BIOLOGY OF REPRODUCTION **79,** 546–561 (2008)
Published online before print 14 May 2008.
DOI 10.1095/biolreprod.108.068106

A Proteomic Comparison of Immature and Mature Mouse Gonadotrophs Reveals Novel Differentially Expressed Nuclear Proteins that Regulate Gonadotropin Gene Transcription and RNA Splicing[1]

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ABSTRACT

The alphaT3–1 and LbetaT2 gonadotroph cell lines contain all the known factors required for expression of gonadotropin genes, yet only the LbetaT2 cells express the beta subunits. We hypothesized that comparison of their nuclear proteomes would reveal novel proteins and/or modifications that regulate expression of these genes. We identified nine proteins with different expression profiles in the two cell lines, of which several were chosen for further functional studies. Of those found at higher levels in alphaT3–1 nuclei, 1110005A23RIK was found associated with the *Fshb* gene promoter and repressed its expression. Transgelin 3 overexpression reduced transcript levels of *Fshb*, and its knockdown elevated *Lhb* and *Cga* transcript levels, indicating an ongoing repressive effect on these more highly expressed genes, possibly through altering levels of phosphorylated mitogen-activated protein kinase. Heterogeneous nuclear ribonucleoprotein A2/B1 repressed splicing of the *Fshb* primary transcript, which it binds in the first intron. Proteins at higher levels in LbetaT2 nuclei included prohibitin, the overexpression of which reduced promoter activity of all three gonadotropin subunits, and appeared to mediate the differential effect of GnRH on proliferation of the two cell lines; its knockdown also altered cell morphology. Two other splicing factors were also found at higher levels in LbetaT2 nuclei: the knockdown of PRPF19 or EIF4A3 decreased splicing of *Lhb*, or of both beta subunit transcripts, respectively. The levels of *Eif4a3* mRNA were increased by activin, and both factors increased *Fshb* splicing. This study has revealed a number of novel factors that alter gonadotropin expression and gonadotroph function, and likely mediate or moderate effects of the regulatory hormones.

differentiation, follicle-stimulating hormone, gonadototroph, gonadotropins, luteinizing hormone, pituitary, proteomics

INTRODUCTION

The two mouse pituitary cell lines, αT3–1 and LβT2, were established by targeted oncogenesis using the promoter of either the common α subunit gene (*Cga*), or that of the luteinizing hormone β subunit (*Lhb* [1]). They have proven to be an invaluable tool in the study of regulation of the gonadotropins, and have allowed a major surge in advancement of this field over the last decade (reviewed in [2, 3]). The cells represent gonadotrophs at approximately 11 or 16.5 days of mouse embryonic development; the immature αT3–1 gonadotroph cells express the common α subunit, CGA, but neither of the gonadotropin β subunit genes, although they do express the GnRH receptor and the transcription factors known to be required for β subunit gene expression. We have shown that both β subunit genes are repressed in the αT3–1 cells by the actions of histone deacetylases (HDACs; [4]), but the mechanism behind the differential recruitment of HDACs to these genes in the two cell lines remains unclear. The more mature LβT2 gonadotroph cells abundantly express *Lhb* at basal levels, and the follicle-stimulating hormone β subunit (*Fshb*) only at low levels [5, 6]. As such, a comparison between these two cell lines should reveal differences in expression or modification of proteins that have a novel and crucial regulatory role in determining gonadotropin β subunit gene expression.

Several groups have taken high-throughput approaches to study these cells, although they have been limited to genomic analyses. Extensive studies by Sealfon et al. [7–10] utilized microarrays to detect genes, the expression of which is altered following 1, 3, or 6 h continuous GnRH treatment in the mature LβT2 cells. Their work provided information regarding a number of signaling molecules and transcription factors whose role in the possible regulation of gonadotropin genes by GnRH has been integrated into a model of regulatory circuits (e.g., [7–10]). A similar microarray study was also carried out by Kakar et al. in the same cells after 1 or 24 h GnRH treatment, in which some, but not all, of the same regulated genes were identified [11]. More recently, the downstream cross-talk of stimulation by GnRH and activin was assessed in LβT2 cells using microarrays to determine how the transcriptional responses of GnRH-targeted genes are affected by activin pretreatment [12]. In perifused LβT2 cells, microarrays contributed to the discovery of a potential mechanism for differential regulation of gene expression by pulse frequency and amplitude [13]. We have also employed a screen of regulated genes in these cells following 8 h GnRH treatment using subtractive hybridization, which revealed that ubiquitin conjugating enzyme 4 transcription is increased by GnRH. We showed that this ubiquitylates the estrogen receptor α (ESR1) in a form of cross-talk regulating activity of the *Lhb* gene promoter [14].

A study of the differential expression of genes in the immature αT3–1 and mature LβT2 cells was made by Quirk et al. [15] using differential display PCR. This led to the identification of a high mobility group-like nuclear phosphoprotein, p8, which is more highly expressed in the mature LβT2 cells, and its expression also coincided with that of the *Lhb* gene in the developing mouse pituitary. Gene knockout of

[1]Supported by funding from the Biomedical Research Council, Singapore, and the Academic Research Fund, National University of Singapore to P.M. and National Institutes of Health grants R01 HD43758 and K02 HD40803 to M.A.L.
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Received: 3 February 2008.
First decision: 22 February 2008.
Accepted: 23 April 2008.

ISSN: 0006-3363. http://www.biolreprod.org

p8 in these cells by antisense RNA diminished the expression the LHB subunit and the activity of the *Lhb* gene promoter. However, it is not found in the adult pituitary, and its overexpression failed to induce *Lhb* gene expression in immature αT3–1 cells, or to enhance activity of the *Lhb* gene promoter in LβT2 cells. Therefore, this protein is necessary, but not sufficient for the expression of the *Lhb* gene, and may only have a temporal function in the terminal differentiation of these cells during embryogenesis, possibly involving modifications of the chromatin. It is also essential for the maintenance of tumorigenicity of LβT2 cells [15, 16].

As an alternative approach in the current study, we have used two-dimensional (2D) gel electrophoresis to profile the nuclear proteomes of the two cell lines in order to identify differentially expressed or modified proteins. In comparison to the high-throughput genomic approaches, this has the obvious advantage of studying the functional molecule rather than just the transcript, and also potentially enables identification of differential posttranslational modifications and/or cellular localization, which affect protein activity. We hypothesized that, using this technique, we could identify novel factors differentially expressed in the two cell types that are responsible for the expression of the gonadotropin β subunit genes in the mature cells, and/or their repression in the immature cells. Having identified differentially expressed nuclear proteins, we went on to carry out functional studies in this context.

MATERIALS AND METHODS

Cell Culture and Nuclear Protein Extraction

LβT2 and αT3–1 cells (gifts from Dr. P. Mellon, University of California, San Diego) were cultured as previously described [14, 17], and some were treated with GnRH (10 nM), activin (100 ng/ml) (both from Sigma, St. Louis, MO), or the MEK inhibitor, PD98059 (50 μM; Calbiochem), as indicated. Cells were washed with PBS, and cell nuclei were isolated with NUCLEI EZ PREP (Sigma) kit, or the nuclear proteins were extracted using the NE-PER kit (Pierce, Rockford, IL). The isolated nuclei (first protocol) were homogenized by sonication (30 W, 30 s) in Tris-HCl buffer (40 mM, pH 8.0), and nuclear proteins were exacted by addition of an equal volume of 8 M urea. For both protocols, nucleic acids and insoluble materials were removed by ultracentrifugation (200 000 × *g*, 1 h, 20°C) after the addition of spermidine (3 mM; only for the Sigma kit extraction). After dilution (to 2 M urea), proteins were precipitated (14 000 rpm, 30 min) following 30 min on ice in 20% trichloroacetic acid. The precipitate was washed four times in 1 ml acetone and the pellet redissolved in 8 M urea buffer before treating with the ProteoPrep Reduction and Alkylation kit (Sigma). The samples prepared using the NUCLEI EZ PREP kit were further desalted by gel-filtration column (Pierce). Protein concentration was measured, and trace bromophenol blue was added before storing samples at −30°C for immediate use.

Two-Dimensional Gel Electrophoresis

Nuclear proteins were dissolved in 300 μl of rehydration buffer 1 (RB1) (7 M urea, 2 M thiourea, and 2% CHAPS). Proper Biolytes (Bio-Rad, Hercules, CA) and DTT were added to a final concentration of 0.2 % and 10 mg/ml, respectively. Trace bromophenol blue was also added to visualize the rehydration process. Two-dimensional gel electrophoresis was then carried out as previously described [18].

After electrophoresis, the gel was fixed and visualized by sliver staining, after which the 2D gels were compared manually for identification of differentially expressed proteins. Those protein spots found differentially expressed in at least three pairs of gels were excised, destained, and equilibrated with 100 mM ammonia bicarbonate for 10 min. The samples were then dehydrated with 50% acetonitrile in 50 mM ammonium bicarbonate for 5 min, followed by acetonitrile for 5 min, and this process repeated three times. The gel pieces were dried in a vacuum centrifuge before rehydration in 100 mM ammonium bicarbonate. They were then dehydrated twice more, and were dried to completion with vacuum centrifuge before reswelling in 15 μl digestion solution containing 12.5 ng/μl trypsin (Sequencing Grade Modified Trypsin; Promega) in 50 mM ammonium bicarbonate at 4°C for 30 min. The digestion was carried out for 15 h at 37°C.

After digestion, the reaction was cooled to room temperature and centrifuged at 6000 × *g* for 15 min, and the supernatant was saved. The gel pieces were washed in 15 μl 20 mM ammonium bicarbonate solution and then treated with 15 μl of 5% formic acid in 50% acetonitrile for 10 min, followed by centrifugation at 6000 × *g* for 10 min. Both wash and extraction solution were pooled, and the procedure was repeated before the pooled solutions were dried to completion. The dried, digested peptides were redissolved and applied to the matrix-assisted laser desorption/ionization plates for peptide mass fingerprinting using the PerSeptive Biosystems Voyager-DE STR BioSpectrometry Workstation (PerSeptive Biosystems, Framingham, MA), as previously described [18], and analyzed by the accompanying Data Explorer software. The masses of the peptides were queried against the National Center for Biotechnology Information (Bethesda, MD) nonredundant database using the Ms-Fit program (http://prospector.ucsf.edu), the Mascot program from Matrix Sciences (http://www.MatrixScience.com), or using Aldente (http://www.expasy.org/tools/aldente/) against the Swiss-Prot/TrEMBL databases. The first hit with a significant molecular weight (MW) search score, protein coverage, and reasonable MW and isoelectric point (pl) was generally accepted as the identity. The MOWSE score (from Ms-Fit) is based on the scoring system of Pappin et al. [19], in which the product of matching peptides is inverted and normalized to an average protein mass. The Mascot score (from Matrix Sciences) is calculated as being equal to −10*Log (P), where P is the probability of the observed matching being random, calculated based on the size of the database being searched; $P < 0.05$ is considered significant.

Western Blotting

Relative protein levels in the nuclear or whole-cell extracts (30 μg protein per well) were assessed by Western analysis [14], using polyclonal antisera to processing factor 19 homolog (PRPF19), prohibitin (PHB), heterogeneous nuclear ribonucleoprotein A2/B1 (HNRNPA2B1), gene overexpressed in skeletal muscle (GEM), MAPK1, phospho-MAPK1 (pMAPK1), glyceraldehyde phosphate dehydrogenase, and ACTB (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA), transgelin 3 (TAGLN3) (NP25; Abnova) and eukaryotic translation initiation factor 4A, isoform 3 (EIF4A3) (Abcam).

