

# Interleukin-6 Transcriptionally Regulates Prohibitin Expression in Intestinal Epithelial Cells\*

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Prohibitin (PHB) is a highly conserved protein that has multiple functions in the cell. We recently demonstrated that PHB plays an important role in combating oxidative stress and its expression is down-regulated in human and animal models of inflammatory bowel disease. Little is known regarding the regulation of PHB expression in intestine or other tissues. In this study we examined the regulation of PHB expression in intestinal epithelial cells using the model cell line Caco2-BBE. We successfully cloned the 1192-bp human PHB promoter region and identified the transcription start site 1594 bp upstream from the translation start site due to an intervening intron. We show that the acute phase cytokine interleukin-6 (IL-6) increases PHB protein and mRNA abundance and induces PHB promoter activation. The IL-6 response element site in the PHB promoter is required for maximal basal promoter activity and responsiveness to IL-6. IL-6 also increases binding of nuclear proteins to the IL-6 response element in the PHB promoter that are supershifted by a STAT3 antibody. Both basal promoter activity and IL-6 responsiveness are attenuated by signal transducer and activator of transcription 3 short interference RNA, suggesting that signal transducer and activator of transcription 3 mediates PHB activity by IL-6. Confirming these *in vitro* results, IL-6<sup>-/-</sup> mice exhibit reduced PHB expression in the colon compared with wild-type mice. These results suggest that IL-6 modulates PHB expression in cultured intestinal epithelial cells and in the intestine *in vivo*.

Prohibitin (PHB)<sup>2</sup> is a ubiquitously expressed protein that has pleiotropic functions, including mitochondrial protein folding (1, 2), inhibition of cell cycle progression and apoptosis (3–6), regulation of transcription (7, 8), modulation of cell motility (9), and ligand binding at the plasma membrane (10).

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) DQ406856.

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<sup>2</sup> The abbreviations used are: PHB, prohibitin; EMSA, electrophoretic mobility shift assay; IL-6, interleukin-6;  $\alpha$ 2-M,  $\alpha$  2-macroglobulin; IL-6RE, interleukin-6 response element; Ct, cycle threshold; siRNA, short interference RNA; STAT, signal transducer and activator of transcription; WT, wild type.

PHB is a potential tumor suppressor gene, and expression of PHB is altered in gastric, cervical, breast, and colorectal cancer (11–15). Evidence also suggests that PHB expression is altered during other disease states. We and other investigators have shown that PHB expression is decreased during inflammatory bowel disease (16–18). In our recent study, we demonstrated that PHB localizes to the mitochondria in a model intestinal epithelial cell line as well as in native human colonic epithelia (18). We demonstrated that PHB increased glutathione levels during oxidative stress and induced the expression of glutathione S-transferase  $\pi$ . Importantly, we showed that PHB was highly effective in protecting epithelial cells from oxidant-induced epithelial barrier dysfunction. Loss of barrier function provided by epithelial cells is thought to be the initial inciting event that underlies injury and inflammation in many intestinal disorders, including shock, trauma, sepsis, and inflammatory bowel disease (19). Hence, PHB may play an important role in the pathogenesis of intestinal inflammation by acting as a potent antioxidant. Little is currently known regarding the regulation of PHB expression in intestine or other tissues. In this study we assessed the regulation of PHB expression by the immunomodulatory cytokine interleukin-6 (IL-6).

IL-6 is an acute phase cytokine and is thought to participate in host defense mechanisms. One of the effects of IL-6 is protection against oxidative stress by inducing antioxidant defenses, including increased glutathione levels, glutathione peroxidase, and metallothionein expression (20–22). IL-6<sup>-/-</sup> mice show increased susceptibility to oxidant-induced hepatocellular injury (23) and alveolar epithelial cell death (24), and exogenous administration of IL-6 protects intestinal epithelial cells against sepsis-induced oxidative stress (25). With respect to the intestine, IL-6 is increased in the serum and in mucosal biopsies of patients with inflammatory bowel disease during the acute phase of inflammation (26–28). Although some studies have shown that IL-6 may be pro-inflammatory, other studies have shown that IL-6 may indeed protect intestinal mucosa from the consequences of systemic inflammation, including permeability alterations. For example, mice given oral administration of IL-6 exhibit decreased permeability and reduced bacterial invasion through the gastrointestinal tract (29–31). The role of IL-6 in protecting against oxidative stress and its protective role in intestinal inflammation known to be associated with oxidative stress prompted us to examine the regulation of PHB by IL-6.

## EXPERIMENTAL PROCEDURES

**RNA Isolation and Quantitative Real-time PCR Analysis**—Total RNA was isolated from monolayers of Caco2-BBE cells and colon from IL-6<sup>-/-</sup> mice and wild-type (WT) mice using

TRIzol reagent (Invitrogen). Total RNA was then reverse transcribed using the Thermoscript reverse transcriptase PCR system (Invitrogen). 50 ng of reverse-transcribed cDNA was amplified by quantitative real-time PCR using 10  $\mu$ M PHB gene-specific primers and iQ SYBR Green Supermix (Bio-Rad) using the following PCR conditions: initial denaturation of one cycle at 95 °C for 10 min, followed by amplification at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s for 35 cycles. Expression level of  $\beta$ -actin was used as an internal control. Raw cycle threshold values ( $C_t$  values) obtained for IL-6-treated cells/IL-6<sup>-/-</sup> and WT mice were deducted from the  $C_t$  value obtained for internal  $\beta$ -actin transcript levels. For graphical representation of quantitative PCR data, the  $\Delta\Delta C_T$  was calculated as follows:  $\Delta\Delta C_T = (C_{t, \text{target}} - C_{t, \beta\text{-actin}})_{\text{treatment/IL-6}^{-/-}} - (C_{t, \text{target}} - C_{t, \beta\text{-actin}})_{\text{non-treatment/WT}}$ , with the final graphical data derived from  $2^{-\Delta\Delta C_T}$ . The primers utilized for quantitative real-time PCR were designed using human nucleotide sequences available in the GenBank<sup>TM</sup> data base. Human PHB (sense: 5'-GGGCACAGAGCTGTCATCTT-3', antisense: 5'-TGACTGGCACATTACGTGG T-3'); mouse PHB (sense: 5'-GCATTGGCGAGGACTATGAT-3', antisense: 5'-CTCTGTGAGGTCATCGCTCA-3');  $\beta$ -actin (sense: 5'-TATGCCAACACAGTGTCTGTCTGG-3', antisense: 5'-TACTCCTGCTTGCTGATCCACAT-3').

