Regulation of the Bone-restricted IFITM-like (Bril) Gene Transcription by Sp and Gli Family Members and CpG Methylation

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Background: BRIL is a bone-specific membrane protein that is involved in osteogenesis imperfecta type V. Bril transcription is activated by Sp1, Sp3, OSX, and GLI2 and by CpG demethylation. Regulation of Bril transcription is important to understand its role in skeletal biology.

Results: Bril transcription is activated by Sp1, Sp3, OSX, and GLI2 and by CpG demethylation.

Conclusion: Regulation of Bril involves trans-acting factors integrating at conserved promoter elements and epigenetic modifications.

Significance: Identification of the mechanisms governing Bril transcription is important to understand its role in skeletal biology.

Bril encodes a small membrane protein present in osteoblasts. In humans, a single recurrent mutation in the 5′-UTR of BRIL causes osteogenesis imperfecta type V. The exact function of BRIL and the mechanism by which it contributes to disease are still unknown. The goal of the current study was to characterize the mechanisms governing Bril transcription in humans, rats, and mice. In the three species, as detected by luciferase reporter assays in UMR106 cells, we found that most of the base-line regulatory activity was localized within ~250 bp upstream of the coding ATG. Co-transfection experiments indicated that Sp1 and Sp3 were potent inducers of the promoter activity, through the binding of several GC-rich boxes. Osterix was a weak activator but acted cooperatively with Sp1 and GLI2 to synergistically induce the BRIL promoter. GLI2, a mediator of hedgehog signaling pathway, was also a potent activator of BRIL through a single GLI binding site. Correspondingly, agonists of the hedgehog pathway (purmorphamine and Indian hedgehog) in MC3T3 osteoblasts led to increased BRIL levels. The BRIL promoter activity was also found to be negatively modulated through two different mechanisms. First, the ZFP35/4 zinc finger protein repressed basal and Sp1-induced activity. Second, CpG methylation of the promoter region correlated with an inactive state and prevented Sp1 activation. The data provide the very first analyses of the cis- and trans-acting factors regulating Bril transcription. They revealed key roles for the Sp members and GLI2 that possibly cooperate to activate Bril when the promoter becomes demethylated.

Bril (bone-restricted ifitm-like, or Ifitm5) was discovered as part of a high throughput screen for cDNAs encoding secreted and membrane proteins in osteoblastic cells (1). BRIL is part of an evolutionarily conserved family of so-called small interferon-inducible transmembrane (IFITM) proteins (2), for which there are at least four closely related members in humans (IFITM1, -2, -3, and -10) (3, 4). The mouse has two other members (IFITM6 and -7). The term “dispanin” has been coined recently to encompass IFITMs into an even larger family of proteins that have two transmembrane passages (4).

The classification of BRIL, however, as part of this family of IFITMs is based on general rather than functional considerations. BRIL, IFITM1, IFITM2, and IFITM3 are all clustered within 25 kb on chromosome 11 (in humans); they all possess a similar gene architecture comprising two small coding exons separated by a short intron and a potentially similar predicted protein topology, having two transmembrane domains. Immunolocalization studies using tagged BRIL (5) and IFITM3 (6, 7) suggested that they both have their N and C termini extruding out into the extracellular space, although this predicted model has been challenged recently at least for IFITM3 (8, 9). Furthermore, other members like IFITM3 seem to localize preferentially into the endosome compartment (8, 9), whereas BRIL localizes mostly to plasma membranes (5). BRIL presents some other features that make it a distinctive member. For instance, unlike other IFITM members, which are highly inducible by type I interferons due to the presence of interferon regulatory elements in their promoter region (10), Bril transcription is not responsive to interferons (11). More importantly, we have shown that expression of Bril is mostly restricted to osteoblasts (5), whereas other Ifitm genes are ubiquitous. Expression of Bril was confirmed to be enriched in bone tissues in humans (12) and in the tammar wallaby (3) and increased under culture conditions favoring osteogenic differentiation (13).

At the functional level, our group was the first to suggest that BRIL is a positive modulator of mineralization via overexpression and knockdown studies in cultured osteoblasts (5). The molecular mechanisms of BRIL action in osteoblasts, however, have yet to be uncovered. Whether BRIL contributes directly to mineralization by interacting with its extracellular environment/matrix and/or indirectly in association with other mem-
brane and intracellular mediators is still unknown. An interesting hypothesis put forward is that BRIL could be involved in calcium binding through its conserved aspartate-rich C-terminal end (2), a domain reminiscent of EF-hand structure (14). Also, BRIL was found to interact with other transmembrane proteins, such as FK506-binding protein 11, an association that appears to modulate complex assembly with the tetraspanin proteins CD9 and CD81 (15). It remains to be determined whether these interactions occur in vivo and contribute to BRIL function.

Studies exploring the function of Bril by genetic approaches in mice have yielded equivocal evidence. A knock-out mouse model for Bril was reported to have breeding problems and displayed only a subtle and transient reduction in bone length and structure in embryos and neonatal mice, without any evidence for changes in bone morphometric parameters (16). In contrast, our own Bril knock-out/LacZ knock-in mouse did not present any developmental and reproductive problems and did not show any appreciable mineralization defects in their skeletons.\(^2\) In addition, genetic ablation in mice of either Ifitm3 alone or the entire locus comprising Ifitm1, -2, and -3 and Bril did not present any apparent physiological phenotype under normal conditions (17). What has emerged recently is the prominent role of IFITM1, -2, and -3 in inhibition in cell entry and infection by various viruses (6, 18–23), a function dependent on palmitoylation of conserved cysteine residues (7, 9).

Clearly, the information gained from existing mouse models has not allowed one to conclusively infer a function for BRIL in the skeleton (15, 17), despite marked effects observed in vitro on osteoblast activity (5). Two independent studies, however, found that a mutation in the 5′-UTR region of the Bril gene is the cause of osteogenesis imperfecta (OI)\(^3\) type V (12, 25). OI type V is inherited in an autosomal dominant fashion, and patients with OI type V display distinct clinical features not usually observed in any other OI type, such as hyperplastic cal-clus formation and interosseous membrane ossification (26). The mutation found (c.−14C→T) creates a novel in frame ATG upstream of the natural coding start of BRIL, resulting in an extension of 5 residues (MALEP) at its N terminus. Our group has now confirmed that this single recurrent mutation is present in 42 individuals with type V OI (27). It has been proposed that the N terminus extension of the mutant BRIL is a gain of detrimental function, but the underlying molecular mechanism is still elusive.

Nothing is presently known about the regulation of the Bril gene regulation. The aim of the current study was to characterize the mechanisms governing Bril transcription. More specifically, we mapped the promoter cis-acting regulatory elements essential for the osteoblast-specific expression of Bril in humans, rats, and mice. Trans-acting factors were identified that either activated or repressed Bril, and evidence indicates that CpG methylation is an epigenetic mode of regulation for Bril.