RT-PCR and Quantitative Real-Time PCR

RNA was isolated from the LβT2 and αT3–1 cells using Trizol (Invitrogen, Carlsbad, CA), and reverse transcribed (5 μg) using oligo-dT(18) primer and the SuperScript One-Step RT-PCR System (Invitrogen). Of the 20 μl cDNA obtained, 1 μl was used for a PCR reaction with specific primers (0.4 μM; Table 1). Except for amplification of the gonadotropin and *Actb* cDNAs, all primers span most of the coding sequence of the respective genes, and all except *1110005A23Rik* contain at least three introns. Primers for detection of the gonadotropin subunit cDNAs all target partial sequences only, but all span one intron (first intron from *Fshb*, second intron from *Lhb* and *Cga*), unless specified otherwise in the figure legend. The primers for *Actb* amplify part of the sequence, and do not span an intron. PCR was carried out initially at 96°C for 30 sec, then cycled at 96°C for 10 sec, 58°C for 10 sec (or melting temperature less 5°C) and 72°C for 30 sec (for *Fshb*, *Lhb*, and *Cga*) or for 1 min (all other targets) over 25–30 cycles, before a final elongation at 72°C for 2 min. Optimal amplifications in the linear range were achieved for *Actb* and the *Cga* after 25 cycles; for *Lhb* and *Fshb*, 28 or 30 cycles, respectively; and for all other transcripts, 30 cycles. PCR samples were run on 1 % agarose, and densitometry readings were calculated using GeneTools software (Syngene, UK), with *Actb* or *Pin1* as internal control to provide semiquantitative analysis of the relative mRNA levels.

PCR products were analyzed initially on agarose gels to ensure specificity of the amplification, after which real-time PCR was carried out using the SYBR green I dye with the ABI Prism 7700 sequence detector (Perkin-Elmer Applied Biosystems, Waltham, MA), essentially as reported previously [14]. The reactions were performed in a 20-μl volume, containing the PCR Master Mix, respective primers as listed in Table 1, and 1 μl of the cDNA template. The samples were heated to 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The templates were serially diluted 5-fold in order to ensure that the dynamic ranges of both the target and reference were similar, and the comparative cycle threshold method was used to compare mRNA levels in the various samples.

Chromatin and RNA Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was carried out as reported previously [14], 48 h after transfection of the HA-1110005A23Rik construct (10 μg DNA per 100 mm plate). The rabbit anti-HA antisersa (Santa Cruz Biotechnology Inc.) was used to precipitate HA-tagged 1110005A23RIK protein, which was expressed in the pXJ40-HA vector (a gift from Boon Chuan Low, National University of Singapore). Primers were used to amplify the *Fshb*

TABLE 1. Oligonucleotides and PCR primers used in this study.

Gene	Sequence
Eif4a3	
Forward	5′-CGGAATCCGGATCATGGCG-3′
Reverse	5′-CGGTGCACCAGCACCAGC-3′
Cloning	
Forward	5′-CGGGATCCGGACCATGGCGGCTAAC-3′
Reverse	5′-CCGAATTCAGATGAGGTCAGCCACATTC-3′
SiRNA	
Forward	5′-ATCCCCGCAGATAATTAAAGGGAGATTCAAGAGATCTCCCTTTAATTATCTGCTTTTTGGAAA-3′
Reverse	5′-AGCTTTTCCAAAAAGCAGATAATTAAAGGGAGATCTCTTGAATCTCCCTTTAATTATCTGCGGG-3′
Prpf9	
Forward	5′-GACTGGCAGCAGCGCTACG-3′
Reverse	5′-GCCCAGAACTGTGAGAAGGC-3′
Cloning	
Forward	5′-ACGGATCCCGCCATGTCCCTGATCTGCTC-3′
Reverse	5′-TGGAATTCAGAACTGTGAGAAGGCATAGG-3′
SiRNA	
Forward	5′-GATCCCCGTACATTGCAGAGAATGGCTTCAAGAGAGCCATTCTCTGCAATGTACTTTTTGGAAA-3′
Reverse	5′-AGCTTTTCCAAAAAGTACATTGCAGAGAATGGCTCTCTTGAAGCCATTCTCTGCAATGTACGGG-3′
1110005A23Rik	
Forward	5′-GGGTAACAAGATGGCGACCGA-3′
Reverse	5′-CATCAGGCAATCCCAAAGCGC-3′
Cloning	
Forward	5′-GGGGATCCACCATGGCGACCGAGACGGTGG-3′
Reverse	5′-GGGAATTCCATCAGGCAATCCCAAAGCGC-3′
SiRNA	
Forward	5′-GATCCCCGATTTGGTTTGAATGTCTCTTCAAGAGAGAGACATTCAAACCAAATCTTTTTA-3′
Reverse	5′-AGCTTAAAAAGATTTGGTTTGAATGTCTCTCTCTTGAAGAGACATTCAAACCAAATCGGG-3′
N-terminal mutant	
Forward	5′-GGGGATCCACCATGGTACTGGGAGATGAAACTGAGG-3′
C-terminal deletion	
Forward	5′-CCTGTAAGCCTGGAGTAGTAAGAAGGCTGCCCGG-3′
Reverse	5′-CCGGGCAGCCTTCTTACTACTCCAGGCTTACAGG-3′
Tubb5	
Forward	5′-CAGTAAACCGTAGCCATGAGGG-3′
Reverse	5′-GCCTTAGGCCTCCTCTTCTGC-3′
Hnrnpa2b1	
Forward	5′-ACGAGTCCCGTGCGCGTCC-3′
Reverse	5′-CCTCTGGGCTCTCATCCTCTCC-3′
SiRNA	
Forward	5′-GATCCCCCGTGCTGTAGCAAGAGAGGTTCAAGAGACCTCTCTTGCTACAGCACGTTTTTA-3′
Reverse	5′-AGCTTAAAAACGTGCTGTAGCAAGAGAGGTCTCTTGAACCTCTCTTGCTACAGCACGGGG-3′
Tagln3	
Forward	5′-GGATGCCGCGTGTGTCCTCTC-3′
Reverse	5′-CGGAACATCCGTCCTCTCTACCA-3′
SiRNA	
Forward	5′-GATCCCCGATGCTGTATAGTGAGTGCTTCAAGAGAGCACTCACTATACAGCATCTTTTTA-3′
Reverse	5′-AGCTTAAAAAGATGCTGTATAGTGAGTGCTCTCTTGAAGCACTCACTATACAGCATCGGG-3′
Phb	
Forward	5′-GGAAGCAACAGAAGGAGTCATGG-3′
Reverse	5′-CTCACTGGGGAAGCTGGAGAAG-3′
3′UTR	
Forward	5′-CACCCCAGAAAATCACTGTGAA-3′
Reverse	5′-GAAGGTCTGGGTGTCATTTATTGAC-3′
SiRNA	
Forward	5′-GATCCCCTGTGGATGCTGGACACAGATTCAAGAGATCTGTGTCCAGCATCCACATTTTTA-3′
Reverse	5′-AGCTTAAAAATGTGGATGCTGGACACAGATCTCTTGAATCTGTGTCCAGCATCCACAGGG-3′
Gem	
Forward	5′-CCAAGATCCCGCAATGACTC-3′
Reverse	5′-GGTGCCTAGAGCACAGACAGG-3′
Cga	
Forward	5′-GCTGTCATTCTGGTCATGCT-3′
Reverse	5′-GTAAATGCTTTGGCCACACA-3′
Fshb exon 3	
Forward	5′-AGCACTGACTGCACCGTGAG-3′
Reverse	5′-CCTCAGCCAGCTTCATCAGC-3′
Fshb exon 1–2	
Forward	5′-GTTCAGCTTTCCCCAGAAGA-3′
Reverse	5′-CCTAGTATAGCAGTAGCCCG-3′
Pin1	
Forward	5′-CCGGAATTCATGGCGGACGAGGAGAAG-3′
Reverse	5′-TGCTCTAGATCATTCTGTGCGCAGGAT-3′

TABLE 1. Continued.

Gene	Sequence
Lhb	
Forward	5′-GCCTGTCAACGCAACTCTGG-3′
Reverse	5′-CAGGCCATTGGTTGAGTCCT-3′
Lhb ChIP	
Forward	5′-CAATCTGGGGGTTCAGCGAG-3′
Reverse	5′-CCTTGGGCACCTGGCTTTAT-3′
Fshb ChIP	
Forward	5′-CACAGCCCATAGGAACAAGA-3′
Reverse	5′-CCAAAGCAGTCTAAATGCC-3′
Actb	
Forward	5′-GCCATGTACGTAGCCATCCA-3′
Reverse	5′-ACGCTCGGTCAGGATCTTCA-3′

or *Lhb* gene proximal promoter using ChIP primers shown in Table 1. ChIP analysis was carried out as above over 30 cycles for the input samples, and 35 cycles for the ChIP product.

RNA immunoprecipitation (RIP) was carried out based on previously described methods [20]. In brief, cells were cross-linked in 1% formaldehyde (10 min) before the reaction was stopped with glycine (0.25 M). After three washes with PBS, the cells were suspended in RIPA buffer (50 mM TrisHCl, pH7.5, 1% NP40, 0.5% sodium deoxycholate, 0.05% SDS, 1 mM EDTA, 150 mM NaCl) and lysed by sonication. Following centrifugation to remove debris, aliquots of cell lysate were precleared by incubation with protein G-Sepharose beads for 1 h at 4°C in the presence of tRNA (100 μg/ml). Additional protein G beads were coated with goat anti-HNRNPA2B1 antibody (SC-10036; Santa Cruz Biotechnology Inc.) for 2 h at 4°C, followed by three washes with RIPA buffer. The beads (20 μl packed volume) were incubated with 0.5 μl RNasin (Promega) for 10 min, and then incubated with the lysate (1 h, room temperature), before six washes with RIPA buffer containing 2 M urea. The washed beads were resuspended in elution buffer (50 mM TrisHCl, pH7, 5 mM EDTA, 10 mM DTT, and 1% SDS), and the samples were reverse cross-linked (70°C, 45 min). RNA was extracted from the elution using Trizol. The residual DNA was removed using the Turbo DNA-free kit (Ambion), and the RNA was reverse transcribed with *Fshb* exon 1–2 reverse primer (Table 1) and MMLV reverse transcriptase (Promega). The *Fshb* RNA associated with HNRNPA2B1 was assayed by PCR with the *Fshb* exon 1–2 primers (Table 1).

Reporter Gene Assays, DNA Constructs, and Transfections

Reporter gene assays were carried out using −1973 to +694 bp of the murine *Fshb* gene promoter, which includes the first intron (*Fshb*IP) or −1305 to +3 of the murine *Lhb* gene promoter, both in pGL2. For the *Cga* promoter, −507 to +46 of the murine *Cga* gene was used [21] for the experiments shown; in all cases, similar results were obtained using the longer 1.5 kb human *Cga* promoter (results not shown) [22]. Alternatively, the SV40-LGALS4-responsive pGLuc-SGALS4 was used to measure activity of the fusion proteins in the pM vector [13]. Cells were transfected using GenePORTER 2 (Gene Therapy Systems, San Diego, CA), and luciferase values were measured and normalized as previously described [14]. The expression vectors were created following RT-PCR from αT3–1 or LβT2 RNA using the primers shown in Table 1. The 1110005A23Rik truncated constructs were created with C-terminal truncation (1–116) or N-terminal deletion (scaffold attachment protein [SAP] domain deletion, 55–210), and were cloned into the pXJ40-HA vector or the pM vector (Clontech). The small interfering RNA (siRNA) constructs were constructed in the pSUPER vector (OligoEngine, Seattle, WA) to include the specific 19 nt target sequences shown in Table 1. Typically, 3 μg DNA construct was transfected into cells at 50% confluency in 60 mm plates and left for 48 h before harvest. The controls for these experiments utilized a similar siRNA construct targeting GFP [14], the transfection of which has no apparent effect on any transcript levels measured, indicating the lack of any global effects on the transcription machinery. Alternatively, the pSUPERpuro was used for stable transfections, following puromycin selection for 2 weeks. The successful knockdown, as compared to untransfected and siGFP-transfected cells, was confirmed by Western analysis.

