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BASIC HELIX-LOOP-HELIX TRANSCRIPTION FACTORS, BHLHB2 AND BHLHB3; THEIR GENE EXPRESSIONS ARE REGULATED BY MULTIPLE EXTRACELLULAR STIMULI

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1. ABSTRACT

The E box sequence (5'-CANNTG-3') is found in the transcriptional regulatory region of a number of genes. Of the basic helix-loop-helix (bHLH) proteins binding to the E box sequence, class B of bHLH proteins, BHLHB2 (also referred to as the DEC1/Eip1/SHARP-2/Stra13/Clast5) and BHLHB3 (also referred to as the DEC2/SHARP-1/SHARP1), are transcription factors that contain a unique orange domain. These transcription factors repress the transcription of target genes not only via binding to the E box sequence but also via protein-protein interactions with other transcription factors. Both the *BHLHB2* and *BHLHB3* genes are widely expressed in both embryonic and adult tissues. Their gene expressions are regulated in a cell type-specific manner by various extracellular stimuli, such as growth factors, serum starvation, hypoxia, hormones, nutrient, cytokines, light, and infection. Therefore, these transcription factors play pivotal roles in multiple signaling pathways that impact many biological processes including development, cell differentiation, cell growth, cell death, oncogenesis,

immune systems, circadian rhythm, and homeostasis. The structural features, functions, and biological roles of the novel bHLH transcription factors, BHLHB2 and BHLHB3, are discussed along with the mechanisms in which the genes encoding these factors are regulated.

2. INTRODUCTION

The E box sequence (5'-CANNTG-3') is found in the transcriptional regulatory region of a number of genes and controls the transcription of these genes in biological events including development, cell differentiation, cell growth, and oncogenesis (1). The majority of basic helix-loop-helix (bHLH) transcription factors, with or without a leucine zipper motif, bind to the E box sequence, thus regulating gene transcription (1). The bHLH proteins are comprised of two classes; one encompasses ubiquitous transcription factors such as E12/E47, upstream stimulatory factor (USF) 1, USF2, and c-Myc, the other, cell type-specific types including MyoD, myogenin, and the NeuroD/β-cell E-box trans-activator 2 (1-7). The majority of bHLH proteins are capable of homodimer and/or heterodimer formation and function as transcriptional activators. On the other hand, it has been reported that some factors interfere with the activity of these bHLH transcription factors via dimerization or repress gene transcription via direct binding to the E box sequence. The former includes inhibitors of DNA binding/differentiation (Id) family proteins that are helix-loop-helix (HLH) proteins lacking the basic region required for binding to DNA (8). Id proteins interact with bHLH transcription factor via an HLH motif and interfere with binding to the E box sequence of the bHLH transcription factors, thus inhibiting their transcriptional activities (8). Recently, another class of bHLH protein including Hairy and Enhancer of Split (HES), the bHLH domain-containing protein, class B (BHLHB) 2, BHLHB3, and the Hairy/E (spl)-related with YRPW motif (HEY)/hairy and enhancer of split related (HESR) family has been identified as the latter ones (9-16). These transcription factors bind to the E box sequence and repress gene transcription. All these proteins contain a unique orange domain, which is highly conserved among the members of the subfamily, in addition to the bHLH motif. The orange domain is a motif of ~35 amino acids present in eukaryotic transcriptional repressors, which regulate cell differentiation, embryonic patterning, and other biological processes. (17). These proteins are classified into the three groups: (group I) HES1, HES2, HES4, and HES6 orthologs; (group II) HEY1, HEY2, HEYL, HESL, HES5, and HES7 orthologs; (group III) HES3, BHLHB2, and BHLHB3 orthologs (18). The focus of this review is on studies of both the *BHLHB2* and *BHLHB3* genes, the expression of which are regulated by development, cell differentiation, cell growth, and various extracellular stimuli.

3. STRUCTURE AND FUNCTION OF BHLHB2 AND BHLHB3

3.1. Molecular cloning of BHLHB2 cDNA and its structural feature

Four independent groups have cloned mammalian BHLHB2 cDNAs (10-13). The human form was designated as the DEC1 or E47 interaction protein 1 (Eip1) (10, 11). The rat form was designated as the enhancer of split- and hairy-related protein-2 (SHARP-2) and mouse form was designated as the stimulated with retinoic acid 13 (Stra13) or Clast5, respectively (12, 13, 19). The nucleotide sequences of the human, rat, and mouse cDNAs appear in the GenBank/DDBJ/EMBL DNA databases under the following accession numbers; NM_003670, NM_053328, and NM_011498, respectively.

Of these, the DEC1 was cloned in a cDNA induced in a cAMP-dependent manner in differentiated human embryo chondrocytes using the subtractive hybridization method (11). E47 is an alternative splicing form of the *E2A* gene and the bHLH protein is widely distributed in human tissues. Eip1 was cloned as an E47-interacting protein using a protein-protein interaction screening method (10). The SHARP-2 was cloned as a bHLH protein expressed in adult rat brain using a combination of degenerate PCR primers corresponding to the residues in helix-1 and helix-2 in bHLH proteins of the HES subfamily (13). Stra13 was cloned as a retinoic acid (RA)-inducible gene during the neuronal differentiation of P19 mouse embryonal carcinoma cells (12).

The amino acid sequences of the human, rat, and mouse forms are compared and their structural motifs are shown in Figure 1. While the DEC1 consists of 412 amino acid residues, SHARP-2 and Stra13 consist of 411 amino acid residues, respectively. All proteins contain a bHLH motif in the N-terminal region, an orange domain in the central region, and a proline-rich domain in the C-terminal region. The tyrosine and serine/threonine residues of the BHLHB2 protein are phosphorylated (20). The protein contains some putative phosphorylation sites by some protein kinases including casein kinase II (12).