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**EXPERIMENTAL PROCEDURES**

**Cell Cultures and Treatment**—The HEK293, UMR106, and MC3T3-E1 (subclone 4, hereafter designated MC3T3) cells were obtained from ATCC and used up to passage 16. HEK293 and UMR106 cells were grown in DMEM, and MC3T3 cells were grown in α-minimum essential medium, all supplemented with 10% fetal bovine serum (FBS) (Invitrogen). For transient transfection experiments, cells were seeded at 100,000 cells/well in 12-well plates (Corning). For differentiation of MC3T3, cells were seeded at 100,000 cells/well in 6-well plates (Sarstedt) and grown for 72 h, at which point they reached confluence. From this point on, which was considered day 0, cells were fed α-minimum essential medium + 10% FBS supplemented with 50 μg/ml ascorbic acid (Sigma) and 3 mM β-glycerophosphate (Sigma). Medium was changed every 2 or 3 days. A stock solution of purmorphamine (PMP) (Cayman Chemical, Ann Arbor, MI), an agonist of the hedgehog pathway, was prepared in DMSO at a concentration of 10 mg/ml and stored at −20 °C. PMP was diluted in differentiating medium to a final concentration of up to 3 μM. Concentration of DMSO was kept constant at 0.05% (v/v) and was also added to control cells. Treatment with PMP was commenced at day 0 and continued throughout the experiment with freshly added PMP at every medium change. Recombinant Indian hedgehog (rIHH) C28II N terminus (R&D Systems, Minneapolis, MN) was diluted directly into medium at the desired final concentration. Mineralization was monitored by alizarin red staining as described previously (5).

**Luciferase (Luc) Reporter Constructs and Expression Plasmids**—The oligonucleotides used in this study are listed in **supplemental Table 1**. The Bril gene promoters were amplified by PCR on genomic DNA extracted from mouse MC3T3 cells, rat UMR106 cells, and human HEK293 cells. Amplification was performed on 25 ng of genomic DNA using Phusion DNA polymerase (New England Biolabs) as recommended by the manufacturer. The sizes of the longest genomic fragments were arbitrarily set at −3913, −1327, and −1434 bp for mice, rats, and humans, respectively. The PCR products extended through the initiator coding ATG of the Bril gene and were cloned directionally into the pGL3-basic (Promega) Luc reporter plasmid. The empty pGL3-basic plasmid was used as a negative control. The promoter regions were progressively deleted from their 5′-ends either using unique restriction sites or through PCR-based amplification (see **supplemental Table 1**). Internal deletions and point mutations were introduced by whole plasmid amplification using Phusion DNA polymerase (New England Biolabs) with phosphorylated primers covering the desired regions, purified on agarose gel, and religated. Plasmids were prepared using the Midiprep Qiagen kit (Qiagen). The identity of all constructs was confirmed by Sanger sequencing on an Applied Biosystems 3730xL DNA Analyzer through the McGill University and Genome Quebec Innovation Centre. For cotransfection studies, all effector transcription factors were overexpressed from plasmids under the regulation of the human CMV promoter, except for AP2α, which was a Rous sarcoma virus-driven promoter. The following plasmids were purchased from Origene: human Sp3 variant 1 (Sp3-L1)
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(rc222027), human Sp3 variant 2 (Sp3-L2) (rc220658), human LEF1 (rc208663), human DLX5 (sc320170), human OSX (osterix) (sc365099), human HIFα (sc119189), human MXS2 (sc118633), human SOX9 (rc208944), mouse Nf1-X (mc200098), mouse Elk1 (mc201893), mouse Zfp354c (mc203431), mouse Zfp521 (mc204047), mouse Hoxa10 (mc206653). The plasmid encoding the short Sp3 isoform (Sp3-S) was generated by digestion of the Sp3-L1 plasmid with BamHI and MfeI, and ends were blunted with T4 DNA polymerase and religated. Other plasmids were obtained from Addgene (Cambridge, MA): human GLI1 (16419), human GLI2 (16420), human AP2α (12100), and human cMYC (16011). The cDNAs for GLI1 and GLI3 were excised from their original pBluescript plasmids and subcloned into pCMV6-Neo (Invitrogen). The cDNAs for mouse β-catenin (βCat), mouse Sp1, and mouse Tcf7 (encoding the TCF1 protein in mice) were obtained by RT-PCR on RNA from MC3T3 with gene-specific primers (supplemental Table 1). Constitutively active βCat was created by consecutively introducing point mutations in codons for serine 33, serine 37, and threonine 41, which were all converted to alanine residues. Expression plasmids for mouse RUNX2 and ATF4 were obtained from Dr. Gerard Karsenty (Columbia University), and expression plasmids for human MEF2C and MEF2D from Dr. Xiang-Jiao Yang (McGill University). The GFP-expressing plasmid pQBiFc3 (Qbiogene) was used as a negative control.

Transient Transfection and Luc Assays—24 h after seeding, medium was changed, and cells were transfected with FuGENE 6 or X-tremeGENE G9 (Roche Applied Science) according to the manufacturer’s instructions. The plasmid DNA/transfection agent ratios were 3:1 for HEK293 and UMR106 and 6:1 for MC3T3. For single transfections in UMR106 cells, 400 ng of Luc reporter plasmid was transfected. For co-transfection studies, a plasmid mix containing 100 ng of reporter and 300 ng of effector were used. For triple co-transfections, the reporter plasmid (100 ng) was mixed with effector plasmids to a total of 300 ng, and when necessary, the effector mix was completed with plasmid encoding GFP. 42 h after transfection, 250 μl of passive cell lysis buffer (Promega) was added per well, and Luc activity was measured using 5 μl of cell extract with 100 μl of the luciferase assay system (Promega) on a Sirius luminometer (Berthold, Oak Ridge, TN). Each transfection was done on duplicate wells and repeated at least three times. As negative controls, the empty pGL3-basic or the GFP-expressing plasmid was used to calculate the -fold induction. Mean values and S.E. are either reported as raw relative luciferase units measured or by -fold induction relative to controls.

RNA Extraction, Reverse Transcription, and Real-time qPCR—Cells were washed twice with PBS, and total cellular RNA was extracted with TRIzol (Invitrogen). Purified RNA was quantified on a NanoDrop spectrophotometer (Thermo Scientific), and 2 μg was reverse transcribed in 20 μl with the High Capacity cDNA synthesis kit (Applied Biosystems). Real-time qPCR was performed on an Applied Biosystems 7500 PCR machine with 0.5–1 μl of cDNA in a 25-μl reaction volume with the 2X Universal PCR Master Mix and the following Taqman probes (Applied Biosystems): Alp Mm00475834_m1, Bglp1 (osteocalcin) Mm03413826_mH, Glil1 Mm00494654_m1, Glil2 Mm01293116_m1, Glil3 Mm00492345_m1, Bril Mm00804741_g1, Mef2c Mm01340842_m1, Runx2 (Runt-related transcription factor 2) Mm00501584_m1, Sp1 Mm00489039_m1, Sp3 Mm00830425_m1, O XB Mm00506474_m1, Tcf7 Mm00493445_m1, Zfp354c Mm00457419_m1. All data are normalized to β-actin (4352993) or ribosomal 18S (Mm03928990_g1), and values were expressed as 2\(^{-ΔΔC_T}\) (28).