Growth Assays

For thiazolyl blue tetrazolium bromide (MTT) assays, cells were split into 96-well plates (10 000 cells/well) and, after overnight incubation, the media were replaced by serum-free media (SFM) and transfections performed (100 ng DNA/well). After 8 h, the media were changed, some of which contained 10 nM GnRH. After a further 48 h, 20 μl of 5 mg/ml MTT in PBS was added to each well, and the reaction was incubated (37°C, 5% CO_2) for 1 h. Media were then removed, and the insoluble MTT metabolic product was resuspended in dimethyl sulfoxide. The absorbance was read at 560 nm, with subtraction of background at 670 nm.

For assessment of bromodeoxyuridine (BrdU) incorporation, cells were treated similarly as for the MTT assay, except that the GnRH treatment was reduced to 16 h. The assay was conducted using the BrdU cell proliferation kit (Roche).

Statistical Analysis

All data are from at least three independent experiments, which were either combined or are shown as a representative figure. Statistical analysis comprising ANOVA followed by either Student or Bonferroni *t*-test was used to confirm differences between means of independently treated cells, and differences considered significant at $P < 0.05$.

RESULTS

Comparison of Proteins Expressed in Immature and Mature Gonadotroph Cell Lines

Several methods were used to enrich for nuclear proteins, two of which were found to produce highly enriched fractions of proteins that focused well. In the first, the nuclei were isolated, after which the proteins were extracted by sonication and urea buffer. The second method involved initial removal of the cytoplasmic proteins by centrifugation, followed by lysis of the nuclei, and solubilization of the nuclear proteins. Extensive desalting was performed to achieve a low salt content suitable for the isoelectric focusing, and proteins were also reduced and alkylated to achieve enhanced focusing and reduce streaking and artifacts [23]. The resulting gels showed profiles that were generally well focused, and were remarkably similar for the two cell lines, although differing somewhat according to the protocol used for nuclear enrichment (Fig. 1, A and B). Extensive analysis of proteins resolved in this way indicated a high degree of enrichment, although, clearly, the various means of extraction do enrich for different classes of proteins.

After running at least three gels for each cell type and using each protocol, comparisons were made, and several protein spots were found consistently at different intensities in the two cell lines (Fig. 1, A and B); these were excised for identification.

Identification and Verification of Differentially Expressed Proteins

The excised proteins (Fig. 2A) were identified: the matching peptides covered 17%–50% of the proteins, and the identifications were considered significant (Table 2). Although the different profiles in the 2D gels could be due to regulation at the level of gene expression, cellular localization, and/or posttranslational modifications, Western blot analysis for those

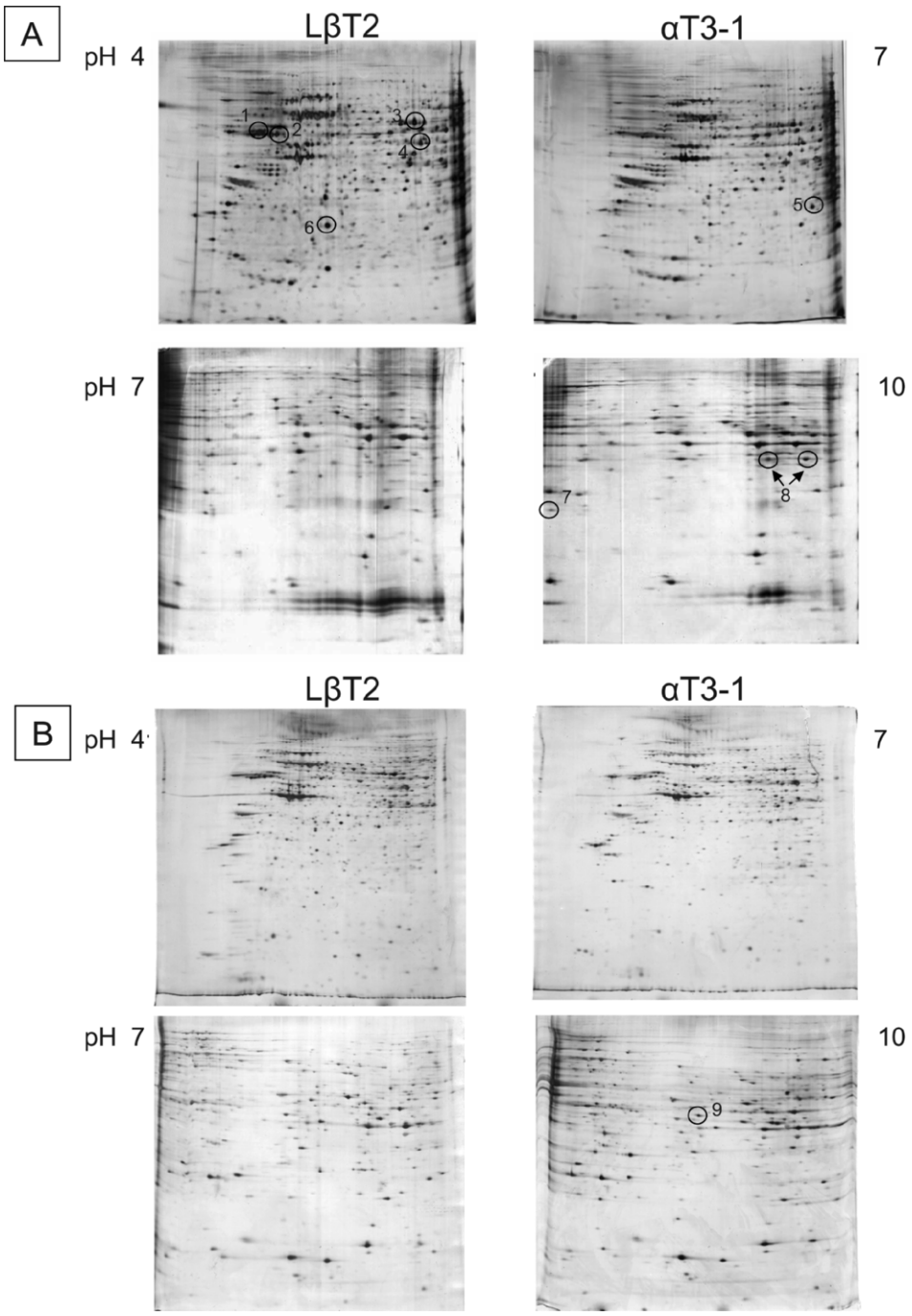

FIG. 1. Two-dimensional gel electrophoresis of proteins from isolated nuclei (**A**) or solubilized nuclear proteins (**B**) of untreated mature LβT2 and immature αT3–1 gonadotrophs over the pI ranges 4–7 or 7–10. Nuclei were prepared as described in the *Materials and Methods*, before resolving over first and second dimensions. Numerous pairs of samples were run and compared, and representative pairs are shown. Protein spots showing differential expression patterns that were isolated for identification are numbered; protein 8 appears in two distinct spots on the gel (as revealed by its subsequent identification).

proteins with available antisera confirmed their different protein levels in the nuclear extracts of the two cell lines (Fig. 2B). Semiquantitative RT-PCR indicated that their gene expression levels also varied accordingly (Fig. 2C), indicating that at least some of the differences between in the two cell lines are in the differential gene expression of these proteins. Western blotting also verified that these proteins are present in the mouse pituitary gland, and are, therefore, not artifacts of the cell lines (data not shown).

The proteins identified as differentially expressed in the two cell lines include those likely involved in signaling, such as RIKEN cDNA 1110005A23 gene (1110005A23Rik, also known as HCC1 or CIP29), PHB, and GTP binding protein (GEM); those involved in RNA processing, such as PRP19/PSO4 pre-mRNA processing factor 19 homolog (*S. cerevisiae*) (PRPF19), EIF4A3, and HNRNPA2B1; and those interacting with actin and/or the cytoskeleton: tubulin, β5 (TUBB5) and TAGLN3 (or NP25). Additional proteins were isolated that could not be identified with confidence. Notably, of all of the proteins that we identified, only one was nonnuclear: the mitochondrial F1 complex β subunit protein, which was isolated following the first method of enrichment. We pursued study of several of these proteins in order to verify whether they are responsible for the differential expression of the gonadotropin genes in these two cell lines.

1110005A23RIK Inhibits Fshb *Gene Expression*

The 1110005A23RIK protein was found at high levels in the αT3–1 gonadotrophs, but was barely detectable in the LβT2 gonadotrophs. Although the *Fshb* gene is expressed only at

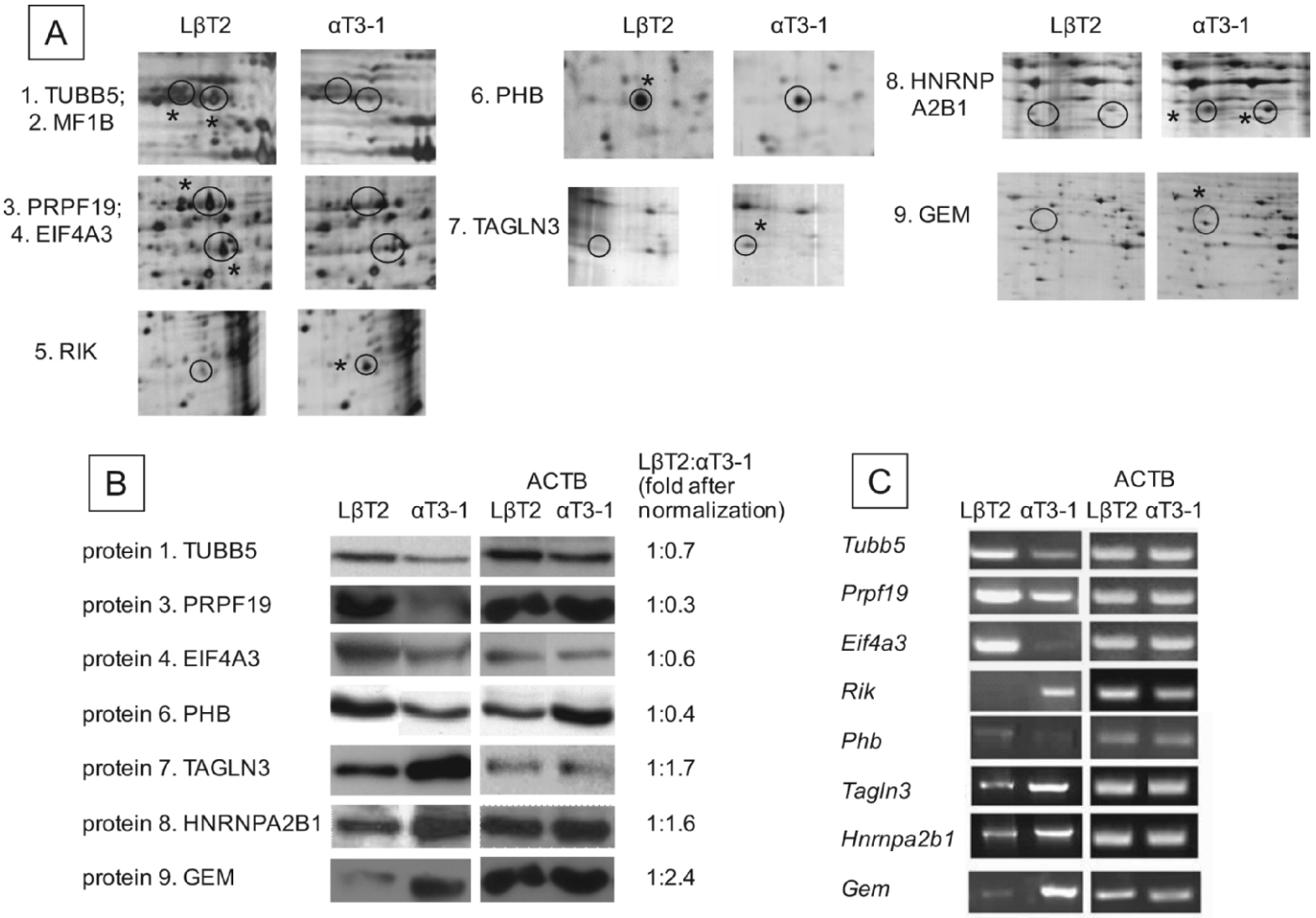

FIG. 2. Identification and verification of differentially expressed proteins. **A**) The differentially expressed proteins marked in Figure 1 are shown magnified; an asterisk marks the spot of higher intensity for each pair. **B**) After the proteins were excised, in-gel digested, and subjected to mass spectrometry, a putative identity was obtained and, where antisera were available, Western analysis was carried out to verify the identification and examine differential expression in nuclear extracts of the two cell lines, with ACTB as internal control. The expression levels of these proteins were assessed, and are shown, after normalization, relative to levels in LβT2 cells. **C**) RT-PCR was also carried out to determine whether the differential protein levels relate to differential gene expression, with *Actb* as internal control. The 1110005A23RIK is abbreviated to RIK in all figures.