3.2. Molecular cloning of BHLHB3 cDNA and its structural feature

BHLHB3 cDNAs were also cloned by several groups (13, 14, 21-23). The human form was designated as DEC2 (14). The rat and mouse forms were designated as the enhancer of split- and hairy-related protein-1 (SHARP-1), and DEC2 and mSHARP-1, respectively (13, 21, 22). The canine form was designated as SHARP1 (23). The nucleotide sequences of the human, rat, mouse, and canine cDNAs are available in the GenBank/DDBJ/EMBL DNA databases under the following accession numbers; NM_030762, NM_133303, NM_024469, and NM_001002973, respectively.

The rat SHARP-1 was cloned using the same method as was used for SHARP-2 (13). SHARP-1 was also cloned as a protein that binds to a transcriptional regulatory region (+166 to +477) within the first intron of the *M1 muscarinic acetylcholine receptor* gene using a one-hybrid system (21). Human and mouse DEC2 cDNAs were isolated by a PCR cloning method using a combination of an EST database search and the 5'- and 3'-rapid amplification of the cDNA end (14, 22). The canine SHARP1 was cloned as a positional candidate for canine

```
                                                                           basic
human   1:MERIPSAQPPPACLPKAPGLEHGDLPGMYPAHMYQVYKSRRGIKRSEDSKETYKLPHRLI 60
rat     1:MERIPSAQPPPTCLPKTPGLEHGDLSGMDFAHMYQVYKSRRGIKRSEDSKETYKLPHRLI 60
mouse   1:MERIPSAQPPPTCLPKAPGLEHGDLSGMDFAHMYQVYKSRRGIKRSEDSKETYKLPHRLI 60
          *********** **** ******** **  ******************************
                 helix 1           loop          helix 2
human  61:EKKRRDRINECIAQLKDLLPEHLKLTTLGHLEKAVVLELTLKHVKALTNLIDQQQQKIIA 120
rat    61:EKKRRDRINECIAQLKDLLPEHLKLTTLGHLEKAVVLELTLKHVKALTNLIDQQQQKIMA 120
mouse  61:EKKRRDRINECIAQLKDLLPEHLKLTTLGHLEKAVVLELTLKHVKALTNLIDQQQQKIIA 120
          ********************************************************** *
                                                   orange
human 121:LQSGLQAGELSGRNVETGQEMFCSGFQTCAREVLQYLAKHENTRDLKSSQLVTHLHRVVS 180
rat   121:LQSGLQAGDLSGRNIEAGQEMFCSGFQTCAREVLQYLAKHENTRDLKSSQLVTHLHRVVS 180
mouse 121:LQSGLQAGDLSGRNLEAGQEMFCSGFQTCAREVLQYLAKHENTRDLKSSQLVTHLHRVVS 180
          ******** ***** * ****************************************

human 181:ELLQGGTSRKPSDPAPKVMDFKEKPSSPAKGSEGPGKNCVPVIQRTFAHSSGEQSGSDTD 240
rat   181:ELLQGSASRKPLDSAPKPVDFKEKPSFLAKGSEGPGKNCVPVIQRTFAPSGGEQSGSDTD 240
mouse 181:ELLQGGASRKPLDSAPKAVDLKEKPSFLAKGSEGPGKNCVPVIQRTFAPSGGEQSGSDTD 240
          *****  **** * ***  * *****  ****************** * *********

human 241:TDSGYGGESEKGDLRSEQPCFKSDHGRRFTMGERIGAIKQESEEPPTKKNRMQLSDDEGH 300
rat   241:TDSGYGGELEKGDLRSEQPYFKSDHGRRFTVGERVSTIKQESEEPPTKKSRMQLSDEEGH 300
mouse 241:TDSGYGGELEKGDLRSEQPYFKSDHGRRFAVGERVSTIKQESEEPPTKKSRMQLSEEEGH 300
          ******** ********** *********   ***   ************ *****   ***
                                  P-rich
human 301:FTSSDLISSPFLGPHPHQPPFCLPFYLIPPSATAYLPMLEKCWYPTSVPVLYPGLNASAA 360
rat   301:FVGSDLMGSPFLGPHPHQPPFCLPFYLIPPSATAYLPMLEKCWYPTSVPLLYPGLNTSAA 360
mouse 301:FAGSDLMGSPFLGPHPHQPPFCLPFYLIPPSATAYLPMLEKCWYPTSVPVLYPGLNTSAA 360
          *  ***  ***************************************** ****** ***

human 361:ALSSFMNPDKISAPLLMPQRLPSPLPAHPSVDSSVLLQALKPIPPLNLETKD       412
rat   361:ALSSFMNPDKIPTPLLLPQRLPSPL-AHSSLDSSALLQALKQIPPLNLETKD       411
mouse 361:ALSSFMNPDKIPTPLLLPQRLPSPL-AHSSLDSSALLQALKQIPPLNLETKD       411
          ***********  *** ******** ** * *** ****** **********
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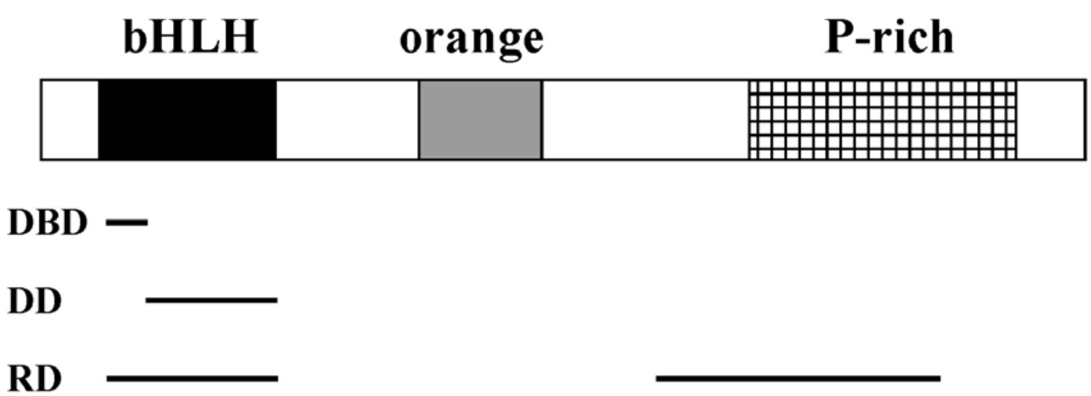

Figure 1. Deduced amino acid sequences of mammalian BHLHB2 and comparisons among species. The asterisks indicate amino acid identity and the dash indicate a gap where no corresponding amino acid sequence between each protein is found. Species names are described at the *left*. A schematic diagram of BHLHB2 is illustrated at the *bottom*. bHLH, basic helix-loop-helix motif; orange, orange domain; P-rich, proline-rich domain; DBD, DNA-binding domain; DD, dimerization domain; RD, repression domain.

early retinal degeneration, which is an autosomal recessive, early onset canine retinal disease (23, 24).

The amino acid sequences of the human, rat, mouse, and canine forms are compared and their structural motifs are shown in Figure 2. While the human DEC2 consists of 482 amino acid residues, the mouse DEC2 contains 410 amino acid residues. Although an open reading frame of SHARP-1 consisting of 253 amino acid residues was initially reported, a deletion of a cytidine