Alkaline Phosphatase Activity and Western Blotting—Alkaline phosphatase (ALP) activity was measured on cell extracts prepared with 100 mm Tris-HCl (pH 9.0) containing 0.1% Triton X-100. Extracts were mixed with p-nitrophenil phosphate (Sigma), and the activity was recorded on an ELx808 96-well microplate reader (BioTek) at 450 nm over 60 min at 37 °C with readings every 5 min. Protein concentrations were measured using the Bradford reagent (Bio-Rad), and specific activity (p-nitrophenil phosphate produced/min/mg of protein) was calculated using a standard curve of p-nitrophenol (Sigma). For Western blotting, total cell extracts were prepared with 50 mm Tris-HCl (pH 7.4), 150 mm NaCl, 1 mm EDTA, 1% (v/v) Nonidet P-40. Insoluble material was pelleted at 13,000 rpm for 10 min at 4 °C, and the supernatant was mixed with 4× Laemmli buffer and boiled. SDS-PAGE and Western blotting were performed as described previously (5). Equal amounts of protein loaded across lanes were verified by immunoblotting with an anti-ACTIN antibody (clone MA1-744) from Affinity Bio-Reagents (Golden, CO). The anti-BRIL antibody was as described previously (5).

Mapping of Transcriptional Start Sites—The transcription start sites of the mouse and rat Bril gene were mapped using the oligonucleotide-capping 5’-rapid amplification of cDNA ends strategy as described previously (1, 29). Briefly, total RNA was extracted from differentiating mouse MC3T3 cells at day 15 and confluent rat UMR106 cells. Poly(A) RNA was purified using the OligoTex kit (Qiagen) and processed as follows. mRNAs were dephosphorylated with bacterial alkaline phosphatase (Takara) and then treated with tobacco acid pyrophosphatase (Epicenter Biotechnologies) to remove the cap structure. An RNA linker was ligated with T4 RNA ligase 1 (New England Biolabs) at the 5’-end of mRNA. The purified RNA was reverse transcribed with Superscript III (Invitrogen) and the Bril-specific reverse primer. The cDNA was then amplified by PCR using a forward primer complementary to the RNA linker and a nested reverse primer specific for Bril. The PCR products were separated on a 1.5% agarose gel, purified from gel on MinElute columns (Qiagen), and cloned into pBluescript plasmid. Plasmids were purified from cultures of bacterial transformants, and DNA was sequenced with T7 primer.

DNase I Footprinting and Electromobility Shift Assay (EMSA)—Nuclear extracts used for both DNase I footprinting and EMSA were prepared from HEK293 cells based on a previously published technique (30). Extracts were obtained 48 h after transient transfection or not with plasmid expressing Sp1 in HEK293. The procedure for the DNase I footprinting assay was essentially as described (31, 32). To generate a rat Bril promoter DNA probe, an oligonucleotide covering positions –265 to –247 relative to the ATG was end-labeled with T4 polynucleotide kinase (New England Biolabs) and 50 μCi of [γ-32P]ATP (3000 Ci/mmol) (PerkinElmer Life Sciences) at
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37 °C for 1 h. Using the rat Bril promoter Luc construct as template, a PCR fragment was amplified with the labeled primer and a reverse primer located at 66 bp within the coding region of Luc. The resulting 331-bp fragment was purified on a 5% acrylamide-TBE gel, and 25,000 cpm was used for binding with nuclear extracts at 25 °C for 10 min. Increasing concentrations of HPLC-pure DNase I (Amersham Biosciences) were added and digested for exactly 2 min at 25 °C. The reaction was stopped and treated with proteinase K for 30 min at 55 °C. After precipitation, the digested probe was separated on a 5% acrylamide-urea-TBE sequencing gel. Gel was dried and exposed to radiography film for 18 h at −80 °C. The naked probe without nuclear extracts was digested in parallel and run side-by-side on the gel. A Maxam-Gilbert G + A chemical sequencing reaction on the labeled probe was performed to obtain a proper ladder. For EMSA, oligonucleotide pairs were end-labeled as above with [γ-32P]ATP, annealed, and purified on Illustra ProQuant G50 microcolumns (GE Healthcare). Labeled double-stranded oligonucleotides (50,000 cpm; 13 fmol) were incubated with nuclear extracts for 10 min at 25 °C and separated on 4% acrylamide-TBE (0.5×) gels. Gels were dried, and autoradiography was performed for 3–15 h.

Gl2 Knock-out Mice and Tissue Processing—All animal experimentation was reviewed and approved by the Shriners Hospital for Children animal care committee and McGill University. The Gl2 heterozygote mice were kindly provided by Dr. Janet Henderson (McGill University) and maintained in the C3H background. The Gl2−/− mice were described previously to be perinatally lethal and shown to have impaired bone development (33, 34). In order to generate the Gl2−/− embryos at embryonic stages E15.5 and E17.5, pregnant females were euthanized by CO2 asphyxia followed by cervical dislocation. Embryos were dissected, and genomic DNA was extracted from tails for PCR genotyping exactly as described previously (33, 34). Hind limbs and heads were dissected and processed for RNA extraction and immunohistochemistry. The hind limbs were skinned and homogenized with a Polytron homogenizer into 1 ml of TRIzol, and total RNA was extracted and processed for RT-qPCR as described above. For immunolocalization of BRIL, hind limbs were fixed with 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.0) at 4 °C for 24 h, washed with PBS, and decalcified (only for E17.5 tissues) at 4 °C for 48 h with a 15% EDTA solution (pH 7.0). After dehydration, tissues were embedded into paraffin, and 5-μm sections were cut. All subsequent steps were performed at room temperature. Sections were deparaffinized with xylene and rehydrated. Sections were sequentially incubated with dual endogenous enzyme block (Dako) for 10 min, 5% skim milk in PBS for 1 h, and anti-Bril affinity-purified antibody (0.02 μg/ml) in PBS with 0.5% skim milk for 1 h. Sections were washed five times with PBS, incubated with Envision+ anti-rabbit HRP polymer (Dako) for 30 min, and washed five times with PBS. Visualization was achieved by incubation with the Liquid DAB+ Substrate Chromogen system (Dako) for 10 min and then rinsing thoroughly with tap water. Sections were briefly counterstained with hematoxylin QS (Vector) and mounted with Microkit (Macalab). Photographs were taken on a Laica microscope equipped with an Olympus DP70 digital camera.

