state of cells and organisms (2). Here, we added ERSE-26, an ER stress-dependent regulatory motif, to the already complex ER stress response. We show that ERSE-26 (CCAAT-N26-CCACG), first identified in the human *PRNP* promoter (34), is transactivated by sXBP1 in a luciferase reporter system and that mutagenesis of CCACG to ATCTA abolishes the ability of sXBP1 to transactivate ERSE-26. Furthermore, we show that ER stressors in mammalian cells increased the levels of mRNA from genes containing ERSE-26 and other ER stress-regulatory elements (*PRNP* and *GADD45B*) and in genes with no other known ER stress regulatory elements (*ERLEC1* and *SESN2*). We also show that expression of sXBP1 in MCF-7 cells is sufficient to significantly increase *PRNP* gene expression and that siRNA against *XBP1* stunts the BFA-mediated increase of *PRNP* mRNA levels. This

TABLE 5
Differential mRNA gene expression of ERSE-26 genes between activated B cells and plasma cells (50)

ID	Adjusted p value	Log_2 Fold Change	Gene Symbol	Gene Title
Genes containing ERSE-26				
ILMN_1724376	1.73e-06	2.37036547	ERLEC1	Endoplasmic reticulum lectin 1
ILMN_1712095	5.47e-07	1.26973932	FOXO4	Forkhead box O4
ILMN_1769388	5.67e-05	0.92537059	GJB2	Gap junction protein, beta 2, 26kDa
ILMN_1719906	1.78e-05	-0.66041363	HADH	Hydroxyacyl-CoA dehydrogenase
ILMN_1667432	1.02e-03	-0.52584261	HYAL3	Hyaluronoglucosaminidase 3
ILMN_1809417	1.12e-03	-0.34495628	LRFN4	Leucine rich repeat and fibronectin type III domain containing 4
ILMN_1765001	2.34e-02	0.22949593	NAT6	N-Acetyltransferase 6 (GCN5-related)
ILMN_1775011	5.28e-04	0.53481764	NOL10	Nucleolar protein 10
ILMN_1737988	2.20e-03	0.43313238	PRNP	Prion protein
ILMN_1795930	1.16e-08	-2.31003976	PTGER4	Prostaglandin E receptor 4 (subtype EP4)
ILMN_1757370	6.95e-03	-0.2921249	SMPD1	Sphingomyelin phosphodiesterase 1, acid lysosomal
ILMN_1651506	3.00e-02	-0.26074315	TGS1	Trimethylguanosine synthase 1
ILMN_1791147	7.77e-08	2.14668332	YPEL3	Yippee-like 3 (Drosophila)
Genes responding to ER stress.				
ILMN_2365465	1.13e-12	4.30665755	XBP1	X-box binding protein 1
ILMN_1814313	2.72e-01	0.13010947	ATF6B	Activating transcription factor 6 beta
ILMN_1672128	5.42e-06	1.1635569	ATF4	Activating transcription factor 4 (tax-responsive enhancer element B67
ILMN_1773865	5.39e-08	1.75110177	HSPA5	Heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)

response is surprising because the canonical ERSE CCAAT-N9-CCACG is transactivated by ΔATF6α, not sXBP1, and disruption of N9 abolishes ΔATF6α transactivation of ERSE (36, 37). In contrast, we found that ATF6α does not interact with or transactivate ERSE-26. Our results suggest that CCAAT-NX-CCACG motifs present in gene promoters may have greater functionality in the response to ER stress than believed previously.

The bioinformatic search for gene promoters containing ERSE-26 identified 38 genes, including *PRNP*. Except for *C6orf20*, *GADD45B*, and *LRRC55*, none of these genes had been identified previously as ERSE-containing or ER stress-responsive genes. The function of the ERSE-26 genes was studied to determine whether these could link the ER stress response via the ERSE-26 motifs with specific functions. One notable functional group was genes related to cell adhesion and synapse function. *GAD2*, *LRFN4*, *LRRC55*, and *SLC38A5* all play a role in this process. *GAD2* is one of two terminal rate-limiting enzymes that cleave glutamic acid into γ-aminobutyric acid (59). *LRFN4*, also known as *SALM3*, binds post-synaptic density

95 protein and induces neurite outgrowth (60). *LRRC55* enhances the activity of calcium-activated potassium channels (61). Finally, *SLC38A5*, also known as *SN2* or *SNAT5*, is an astrocyte-expressed amino acid transporter that permits astrocytes to release glutamic acid for neurons to cleave to γ-aminobutyric acid (62). Together with the suspected role of PrP at the synapse (reviewed in Ref. 63), these results raise the possibility that ER stress regulates synaptic function in neurons through the ERSE-26 motif.

Another group has identified oxidative stress function. Because protein folding is a redox state-dependent, energy-requiring process, an overlap between the ER stress response and the oxidative stress response is logical. The genes *NUDT9*, *TIMM44*, *SESN2*, and *GADD45B* are localized in the mitochondria and respond to oxidative stress (64–67). *GADD45B* is an activator of pro-survival p38 MAPK signaling (66, 68, 69). Both *TIMM44* and *NUDT9* localize to the mitochondria, and *TIMM44* acts as a translocase to allow import of peptides/proteins to the inner mitochondrial membrane (70). *SESN2* and *GADD45B* are both regulated by p53 in response to environmental stresses (66, 71). Interestingly, our results included a transcription factor, *FOXO4*. The FoxO family of transcription factors regulates sestrin family members, which suggests a possible feedback loop in ERSE-26 genes (64). Finally, *PRNP* contains a p53 binding site, is induced in oxidative stress, and protects cells against oxidative stress (8–10, 72–74). Therefore, ER stress regulation via ERSE-26 in these genes may allow functions related to oxidative stress.

There is also evidence that some of the ERSE-26 genes are up-regulated in an XBP1-dependent manner during B cell differentiation into plasma cells. These results support a role for ERSE-26-dependent gene expression in a human physiological system.

Our results show that the -fold increase in ER stress-mediated gene expression is highly variable and can be regulated by genetic background, cell type, and ER stressor. Furthermore, despite clear evidence that XBP1 regulates ERSE-26 in the *PRNP* and *ERLEC1* promoters, not all ERSE-26-containing genes are up-regulated by XBP1 only. Elucidating how and why the ER stress response is so variable remains a future goal, but these results illustrate once again the complexity of the ER stress response. Nevertheless, our data identify a novel XBP1-regulated ERSE and reveal previously unknown ER stress-regulated genes.

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