```
                                                                                                basic
human          1 MDEGIPHLQERQLLEHRDFIGLDYSSLYMCKPKRSMKRDDTKDTYKLPHRLIEKKRRDRI     60
rat            1 MDEGIPHLQERQLLEHRDFIGLDYSSLYMCKPKRSLKRDDTKDTYKLPHRLIEKKRRDRI     60
mouse          1 MDEGIPHLQERQLLEHRDFIGLDYSSLYMCKPKRSLKRDDTKDTYKLPHRLIEKKRRDRI     60
canine         1 MDEGIPHLQERQLLEHRDFIGLDYSSLYMCKPKRSMKRDDSKDTYKLPHRLIEKKRRDRI     60
                 ********************************** ****.******************
                    helix 1        loop          helix 2
human         60 NECIAQLKDLLPEHLKLTTLGHLEKAVVLELTLKHLKALTALTEQQHQKIIALQNGERSL    120
rat           60 NECIAQLKDLLPEHLKLTTLGHLEKAVVLELTLKHLKALTALTEQQHQKIIALQNGERSL    120
mouse         60 NECIAQLKDLLPEHLKLTTLGHLEKAVVLELTLKHLKALTALTEQQHQKIIALQNGERSL    120
canine        60 NECIAQLKDLLPEHLKLTTLGHLEKAVVLELTLKHLKALTALTEQQHQKIIALQNGERSL    120
                 ************************************************************
                                          orange
human        120 KSPIQSDLDAFHSGFQTCAKEVLQYLSRFESWTPREPRCVQLINHLHAVATQFLPTPQLL    180
rat          120 KSPVQADLDAFHSGFQTCAKEVLQYLARFESWTPREPRCAQLVSHLHAVATQLL-TPQVT    179
mouse        120 KSPVQADLDAFHSGFQTCAKEVLQYLARFESWTPREPRCAQLVSHLHAVATQLL-TPQVP    179
canine       120 KSPIQSDLDAFHSGFQTCAKEVLQYLSRFESWTPREQRCVQLINHLHAVATQFLPTPQLL    180
                 *** * ******************** *********.** **  ******** * ***

human        180 TQQVPLSKGTGAPSAA---GSAAAPCLERAGQKLEPLAYCVPVIQRTQPSAELAAENDTD    237
rat          179 PGRGPGRAPCSAGAAAASGSERVARCV-PVIQRTQPGTEPEHDTDTDSGYGGEAEQGRAA    238
mouse        179 SGRGSGRAPCSAGAAAASGPERVARCV-PVIQRTQPGTEPEHDTDTDSGYGGEAEQGRAA    238
canine       180 TQQVPLSKGAGAPSAAAPAGSAAAPCLERAGQKLEPLAHCVPVIQRTQPSAELAAENDTD    240
                    .      *  **.      * *     *   *                  *

human        237 TDSGYGGEAEARPDREKGKGAGASRVTIKQEPPGEDSPAPKRMKLDSRGGGSGGGPGGG-    296
rat          238 VKQEPPGDPSPAPKRLKLEARG-------------------------------------    260
mouse        238 VKQEPPGDSSPAPKRPKLEARG-------------------------------------    260
canine       240 TDSGYGGEAEARPDRDKGKGSGAGRVTIKQEPPGEDSPAPKRMKLDSRGGGGGGG-GGGL    299
                       *     * * *    *
                                          A/G-rich
human        296 -------------------AAAAAAALLGPDPAAAAALLRPDAALLSSLVAFGGGGGAPF    337
rat          260 ------------------------------------ALLGPEPALLGSLVALGGG--APF    282
mouse        260 ------------------------------------ALLGPEPALLGSLVALGGG--APF    282
canine       299 GGGGGGGLGGGGGGGLGGGAAAAAAALLGPDPAAAAALLRPDAALLSSLVAFGGGGGAPF    359
                                                     *** *  *** **** ***  ***

human        337 PQPAAAAAPFCLPFCFLSPSAAAAYVQPFLDKSGLEKYLYPAAAAAPFPLLYPGIPAPAA    397
rat          282 AQPAAA--PFCLPFYLLSPSAAA-YVQPWLDKSGLDKYLYPAAAA-PFPLLYPGIPA---    335
mouse        282 AQPAAA--PFCLPFYLLSPSAAA-YVQPWLDKSGLDKYLYPAAAA-PFPLLYPGIPA---    335
canine       359 AQPAAAAAPFCLPFYFLSPSAAAAYVQPFLDKSGLEKYLYPAAAAAPFPLLYPGIPAPAA    419
                 .*****  ******. ******* **** ****** ********* ***********