**SDS-PAGE and Western Immunoblot Analysis**—Total protein was isolated from polarized Caco2-BBE cells plated on permeable supports and treated with 100 ng/ml recombinant human IL-6 (R&D Systems, Minneapolis, MN) on the basolateral side because previous studies have shown that IL-6 receptors are predominantly expressed on the basolateral side in these cells (32). The samples were separated by SDS-PAGE using Laemmli's 2 $\times$  SDS sample buffer and 4–20% gradient polyacrylamide gels followed by electrotransfer to nitrocellulose membranes. Membranes were incubated with primary antibodies at 4 °C overnight and subsequently incubated with corresponding peroxidase-conjugated secondary antibodies. Rabbit polyclonal PHB antibody was obtained from Lab Vision Corp. (Fremont, CA). Rabbit polyclonal phospho-STAT3 and total STAT3 antibodies were obtained from Cell Signaling (Danvers, MA). Membranes were washed and immunoreactive proteins were detected using enhanced chemiluminescence (Denville Scientific Inc., South Plainfield, NJ) and exposed to high performance chemiluminescence film (Denville). Blots were reprobed with anti- $\beta$ -tubulin (Sigma) antibody as a loading control. Films were analyzed by densitometry, and signal intensity was quantitated using a gel documentation system (Alpha Innotech, San Leandro, CA).

**Isolation of the 5'-Flanking Region of the Human PHB Gene**—The 5'-flanking region of the human PHB gene was amplified by PCR using human chromosome 17 genomic DNA (clone RP11-1079K10; BacPac Resources, Children's Hospital Oakland Research Institute, Oakland, CA) as a template and using the following primers: 5'-GCAAAAGCTTCCTCACAAGTCGGACTCACGC-3' (underlined nucleotides indicate a HindIII site); 5'-GCAACTCGAGGGAGAAACCCCGTCTCTAC-3' (underlined nucleotides indicate a XhoI site). After sequence confirmation, the 1192-bp PCR product was cloned into pGL3 luciferase reporter vector (Promega, Madison, WI) using XhoI

and HindIII restriction sites. The DNA sequence of human PHB promoter region has been submitted to GenBank<sup>TM</sup> and is available under accession DQ406856. Putative transcription factor binding sites within the full-length human PHB promoter were identified using the Web-based search program Transcription Element Search System (TESS; www.cbil.upenn.edu/teess/).

**5'-Rapid Amplification of cDNA Ends (RACE)**—The GeneRacer<sup>TM</sup> kit (Invitrogen) was used to obtain the full-length cDNA sequence at the 5'-end of PHB. Briefly, 5'-racing cDNA was prepared from 4  $\mu$ g of total RNA isolated from Caco2-BBE intestinal epithelial cells as described in the manufacturer's protocol. Subsequent PCR was performed using the antisense human-specific PHB oligonucleotide (5'-CCACAATGTCCTGCACTCCACGG-3') corresponding to nucleotides +126 to +148, the GeneRacer<sup>TM</sup> 5'-primer, 0.2 mM dNTPs, and SurePol<sup>TM</sup> DNA polymerase (Denville) using the following PCR conditions: initial denaturation of one cycle at 94 °C for 2 min, followed by amplification at 94 °C for 30 s, 69 °C for 30 s, and 72 °C for 2 min for 25 cycles. The PCR products were cloned into the Zero Blunt TOPO vector (Invitrogen) for sequencing to determine the transcription start site.

**Comparison of the Human PHB Promoter with the Mouse PHB Promoter**—To determine homology across the human and mouse PHB promoter regions, the 1054-bp region upstream from the transcription start site of the mouse PHB gene was obtained from the mouse chromosome 11-nucleotide sequence available in the GenBank<sup>TM</sup> data base (accession number AL732490). Alignment of the human PHB promoter and the mouse PHB promoter sequences and calculation of percent homology were performed using Vector NTI Advance 10 software (Invitrogen). The mouse PHB promoter sequence was submitted to TESS to determine whether the IL-6 response element present in the human PHB promoter is preserved in the mouse PHB promoter.

**Deletion Constructs, Mutagenesis, and Reporter Gene Assay**—To determine the putative regulatory site(s) necessary for basal PHB promoter activity, deletion constructs were obtained by internal restriction enzyme digest within the full-length (–1054/+138 bp) PHB promoter construct. The truncated fragments –949/+138 bp, –189/+138 bp, and –35/+138 bp were obtained using restriction enzymes SmaI, SacII, and SacI, respectively. The truncated fragments were digested with Klenow enzyme to remove overhangs if necessary and recircularized using the Quick Ligation kit (New England Biolabs, Ipswich, MA). To determine the importance of the IL-6 response element (IL-6RE) in PHB promoter activity, site-specific mutation was introduced into the wild-type –1054/+138-bp PHB promoter in pGL3 by PCR amplification using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) and sense and antisense primers of the same sequence possessing a mutation in the IL-6RE: wild-type sense, 5'-CTGTAATCTCAGCTATTCTGGGAGGGTGAGGCAGGAGAAT-3'; mutant sense, 5'-CTGTAATCTCAGCTATTCTCCATGGGTGAGGCAGGAGAAT-3'; mutant antisense, 5'-ATTCTCCTGCCTCACCCATGGAGAATAGCTGAGATTACAG-3'. Nucleotide substitutions are indicated in bold, and the wild-type IL-6RE sequence is underlined. The PCR

## IL-6 Regulates Prohibitin Expression

product was sequenced to ensure nucleotide substitution at the IL-6RE site.