very low levels in the unstimulated LβT2 gonadotrophs, overexpression of 1110005A23RIK in these cells reduced the level of the *Fshb* mRNA until it was undetectable, while not having the same effect on the *Lhb* transcript (Fig. 3A). This ability of 1110005A23RIK to repress transcription of *Fshb*, but not *Lhb*, was confirmed in both a reporter gene assay and real-time quantitative PCR analysis, in which the activity of the *Fshb*, but not the *Cga* nor *Lhb* promoter, was reduced following 1110005A23RIK overexpression, and the mRNA levels were similarly affected (Fig. 3, B and C). Conversely, transfection of an siRNA construct that reduces *1110005A23Rik* expression, increased activity of the *Fshb* gene promoter, while activity of the *Lhb* and *Cga* promoters appeared unaffected (Fig. 3D).

The 1110005A23RIK protein contains a SAP domain at its N terminus that is likely responsible for its binding to the DNA, and a lysine-arginine-rich domain at the C terminus. In order to test the role played by these domains in the repressive effect, a LGALS4-1110005A23RIK fusion protein was created, enabling assessment of the effect of this protein or its truncated mutants on the activity of a LGALS4-responsive reporter gene, which, driven by SV40, has quite high basal levels of expression. Deletion of the SAP domain did not affect the repressive actions of this fusion protein, but deletion of the C terminus abolished the repressive effect (Fig. 3E). Similar effects were seen on the activity of the 1110005A23RIK protein devoid of its C terminus on *Fshb* promoter activity (Fig. 3F), indicating a crucial role for this domain in the repression of gene expression.

In order to verify whether 1110005A23RIK exerts a direct effect at the level of the *Fshb* gene promoter, 1110005A23RIK fused to an HA tag was overexpressed. The tagged protein was detected at the *Fshb* gene proximal promoter by ChIP using antisera to HA, which did not precipitate any endogenous proteins associated with the promoter in these cells (Fig. 3G). We tested the effect of GnRH on *1110005A23Rik* levels in LβT2 cells using semiquantitative RT-PCR, which indicated an increase in *1110005A23Rik* transcript levels after GnRH exposure (Fig. 3H). Given that derepression of gene expression often employs posttranslational modification of the repressor to cause its dissociation from the DNA, ChIP analysis was also carried out following GnRH treatment. This confirmed 1110005A23RIK association with the *Fshb* gene promoter, which is still apparent, and possibly even increased, following GnRH treatment. There was no apparent association of 1110005A23RIK with the *Lhb* promoter (Fig. 3I).

TABLE 2. Identification following mass spectrometry of the proteins found differentially expressed.

Protein name (symbol)	NCBI accession No.	MOWSE or Mascot score, or *P* value	Protein coverage (length, no. of matching peptides)	Location of matching peptides (amino acid)	Theoretical MW/pI	Expression pattern on 2D gel
Tubulin, beta 5 (TUBB5)	GI: 12846758	2.41e+009	40% (444, 17)	47–58, 63–77, 78–103, 104–121, 155–162, 217–241, 242–251, 242–252, 252–262, 253–262, 263–276, 283–297, 310–318, 351–359, 381–390	49640.1/4.78	αT3<LβT2
Mitochondrial F1 complex, beta subunit (ATP5B)	GI: 31980648	3.53e+008	38% (529, 16)	95–109, 110–121, 125–143, 134–143, 144–155, 226–239, 240–259, 265–279, 282–294, 295–310, 295–324, 311–324, 325–345, 388–406, 407–422	56300.8/5.19	αT3<LβT2
PRP19/PSO4 pre-mRNA processing factor 19 homolog (S. cerevisiae) (PRPF19)	GI: 19527358	1.49e+005	20% (504, 7)	57–76, 77–93, 101–115, 193–206, 266–289, 267–289, 290–303	55239.2/6.14	αT3<LβT2
Eukaryotic translation initiation factor 4A, isoform 3 (EIF4A3)	GI: 26344810	3.71e+003	22% (411, 8)	52–67, 52–70, 106–116, 152–166, 153–166, 199–206, 261–287, 330–339, 340–358	46768.2/6.57	αT3<LβT2
RIKEN cDNA 1110005A23 gene (1110005A23Rik; AKA HCC1)	GI: 13384730	80 (Mascot)	24% (210, 5)	74–80, 93–102, 110–120, 154–166, 157–166 84–93, 94–105, 106–117, 134–143, 187–195,	23291/6.29	αT3>LβT2
Prohibitin (PHB)	GI: 6679299	1.09e+005	34% (272, 10)	196–202, 220–239, 240–253	29820/5.57	αT3<LβT2
Transgelin 3 (TAGLN3)	GI: 9790125	68 (Mascot)	50% (199, 11)	1–12, 5–12, 5–17, 65–84, 99–106, 107–127, 128–139, 140–153, 146–153, 160–168, 161–168	22627/6.84	αT3>LβT2
Heterogeneous nuclear ribonucleoprotein A2/B1 (HNRNPA2B1)	GI: 109134362	151 (Mascot)	41% (341, 10)	10–26, 27–34, 102–117, 118–135, 142–161, 162–173, 162–174, 192–201, 202–216, 274–298	36014/8.67	αT3>LβT2
GTP binding protein (gene overexpressed in skeletal muscle) (GEM)	GI: 38258884	1.4e-5 (*P* value)	17% (295, 4)	44–61, 44–62, 84–102, 198–209	33753/8.7	αT3>LβT2

Prohibitin Represses Activity of All Three Gonadotropin Subunit Gene Promoters and Reduces Cell Numbers

PHB is found at higher levels in LβT2 cells than in the αT3–1 cells. Initially, we addressed the possibility of its affecting transcription of the gonadotropin subunits by overexpressing the protein or its 3′ untranslated region (UTR) in LβT2 cells. The 3′UTR has previously been shown to have similar effects as PHB itself [24, 25], and thus provides a confirmation of the PHB effect. The outcome was evaluated by RT-PCR, and the amplicons quantified by densitometry. Despite being only a semiquantitative technique, this revealed that PHB and its 3′UTR overexpression had similar and significant effects on the *Lhb* and *Cga* transcripts, reducing them by half, while not affecting those of *Fshb*, which are already very low in these cells (Fig. 4A). The mRNA levels were also measured by real-time quantitative PCR, which showed a reduction in all three transcripts following PHB overexpression (Fig. 4B). Finally, reporter gene assays, using the promoters of the three gonadotropin genes, were carried out, and the activities of all three promoters were reduced by the PHB (Fig. 4C). The efficacy of the overexpression in these and the αT3–1 cells was confirmed by Western analysis (Fig. 4D).

Based on previous reports that PHB can alter cell proliferation and/or apoptosis, we examined the effects of overexpression of PHB or its 3′UTR on cell numbers using an MTT assay. Both cell types were cultured in SFM, and some were exposed to GnRH for 48 h. In untreated LβT2 cells, overexpression of PHB or its 3′UTR reduced the cell number. GnRH treatment alone nearly halved the cell number, but this reduction was not affected further by overexpression of either PHB or its 3′UTR. However, in untreated αT3–1 cells, cell number was not affected by overexpression of either factor, but the GnRH-induced increase was reduced by PHB or its 3′UTR (Fig. 4E). We considered that the likely effect of the 3′UTR overexpression was in stabilizing the transcript, resulting in increased protein expression. We therefore examined the effect of the 3′UTR expression on PHB levels by Western blot. This revealed an increase in PHB levels in the nucleus, although the same effect was not obvious in the cytoplasm (Fig. 4F).

In order to further explore the role of PHB in the LβT2 cells, we knocked down its levels using siRNA in a stable transfection, which produced two clones with different levels of PHB expression (Fig. 5A). The effects of GnRH on cell proliferation in these knockdown LβT2 cells, as well as in control LβT2 and αT3–1 cells, were assessed by measuring BrdU incorporation. In αT3–1 cells, GnRH stimulated an increase in BrdU incorporation, while, in the control LβT2 cells, the opposite effect was seen. Interestingly, the knockdown LβT2 cells expressing lower levels of PHB (siPHB1) responded to GnRH by increasing DNA synthesis similarly to the αT3–1 cells, while the clone with higher PHB levels (siPHB2) responded to GnRH, as did the control LβT2 cells, by decreasing DNA synthesis (Fig. 5B). Also notable was the altered cell morphology in both siPHB1 and siPHB2 cells, which was similar to the αT3–1 cells, being more marked in the cells with less PHB: the αT3–1 and siPHB1 cells have more polarized projections, while the wild-type LâT2 cells and the siPHB2, which have higher levels of PHB, are more cuboidal, with less apparent attachment and cell contact (Fig. 5C).