human        397 AAAAAAAAAAAAAAFPCLSSVLSPPPEKAGAAAAT-LLPHEVAP---LGAPHPQ------    447
rat          335 -----AAAAAAAAAFPCLSSVLSPPPEKAGSAAGAPFLAHEVAPPGSLRPQHAHSRTHLP    390
mouse        335 -----AAAAAAAAAFPCLSSVLSPPPEKAGATAGAPFLAHEVAPPGPLRPQHAHSRTHLP    390
canine       419 AAAAAAAAAAAAA-FPCLSSVLSPPPEKAGAAAAT-LLPHEVAPPGALHPPHPHPHPHPH    477
                       ********.****************..*    * *****.. * . * .   .

human        447 -HPHGRTHLPFAGPREPGNPESSAQEDPSQPGKEAP                          482
rat          390 HAVNPESSQEDATQPAKDAP----------------                          410
mouse        390 RAVNPESSQEDATQPAKDAP----------------                          410
canine       477 PHPHGRTHLAFAGAREPGNPESSAQEDPSQPGKEAH                          513
                           *       *
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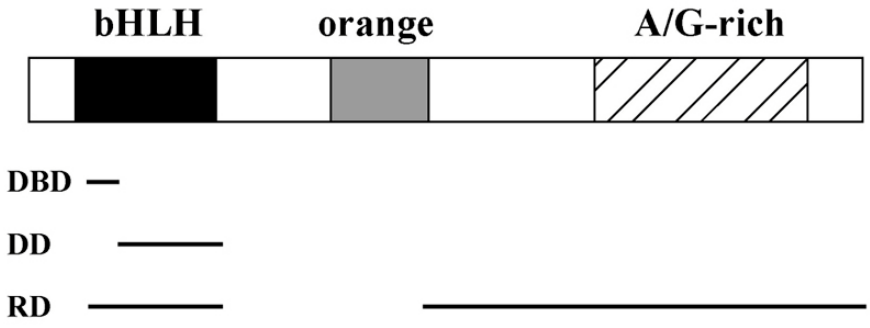

Figure 2. Deduced amino acid sequences of mammalian BHLHB3 and comparisons among species. The asterisks indicate amino acid identity and the dash indicate a gap where no corresponding amino acid sequence between each protein is found. Species names are described at the *left*. A schematic diagram of BHLHB3 is illustrated at the *bottom*. A/G-rich, alanine/glycine-rich domain.