Bisulfite Sequencing and in Vitro Methylation of Plasmids—MC3T3 cells were grown to confluency and differentiated for 3 days in the absence or presence of 1 μM PMP. Total genomic DNA was isolated and purified. 2 μg of genomic DNA from control and PMP-treated cells were treated using the MethylCode Bisulfite Conversion Kit (Invitrogen) and subsequently purified as recommended by the manufacturer. Converted genomic DNA was then amplified by PCR using two different primer pairs designed with the MethPrimer software (35). The primer sequences are matching fully converted DNA, where all Cs have been converted to Ts, and do not cover any CpG dinucleotides. They amplify two overlapping fragments of the mouse Bril gene of 172 and 250 bp. In total, the analysis looked at 12 CpG sites located on a 408-bp segment starting from −242 to +166 in relation to the ATG (position set as +1). Each PCR contained 600 ng of converted genomic DNA, a 0.5 μM concentration of each primer, 0.2 mM dNTP, 3 units of recombinant TaqDNA polymerase (New England Biolabs) in a final reaction volume of 25 μl. The PCR program was as follows: 95 °C for 2 min (one cycle); 94 °C for 30 s, 56 °C for 25 s, 72 °C for 25 s for 35 cycles. The PCRs were loaded on a 2% agarose gel, stained with ethidium bromide, and the products were excised from the gel and purified on Minelute columns (Qiagen). The purified products were then ligated into pBluescriptKS having T/A overhangs. After transformation into DH10B bacteria, randomly selected colonies were picked for plasmid preparation. Sequencing was done with the T7 primer on a total of 72 independent clones (18 each for the control and PMP-treated cells, for both primer pairs). The human −1434 bp promoter Luc reporter plasmid was methylated in vitro using the M.SssI CpG methyltransferase (New England Biolabs). Briefly, 6 μg of plasmid was incubated for 4 h at 37 °C in a final volume of 120 μl with 40 units of M.SssI in the presence of 160 μM S-adenosylmethionine. As control, the plasmids were processed under identical conditions but without enzymatic treatment. The treated and non-treated plasmids were purified on Minelute columns (Qiagen) and digested with HpaII (cleavage inhibited by methylation) or MspI (cleavage insensitive to methylation) to confirm the methylation efficiency. Plasmids (100 ng) were then co-transfected into HEK293 cells together with 300 ng of either GFP or Sp1. Luc activity was assayed 42 h later.

RESULTS

Characteristics of the Bril Gene and Promoter Region—In order to search for potential regulatory regions present in the vicinity of the BRIL gene and common between humans (Fig. 1A), rats, and mice, the genomic sequences were analyzed in silico using the ECR Browser (available on the World Wide Web). The highest regions of similarity found were those covering the exons and intron (Fig. 1B). A very high region of identity (red area, up to 75%) was also observed on a short stretch upstream of exon 1 covering about 400 bp, which probably corresponds to the proximal promoter (Fig. 1C). Further upstream, the sequence homology abruptly became very divergent and less likely to contain conserved regulatory elements. Another highly conserved region covering about 70 bp was also identi-
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FIGURE 1. Structure, conservation, and promoter region annotation of the human and rat Bril genes. A, schematic representation of the human BRIL gene locus, flanked with the IFITM2 and ATHL1 genes. B, evolutionarily conserved regions between the human and the rat and mouse genes are graphically represented. Plots illustrate regions with the highest degree of similarity (percentage indicated at the right) for the genomic sequence of rats and mice as compared with humans (top). The regions indicated in red cover the putative promoter (left) and the 3′-intergenic region (right). C, sequence alignment of the human (h) and rat (r) genomic sequences extending about 360 bp upstream of the coding ATG in exon 1. Transcriptional start sites are indicated by bent arrows below and above the sequences for rats and humans, with numbers indicative of transcripts sequenced. Identical bases are shaded gray, and putative TATA elements are boxed. Conserved putative binding sites for transcription factors are represented by arrows.

fied in the 3′-intergenic region (red area). The transcriptional start site for rats was experimentally mapped using oligonucleotide-capping methodology in UMR106 cells. The results indicated that transcription initiates at different sites, with a variable 5′-untranslated region of about 70–100 bp (Fig. 1C, bent downward arrows). Very similar results were observed in mice, where transcription start sites in MC3T3 cells are within 70 bp upstream of the coding ATG (data not shown). For human BRIL, inspection of expressed sequence tags found at UniGene (available on the NCBI, National Institutes of Health Web site) revealed the transcription start sites clustered at around −30 bp relative to the ATG (Fig. 1C, bent upward arrows). Among the putative DNA regulatory element transcription factor binding sites found most frequently, irrespective of species, were Sp1-like, GC-rich binding sites (Fig. 1C). At least four such GC-rich sequences are present within the proximal promoters of rats, humans, and mice. This is perhaps inconsistent with the high GC content (70%) feature of this region being annotated as a CpG island in the human BRIL gene. Of special interest was also the presence of elements matching the binding site for GLI, TCF/LEF, and two putative TATA boxes (Fig. 1C, boxed). Other putative elements for ZFP354c, AP2α, and RUNX2 were also variably found interspersed in rats, humans, and mice.

Mapping of Rat and Human Bril Promoter Regulatory Elements in UMR106 Cells—In order to map the regulatory regions important to drive expression of Bril gene transcription, genomic fragments for mice (−3913), rats (−1327), and humans (−1434) were cloned in the pGL3-basic Luc reporter plasmid. Transient transfection experiments were first conducted in osteosarcoma UMR106 cells (Fig. 2) that were found to express Bril constitutively (5). The activities of all full-length promoter constructs tested were highly active in UMR106 cells (~300,000 relative light units) as compared with the empty pGL3-basic plasmid (~20,000 relative light units). Progressive deletions from the 5′-end of the promoter fragments indicated a significant loss of activity at −213 for rats (Fig. 2A, gray bars) and at −134 for humans (Fig. 2B, gray bars). The activity further declined to background levels for the −82 and −53 fragments (Fig. 2, A and B). Internal deletions covering the same regions displayed a similar loss in activity (Fig. 2, A and B, black bars). These data suggested that crucial regulatory elements are located within −250 bp upstream of the ATG. Very similar results were obtained with the mouse Bril promoter region, and longer upstream sequences up to 3.9 kb did not afford more activity to the promoter (supplemental Fig. 1).

Sp1 Binds Several Rat Promoter GC-rich Elements in Vitro—Because of the high GC-rich content of the promoter region, we further investigated whether the Sp1 transcription factor could physically interact with the rat proximal fragment in vitro using DNase I footprinting and EMSAs (Fig. 3). A rat −283 bp end-labeled fragment was incubated or not (nude probe) with nuclear extracts prepared from wild type or HEK293 cells. Because of the high GC-rich content of the promoter region, we further investigated whether the Sp1 transcription factor could physically interact with the rat proximal fragment in vitro using DNase I footprinting and EMSAs (Fig. 3). A rat −283 bp end-labeled fragment was incubated or not (nude probe) with nuclear extracts prepared from wild type or HEK293 cells. To further delineate which rat sequences can interact with Sp1, oligonu-
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Figure 2. Transcriptional activity of the Bril promoter in UMR106 cells.

The rat (A) and human (B) promoter regions were cloned into the pGL3-basic reporter plasmid, and Luc activity was measured 48 h after transient transfection in UMR106 cells. The longest constructs tested extended 1327 and 1434 bp upstream of the coding ATG for the rat and human, respectively. Progressive truncations from the 5’-end (gray bars) and internal deletions (back bars) indicated that maximal regulatory activity resides within −265 bp (rat) and −199 bp (human), with complete loss of activity down to −82 bp. Data represent relative Luc activity as compared with the −633 and −1434 bp for the rat and human constructs, respectively. Promoterless pGL3-basic was used as a negative control. Results shown are mean ± S.E. (error bars) (n = 4).