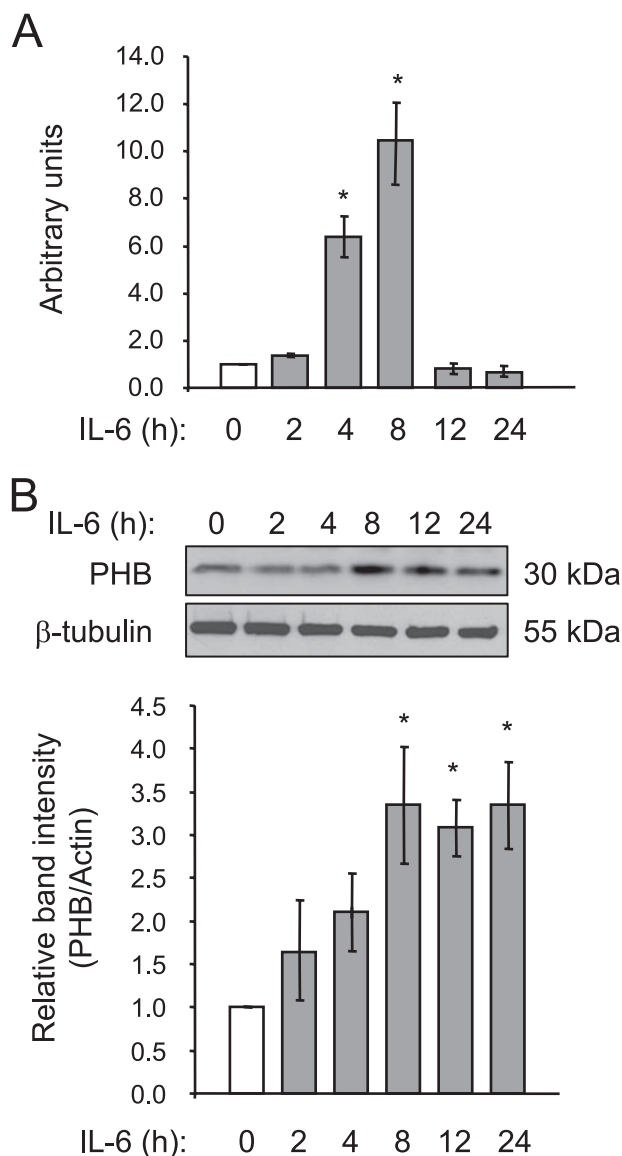
For reporter gene assays, Caco2-BBE cells were plated onto permeable supports and co-transfected with 1.6  $\mu$ g of the reporter construct and 20 ng of pRL-CMV (*Renilla* luciferase; Promega) as an internal control. Cells were transfected using Lipofectamine 2000 (Invitrogen) and were harvested 72 h post-transfection. Cells treated with IL-6 were serum-deprived overnight and treated with 100 ng/ml IL-6 for 6 h. Luciferase activity was measured using the Dual-Luciferase Reporter Assay system (Promega) and a Luminoskan Ascent luminometer (Thermo Electron Corp., Waltham, MA). Relative luciferase was calculated by normalizing firefly luciferase activity to *Renilla* luciferase activity of the pRL-CMV vector.

The  $\alpha$ 2-macroglobulin ( $\alpha$ 2-M) promoter was used as a positive control because it contains a consensus-type II IL-6RE. The  $-1151/+54$ -bp  $\alpha$ 2-M promoter in pGL3 luciferase reporter vector was a generous gift from Dr. James E. Darnell, Rockefeller University, New York, NY.

**Effect of STAT3 siRNA on PHB Promoter Activity**—STAT3 siRNA (5'-CCGGAAGAGAGUGCAGGAUCUAGAA-3') was obtained from Invitrogen and co-transfected with the full-length ( $-1054/+138$ -bp) PHB promoter construct and pRL-CMV for 48 h because this time point showed highest RNA interference by Western blot for STAT3. Cells were serum-deprived overnight, treated with 100 ng/ml IL-6 for 6 h, and assayed for luciferase activity as described above. Scramble RNA was used as a negative control (Invitrogen).

**Electrophoretic Mobility Shift Assay (EMSA) and STAT3 Supershift**—Nuclear protein extracts were isolated from serum-deprived polarized Caco2-BBE cells treated with 100 ng/ml IL-6 on the basolateral side for 0, 0.5, 1, 2, and 8 h. 10  $\mu$ g of nuclear protein was assayed for DNA binding to biotin-labeled, double-stranded oligonucleotides corresponding to the IL-6RE binding site (5'-GCTATTCTGGGAGGGTGA-3') in the PHB promoter obtained from Integrated DNA Technologies (Coralville, IA). The IL-6RE binding site is underlined. The oligonucleotides were end-labeled using the Biotin 3'-end DNA labeling kit (Pierce) according to the manufacturer's instructions. EMSAs were performed using the Lightshift Chemiluminescent EMSA kit (Pierce). 20 fmol biotin-labeled oligonucleotide was incubated with 10  $\mu$ g of nuclear proteins for 20 min at room temperature in binding buffer (50 mM Tris, pH 7.4, 2.5 mM EDTA, 0.25 mg/ml poly(dI-dC), 250 mM NaCl, 2.5 mM dithiothreitol, 5 mM MgCl<sub>2</sub>, and 20% glycerol). Binding was competed by 8-fold excess unlabeled IL-6RE oligonucleotides (cold). For STAT3 supershift, 8  $\mu$ g of STAT3 antibody (Santa Cruz) was added after incubation of the nuclear proteins with the biotin-labeled oligonucleotides. IL-6RE binding complexes were resolved by electrophoresis using 5% TBE Criterion gels (Bio-Rad), transferred to Biodyne B pre-cut modified nylon membranes (Pierce), UV cross-linked, and visualized using the Chemiluminescent Nucleic Acid Detection system (Pierce).

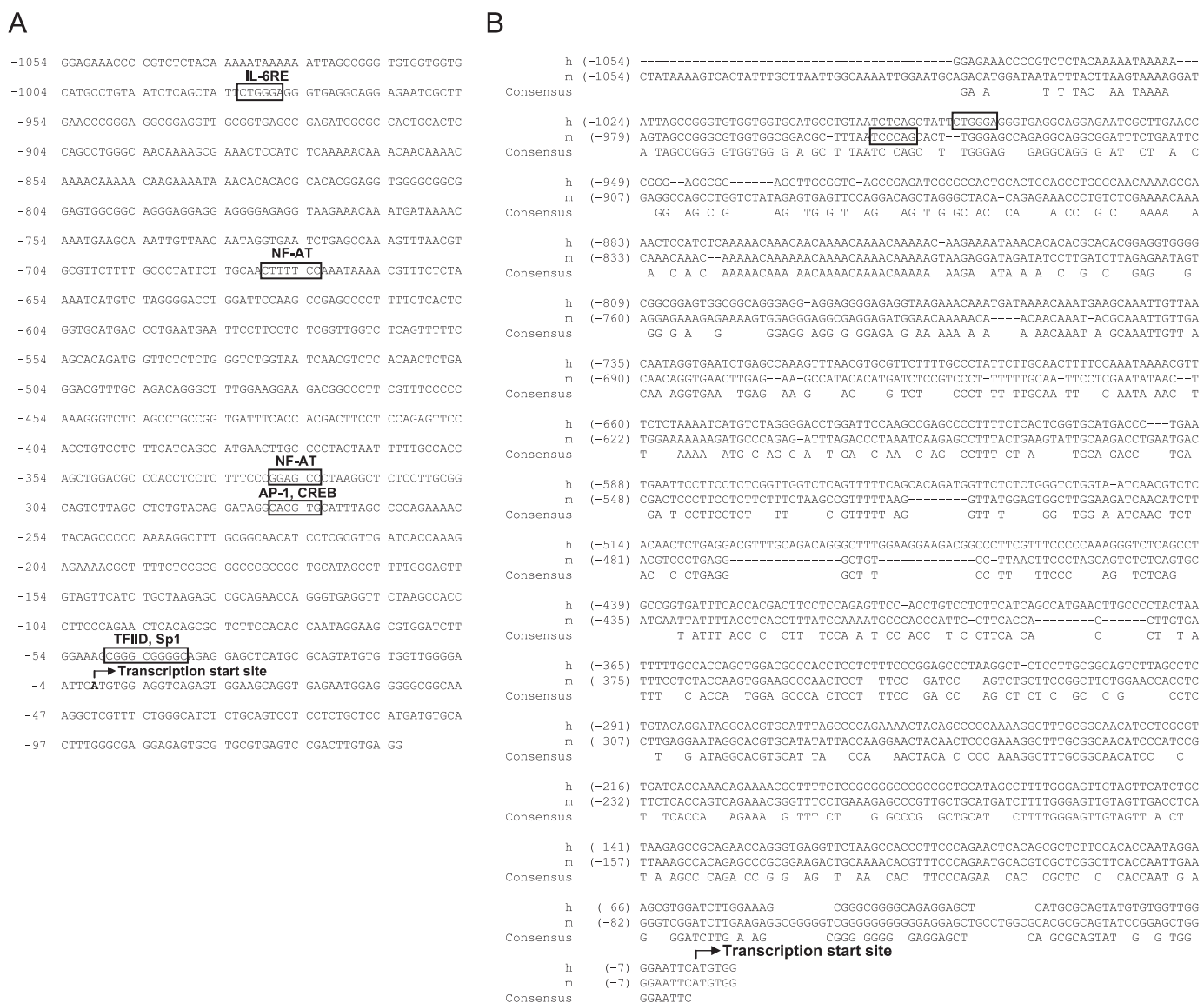
**IL-6<sup>-/-</sup> Mice**—Male wild-type (WT) and IL-6<sup>-/-</sup> mice (6–8 weeks, 12–16 g) on the inbred C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were group-housed in standard cages under a controlled