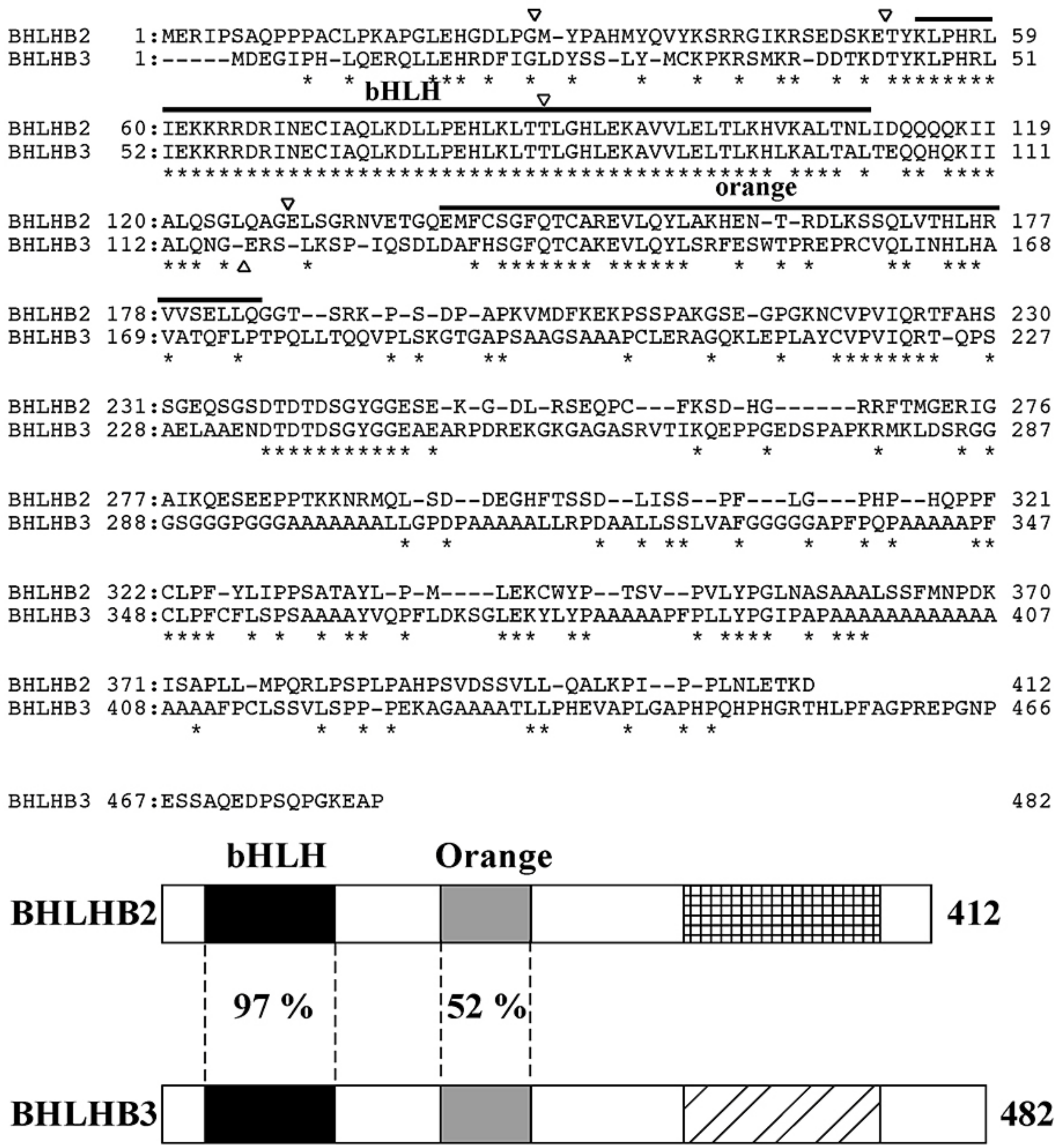
BHLHB2 1:MERIPSAQPPPACLPKAPGLEHGDLPGM-YPAHMYQVYKSRRGIKRSEDSKETYKLPHRL 59
BHLHB3 1:-----MDEGIPH-LQERQLLEHRDFIGLDYSS-LY-MCKPKRSMKR-DDTKDTYKLPHRL 51
bHLH
BHLHB2 60:IEKKRRDRINECIAQLKDLLPEHLKLTTLGHLEKAVVLELTLKHVKALTNLIDQQQQKII 119
BHLHB3 52:IEKKRRDRINECIAQLKDLLPEHLKLTTLGHLEKAVVLELTLKHLKALTALTEQQHQKII 111
orange
BHLHB2 120:ALQSGLQAGELSGRNVETGQEMFCSGFQTCAREVLQYLAKHEN-T-RDLKSSQLVTHLHR 177
BHLHB3 112:ALQNG-ERS-LKSP-IQSDLDAFHSGFQTCAKEVLQYLSRFESWTPREPRCVQLINHLHA 168
BHLHB2 178:VVSELLQGGT--SRK-P-S-DP-APKVMDFKEKPSSPAKGSE-GPGKNCVPVIQRTFAHS 230
BHLHB3 169:VATQFLPTPQLLTQQVPLSKGTGAPSAAGSAAAPCLERAGQKLEPLAYCVPVIQRT-QPS 227
BHLHB2 231:SGEQSGSDTDTDSGYGGESE-K-G-DL-RSEQPC---FKSD-HG------RRFTMGERIG 276
BHLHB3 228:AELAAENDTDTDSGYGGEAEARPDREKGKGAGASRVTIKQEPPGEDSPAPKRMKLDSRGG 287
BHLHB2 277:AIKQESEEPPTKKNRMQL-SD--DEGHFTSSD--LISS--PF---LG---PHP--HQPPF 321
BHLHB3 288:GSGGGPGGGAAAAAAALLGPDPAAAAALLRPDAALLSSLVAFGGGGGAPFPQPAAAAAPF 347
BHLHB2 322:CLPF-YLIPPSATAYL-P-M----LEKCWYP--TSV--PVLYPGLNASAAALSSFMNPDK 370
BHLHB3 348:CLPFCFLSPSAAAAYVQPFLDKSGLEKYLYPAAAAAPFPLLYPGIPAPAAAAAAAAAAAA 407
BHLHB2 371:ISAPLL-MPQRLPSPLPAHPSVDSSVLL-QALKPI--P-PLNLETKD 412
BHLHB3 408:AAAAFPCLSSVLSPP-PEKAGAAAATLLPHEVAPLGAPHPQHPHGRTHLPFAGPREPGNP 466
BHLHB3 467:ESSAQEDPSQPGKEAP 482
bHLH
Orange
BHLHB2
412
97 %
52 %
BHLHB3
482

Figure 3. A comparison of the amino acid sequences of human BHLHB2 with BHLHB3. The locations of introns are indicated by triangles. Schematic drawings of BHLHB2 and BHLHB3 are illustrated at the *bottom*. Homology in each bHLH or orange domain is calculated and shown as the percentage.

residue in the coding region was found in comparison with the nucleotide sequence of both cDNA and rat genomic DNA (13, 14). Therefore, SHARP-1 cDNA should encode 410 amino acid residues as well as the mouse form. The canine SHARP1 consists of 513 amino acid residues (23). All of the above proteins contain a bHLH motif in the N-terminal region, an orange domain in the central region, and an alanine/glycine-rich domain in the C-terminal region.

3.3. Structural similarity between BHLHB2 and BHLHB3 and their function

The amino acid sequences between human BHLHB2 and BHLHB3 are compared in Figure 3. The overall homology between the two proteins is 42 %. However, the amino acid sequences of the bHLH domain are highly conserved, with a homology of 97 %. While the basic region of the bHLH domain is a DNA-binding domain, the HLH region is a dimerization domain (Figures

1 & 2). Therefore, the high homology in these regions suggests that these proteins form a homodimer and/or a heterodimer and regulate the expression of similar downstream target genes. In contrast, the amino acid sequences of the orange domain are only moderately conserved, with a homology of 52 %. The amino acid sequences of their C-terminal regions are more divergent. Unlike the HES and HEY/HESR family, both BHLHB2 and BHLHB3 proteins lack the WRPW motif for recruitment of the Groucho co-repressor in their C-terminal regions.