Cleotides containing GC-rich elements (Fig. 3B, top) were incubated with nuclear extracts from HEK2993 cells overexpressing Sp1 and analyzed by EMSA. All oligonucleotides except −117 yielded an Sp1-specific complex (Fig. 3B, bottom) but with different affinities. Intensity for −160, −142, and −82 was similar to that observed with the oligonucleotide containing the consensus Sp1 binding site (Fig. 3, Sp1). The mutant Sp1 oligonucleotide having two mutated bases (Fig. 3, mSp1) did not bind Sp1. Interestingly, footprints FP1, FP2, and FP3 observed in Fig. 3A matched oligonucleotide sequences −61, −82, −142, and −160, respectively.

Sp Family Members Can Transactivate the Bril Promoters in Vivo—A co-transfection assay was next performed to screen a selected set of transcription factors that could activate the human −1434 bp Luc reporter in HEK2993 and MC3T3 cells (supplemental Fig. 2). Among 15 transcription factors tested, Sp1 had some of the strongest transactivating properties. To further determine whether other Sp family members could activate the Bril promoters for rats (−1327), mice (−1630), and humans (−1323), they were co-transfected with Sp1, Sp3, or OSX in MC3T3 (Fig. 4A) and HEK2993 (Fig. 4, B and C). Three different isoforms of Sp3 (Sp3-L1 (residues 1–781), Sp3-L2 (residues 69–781), and Sp3-S (residues 286–781) were also tested because of the reported stimulatory (Sp3-L1 and Sp3-L2) and inhibitory effects (Sp3-S). In both cell types, the Bril promoter activity was most dramatically induced by Sp3-L1 > Sp1 > Sp3-L2, to more than 60- and 700-fold relative to the negative GFP control, in MC3T3 (Fig. 4A) and HEK2993 (Fig. 4B), respectively. Sp3-S did not induce a significant increase in activity, whereas OSX only had marginal effects compared with Sp1 and Sp3. OSX was more active in MC3T3 cells, reaching an up to 10-fold increase relative to background for the human promoter (Fig. 4A). Of note is the extremely high -fold increase attained in HEK2993 cells compared with MC3T3, probably reflecting much better transfection efficiencies in HEK2993 (>50%), in contrast to MC3T3 (<5%). The effects of combinations of Sp1 with other Sp members on the activity of the human BRIL promoter were also tested in HEK2993 (Fig. 4C). Two important findings were observed. First, doubling the amount of transfected Sp1 (from 150 to 300 ng) resulted in the promoter being 3.7-fold more active, suggesting a synergistic mode of activation. Second, co-transfection of Sp1 with Sp3-L1, Sp3-L2, and surprisingly Sp3-S or OSX resulted in -fold inductions greater than simple additive effects. These data suggest that Sp members are potent transactivators of Bril and that they potentially interact together and with OSX to give synergistic effects.

Contribution of TATA Boxes and Individual Sp1 Elements to Bril Promoter Activity—The human BRIL promoter was selected for subsequent studies because of its higher responsiveness to transcriptional regulators, at least of the Sp family. We next analyzed the contribution of the two TATA-like boxes and of the four Sp1-like sites. To abolish activity of the respective elements, as depicted in Fig. 5A, we introduced either internal deletions in the putative TATA boxes or two point mutations in the Sp1 elements that abolished binding activity (see Fig. 3B). Co-transfection experiments were performed in HEK2993 cells to assess the base-line (with GFP) and stimulated (with Sp1) activity of the human −452 promoter, which retains full activity compared with the −1434 bp promoter (see Fig. 1B). Deletion of the proximal TATA (Δ-pTATA) caused a 67% decrease in base-line activity (Fig. 5B, left). Induction by Sp1 was also dramatically reduced in the construct lacking the proximal TATA box (Fig. 5B, right). Deletion of the distal TATA box (Δ-dTATA) did not impact the base-line and Sp1-induced activity of the reporter (Fig. 5B). The constructs carrying single, double, triple, or quadruple mutations in the four Sp1 elements were tested for activation by Sp1 in HEK2993 cells (Fig. 5C). Each of the four sites contributed to a different extent to Sp1 responsiveness, with site 1 (at −83) and sites 2, 3, and 4 contributing to about 60 and 25% of the reporter activity, respectively. The double and triple site mutants had activities ranging from 50% to more than 80% reduction in activity, whereas the quadruple mutant had less than 10% of residual activity (Fig. 5C). These data suggest that induction by Sp family members is dependent on the presence of multiple binding sites in conjunction with the proximal TATA box.
**Zfp354C Can Repress Base-line and Sp1-mediated Activation**—The *in silico* search for potential binding sites of the *Bril* promoter revealed the presence of several consensus binding sites (CCAC) for ZFP354C. For instance, in the proximal promoter segments shown in Fig. 1B, there are 10 and 8 potential CCAC elements, respectively, for humans and rats. This is of particular interest because ZFP354C (also called KID3A and AJ18) is a C2H2 zinc finger transcription factor of the KRAB (Kruppel-associated box) family, known for its general repressive functions on transcription, particularly on RUNX2-mediated regulation of the osteocalcin promoter in osteoblasts. When tested on the rat, mouse, and human promoter segments, Zfp354C systematically repressed the base-line activity in HEK293 cells down to as much as 76% (Fig. 6A). In a cotransfection experiment in HEK293 cells, ZFP354C was also found to be a potent inhibitor of Sp1-mediated transactivation, up to 99% repression at equal quantities (Fig. 6B). Expression of Zfp354C measured by RT-qPCR was 60-fold greater in cells not expressing endogenous *Bril* (HEK293 and NIH3T3) relative to those expressing (UMR106) or committed to express it (MC3T3) (data not shown).

**The Conserved 3′-Intergenic Region of BRIL Is Not a Direct Target of RUNX2**—Consistent with the absence of the canonical RUNX2 binding site (ACCACA) within the promoter region of *Bril*, the human −1434 bp promoter was not inducible by RUNX2 (supplemental Fig. 2). However, a highly conserved segment of genomic DNA located in the 3′-intergenic region (Fig. 1B) was found to contain two perfect RUNX2 binding elements located 172 and 246 bp downstream of the 3′-UTR region (supplemental Fig. 3A). To test the possibility that these sites might be functionally operative, the synthetic SV40 polyadenylation cassette of the human *BRIL* 3′-UTR region remained at base-line levels after cotransfection experiments in HEK293 cells overexpressing Sp1, separated on PAGE, and visualized by autoradiography.
co-transfection with a RUNX2 expression plasmid, suggesting that this region was not functional at least in this plasmid configuration (supplemental Fig. 3). The BRIL 3’-UTR plasmid was still fully responsive to Sp1 (supplemental Fig. 3).