**FIGURE 1. IL-6 induces expression of PHB in Caco2-BBE cells.** A, total RNA from polarized Caco2-BBE cells treated with IL-6 for various amounts of time was subjected to reverse transcription followed by quantitative real-time PCR amplification using PHB-specific primers. Histograms show mean  $\pm$  S.E. relative to 0-h time point. \*,  $p < 0.05$  versus control;  $n = 3$ /time point performed in triplicate. B, representative Western immunoblots showing PHB and  $\beta$ -tubulin (loading control) expression in total protein lysates from Caco2-BBE cells treated with IL-6. Histograms show mean  $\pm$  S.E. relative to no-treatment control cells. \*,  $p < 0.05$  versus control;  $n = 3$ /treatment.

temperature (25  $^{\circ}$ C) and photoperiod (12:12-h light/dark cycle) and were allowed standard chow and tap water *ad libitum*. They were allowed to acclimate to these conditions for at least 7 days before inclusion in experiments. All procedures were in accordance with the Emory University Institutional Animal Care, authorization number 146–2002. Total colon was isolated; a small distal portion was snap-frozen in Optimal Cutting Temperature and stored at  $-80^{\circ}$ C for confocal staining, while the remaining colon was split into two pieces with one half homogenized in phosphate-buffered saline containing 1% Triton X-100, 1% Nonidet P-40 (v/v), 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and 1  $\mu$ l/ml protease inhibitor mixture III (Roche Applied Science) to obtain protein





**FIGURE 2. Characterization of the cloned 5'-flanking region of the human PHB gene and comparison of the human and mouse PHB promoter sequences.** A, sequence of the cloned 5'-flanking region of the human PHB gene (GenBank™ accession number DQ406856). Putative transcription factor binding sites, which are boxed in the sequence, were identified using the program TESS. B, sequence alignment of the human (h) and mouse (m) PHB promoter regions. IL-6RE sites present in both promoter regions are boxed.

extracts for Western immunoblotting and the other half homogenized in TRIzol for RNA isolation.

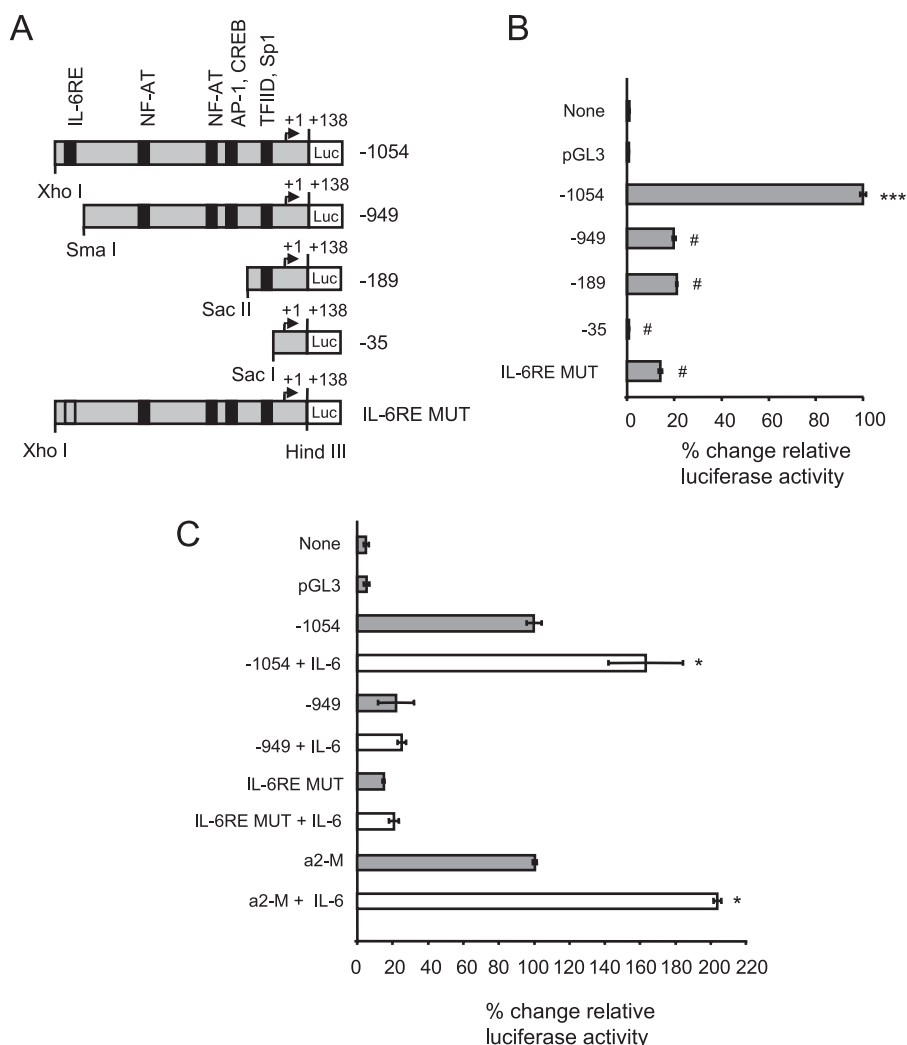
**Confocal Microscopy**—10- $\mu$ m cryostat sections of mouse colon were fixed in buffered 4% paraformaldehyde for 20 min, blocked in 2% bovine serum albumin, incubated with rabbit PHB antibody (Lab Vision Corp.) overnight at 4 °C, washed with phosphate-buffered saline, and subsequently incubated with fluoresceinated secondary antibody for 1 h at room temperature. Colon sections were stained with rhodamine/phalloidin (Molecular Probes, Carlsbad, CA) to visualize actin. Samples were mounted in *p*-phenylenediamine/glycerol (1:1) and analyzed by confocal microscopy (Zeiss dual-laser confocal microscope) as previously described (33).