Ribonucleoprotein A2/B1 Represses Splicing of Fshb

HNRNPA2B1 has a higher expression level in αT3–1 cells. Given that an HNRNP consensus binding site (tagagt [26]) is found on the first intron of the *Fshb* gene, we initially

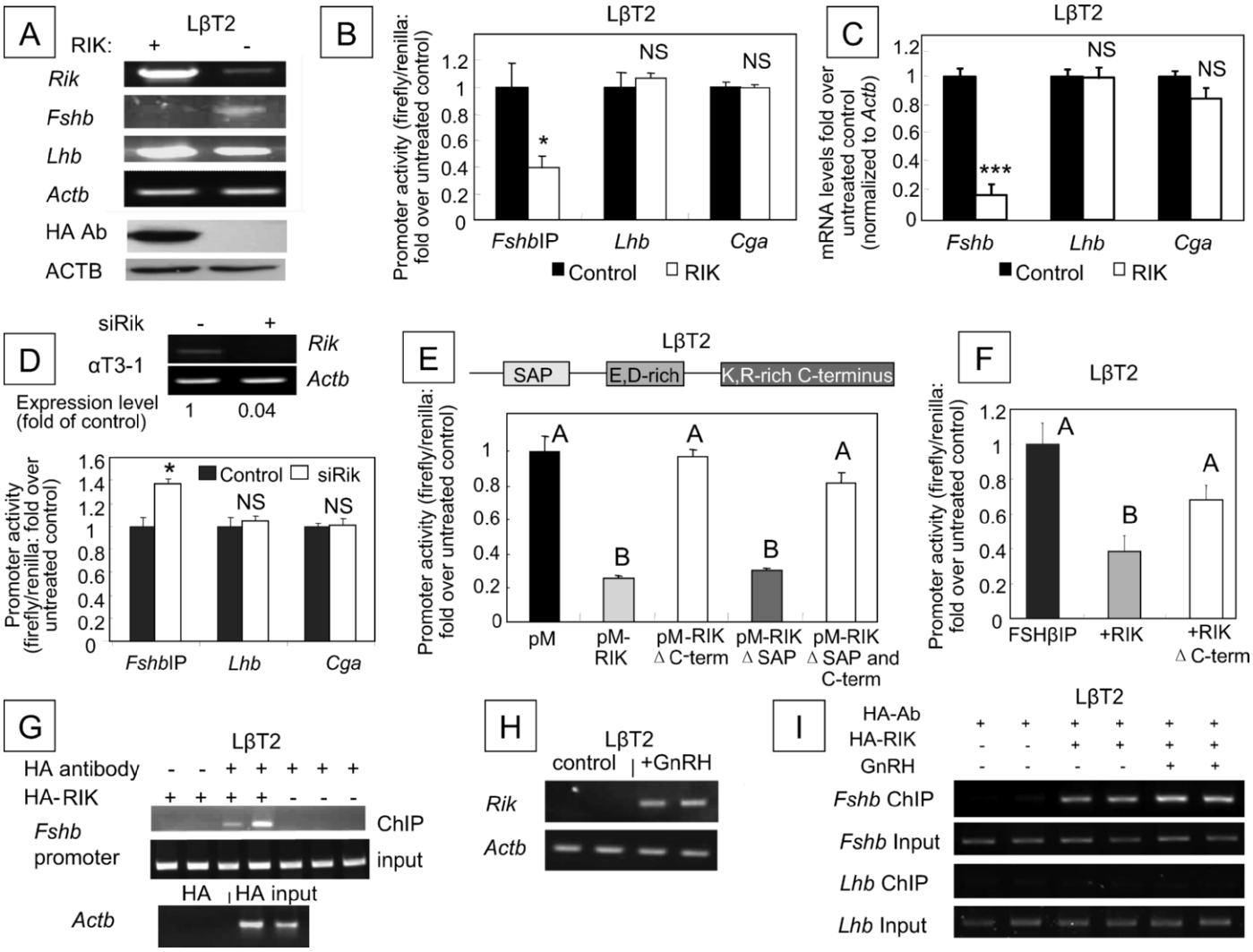

FIG. 3. 1110005A23RIK represses expression of the *Fshb* gene. **A**) An 1110005A23RIK expression vector (RIK) was transfected into LβT2 cells, and RT-PCR carried out for *1110005A23Rik*, *Fshb*, and *Lhb* mRNAs, and the levels of 1110005A23RIK overexpression were evaluated using antisera to the HA tag in Western analysis. The effect of 1110005A23RIK overexpression was tested in LβT2 cells on transiently transfected mouse *Fshb* (intron and promoter (IP): includes first intron), *Lhb*, and *Cga* promoter-luciferase reporter genes with pRL-SV40 as internal control (**B**), or endogenous mRNA levels by real-time PCR, using *Actb* as internal control (**C**). For both types of experiment, levels are expressed relative to those in control cells after normalization with levels of the internal controls. Mean ± SEM; n = 3–4. Student *t*-test compared the promoter activity of each gene with or without 1110005A23RIK overexpression; $*P < 0.05$; $***P < 0.001$, NS: $P > 0.05$. **D**) An siRNA construct targeting *1110005A23Rik* (siRik) was transfected into αT3–1 cells together with each of the subunit promoter-luciferase constructs. Luciferase activity was measured after 48 h and is presented as in **B**. The effect of the knockdown was verified by RT-PCR and quantified relative to the controls after normalization with the levels of *Actb*. **E**) The 1110005A23RIK contains three domains, two of which were removed individually and in combination, and truncated fusion proteins created fused to LGALS4 DBD (pM vector), as shown before testing their effects on a SV40-LGALS4 reporter gene; mean ± SEM; n = 4–6. ANOVA compared means; those that are not significantly different ($P > 0.05$) share the same letter. **F**) The effect of the wild-type or C-terminus-truncated 1110005A23RIK construct on the *Fshb* promoter activity was tested similarly in LβT2 cells; mean ± SEM; n = 4–6. Statistical analysis is as in (**E**). **G**) The 1110005A23RIK was overexpressed in LβT2 cells before ChIP using antisera to the HA tag to detect association with the *Fshb* proximal promoter. Also shown are the input samples before precipitation, and the negative *Actb* control, which was not precipitated by the antisera. **H**) The effect of GnRH (10 nM, 24 h) on the *1110005A23Rik* expression level in LβT2 cells was tested using RT-PCR. **I**) The ChIP analysis was repeated as in **G** after GnRH treatment (10 nM, 4 h), and the association of *Fshb* and *Lhb* proximal promoters was assessed.

overexpressed it in LβT2 cells, and the effect on *Fshb* mRNA splicing was measured by RT-PCR using primers that span the first intron. The HNRNPA2B1 overexpression resulted in an increase in the ratio of the unspliced to the spliced *Fshb* transcript level of 1.75 ± 0.2-fold over that in the untreated control, as assessed by densitometric analysis of the RT-PCR products ($P < 0.05$; Fig. 6A). RIP was carried out, and confirmed the association of HNRNPA2B1 with the first intron of the *Fshb* gene (Fig. 6B).

To confirm further the role of HNRNPA2B1 in the splicing of the *Fshb* gene, we knocked down its expression levels using siRNA (targets both isoforms). The total level of *Fshb* spliced and unspliced transcripts was unaffected ($P > 0.05$; data not shown); however, the effect of the knockdown, even though only partial, obviously increased the levels of the spliced *Fshb*, while the *Cga* and *Lhb* transcripts appeared unaffected (Fig. 6C).

EIF4A3 and PRPF19 Promote Splicing of the Fshb *and/or* Lhb *Primary Transcripts*

Both EIF4A3 and PRPF19 are expressed at higher levels in LβT2 cells than in αT3–1 cells. We therefore tested the effect of their knockdown using siRNA. The ability of these constructs to reduce the respective protein levels was shown

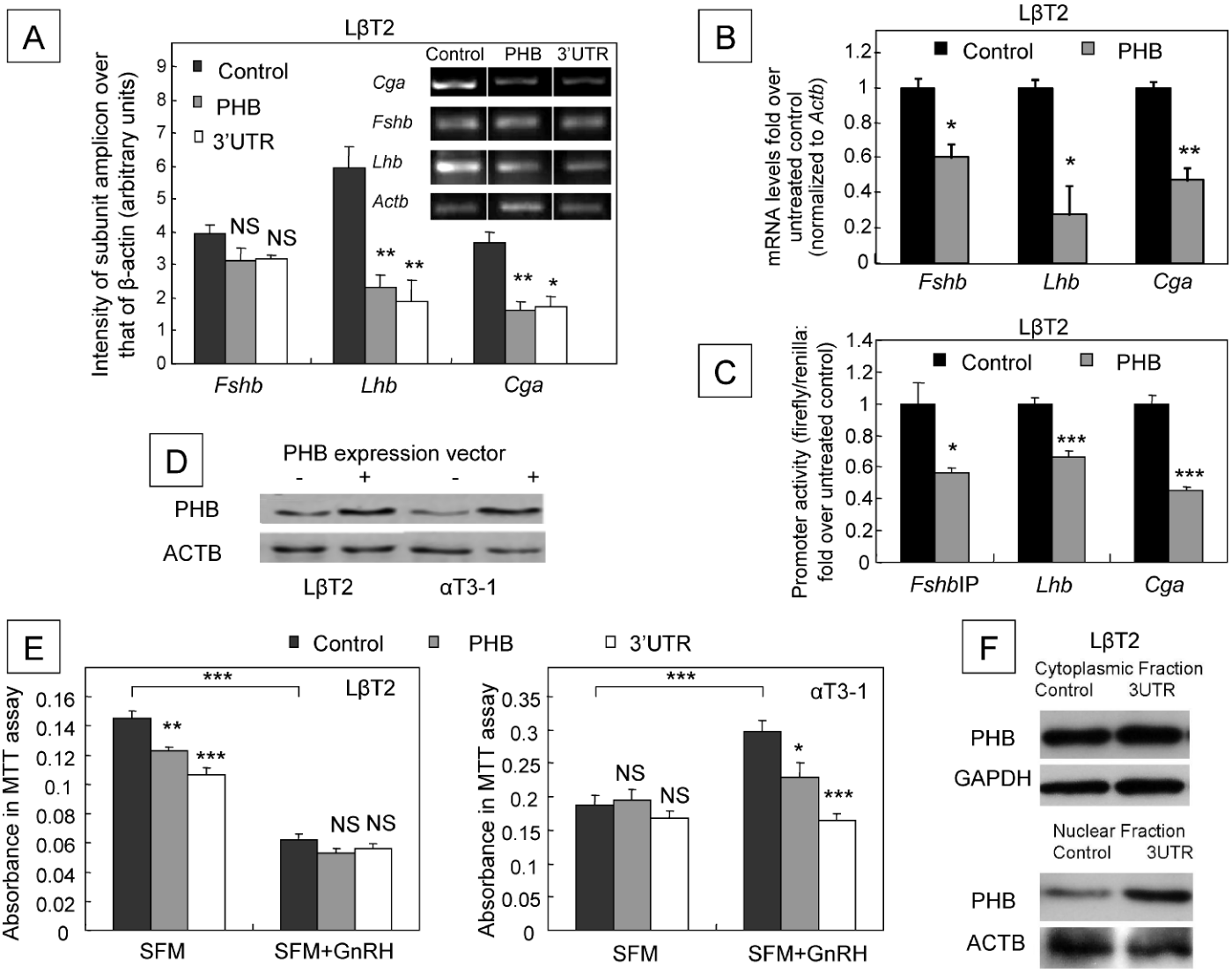

FIG. 4. Prohibitin represses promoter activity of all three gonadotropin subunits, and moderates cell numbers differently in the two cell lines. **A**) PHB or its 3′UTR were overexpressed in LβT2 cells, and the effects on levels of transcripts of the three gonadotropin subunits were assessed by RT-PCR and quantitated after normalization to levels of *Actb*. A sample gel is shown, as well as the quantitated densitometry readings relative to levels of *Actb*; mean ± SEM; n = 3. Student *t*-test compared means from the untreated control with activity of the same promoter in PHB- or 3′UTR-transfected cells; NS: $P > 0.05$; *$P < 0.05$; **$P < 0.01$. The effect of PHB overexpression on the three gonadotropin subunits was also tested by real-time PCR (**B**) and promoter assays (**C**), as in Figure 3B. Mean ± SEM; n = 3–4. Statistical analysis was as in (**A**); ***$P < 0.001$. **D**) Western analysis confirms the level of overexpression of PHB following transfection of the expression vector in the two cell lines. **E**) MTT cell growth assays were carried out in LβT2 and αT3–1 cells to evaluate the effect of PHB or its 3′UTR overexpression on cells in SFM with or without GnRH treatment (10 nM) for 48 h. Mean ± SEM; n = 6. Statistical analysis was as described in (**A**) and (**C**), with additional comparison between untransfected controls with and without GnRH treatment. **F**) The effect of overexpression of the 3′UTR on PHB protein levels was assessed by Western analysis on cytoplasmic or nuclear fractions from LβT2 cells.

by Western analysis (Fig. 7A). RT-PCR was then employed to test the effects of the knockdown on *Fshb* and *Lhb* transcripts. The *Eif4a3* mRNA was almost completely knocked down in one of the samples, and only partially so in the other. Its reduced levels correlated with a marked decrease in the spliced *Fshb* transcript, while the unspliced transcript spanning intron 1, which was not detected in the control cells, was also visible in these cells. The effect on the *Lhb* transcript appeared similar, with an increase in the amount of unspliced primary transcript detected. Conversely, the knockdown of PRPF19, which appeared highly effective, did not appear to affect the transcript levels or the splicing of the *Fshb* gene, but did increase the amount of unspliced *Lhb* primary transcript (Fig. 7B).