GLI2 Regulates Bril in Conjunction with Sp Members in MC3T3—Another putative element identified in the Bril promoter is a canonical binding site for the GLI transcription factors, located in the reverse orientation at −191 and −228 in humans and rats, respectively (Fig. 1B). The consensus binding site for GLI has a core of 10 nucleotides (GACCACCCAC-NNG) (Fig. 7A, top), which becomes a high affinity site when a G is present at +4 relative to the core (36, 37). The human site is an almost perfect match to the high affinity consensus except for a single base change at the last position of the core, which is known to be divergent at that position (GACCACCCACGcagG). The rat GLI site also is a perfect match except at the +4G (GACCACCCACcagA). The functionality of the GLI site to the activity of the human promoter was tested by co-transfection experiments in MC3T3 (Fig. 7A). The wild type human 1434 bp promoter was responsive to all three human Gli family members but with the following magnitude of potency: GLI2 > GLI3 > GLI1 (Fig. 7A). The 1434 bp promoter having two point mutations introduced within the GLI binding element (Fig. 7A, top), became unresponsive to GLI1 and GLI3 but was still slightly activated (~3-fold) by GLI2. Another putative GLI site located further upstream at position −763 (GACCAACCCACcaca), could have contributed to this residual activity, but this was not investigated.
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To further delineate the responsiveness of the human BRIL promoter to GLI2, a kinetic and dose-response experiment was performed in MC3T3 (Fig. 7B). Activity of the human −1434 bp promoter to GLI2 was significantly different in both the kinetics and magnitude of induction as compared with Sp1 (Fig. 7B), peaking at 15 h post-transfection with an increase up to 80-fold. The potency of GLI2 was greater than Sp1, with significant induction being detected with only 50 ng of plasmid transfected, above those recorded at 15 and 25 h for Sp1 used at 300 ng (Fig. 7B, filled symbols). Co-transfection of GLI2 with either OSX or Sp1 also resulted in activities that were significantly higher than the sum of individual responses (Fig. 7C), suggesting again a synergistic control of BRIL activation. This was not a generalized response because TCF1, when used in combination with GLI2, did not significantly affect its transcriptional readout. Last, endogenous Bril expression in MC3T3 transfected with GLI2 was significantly increased by 2.7-fold at 25 h (Fig. 7D), suggesting that GLI2 can directly modulate Bril.

Hedgehog Signaling in MC3T3 Stimulates Bril Expression, Differentiation, and Mineralization—We next confirmed whether activation of the hedgehog signaling pathway, which leads to GLI2 activation, could regulate endogenous Bril expression in osteoblasts. MC3T3 cells were grown to confluence (day 0) and incubated in differentiating media in the absence or presence of PMP. PMP is a direct agonist of Smoothened (SMO), the transmembrane receptor that ultimately transmits intracellular signals to modulate gene expression through GLI transcription factors. SMO is normally kept in check by the repressive action of another transmembrane protein called Patched (PTCH), the receptor of hedgehog proteins. Hedgehog binding to PTCH relieves the inhibition of SMO, the signaling effector of the cascade. MC3T3 continuously treated with 1 μM PMP had an accelerated mineralization, as determined by alizarin red staining (Fig. 8A). Compared with control cells receiving DMSO only, which started mineralizing at around day 10, PMP-treated cells displayed almost maximal alizarin red staining at day 7 and continued to increase at day 10. ALP activity was also induced 2–9-fold starting on day 2 after treatment (Fig. 8B). Western blotting also revealed markedly increased levels of BRIL from day 2 onward (Fig. 8C), when levels of BRIL in control cells are still undetectable. Maximal expression of BRIL peaked at day 4, as compared with days 7 and 10 for the untreated cells. The effect of PMP on BRIL at day 4 was also concentration-dependent, being effective as low as 0.3 μM and maximal at 3 μM (Fig. 8D, left). Treatment of MC3T3 for 4 days with rIHH also induced strong expression of BRIL at both concentrations tested (Fig. 8D, right), suggesting that the entire PTCH-SMO axis was operative.

Gene Expression Pattern in MC3T3 Treated with PMP—The gene expression signature of control and PMP-treated MC3T3 was investigated in more detail by qPCR (Fig. 9). In comparison with control cells, the expression of Bril was up-regulated 2-, 29-, 33-, and 17-fold at day 1, 2, 3, and 4, respectively. By day 10, Bril expression had returned to control levels. The general kinetic pattern of Bril transcripts paralleled those observed at the protein level (Fig. 8D). This profile of Bril expression during MC3T3 differentiation was overall similar to that of markers of differentiation, Alp and Ibsp (integrin binding sialoprotein) (Fig. 9, first row). In contrast, expression of osteopontin (Sp1) and osteocalcin (Ocn) were mostly unaffected at the early time points (days 1–4) but increased significantly at days 7 and 10. A subset of transcription factor gene expression was also monitored to see which would correlate with that of Bril. Among those tested, Gli1 was most responsive to PMP and was induced at the earliest time point tested (19-fold at day 1 up to more than 50-fold at days 2, 3, and 4) (Fig. 9, second row). Gli2 also was increased 2-, 13-, and 20-fold by day 1, 2, and 3, respectively. Gli3 levels remained constant, and more modest increases (2–3-fold) were noted for Runx2 throughout the experiment. Marginal to no inductions were observed for Sp1, Sp3, and Zfp354C. Interestingly, the Mef2c and Tcf7 transcription factors were also up-regulated by PMP, in the range of 2–10-fold. It should be noted that expression profiles for Gli2, Osx, Mef2C, and Tcf7 in non-treated cells also increased steadily up to about days 7–10, in a similar fashion to those of Bril. These data suggest that PMP promoted gene expression of Bril and accelerated differentiation of MC3T3 cells.

Bril Expression Domain Is Reduced in Long Bones of Gli2 Knock-out Mice—In order to verify whether GLI2 is essential for Bril expression in vivo, we examined the Gli2 knock-out (KO) mouse model (33, 34), having a deletion in exons encoding for the zinc fingers 3–5. Expression of Gli2 and Bril was
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FIGURE 7. Activation of the human BRIL promoter and endogenous mouse Bril gene by GLI2 in MC3T3 cells. A, the human BRIL promoter contains an element between −191 and −204 matching the GLI consensus binding site (top, core binding site highlighted). The WT and mutant (having a 2-base substitution within the GLI core, lowercase at top) human −1434 bp promoter Luc constructs (100 ng) were co-transfected in MC3T3 cells with 300 ng of plasmids encoding GFP, GLU1, GLU2, or GLU3. 42 h post-transfection, Luc activity was measured, and results are presented as -fold increase relative to GFP. Values above bars represent -fold increases. B, the human −1434 bp promoter Luc construct (100 ng) was co-transfected in MC3T3 cells with increasing amounts of plasmid encoding GLI2 (open symbols) or with 300 ng of Sp1 (closed squares). Luc activity was measured 15, 25, and 42 h post-transfection and expressed as -fold increase relative to a co-transfection with GFP. C, MC3T3 cells were co-transfected with the human −1434 bp promoter Luc construct (100 ng) with 150 ng each of GFP or GLI2, in combination with OSX, Sp1, or TCF1, and Luc activity was recorded after 25 h. D, real-time quantitative PCR analysis of endogenous Bril expression 25 h post-transfection with plasmids encoding GFP, GLI2, or Sp1. Values are normalized to β-actin and expressed as 2^−ΔΔCt relative to the GFP control. Results shown are mean ± S.E. (error bars) (n = 3–5).
restriction at CCGG sites by HpaII (Fig. 11C). When assayed by co-transfection in HEK293 cells, both the base-line and Sp1-induced activities of the methylated promoter were reduced down to less than 10% (Fig. 11D).