**Statistical Analysis**—Values are expressed as mean  $\pm$  S.E. Statistical analysis was performed using unpaired Student's *t* test. A *p* value <0.05 was considered statistically significant in all analyses.

## RESULTS

**IL-6 Induces Expression of PHB in Caco2-BBE Cells**—Caco2-BBE cells were treated with IL-6 for various amounts of time and assayed for PHB mRNA and protein expression. As shown by quantitative real-time PCR, PHB mRNA expression is significantly increased by IL-6 treatment at 4 and 8 h and returns to basal levels at 12 h (Fig. 1A). PHB protein expression is significantly increased after 8 h of IL-6 treatment and sustained through 24 h (Fig. 1B).

**Isolation of the 5'-Flanking Region of the Human PHB Gene and Comparison of the Human and Mouse PHB Promoter Sequences**—To determine whether IL-6 induces PHB mRNA and protein expression via transcriptional responses, the 1192-bp promoter region of PHB was cloned (Fig. 2A). The 5'-flanking region of the human PHB gene isolated from Caco2-BBE cells was confirmed by sequence comparison with



**FIGURE 3. The IL-6 response element is essential for basal PHB promoter activity, and the IL-6RE is required for IL-6 responsiveness.** *A*, schematic representation of the putative regulatory sites in the full-length PHB promoter and deletion constructs. Restriction enzymes used to make the deletion constructs are shown. *B*, the full-length promoter (–1054 bp) of the PHB gene and sequential deletion promoter constructs (–949, –189, –35 bp) were transfected into Caco2-BBE cells, and the basal relative luciferase activity was measured after 72 h. An IL-6 response element (IL-6RE) that is located at –977 to –982 bp from the transcription start site was mutated by site-directed mutagenesis (IL-6RE MUT), transfected into Caco2-BBE cells, and assessed for luciferase activity. All data presented represent the mean  $\pm$  S.E. for firefly luciferase activity normalized to *Renilla* luciferase activity from two independent experiments ( $n = 6$ /treatment for each experiment). \*\*\*,  $p < 0.0001$  versus pGL3; #,  $p < 0.0001$  versus –1054. *C*, relative luciferase activity was measured in serum-deprived Caco2-BBE cells transfected with pGL3 vector, full-length PHB promoter (–1054), full-length PHB promoter with mutated IL-6RE site (IL-6RE MUT), –949 deletion construct, or  $\alpha$ 2-macroglobulin ( $\alpha$ 2-M) promoter (as a positive control) and treated with IL-6 (100 ng/ml) for 6 h. All data presented represent the mean  $\pm$  S.E. for normalized luciferase activity from two independent experiments ( $n \geq 3$ /treatment for each experiment). \*,  $p < 0.05$  versus –1054.

the human genomic clone of chromosome 17 (GenBank<sup>TM</sup> accession number AC091180). To localize the transcription start site(s) for the PHB gene, 5'-rapid amplification of cDNA ends experiments were performed with total RNA isolated from Caco2-BBE cells. Subsequent PCR produced a single 215-bp DNA fragment using a PHB-specific primer located downstream of the start ATG and the GeneRacer<sup>TM</sup> 5'-primer (data not shown). Sequencing revealed that this fragment mapped 1594 bp upstream from the start of translation codon (ATG) due to an intervening intron (Fig. 2A). The first base of this fragment is considered the transcription start site and will be referred to as nucleotide +1. A putative IL-6RE site is located at –977 to –982 bp from the start of transcription, two nuclear

factor of activated T-cells sites are located at –679 to –673 bp and at –323 to –328 bp, and an activator protein-1 (AP-1)/cAMP response element-binding protein site is located at –273 to –278 bp (Fig. 2A). A putative TFIID/Sp1 site is located at –39 to –48 bp from the transcription start site, indicating that the general transcription initiation factor is present in the region cloned.

To determine homology across the human and mouse PHB promoter regions, the 1054-bp region upstream from the transcription start site of the mouse PHB gene was aligned with the human PHB promoter sequence (Fig. 2B). The two promoter regions share 56.4% homology. To determine whether the mouse PHB promoter also possesses an IL-6RE in the same region as the human PHB promoter, the mouse PHB promoter sequence was submitted to TESS for analysis. An inverse IL-6RE of identical sequence is located in the mouse PHB promoter 11 base pairs upstream from the IL-6RE in the human PHB promoter, indicating that the IL-6RE is preserved.

*The IL-6 Response Element Is Essential for Basal PHB Promoter Activity*—To identify regions involved in regulating PHB gene transcription, a series of sequential deletion reporter constructs (–949, –189, –35 to +138 bp) were cloned into the luciferase-containing vector pGL3 (Fig. 3A). Constructs were transiently transfected into Caco2-BBE cells, and relative luciferase activity was compared with the full-length promoter (–1054 to +138).

Cells transfected with the full-length promoter showed an 80-fold induction of relative luciferase activity compared with cells transfected with pGL3 vector (Fig. 3B). The –949 and –189-bp deletion constructs showed an 80% decrease in relative luciferase activity compared with the full-length promoter, suggesting that the regulatory site removed in the –949-bp construct is essential for maximal PHB promoter activity. The regulatory site present between –1054 and –949 bp is an IL-6RE (–977 to –982 bp). The –35 deletion construct showed no induction of relative luciferase.