Further studies on the EIF4A3 revealed that its overexpression in αT3–1 cells was sufficient to increase the level of *Fshb* transcript (without differentiating between the spliced and unspliced transcripts; Fig. 7C). Real-time quantitative PCR revealed a 4.2-fold increase ($P < 0.05$) in *Fshb* transcript levels after the overexpression (data not shown). Furthermore, we found that exposure of LβT2 cells to activin (100 ng/ml, 24 h) increased the levels of *Eif4a3* expression, which was accompanied by an increase in the levels of the *Fshb* mature transcript, with a corresponding reduction in levels of the primary unspliced transcript (Fig. 7D). Total levels of *Fshb* transcripts (after normalization with levels of *Actb*) increased 1.6-fold following activin treatment (data not shown).

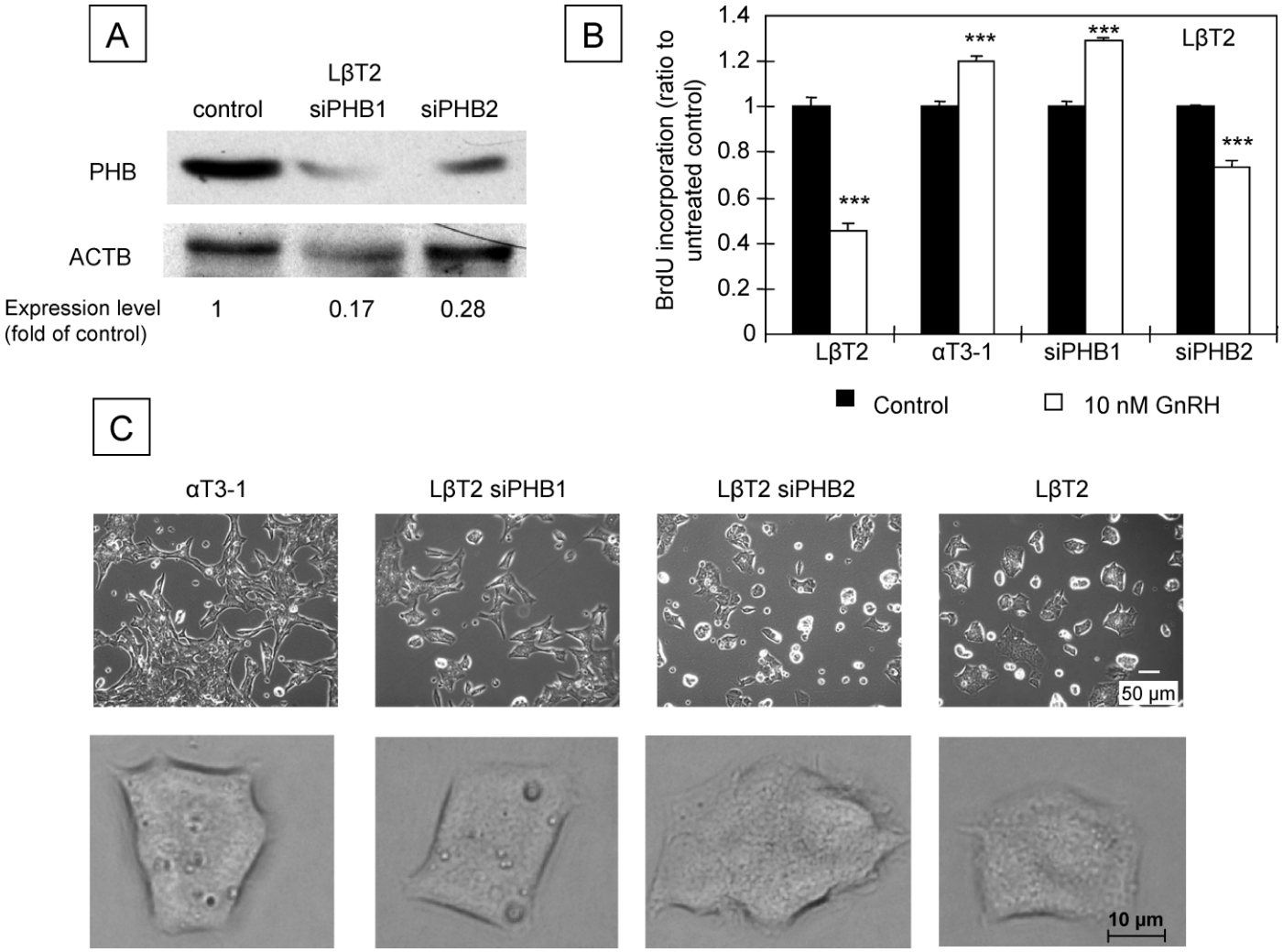

FIG. 5. Prohibitin prevents GnRH-induced cell proliferation and alters cell morphology. **A**) LβT2 cells were stably transfected with siRNA to knockdown PHB expression, and Western analysis used to confirm the degree of knockdown in two of the clones (siPHB1 and siPHB2). The degree of knockdown was quantified relative to the controls after normalization with levels of ACTB in the same samples. **B**) The same two clones, as well as nontransfected αT3–1 and LβT2 cells, were cultured, and some of the cells exposed to GnRH (10 nM, 16 h) before carrying out a BrdU assay to evaluate cell proliferation. BrdU incorporation is expressed as a ratio to that in untreated cells of the same type. Mean ± SEM; n = 6. Statistical analysis was as described in Figure 4. **C**) Wild-type αT3–1 and LβT2 cells, as well as the LβT2, PHB1, and PHB2 knockdown cells, were photographed at two magnifications showing their different morphologies.

TAGLN3 Represses Fshb *and* Lhb *Transcript Levels in LβT2 Cells*

TAGLN3 has higher expression levels in αT3–1 cells, and its overexpression in LβT2 cells reduced the promoter activity of all three subunit genes, most notably for *Fshb* (Fig. 8A). The effect on the *Fshb* gene was confirmed by quantitative real-time PCR analysis, although the endogenous levels of *Lhb* and *Cga* were unaffected by the overexpression (Fig. 8B). A functional role for TAGLN3 in repressing expression of the *Lhb* and *Cga* genes was confirmed through transfection of an siRNA construct, which reduced TAGLN3 expression (Fig. 8C) and increased mRNA levels of *Lhb* and *Cga*, while levels of *Fshb*, which are already very low, were not affected (Fig. 8D).

In order to examine a possible role for TAGLN3 in altering MAPK signaling, the effects of its overexpression or knockdown in LβT2 cells was assessed by Western analysis. In untreated cells, pMAPK1 was barely detectable, but its levels were clearly increased following TAGLN3 knockdown (Fig. 8E). In GnRH-treated cells, the same effect of TAGLN3 knockdown was seen, albeit rather less obviously, whereas the TAGLN3 overexpression clearly reduced the GnRH-activated levels of pMAPK1 (Fig. 8F). In order to confirm the role of the MAPK activation in mediating the effects of TAGLN3, cells were treated with a MEK inhibitor, which was seen to abolish the effects of the siTAGLN3 construct on the promoter activity of the *Lhb* and *Cga* gonadotropin subunit genes (Fig. 8G).

DISCUSSION

Two-Dimensional Gel Electrophoresis of Gonadotroph Nuclear Proteomes

In the current study, enrichment of nuclear proteins was attained using two different approaches, both of which allowed good resolution of the proteins on the 2D gels, but clearly

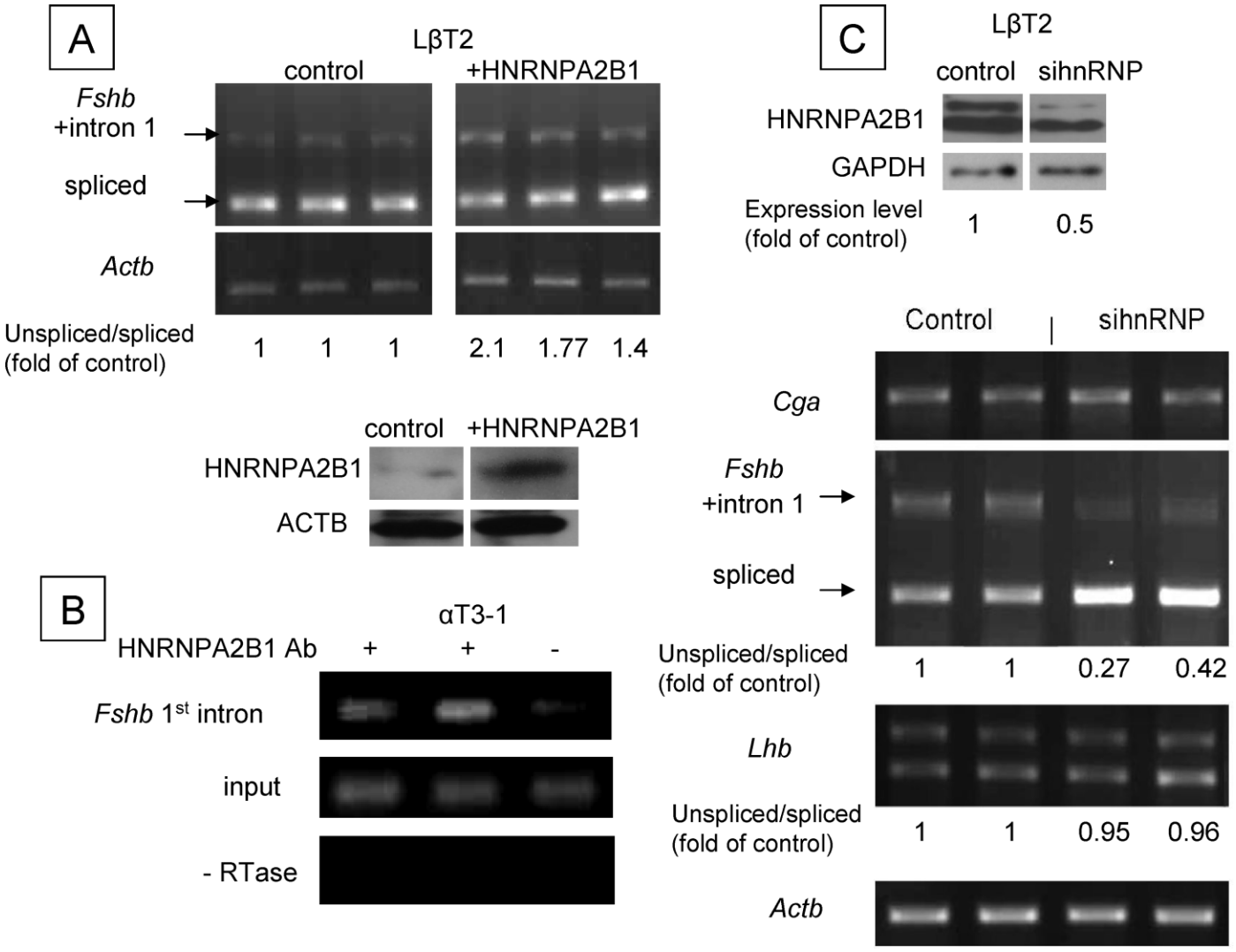

FIG. 6. Ribonucleoprotein A2/B1 represses *Fshb* first intron splicing. **A**) After overexpression of HNRNPA2B1 in LβT2 cells, RT-PCR was carried out to evaluate the spliced and unspliced *Fshb* mRNA using primers on the first and second exons. The relative levels of unspliced:spliced transcripts were measured by densitometry readings, and are shown as fold difference of the level in control cells. The level of HNRNPA2B1 overexpression, assessed by western analysis, is also shown. **B**) RIP was carried out in αT3–1 cells using antisera to HNRNPA2B1 and primers that span the first intron of *Fshb*. **C**) SiRNA constructs targeting *Hnrnpa2b1* were transfected into LβT2 cells, and Western blots confirm the reduced levels of HNRNPA2B1; also shown are the quantified levels relative to those in controls, after normalization to glyceraldehyde phosphate dehydrogenase (a sum of both isoforms). The effects of these constructs on each of the gonadotropin subunits were assessed by RT-PCR using primers that span an intron, and quantified as in **A**. The band for the Cga transcript is the spliced form (due to the larger intron and the short amplification time used in the PCR).

enrich for distinct sets of proteomes, highlighting the value of utilizing more than one enrichment technique. Notably, all of the proteins that we have identified, with only one exception, were either nuclear, pan-cellular, or of previously unknown localization. The nuclear proteomes of the two cell lines were highly similar using either method, which is expected, because the two cell lines were from the same cell lineage, and differ only in a few days of embryonic development [1]. However a number of differences were identified and studied further in an attempt to reveal their specific effects on gonadotropin gene transcription that might explain the differences in phenotypes of these cells.