**DISCUSSION**

Starting from an *in silico* analysis of the conserved Bril sequences in rats, mice, and humans, it became obvious that many different elements located within the proximal promot-
promoter context, Sp3 can either synergize or repress Sp1-mediated transcription (44, 45). However, the long forms of Sp3 were found to be equally effective as Sp1 at activating the Bril promoters, whereas the short form had no transacting properties on its own. Furthermore, co-transfection studies indicated that the human BRIL promoter responded in a synergistic fashion to Sp1 alone or in combination with all three Sp3 isoforms. The nature of the response is in line with the known transactivating properties of Sp1, which can be increased through direct or indirect interaction with a plethora of molecules, including itself (44, 45). Sp1 oligomers can thus synergistically transactivate at promoters containing multiple copies of its DNA binding element, through cooperative DNA binding, which was also observed on the human BRIL promoter. Sp1 and Sp3 are considered rather ubiquitous transcription factors, yet they have been found to contribute to the tissue-specific activation of a number of genes expressed in bones, such as Runx2, Osx, Alp, Coll1a2, Rankl, and Ocn (46–51). Consistent with this, we have shown that the levels of Sp1 and Sp3 in differentiating MC3T3 remained relatively constant. Although they are not considered to be controlling major pathways specific for bone development and formation, Sp3 null mice do show bone and tooth formation defects (52, 53). On the other hand, the OSX transcription factor, a member of the Sp protein family called Sp7, represented a more probable candidate regulator of Bril. OSX is known to act downstream of RUNX2 in osteoblastic differentiation, and both factors are considered as master regulators of osteoblastogenesis (54, 55). Through binding GC-rich elements, OSX orchestrates expression of many genes involved in bone formation (56). Despite the presence of several functionally active Sp1 sites, OSX turned out to be totally inactive in HEK293 cells with either GFP or Sp1. Luc activity was measured 42 h later and normalized to the control GFP values. Error bars, S.E.
noprecipitation indicated a physical interaction between OSX and Sp1 (57). Such a mechanism could be operative at the Bril promoter. Considering the complex mode of OSX function, it is also possible that conditions inherent to our experimental setup may have contributed in part to the weak response. For instance, OSX transcriptional properties were shown to be modulated, positively and negatively, by interaction with the co-activator NFATc1 (58) and the lysine histone demethylase NO66 (59) and further modulated by phosphorylation (56). The same could apply to RUNX2, which was inactive on Bril, even when tested in the context of the 3’-conserved region that contained two canonical RUNX2 binding elements. Bril was previously reported to be up-regulated by overexpression of RUNX2 in limb bud cultures to the same extent as Osx, Phex, Ocn, and Ibsp (60). In that same study, Bril was found to be down-regulated in the limbs of Osx knock-out mice, but the reported effects may have been indirect, due to either increased or decreased differentiation of osteoblasts.

One of the most frequent putative binding sites found on the Bril promoter region (CCAC) was that of the ZFP354C transcription factor. Our results showed that ZFP354C reduced the basal level of the BRIL promoter activity and inhibited the Sp1-mediated induction, even when combined at ratios of as low as 1:6, and totally abrogated the Sp1 effect at 1:1. ZFP354C is a KRAB domain protein containing 11 C2H2-type zinc fingers. ZFP354C is expressed in many different tissues, including bone (61, 62). Like the other family members ZFP354A and ZFP354B (63), ZFP354C was shown to act as a transcriptional repressor (64). The core consensus DNA binding element of ZFP354C resembles that of the RUNX2 binding site (62, 65). ZFP354C was reported to antagonize RUNX2-mediated transcriptional activation on a multimerized RUNX2 element (62). Functionally, very little is known about ZFP354C, yet it has been suggested to act as a negative regulator of osteogenesis, because its overexpression in C3H10T1/2 reduced BMP4-induced osteogenic differentiation and expression of Alp (62). However, opposite effects were observed in stably expressing rat stromal bone marrow cells, with ZFP354C promoting differentiation and mineralization (61). Although we have not directly investigated the mechanisms involved, we speculate that ZFP354C could bind directly to the putative CCAC elements within the BRIL proximal promoter. In fact, there are a total of 10 CCAC elements within the human BRIL promoter, at least two overlapping with the functional Sp1-2 and Sp1-3 sites. Binding of ZFP354C could impede subsequent binding of other factors. Although the levels of ZFP354C did not change appreciably during MC3T3 cell differentiation, we found that relative expression of Zfp354c was 60 times greater in non-expressing HEK293 than in expressing UMR106 and MC3T3 cells (data not shown). Our results suggest that a delicate balance between activators and repressors would contribute to establishing Bril expression.