To determine whether the IL-6RE is necessary for maximal PHB transcriptional activation, a mutation was introduced into the IL-6RE by site-directed mutagenesis. As shown in Fig. 3B,

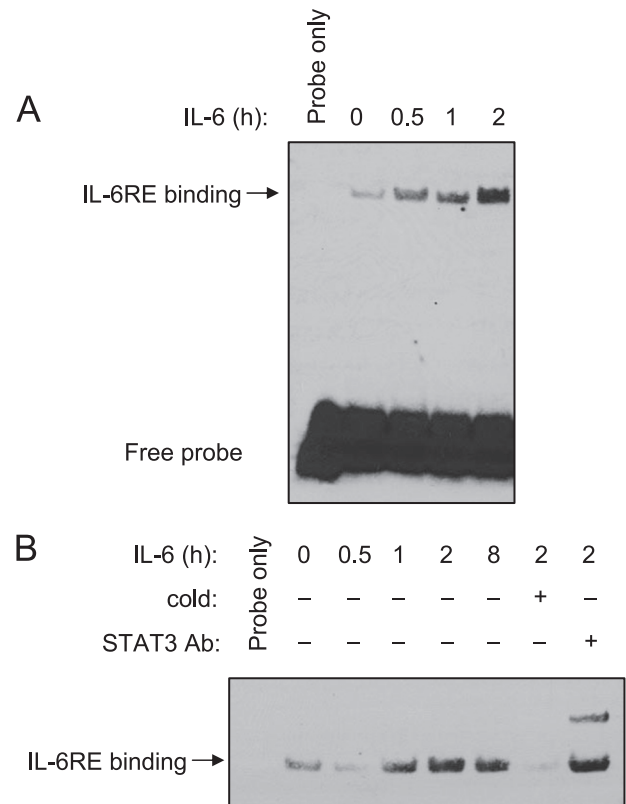
mutation of the IL-6RE resulted in 85% decrease in relative luciferase activity compared with that observed with the  $-1054$  wild-type construct, which was similar to that found with removal of the IL-6RE in the  $-949$  deletion construct. Together, these results suggest that the IL-6RE is the regulatory site necessary for maximal basal PHB promoter activation in intestinal epithelial cells.

**IL-6 Increases PHB Promoter Activation and the IL-6RE Is Required for IL-6 Responsiveness**—To determine whether IL-6 increases PHB promoter activation in Caco2-BBE cells, cells transfected with the full-length construct ( $-1054$  bp) were treated with IL-6 for 6 h and compared with no treatment control cells. As shown in Fig. 3C, cells treated with IL-6 ( $-1054 + \text{IL-6}$ ) showed a 60% increase in promoter activity compared with vehicle-treated cells transfected with full-length promoter ( $-1054$ ), indicating that IL-6 stimulates PHB promoter activation. To identify whether the IL-6RE site was essential for responsiveness to IL-6, Caco2-BBE cells were transfected with the  $-949$ -bp deletion or the IL-6RE mutant (IL-6RE MUT) construct and treated with IL-6 simultaneously with cells transfected with the full-length construct. Both the  $-949$ -bp deletion construct and the IL-6RE MUT showed an 80–85% reduction in basal relative luciferase compared with the full-length construct, as shown previously in Fig. 3B. The  $-949$ -bp construct and the IL-6RE MUT showed no induction of luciferase activity by IL-6 treatment, indicating that the IL-6RE site is necessary for PHB promoter responsiveness to IL-6.

The  $\alpha 2$ -M promoter was used as a positive control because it contains a consensus-type II IL-6RE. The  $-1151/+54$ -bp  $\alpha 2$ -M promoter showed a 2-fold induction of relative luciferase with IL-6 treatment compared with no treatment (Fig. 3C).

**IL-6 Induces STAT3 Binding to the IL-6RE Binding Site Located in the PHB Promoter**—Because the IL-6RE is essential for PHB promoter responsiveness to IL-6, we next performed EMSA to determine whether IL-6 stimulates transcription factor binding to the IL-6RE. Nuclear protein extracts show increased binding to the IL-6RE present in the PHB promoter after 1 h of IL-6 treatment, with maximum binding after 2 h (Fig. 4). Binding is competed by unlabeled IL-6RE oligonucleotides (Fig. 4B, *cold*). To determine whether STAT3 was binding to the putative PHB promoter IL-6RE site, we performed STAT3 supershift using a specific STAT3 antibody. As shown in Fig. 4B, the binding complex is indeed shifted when the antibody is included, indicating that STAT3 is binding to the IL-6RE located in the PHB promoter.

**STAT3 siRNA Down-regulates Basal and IL-6-stimulated PHB Promoter Activity**—We and others have previously demonstrated that IL-6 receptors are abundantly expressed at the basolateral surface of epithelial cells (32). IL-6 modulates its downstream effect through activation of STAT3 and NF- $\kappa$ B signaling in intestinal epithelial cells (32, 34). Given that STAT3 binds to the IL-6RE in the PHB promoter as shown by STAT3 supershift, we determined the effect of STAT3 siRNA on PHB promoter activity. To determine whether STAT3 is involved in PHB promoter activity, cells were transiently co-transfected with full-length PHB promoter construct ( $-1054$ ) and STAT3 siRNA or scramble RNA as a control, treated with IL-6, and assayed for relative luciferase activity. STAT3 siRNA abolishes



**FIGURE 4. IL-6 induces STAT3 binding to the IL-6RE binding site located in the PHB promoter.** A, EMSA showing binding of nuclear proteins from Caco2-BBE cells treated with IL-6 for various amounts of time. B, EMSA showing maximal binding of nuclear proteins from Caco2-BBE cells treated for 2 h with IL-6. Addition of STAT3 antibody to the binding reactions results in a supershift of the complexes. Binding is competed by unlabeled IL-6RE oligonucleotides (*cold*).

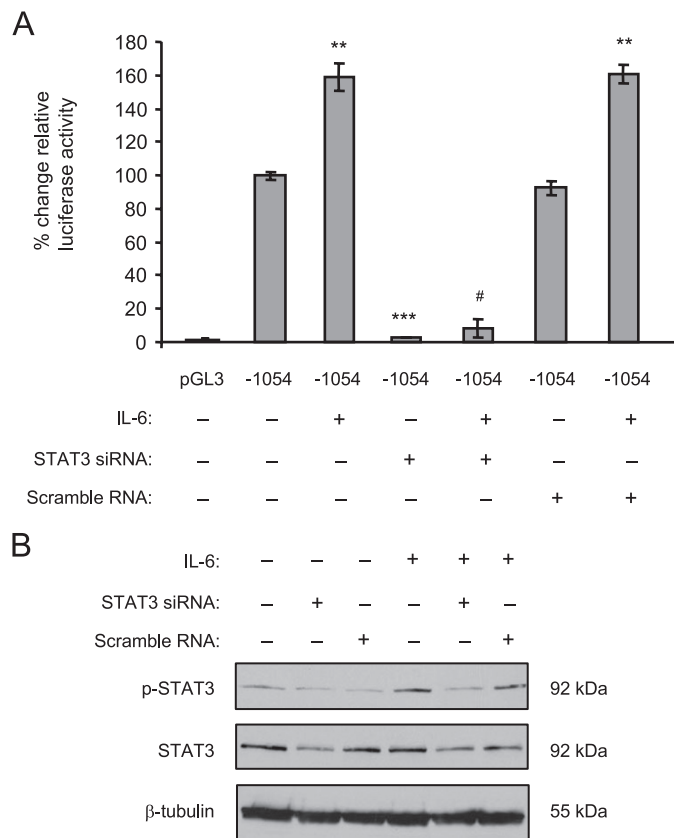
basal PHB promoter activity and stimulation by IL-6 (Fig. 5A). Cells transfected with scramble RNA show similar results to those transfected with full-length PHB promoter construct ( $-1054$ ).