Differentially Expressed Signaling Molecules

The 1110005A23RIK, which is expressed at higher levels in αT3–1 than in LβT2 cells, decreased *Fshb* mRNA levels and repressed promoter activity, and we have shown using ChIP analysis that it is associated with the *Fshb* promoter. This suggests a novel function as a gene-specific transcriptional repressor for this protein that was originally identified in hepatocarcinoma cells, and found to be up-regulated by cytokines in hematopoetic cells [27, 28]. Two groups noted that it inhibits cell proliferation by inducing G2/M arrest, or by inducing apoptosis [29, 30]. Based on our current findings, we propose that this protein acts as a sequence-specific DNA binding factor through its N-terminal SAP domain, which was previously shown to bind A-T-rich scaffold attachment regions, and is thought to be involved in chromosomal organization [29, 31]. Given that removal of the lysine-arginine-rich C terminus also reduced the repression, it is likely that this domain mediates protein-protein interactions with other corepressors. Recent reports have shown that this protein interacts with the DEAD (Asp-Glu-Ala-Asp)-box RNA helicase, and also with the RNA binding protein FUS (fusion, derived from t(12;16) malignant liposarcoma), which is associated with both DNA and RNA [32], thus confirming its likely role in regulating

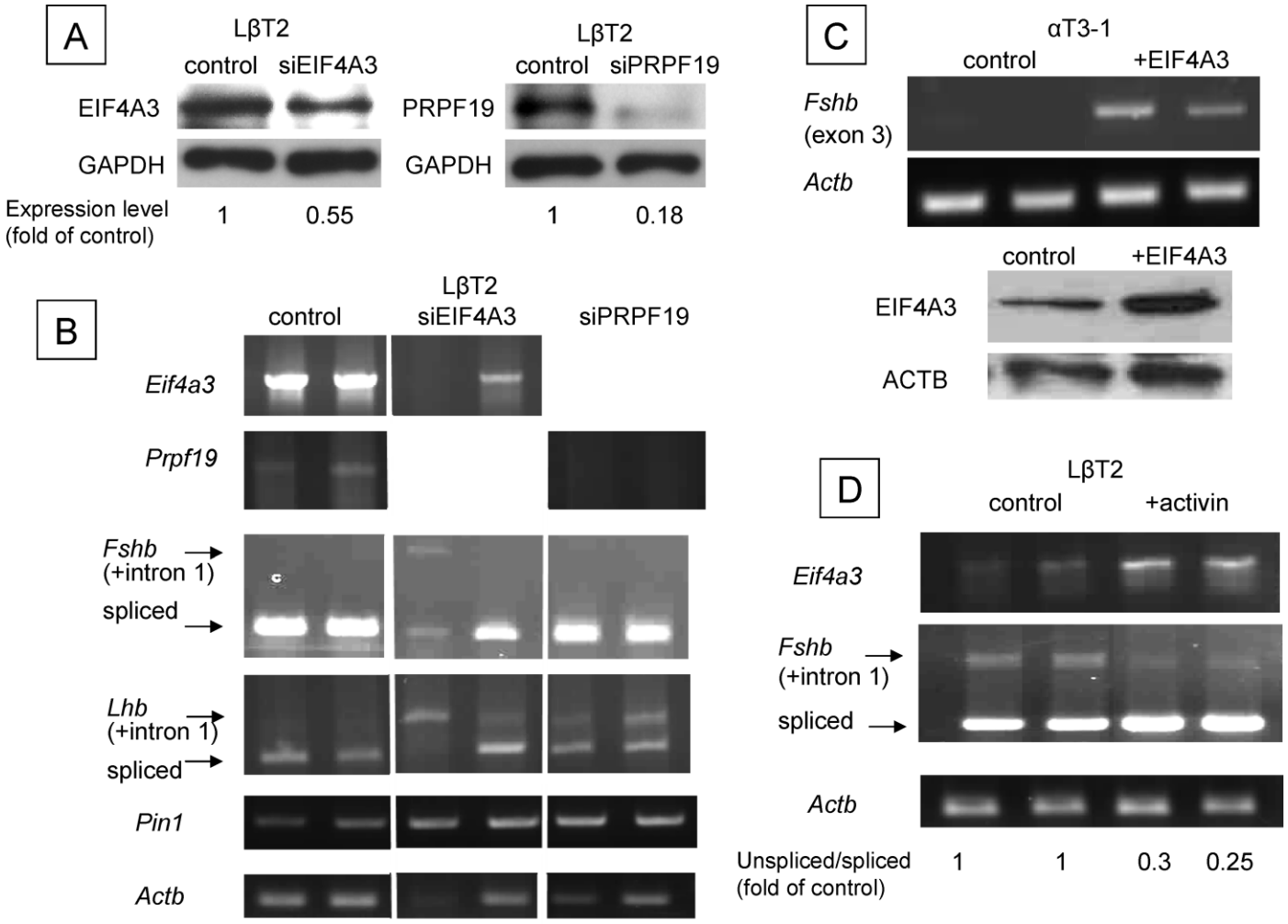

FIG. 7. EIF4A3 and PRPF19 alter splicing of the *Fshb* and/or *Lhb* transcripts. **A**) SiRNA constructs targeting *Eif4a3* or *Prpf19* transcripts were transfected into LβT2 cells, and the respective protein levels assessed by Western analysis and quantified as in Figure 6C. **B**) The effect of these siRNA constructs on the *Fshb* and *Lhb* transcripts, as well as those of the targets, were measured by RT-PCR. **C**) The effect of overexpression of EIF4A3 on the *Fshb* transcript (representing spliced and unspliced forms, the primers amplify only exon 3, and do not span an intron) was measured similarly by RT-PCR in αT3–1 cells; the degree of overexpression was assessed by Western analysis (lower panel). **D**) The effect of activin (100 ng/ml, 24 h) on *Eif4a3* mRNA levels and on the splicing of the *Fshb* primary transcript in LβT2 cells was assessed by RT-PCR using primers spanning the first intron, and relative levels of unspliced:spliced transcripts measured as in Figure 6A.

gene expression. However, we currently have little information on additional interacting proteins from which to postulate the mechanism of repression on the *Fshb* gene. Notably, however, its association was not lost following treatment with GnRH, which activates this gene, thus indicating that its role as a repressor might be regulated through posttranslational modifications. Further study is clearly required to elucidate these events and their role in regulating *Fshb* gene expression.

PHB was found at higher levels in the LβT2 cells, and was shown previously to be necessary for activation of Raf/MEK/MAPK by Ras [33, 34]. GnRH activates Ras in αT3–1 cells, but its activation of MAPK, which is essential for activation of the β subunit genes, is Ras independent in these cells [35–37]. This, therefore, unlikely explains the reduced gonadotropin gene expression in αT3–1 cells, although the paradoxical finding of its higher expression levels in the LβT2 cells, which express these genes, yet its ability to reduce gonadotropin expression, remains puzzling.

PHB modulates transcription of many genes directly through interactions with corepressors and HDACs, as well as transcription factors, including steroid receptors [38–40]. In this study, PHB overexpression reduced promoter activity of all three gonadotropin genes, and also the transcript levels of *Cga* and *Lhb*. It is quite possible that PHB acts as a corepressor or scaffold factor to assemble such repressive complexes at the promoter. This might explain its weaker effect on the endogenous *Fshb* gene in LβT2 cells, which is already repressed by a HDAC complex [4]. Our ongoing studies suggest that PHB may play an important role in the activation of β subunit transcription by GnRH in LβT2 cells, and that it is regulated by GnRH, but behaves differently in LβT2 and αT3–1 cells (J.F. and P.M., unpublished data). It is therefore quite plausible that PHB's ability to alter gonadotropin gene transcription is highly regulated by GnRH, hence its paradoxical presence at higher levels in the β subunit-expressing cells.

PHB overexpression reduced cell number in untreated LβT2 cells, or in GnRH-treated αT3–1 cells, likely involving a reduction in cell proliferation. A repressive effect on growth has been shown previously in other cell types, and is reportedly mediated through a reduction in activity of the E2F proteins and an enhancement of p53-mediated transcription [38, 41, 42]. The significance of this is not yet clear; however, the different expression levels of PHB in these two cell lines, as noted by