It has been reported that both BHLHB2 and BHLHB3 form homo- and hetero-dimers and function as nuclear transcriptional repressors (12, 21, 22, 25-32). While BHLHB2 recognizes and binds to a canonical E box sequence, 5'-CACGTG-3', in a high affinity manner, it also binds to a 5'-CATGTG-3', 5'-CACGTN-3' (where N is any nucleotide sequence), and 5'-CACGCG-3' in a low affinity manner (26, 27). HES proteins can also bind to the N box sequence (5'-CACNAG-3'), a variant of the canonical E box sequence (9). In contrast, both BHLHB2 and BHLHB3 do not bind to the N box sequence (12, 22). A characteristic proline residue is located in the basic region of HES proteins. However, a proline residue in the basic region of both the BHLHB2 and BHLHB3 proteins is located two amino acid residues more amino-terminal in comparison with the HES proteins. The transcriptional repression activity of BHLHB2 is decreased by deletion of the basic region or introduction of mutation in the basic region, indicating that binding to the E box sequences is required for transcriptional activity (Figures 1 &2) (12, 26, 29, 33, 34). C-terminal truncated forms of BHLHB2 with a bHLH region also have a decreased transcriptional repression activity (Figures 1 &2) (12, 26, 29, 32, 33, 35). Thus, at least two regions are required for transcriptional repression activity of BHLHB2. BHLHB2 represses promoter activities of both the *BHLHB2* and *c-myc* genes (25). Interestingly, a decrease in promoter activity of the *BHLHB2* gene but not the *c-myc* gene is restored by treatment with trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor. This indicates that BHLHB2 represses transcription through HDAC-dependent and HDAC-independent pathways. Indeed, a C-terminal region of BHLHB2 physically interacts with the co-repressor HDAC1, NCoR, and mSin3A and subsequently represses the transcription of the *BHLHB2* gene. In contrast, an interaction of BHLHB2 with a component of the basic transcriptional machinery, TFIIB, is required for a decrease in the promoter activity of the *c-myc* gene (25). It has recently been reported that a truncated form of BHLHB2 has the ability to activate gene transcription and that BHLHB2 activates transcription with signal transducers and activators of transcription (STAT) 3 in a STAT-binding sequence-dependent manner (35, 36). These findings indicate that BHLHB2 is able to function as a transcriptional activator under certain circumstances.

BHLHB3 functions as a transcriptional repressor of both the *M1 muscarinic acetylcholine receptor* and *BHLHB2* genes (21, 22). The domains required for transcriptional repression activity on both the bHLH and the C-terminal regions as well as BHLHB2 have been mapped (21). Of these, the activity of the C-terminal region is inhibited by TSA. Thus, BHLHB3 also represses gene transcription by HDAC-dependent (C-terminal) and HDAC-independent (bHLH region) mechanism. At this time, no reports on interactions of BHLHB3 with co-repressors and basal transcriptional machinery have appeared.

Putative target genes of BHLHB2 and BHLHB3, in which endogenous mRNA levels are decreased by the forced expression of both BHLHB2 and BHLHB3 and decreased promoter activity by a co-transfection analysis, and which exhibited an altered expression in *BHLHB2* gene-knock out mice, are listed in Table 1.

Both BHLHB2 and BHLHB3 not only bind to the E box sequence and repress gene transcription, but also contribute to repressing the transcription of the target gene via interactions with other proteins including transcription factors. Interacting proteins of BHLHB2 and BHLHB3 and each interaction domain are also listed in Table 2.

4. REGULATION OF GENE EXPRESSION OF BHLHB2 AND BHLHB3

The expression of both the *BHLHB2* and *BHLHB3* genes is regulated by development, cell differentiation, cell growth, oncogenesis, and cell death as well as multiple extracellular stimuli including growth factors, serum starvation, hypoxia, hormones, nutrient, cytokines, light, and infection. These are listed in Tables 3 and 4.

4.1. Tissue distribution of BHLHB2 and BHLHB3 mRNAs

The sizes of the BHLHB2 and BHLHB3 mRNAs are 3.1- and 3.6-kb, respectively. BHLHB2 mRNAs are expressed ubiquitously in adult tissues (11, 13). The expression of BHLHB3 mRNA in comparison with that of BHLHB2 mRNA occurs in a cell-type restricted manner. BHLHB3 mRNA is expressed in the heart, skeletal muscle, and brain at high levels and the lung, placenta, pancreas, and kidney at low levels (13, 14).

4.2. Developmental regulation of BHLHB2 and BHLHB3 mRNAs

During embryogenesis, BHLHB2 mRNA is expressed in the neuroectoderm, as well as in a number of mesodermal and endodermal derivatives (12). The expression of BHLHB2 mRNA is also induced during cytotrophoblast differentiation in the placenta (37). At E14.5-E18.5 during endochondral bone development in the fetus, the expression of BHLHB2 mRNA is induced and its expression pattern is similar to that of vascular endothelial cell growth factor (VEGF) mRNA (38). BHLHB3 mRNA is expressed in specific dorsal regions of the developing brain, the heart, the developing eye, the olfactory system, and limb buds in the early embryonic stage (E8.5-E12.5) (39). At later stages (E12.5-E16.5), BHLHB3 mRNA is expressed in the liver, prevertebrae, and the developing adrenal and thyroid glands. While the levels of both