**Colonic PHB Protein and mRNA Expression Are Reduced in IL-6 $^{-/-}$  Mice Compared with WT Mice**—Given our results suggesting that IL-6 modulates PHB expression in cultured intestinal epithelial cells, we assessed the effect of IL-6 deficiency on PHB expression *in vivo*. For these experiments we used colon isolated from IL-6 $^{-/-}$  mice. Our data demonstrate that IL-6 $^{-/-}$  mice have significantly reduced PHB protein expression in colonic extracts compared with WT control mice (Fig. 6A). Western blots using extracts from colonic tissue show an additional band above PHB. We are unsure of the identity of the upper band, but it is likely nonspecific binding of the PHB antibody because total colonic extracts contain many other cell types in addition to intestinal epithelial cells as compared with extracts from Caco2-BBE cells.

Quantitative real-time PCR was used to assay PHB mRNA expression in total RNA isolated from colon of IL-6 $^{-/-}$  and WT mice. IL-6 $^{-/-}$  mice show  $\sim 50\%$  less PHB mRNA expression in colon compared with WT mice (Fig. 6B).

We next localized PHB expression in colon sections by immunofluorescence and confocal microscopy. PHB predominantly localizes to epithelial cells with little staining in the underlying lamina propria in IL-6 $^{-/-}$  and WT colon (Fig. 6C,





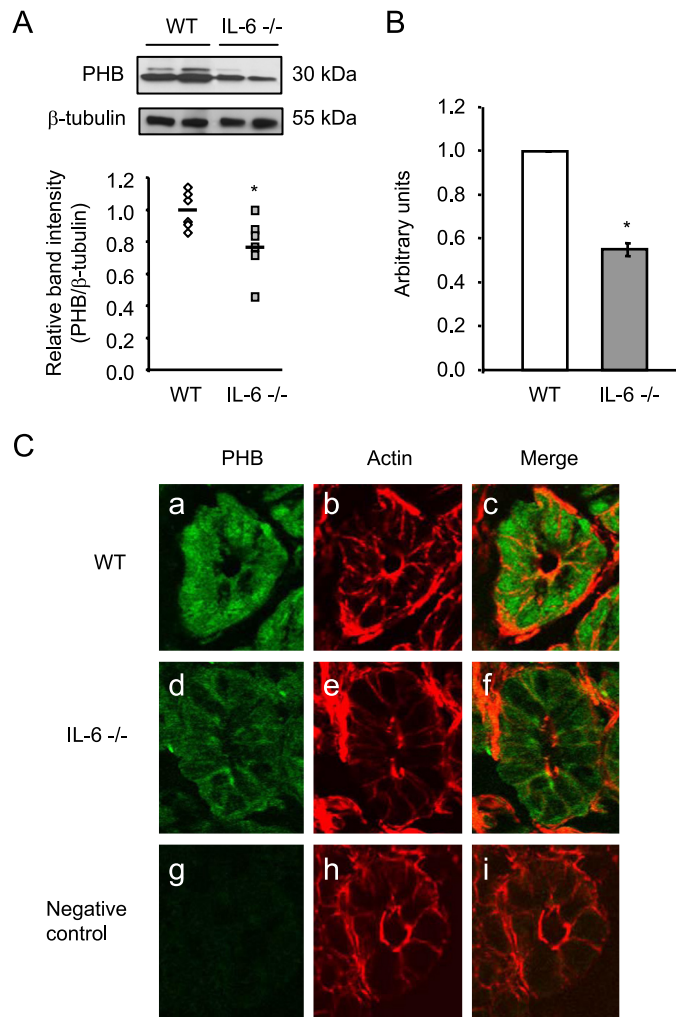
**FIGURE 5. STAT3 siRNA down-regulates basal and IL-6-stimulated PHB promoter activity.** A, relative luciferase activity was measured in serum-deprived Caco2-BBE cells co-transfected with the full-length PHB promoter construct (–1054), STAT3 siRNA, or scramble RNA and treated ± IL-6 (100 ng/ml) for 6 h. All data presented represent the mean ± S.E. for normalized luciferase activity. \*\*,  $p < 0.005$  versus –1054; \*\*\*,  $p < 0.0001$  versus –1054; #,  $p < 0.0001$  versus –1054 + IL-6;  $n = 6$ /treatment. B, total STAT3 and phospho-STAT3 (p-STAT3) were assayed in cells transfected with STAT3 siRNA to determine efficiency of RNA knock down. Cells were treated with 100 ng/ml IL-6 for 1 h. β-tubulin was measured to ensure equal protein loading.

panels c and f), similar to our previous findings (18). Confirming our Western blot results, confocal staining reveals less PHB staining in IL-6<sup>-/-</sup> colon compared with WT colon using the same microscope settings to obtain the images (Fig. 6C, panel a versus panel d).

## DISCUSSION

Although it is well established that the levels of PHB expression are altered in multiple types of cancer and during inflammatory bowel disease (11–18), the regulation of PHB expression in intestine and other tissues was not well characterized. Here we show that the acute phase cytokine IL-6 increases PHB protein and mRNA expression and induces PHB promoter activation in cultured intestinal epithelial cells. Confirming these *in vitro* results, IL-6<sup>-/-</sup> mice exhibit reduced PHB expression in the colon compared with WT mice. These results suggest that IL-6 modulates PHB expression in cultured intestinal epithelial cells and in the intestine *in vivo*.