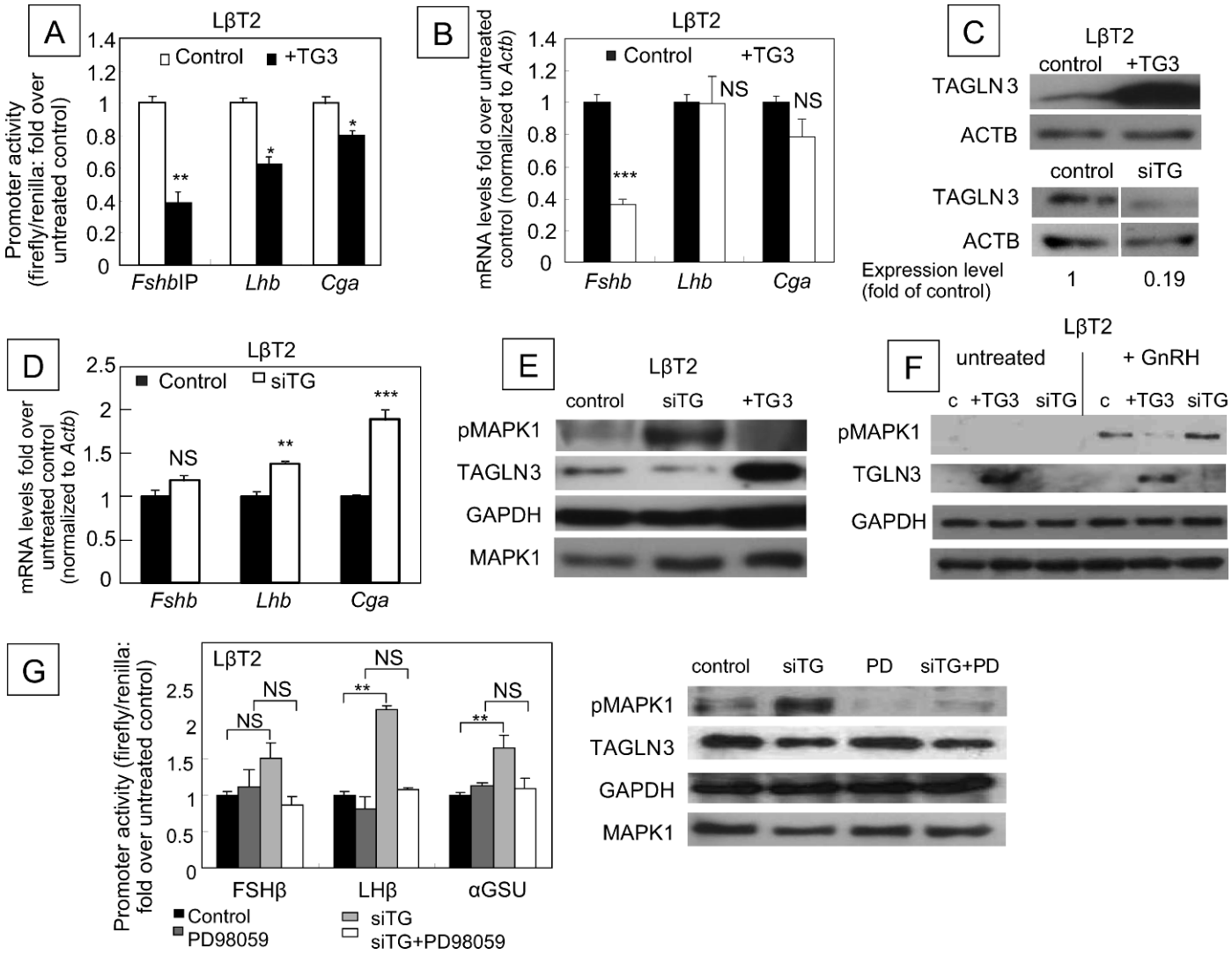

FIG. 8. TAGLN3 represses *Fshb* and *Lhb* transcript levels in LβT2 cells. The effect of TAGLN3 overexpression (+TG3) in LβT2 cells on gonadotropin promoter activity (**A**) and endogenous mRNA levels (**B**) was assayed using reporter gene assays and real-time PCR, as in Figure 3; mean ± SEM, n = 3–4. **C**) The degree of TAGLN3 overexpression (top) or its knockdown using an siRNA construct (siTG; bottom) in LβT2 cells was assessed by Western analysis, both with ACTB as internal control. The efficiency of the knockdown was assessed as in Figure 5A. **D**) The effects of the siRNA construct targeting *Tagln3* on all three gonadotropin subunits was similarly tested by real-time PCR, as in Figure 3; mean ± SEM, n = 3. The effects of TAGLN3 overexpression or knockdown on pMAPK levels were also assessed in untreated (**E**) or untreated and GnRH-treated (10 nM, 5 min) (**F**) LβT2 cells using 60 μg (**E**) or 30 μg (**F**) protein. **G**) To determine whether the effects of TAGLN3 related to MAPK inactivation, promoter assays were carried out (as in Fig. 3) in LβT2 cells, some of which were incubated with a MEK inhibitor, PD98059, with or without transfection of the siRNA TAGLN3 construct. Luciferase assays were carried out, and data are presented as in Figure 3. A Western blot, revealing the effect of the siTAGLN3 construct transfected with or without the MEK inhibitor (PD [PD98059]), is shown on the right.

most groups working with them, might perhaps be responsible for the slower proliferation rates of LβT2 as compared to αT3–1 cells, although we have yet to show this. The mechanisms through which GnRH exerts opposing effects on cell number in these two cell lines are not yet clear, but are currently being addressed in a separate study.

Also noted was the effect of PHB on cell morphology, which has been reported following its knockdown in HeLa cells. A comparison between the LβT2 cells in which PHB was knocked down, and the αT3–1 cells in which the nuclear levels are naturally lower, strongly suggests that the difference in PHB expression levels might well be responsible for the respective morphologies of these cells. Although we have not specifically examined the implications of this morphology, it was previously reported to correlate in HeLa with increased intercellular adhesion, associated with increased levels of cadherins and β catenin at the regions of cell contact, and with changes in the responses of cell motility to epidermal growth factor [33, 34].

Levels of GEM, a Ras-related GTPase that inhibits calcium channel activity, were found to be higher in the αT3–1 cells. GnRH was previously noted to up-regulate GEM transcript levels in LβT2 cells, and was suggested to provide a negative feedback loop by decreasing Ca^{2+} influx through L-type channels [7, 10]. However, it is found primarily in the nucleus in various cell lines, and its role there is not yet clear. Its role in regulating gonadotropin gene expression is being pursued in a separate study.

Proteins Involved in RNA Processing

We have previously noted that, although we can induce expression of both the *Lhb* and *Fshb* genes in αT3–1 cells and

Fshb in the LβT2 cells, these genes are not fully spliced [4]. Given this observation of aberrant splicing, we were interested to note that three of the differentially expressed proteins in these cell lines are reported to be involved in RNA processing, and specifically appear to regulate the splicing of these genes.

The HNRNP proteins have roles in RNA splicing and mRNA localization, and HNRNPA2B1 was found at higher levels in the αT3–1 cells. HNRNPA2B1 acts as a splice repressor by directly binding to the RNA and antagonizing RS (Arg-Ser) domain proteins. It also induces secondary structure, and causes exon skipping by binding either side of the exon, which is then looped out [43–46]. Interestingly, the related protein, HNRNPA1, is regulated by MAPK14 (p38MAPK), which controls its translocation into the cytoplasm, and likely determines the alternative splicing of CD44 [26, 47, 48]. The presence of an HNRNP-binding site on the first intron of the *Fshb* primary transcript, together with our experimental data, strongly suggest that it is involved in regulating splicing of the *Fshb* gene, while it did not have any obvious effects on the *Lhb* or *Cga* transcripts. Interestingly, this protein was found at lower levels in pituitaries of mice with targeted overexpression of LH than in their wild-type littermate controls [16], although the reason for this is not clear. A reduction in its expression with development has been noted previously in other tissues [49, 50], while its overexpression is also considered an early marker for various cancers [51–53].

The EIF4A3 protein is expressed at lower levels in the αT3–1 cells, and its knockdown in the LβT2 cells clearly reduced the amount of spliced transcript for both β subunit genes, while also apparently reducing FSHβ transcription. This protein belongs to the DExH/D box family of RNA helicases, and localizes predominantly in the nucleus, unlike EIF4A1 and EIF4A2, which are translation initiation factors [54]. A number of recent studies have shown that EIF4A3 has an essential role in nonsense-mediated mRNA decay of transcripts that have not been properly spliced and contain a premature stop codon [55–57]. It also remains associated with the mRNA product as part of the exon junction complex on newly spliced mRNAs, and helps recruit additional RNA processing factors [56, 58, 59].

The level of *Eif4a3* mRNA was markedly increased following activin treatment, which induced an increase in spliced transcripts and a decrease in unspliced transcripts of *Fshb*. Notably, the homologous *Xenopus* EIF4A3 is up-regulated by another TGFβ family member, bone morphogenetic protein-4, which induces epidermal differentiation and inhibits neural differentiation [60]. These findings indicate that the effects of activin on expression of the *Fshb* gene are not limited to its well-recognized activation of transcription [6, 61–63], but also likely through the regulation of the pre-mRNA splicing.

The final RNA processing factor that we found differentially expressed and at higher levels in the mature LβT2 cells was PRPF19, which contains an N-terminal U-box domain and a C-terminal seven-repeat WD40 domain, and is homologous with an essential component of the yeast spliceosome, PRP19 [64, 65]. Our study indicated that this protein might be involved in splicing of the *Lhb* gene, but not that of *Fshb*. Currently, little is known about the mechanisms of regulating splicing [48, 66], and no such studies have been carried out for the gonadotropin genes. Although there is no evidence that these genes are expressed specifically as splice variants, the requirement for "correct" splicing in facilitating further mRNA processing and transport, leading to translation [67, 68], indicates that regulation at the level of splicing could be an additional control mechanism determining expression levels of these genes.

Actin Binding/Cytoskeletal Proteins

Several recent reports indicate that actin and its binding proteins play a crucial role in gene transcription [69–72]. Notably, a number of actin-binding proteins interact with steroid receptors and modify their activity; some of these actions are enhanced further by actin (reviewed in [72]). It has been hypothesized that this ability to enhance transcription is via their recruitment of actin to the target promoters, which supports RNA polymerase activity (reviewed in [72]). TUBB5 is one such protein, which is involved in intracellular transportation, cell division, and cell movement, and has been shown to be expressed differentially during development [73], possibly explaining its differential profile in the nuclear extracts of these two cell types. However, we have not pursued study of this putative role.

TAGLN3, which we found at lower levels in the LβT2 cells, was initially identified as an actin-binding protein involved in cytoskeletal organization in fibroblasts, smooth muscle, and other normal mesenchymal cells [74, 75]. Its overexpression in LβT2 cells indicates that TAGLN3 can exert a repressive effect on promoter activity of all three subunits, which was more notable on *Fshb*, and was also seen on the endogenous *Fshb* transcript levels, but not on the *Lhb* or *Cga*. However, TAGLN3 may well already be exerting its maximal inhibitory effects on the *Lhb* and *Cga* levels in these cells, as its knockdown increased these transcript levels, but did not affect the *Fshb*. In fact, the effects of TAGLN3 appear to correlate negatively with the expression levels of these three genes and/or their promoter activity (*Cga* is expressed at highest levels and *Fshb* at lowest levels). This may be attributed to the inhibitory effect of TAGLN3 on pMAPK1 levels that regulates expression of all three genes, but likely plays a more predominant role in the genes expressed at a higher level.

Previous reports suggest, conversely, that PKC, MAPK1/2, MAPK8 (JNK), and MAPK14 kinases, each of which is activated by GnRH, can affect activity and/or expression of a related transgelin, TAGLN, or Sm22α, [37, 76, 77]. Furthermore, the ability of this TAGLN to repress transcription of the type IV collagenase (MMP9) was shown to occur through AP1 factors, which also regulate the *Fshb* gene and *Lhb* genes in some species, possibly by attenuating MAPK signaling [78–80]. TAGLN3 and TAGLN expression were previously seen to increase following GnRH treatment in LβT2 cells [7, 16], indicating a likely role in the regulation of these genes by GnRH. While the function and role of TAGLN3 in regulating gonadotropin gene expression clearly warrants further study, it could be hypothesized that its repressive effect forms an integral part of activation by some of the regulatory hormones, possibly through a self-regulatory mechanism controlling GnRH signal transduction.

In conclusion, by comparing nuclear proteomes of two closely related cell lines representing distinct developmental stages of a single cell type, we have identified novel factors that contribute to their differentiated phenotype. Proteins 1110005A23RIK, HNRNPA2B1, and TAGLN3 are all repressors of gonadotropin gene expression, and are higher in the immature αT3–1 cells. Proteins identified as being enriched in the mature LβT2 cells (EIF4A3 and PRPF19) enhance splicing of the gonadotropin gene transcripts, while PHB, a repressor of gonadotropin subunit gene expression, was also higher in LβT2 cells, but its activity may be regulated by GnRH. We have thus established a likely role for several of these proteins in regulating gonadotropin gene expression at the level of transcription or processing the transcript. For some of these proteins, their effect appears paradoxical, indicating a

likely fine-tuning mechanism in regulating gonadotropin gene expression, the precise mechanisms of which require more in-depth study. The involvement of these factors in various stages of the regulation of gene expression emphasizes a connection between transcription and RNA processing in determining the levels of gonadotropin gene expression, while simultaneously highlighting the complexity of differentiated cell function. Examination of the regulation of these key proteins in response to the hormones and growth factors that determine the differentiation of specific cell types in the pituitary will shed further light on the role of these regulatory factors in promoting differentiation of the gonadotroph cell lineage. Furthermore, application of these approaches to the study of GnRH regulation of the mature gonadotroph will contribute to the formation of an integrated model of the regulation of gene expression through transcriptional, translational, and posttranslational mechanisms.

ACKNOWLEDGMENTS

We would like to thank Pamela Mellon for the cells and Boon Chaun Low for the pXJ40-HA vector.

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