Another mode of regulation identified here that intersected at least in part with the Sp1 regulation is the methylation status of the Bril promoter. The Bril gene promoters are all GC-rich (up to 70% on 50–70-bp stretches) and contain several CpG dinucleotides covering the promoter and coding region of exon 1. Furthermore, the human BRIL is predicted to contain a bona fide CpG island. CpG islands clustered at most gene promoters are usually kept unmethylated, thus creating a transcriptional permissive state (66). Reciprocally, methylation of CpG dinucleotides is often associated with silent chromatin, being refractory to transcriptional initiation (66). Usually, promoters having CpG islands are relatively nucleosome-deficient, allowing greater accessibility of the underlying DNA to transcriptional regulators. However, the BRIL gene was found to be fully methylated in non-expressing HEK293 cells and also in permissive but non-differentiated MC3T3 cells. Accelerating differentiation of MC3T3 cells with PMP was associated with a considerable demethylation of CpG sites in the promoter and 5′-UTR region but not over the coding region of exon 1. Consistent with the negative effects of promoter methylation, treatment of the human promoter with CpG methylase in vitro resulted in a considerable reduction of its base-line activity and a total abrogation of responsiveness to Sp1. Studies looking at other gene promoters have also reported the repressive effect of CpG methylation on Sp1/Sp3-mediated gene activation (67–69). Thus, in non-expressing cells, methylation of the Bril promoter region would restrict accessibility to activators. Demethylation alone did not appear to be sufficient to activate Bril expression, because treatment with 5-aza-2’-deoxycytidine, a drug that prevents DNA methylation, did not activate Bril in HEK293 and NIH3T3 cells (data not shown). The exact sequence of events, however, that triggers demethylation of the Bril promoter is unclear, but could involve modulated expression of different molecules having such activities. In a recent study (70), DMSO treatment of MC3T3 cells was found to globally reduce CpG methylation. DMSO was found to down-regulate gene expression and protein levels of methylation prone molecules (DNMT1, DNMT3, and HELLs) and to up-regulate enzymes (TET and GADD45) sequentially involved in the conversion of methylcytosine to 5-hydroxymethylcytosine, an intermediate to unmethylated CpG. Although Dlx5 and Fas genes and promoter methylation were clearly affected by DMSO treatment, a global gene expression profile monitored by microarrays did not identify Bril as being changed after 5 days of culture (70). Another study previously implicated the MEF2C transcription factor in the DMSO-induced MC3T3 differentiation and mineralization (71). In that study, microarray analysis of DMSO-treated MC3T3 cells identified Bril as being significantly up-regulated 1.6-fold and down-regulated 4.2-fold after knockdown of Mef2c. Our expression data in MC3T3 confirmed that MEF2C was increased along the differentiation program and was further enhanced by PMP treatment. MEF2C was a strong inducer of the Bril promoter activity (>25-fold) in HEK293 cells but only weakly activated in MC3T3 cells (~3-fold) (supplemental Fig. 1). We cannot formally rule out the possibility that MEF2C could have contributed to Bril expression, independent on the effects of DMSO because the concentration used under our current experimental conditions (0.05%) was 3 times less than the minimal effective concentration that elicited a response in the preceding study. Regulation of gene transcription through DNA methylation is not unique to Bril, because this type of epigenetic event has been detailed previously for a number of bone-related genes, Dlx5 (72), Alp (73), Sost (74), and Osx (75).
Last, we have provided several lines of evidence indicating that the hedgehog pathway participates in the regulation of \( Bril \) expression. A high affinity binding site for GLI1 (36, 37) was found in the proximal promoter across species. We found that this element was essential to mediate strong transactivation of the \( Bril \) promoter to GLI2 and much less to mediate transactivation to GLI1 and GLI3. GLI2 was also found to be much more potent than Sp1, activating \( Bril \) at maximal levels very early after transfection and requiring much smaller quantities of plasmid DNA. Furthermore, co-transfection experiments of GLI2 with Sp1 or OSX indicated a synergistic mode of activation at the \( Bril \) promoter. Sp1 is known to interact with a vast number of different proteins (44, 45), but an interaction with GLI2 has not been reported previously, and future work is required to reveal whether they can physically interact. The up-regulation of the endogenous \( Bril \) after overexpression of GLI2 in MC3T3 is compelling evidence of a direct effect, at least \textit{in vitro}. This is also supported by the activation of the \( Bril \) gene after treatment of MC3T3 with agonists of the hedgehog pathway, either with PMP or rIHH in a time- and concentration-dependent manner. Hedgehog signaling is initiated by binding PTCH, thus relieving its inhibitory effect on SMO, the primary effector of the signaling cascade. Through a series of cytoplasmic events, SMO controls the proteolytic processing, activity, and subcellular localization of GLI proteins that regulate downstream gene targets (76–78). PMP is a small molecule agonist of SMO that was reported to promote osteoblastic differentiation using ALP activity as a readout (79–81). The positive effects of PMP and rIHH on \( Bril \) expression indicated that the PTCH-SMO-GLI pathway is operative in our system. IHH, a soluble factor secreted by prehypertrophic chondrocytes of the growth plate, is essential for osteoblast differentiation \textit{in vivo}, where it signals to the perichondrial cells adjacent to hypertrophic chondrocytes of the bone collar region to induce their differentiation into fully mature mineralizing osteoblasts (78, 82–85). In fact, the expression and localization of \( Bril \) in embryonic long bones that we have previously reported (5) and shown here (Fig. 10) exactly coincides with the domain of osteoblasts responsive to IHH cues. Last, PMP treatment not only increased Alp activity but also dramatically accelerated and increased the extent of mineralization, suggesting that the effects may have been mediated in part by increased differentiation. In support of this, our gene expression profiling indicated that PMP favored increased expression of several gene markers (\( Ibsp \), \( Alp \), \( Osx \), \( Gli2 \), and \( Tcf7 \)) of osteoblastic differentiation with a kinetics very similar to that of \( Bril \). The observation that \( Runx2 \) levels were doubled is also suggestive of increased and/or accelerated differentiation. Robust increases in \( Gli1 \) were observed only after 24 h of PMP treatment. Because \( Gli1 \) is considered a downstream target of \( Gli2 \), this would suggest a feed forward mechanism driven by endogenous \( Gli2 \) in MC3T3. Possible mechanisms leading to increased differentiation and mineralization after hedgehog stimulation have been proposed to involve \( Bmp2 \) in C2C12 and 2T3 cells (78), \( Osx \) in MC3T3 (86), and \( Runx2 \) in C3HT101/2 (87).

To determine whether \( Bril \) is a direct target of GLI2 \textit{in vivo}, we studied its expression in \( Gli2 \) mutant mice in which the DNA binding function of GLI2 is disrupted after deletion of zinc fingers 3–5 (34). These mice die in late gestation and perinatally due to a number of tissue defects and display skeletal growth retardation due to improper development of the growth plate during enchondral ossification (33). Congruent with the possibility of \( Bril \) being directly modulated by the hedgehog \textit{pathway in vivo}, we found that expression levels of \( Bril \) in hind limbs at E15.5 and E17.5 were reduced to less than half. As expected based on our earlier work (5), immunohistochemical localization of \( Bril \) in the tibiae of wild type mice confirmed that it is restricted to the forming perichondrium adjacent to the growth plate, to the developing bony collar, and to primary spongosia. In contrast, the surface area containing cells immunoreactive for \( Bril \) was reduced in the tibia of \( Gli2 \) knock-out mice, with an almost total absence of \( Bril \) in the forming trabeculae at the midshaft region. However, the intensity of the signal was qualitatively similar in \( Gli2 \) knock-out relative to wild type mice, especially in differentiating osteoblasts along the bone collar. These results would suggest that other factors may have compensated for the lack of GLI2 and contributed to \( Bril \) expression. The small yet significant stimulation of the \( Bril \) promoter by GLI1 would support such a hypothesis. Studies aiming to understand the regulation of osteogenesis by GLI family members have revealed a complex scenario, pointing to the overall effects of hedgehog on osteoblasts being highly dependent on the timing, location, and strength of signaling. All three \( Gli \) genes are expressed in overlapping subsets of cells in the developing long bones, including osteoblasts (88). It is not entirely clear whether the GLI proteins can functionally substitute for each other or work in combination, but certainly the activator function of GLI2 is necessary for osteoblast differentiation (89). At the same time, the primary repressor function of GLI3 needs to be alleviated for proper osteogenesis, because it directly interfered with DNA binding activity of \( Runx2 \) at osteogenic target genes (90). Although the activating function of GLI1 was found to be dispensable \textit{in vivo} for mouse development (91), recent evidence has indicated that it is also important for endochondral ossification (24).

In conclusion, we have provided the first detailed analysis of the transcriptional regulation of the \( Bril \) gene. Commonalities were found between the three species studied, suggesting that these are genuine pathways likely to control \( Bril \) transcription. Unexpectedly, different transcription factors were identified that either activated or repressed transcription, some of them acting in a coordinated and synergistic manner. The contribution of potential distal enhancers to \( Bril \) expression is another possibility that we have not yet explored. We also uncovered an epigenetic mode of regulation, through CpG demethylation of the \( Bril \) promoter region, triggering a transcriptionally permissive state. Altogether, these data provide basic mechanisms controlling the osteoblast-specific nature of \( Bril \), an important player in bone physiology.

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