Deletion mapping analysis of the full-length PHB promoter indicated the presence of an IL-6RE (–977 to –982 bp) that was required for maximal basal promoter activity and promoter responsiveness to IL-6. IL-6 also increases binding of nuclear



**FIGURE 6. Colonic PHB protein and mRNA expression are reduced in IL-6<sup>-/-</sup> mice compared with WT mice.** A, Western immunoblots showing PHB or β-tubulin (loading control) expression in total protein lysates from colon of wild-type (WT) and IL-6<sup>-/-</sup> mice. Histograms show mean ± S.E. Each animal is plotted as an individual dot in the histogram. \*,  $p < 0.05$  versus WT;  $n = 6$ . B, total RNA from colon of IL-6<sup>-/-</sup> and WT mice was subjected to reverse transcription followed by quantitative real-time PCR amplification using PHB-specific primers. Histograms show mean ± S.E. relative to control. \*,  $p < 0.05$  versus control;  $n = 4$  animals/group performed in triplicate. C, confocal staining of PHB localization in a cross-section of a colonic crypt from WT (panels a–c) and IL-6<sup>-/-</sup> mice (d–f). Sections were counterstained for rhodamine/phalloidin to visualize actin. PHB staining is reduced in IL-6<sup>-/-</sup> epithelium compared with WT (panels a versus d and c versus f) using the same microscope settings to obtain the images. WT sections incubated with normal rabbit serum and rhodamine/phalloidin antibody to visualize the tissue were used as a negative control (panels g–i). Magnification, ×40.

proteins to the IL-6RE in the PHB promoter as shown by EMSA. The IL-6RE present in the PHB promoter is a type II IL-6RE with the consensus sequence CTGGGA (35, 36). Type II IL-6REs are found in some acute phase proteins such as α 2-macroglobulin and fibrinogen (35, 37) and in several immediate early genes, including ICAM-1 and *junB* (38). The nuclear factors that bind to the type II IL-6RE include the IL-6 response element-binding protein (IL-6REBP), STAT3, and STAT5a/b (37, 39–41). The IL-6RE and flanking nucleotides (TTCTGGGAG; IL-6RE is underlined) in the PHB promoter show high sequence homology with the consensus STAT3 DNA binding motif TTC(C/T)GGGAA (42). Compared with the α 2-macro-

globulin promoter that contains the consensus STAT3 binding motif and shows 2-fold induction of activity by IL-6 in Caco2-BBE cells, the PHB promoter shows 1.6-fold induction by IL-6. This could be due to lack of the A nucleotide in the IL-6RE of the PHB promoter. However, STAT3 supershift of nuclear proteins binding to IL-6RE oligonucleotides in combination with results showing that STAT3 siRNA abolishes basal and IL-6-induced PHB promoter activity suggests that STAT3 binds to the IL-6RE located in the PHB promoter and is crucial for promoter activity in Caco2-BBE cells.

The finding that the IL-6RE is necessary for maximal basal PHB promoter activity is somewhat surprising given that levels of IL-6 under control conditions are relatively low in intestine. Our results reflect the role of the IL-6RE in the regulation of baseline PHB expression in intestinal epithelial cells and not in other cell types because PHB regulation may vary from tissue to tissue. Despite relatively low levels of IL-6 during control conditions, IL-6 is secreted by intestinal epithelial cells (43) as well as pericryptal fibroblasts that are in intimate contact with epithelial cells under physiological conditions. Intestinal epithelial cells possess the IL-6 receptor at the same density or higher than monocytes under physiological conditions (32, 44, 45). Although under pathological conditions excessive secretion of IL-6 may play a major role in the pathogenesis of many diseases, including inflammatory bowel disease (26, 46), under physiological conditions IL-6 expression is important for the host response to a number of infections (47). Similar to our results using PHB deletion constructs, deletion of the IL-6RE from the rat  $\alpha$  2-macroglobulin gene resulted in attenuated basal promoter activity (36). Perhaps the relatively low levels of IL-6 under control conditions are enough to basally stimulate the PHB promoter or STAT3 that binds the IL-6RE is stimulated by other factors besides IL-6 during basal conditions. Approximately 20% of PHB promoter activity resides with deletion or mutation of the IL-6RE, indicating that factors that bind to other transcriptional regulatory sites are also involved in regulating a portion of PHB promoter activity. Regardless, our results indicate that the IL-6RE is responsible for maximal basal and IL-6-stimulated PHB promoter activity in these cells.

Interestingly, our *in vitro* results in intestinal epithelial cells are confirmed in intestine of IL-6<sup>-/-</sup> mice. PHB mRNA and protein expression is attenuated in colon from IL-6<sup>-/-</sup> mice compared with WT control mice, suggesting that IL-6 is a predominant regulator of PHB expression in intestine *in vivo*. PHB protein expression in liver did not vary between IL-6<sup>-/-</sup> and WT mice,<sup>3</sup> indicating that the effect of IL-6 on PHB regulation may be specific to the intestine. Our results showing decreased PHB expression in IL-6<sup>-/-</sup> colon are especially interesting given that the mouse PHB promoter contains an inverse IL-6RE of identical sequence 11 bp upstream from the IL-6RE in the human PHB promoter. Because PHB expression is not completely absent in IL-6<sup>-/-</sup> mice, other factors must modulate PHB expression in addition to IL-6 or compensate in the absence of IL-6. Multiple studies suggest that IL-6 may be protective against mucosal barrier dysfunction by modulating wound healing and combating oxidative stress. IL-6<sup>-/-</sup> mice

exhibit more severe erosion of the intestinal epithelium subsequent to impaired mucosal wound healing when treated with dextran sodium sulfate to induce colitis (48). *In vivo* administration of IL-6 in septic rats was shown to protect mucosal epithelial cells from sepsis-induced oxidative damage (25). Furthermore, IL-6 was shown to be protective against oxidative stress in other organs, including the liver and lung (23, 24). Our recent findings in intestinal epithelial cells suggest that PHB overexpression protects against oxidative stress (18). Further studies are needed to determine whether PHB is a downstream mediator of IL-6 signaling, leading to these functional responses in the intestinal epithelium of IL-6<sup>-/-</sup> mice and mice administered exogenous IL-6.

In conclusion, this study assessed the regulation of PHB expression in intestinal epithelial cells. IL-6 increases PHB protein and mRNA abundance and stimulates PHB promoter activation. Promoter deletion analysis revealed that the IL-6RE is the essential transcription regulatory site for maximal basal and IL-6-induced PHB promoter activity. STAT3 mediates basal and IL-6-induced PHB transcription and binds to the IL-6RE in the PHB promoter. Moreover, IL-6<sup>-/-</sup> mice show decreased PHB expression in colon. Together, these results suggest that IL-6 is the predominant modulator of PHB expression in cultured intestinal epithelial cells and in the intestine *in vivo*. Given that PHB levels are altered in inflammatory bowel disease, this study provides important insights into the potential regulation of PHB expression by IL-6.

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