Table 1. A list of potential BHLHB2- and BHLHB3-target genes

BHLHB2-target genes		
Name	**Level**	**References**
PPAR-gamma2	Promoter	33
c-myc	Promoter	25
BHLHB2	Promoter	25
BHLHB3	Promoter	29, 63
STAT-binding sites	Promoter	36
FAS	Promoter (with STAT3)	36
PEPCK	Promoter	67
Per1	Promoter	75
Per2	Promoter	63
Dbp	Promoter	63
Hemeoxygenase-1	mRNA	65
NADPH oxidase	activity	65
3-hydroxy-3-methylglutaryl-coenzyme A reductase, Hydroxysteroid dehydrogenase, Arylacetamide deacetylase, Enoyl coenzyme A hydratase 1, Tyrosine aminotransferase, Ornithine aminotransferase, Branched chain ketoacid dehydrogenase E1-beta, Cytochrome P-450 2c70, Glutathione *S*-transferase, Cytochrome P-450 2a4, Aminolevulinic acid synthase 1, Insulin-like growth factor-binding protein 1, v-erb-b2 viral oncogene homolog 3, Enhancer of rudimentary homolog, Retinoic acid early transcript gamma, Uniquitin-conjugating enzyme E2E 1, *Mus musculus* 12 days embryo spinal cord cDNA, Coagulation factor XII, Serum amyloid A 2, CD59a antigen, B-cell translocation gene 3, Cathepsin S, Renin 1 structural, S-Adenosylhomocysteine hydrolase, RNA-binding motif protein 3, Aquaporin 4, Erythroid differentation regulator, RIKEN cDNA 0610005C13 gene, RIKEN cDNA 2410004N09 gene, RIKEN cDNA 9430020E02 gene, RIKEN cDNA 3110038L01 gene, RIKEN cDNA 1810073K19 gene, Kidney expressed gene 1	Knockout mouse	64
BHLHB3-target genes		
BHLHB2	Promoter	22
BHLHB3	Promoter	85
Per1	Promoter	35, 75
M1 muscarinic acetylcholine receptor	Promoter	21
Cyp7a	Promoter	77
Cyp8b	Promoter	77
Cyp51	Promoter	77

BHLHB2 and BHLHB3 mRNAs in the central nervous system (CNS) are low in embryonic stages, they increase as postnatal development proceeds (13).

4.3. Expression in cell differentiation and growth

The expressions of both the *BHLHB2* and *BHLHB3* genes are regulated in the process of cell differentiation and growth. The forced expression of these transcription factors leads the cells to promote or inhibit cell differentiation and growth.

4.3.1. Trophoblast differentiation

The level of BHLHB2 mRNA is increased during the differentiation of trophoblast stem cells to trophoblast giant cells (12, 40, 41). The overexpression of BHLHB2 in trophoblast stem cells induces their differentiation into trophoblast giant cells (40). Moreover, when ectoplacental cone trophoblasts are treated with parathyroid hormone-related protein and then induced secondary trophoblast giant cell differentiation, the expression of BHLHB2 mRNA is also induced (42).

4.3.2. Neuronal differentiation

The expression of BHLHB2 mRNA is rapidly induced during neuronal differentiation by treatment with nerve growth factor in pheochromocytoma PC12 cells or retinoic acid in P19 mouse embryonal carcinoma cells (12, 13). The overexpression of BHLHB2 in P19 cells promotes neuronal differentiation and represses mesodermal/endodermal differentiation (12).

4.3.3. Chondrocyte differentiation

During the cAMP-induced differentiation of human embryo chondrocytes, the level of BHLHB2 mRNA is rapidly increased (11). Mouse embryo prechondrogenic ATDC5 cells differentiate into chondrocytes when treated with insulin, transforming growth factor-β (TGF-β), or bone morphogenetic protein-2. In this process, BHLHB2 mRNA levels are also induced (43). In addition, the overexpression of BHLHB2 in ATDC5 cells promotes chondrocyte differentiation (43).

4.3.4. Adipocyte differentiation

BHLHB2 mRNA level in 3T3-L1 preadipocytes is rapidly increased within an hour by the differentiation stimulus, then gradually decreased, and attenuated within 24 h (44). In 3T3-F442A preadipocytes, the level of BHLHB2 mRNA is also rapidly induced by treatment with

Table 2. BHLHB2- and BHLHB3-interacting proteins

BHLHB2-interacting protein				
Name	**Classification**	**Domain of BHLHB2 or BHLHB3**	**Domain of interacting protein**	**References**
BHLHB2	bHLH protein	delta basic	Full length	26
BHLHB3	bHLH protein	delta basic	Full length	32
E47	bHLH protein	7-412	Full length	10
E12	bHLH protein	Full length	Full length	12
MASH1	bHLH protein	Full length	Full length	12
USF	bHLH protein	135-343	Basic region	95
BMAL1	bHLH protein	1-139	PAS-B domain	32, 75
HDAC1	Co-repressor	111-343	Full length	25
NCoR	Co-repressor	111-343	1586-2453	25
mSin3A	Co-repressor	111-412	Full length	25
TFIIB	Pol II	Full length	Full length	25
TBP	Pol II	Full length	Full length	12
STAT3beta	Transcription factor	65-108, 300-412	Full length	36
MSP58	Transcription factor	1-68	FHA domain	20
UBC9	Ubiquitinization	293-412	Full length	50
BHLHB3-interacting protein				
BMAL1	bHLH protein	N-terminal region	Full length	75
MyoD	bHLH protein	Full length	Full length	22
E47	bHLH protein	Full length	Full length	22
BHLHB2	bHLH protein	Full length	delta basic	32
BHLHB3	bHLH protein	Full length	Full length	21
Sp1	Transcription factor	Full length	Full length	22

delta basic, basic region-deleted protein

growth hormone, another adipogenic stimulus (45). Interestingly, when 3T3-L1 preadipocytes are cultured under conditions of hypoxia, differentiation into adipocytes is abrogated, suggesting that a mechanism exists for the regulation of adipogenesis by hypoxia (33). In this process, the BHLHB2 mRNA levels are induced (33). In addition, in BHLHB2-overexpressed 3T3-L1 preadipocytes, differentiation into adipocytes is inhibited. In these cells, the expression of the adipogenic master gene, peroxisome proliferator-activated receptor (PPAR) γ is down-regulated since BHLHB2 represses transcription from the PPARγ gene promoter (285 to 116 bp upstream of a transcription initiation site). This inhibitory activity is required for the N-terminal region of the BHLHB2 containing a bHLH domain. In contrast, in BHLHB2-overexpressed 3T3-F442A cells, no inhibitory effect on differentiation is observed (45). The reason for these controversial results is not clear, at present. 3T3-F442A cells but not 3T3-L1 cells can be cultured in serum-free medium. Thus, serum factors may be required for this effect.

4.3.5. Muscle differentiation

BHLHB3 mRNA is expressed in proliferating C2C12 cells and is down-regulated during myogenic differentiation (46). When the MyoD bHLH protein, a master gene of muscle lineage, is overexpressed in these cells, the cells differentiate into myotube cells. However, when both MyoD and BHLHB3 are co-expressed in C2C12 and 10T1/2 myoblast cells, differentiation into myotube cells is blocked (22, 46). In these cells, the level of MyoD protein is constant but the induction of differentiation-specific marker genes such as myogenin, MEF2C, and myosin heavy chain is impaired. This suggests that a protein-protein interaction between BHLHB3 and MyoD interferes with MyoD-dependent transcriptional stimulation.

4.3.6. Development of mammary gland

The development of the mammary gland is a dynamic process involving cyclical proliferation, cellular differentiation, and cell death. During mammary gland development, BHLHB2 mRNA but not BHLHB3 mRNA is highly expressed in epithelial cells during puberty, and strongly induced in both the ducts and alveoli during early involution (47). In contrast, BHLHB3 mRNA is highly expressed only during late stages of involution. The expression of both BHLHB2 and BHLHB3 mRNAs are dramatically regulated in the epithelial compartment during involution in a mutually exclusive manner. These transcription factors may function in this tissue to regulate apoptosis.

4.3.7. Expression in cancer

BHLHB2 mRNA levels are up-regulated in many tumors tissues including pancreas, lung, breast cancer, and colon carcinoma, and tumor-derived cell lines (28, 30, 34, 48-52). In non-small cell lung cancer, the expression of BHLHB2 is associated with up-regulation of basic fibroblast growth factor receptors but not with the expression of VEGF and thymidine phosphorylase (28). In contrast, in primary human breast carcinomas, the expression of BHLHB2 is associated with that of hypoxia inducible factor (HIF)-1α, VEGF-D, and angiogenin (51, 52). In tumors, BHLHB2 strikingly expresses in connection with areas of necrosis, but does not express

Table 3. Regulation of BHLHB2 and BHLHB3 gene expression in various biological processes

Biological process	Gene	Level	Effect	Cells or Tissues	References
Cell differentiation					
Trophoblast giant cell differentiation	B2	mRNA, Protein	up	Trophoblast stem cell, Cytotrophoblast, 1	37, 40, 41, 42
Neuronal differentiation	B2	mRNA	up	P19 cells, PC12 cells, 1	12, 13
Chondrocyte differentiation	B2	mRNA	up	Human embryo chondrocytes, 1	11, 43
Adipocyte differentiation	B2	mRNA	up, down	3T3-L1 cells, 3T3-F442A cells	33, 44, 45
Muscle differentiation	B3	mRNA	down	C2C12 cells, 10T1/2 cells	22, 46
Mammary gland differentiation	B2,B3	mRNA, Protein	up, down	Mammary gland	47
B cell differentiation	B2	mRNA	up, down	Unstimulated splenic B cells	19, 56
Cell growth/oncogenesis					
Cancer, Tumor	B2,B3	mRNA, Protein	up, down	Colon cancer, Pancreas cancer, Lung cancer, Breast cancer, VHL-deficient cells	28, 29, 30, 34, 48, 49, 50, 51, 52
Growth arrest					
Serum starvation	B2	Protein	up	P19 cells	25
Others					
Ischemia-reperfusion	B2	mRNA	up	Forebrain-Hippocampus	58
Neuronal injury	B2,B3	mRNA	up, down	Corticospinal neuron, Dorsal root ganglion neuron	59
Hypoxia	B2,B3	mRNA, Protein	up	A549 cells, ATDC5 cells, 293T cells, Pancreatic cancer cell, Adipocytes	33, 48, 49, 50, 61
Circadian rhythm	B2,B3	mRNA	up, down	SCN, Peripheral tissues	64, 75, 76, 77, 78
Light	B2	mRNA	up	SCN	75
Infection	B2	mRNA	up	Airway cells	80

Up, up-regulation; down, down-regulation; 1, mouse embryo development

within a zone of morphologically viable cells immediately adjacent to the necrotic zone (30). While BHLHB2 mRNA is highly expressed in colon carcinomas but not in the adjacent normal tissues, the expression of BHLHB3 mRNA is the opposite (29, 34). The overexpression of BHLHB2 causes an inhibition in proliferation and blocks apoptotic pathways initiated via mitochondria by selectively inhibiting the activation of procaspases 3, 7, and 9 (34). However, in 293 T cells, it has been reported that BHLHB2 induces apoptosis by stimulating the activity of the *FAS* gene promoter (36). This body of evidence suggests that controversial events, such as the stimulation or suppression of cell differentiation/growth may depend on the environment of cells expressing BHLHB2 and BHLHB3.

4.4. Expression by extracellular stimuli

Expressions of both the *BHLHB2* and *BHLHB3* genes are regulated by multiple extracellular stimuli (Table 4).

4.4.1. Cell growth and growth arrest

The expression of BHLHB2 mRNA is induced as an immediate early gene by the activation of platelet-derived growth factor receptor in 3T3 cells (53). In cultured HC11 mammary epithelial cells, treatment with epidermal growth factor rapidly induces an accumulation of BHLHB2 protein, which is blocked by glucocorticoid treatment (47). Since the alterations are not associated with any changes of the mRNA level, they are regulated at the post-transcriptional level. Indeed, another group has reported that BHLHB2 interacts with the ubiquitin-conjugating enzyme, UBC9, an increase in BHLHB2 ubiquitinization and its subsequent degradation are invoked, and that the BHLHB2 protein is stabilized by adding a proteasome inhibitor (50). Growth arrest stimuli including TGF-□, TSA, RA, and serum starvation also cause an increase in BHLHB2 mRNA levels (12, 25, 27, 43, 54).

4.4.2. Expression in immune systems

BHLHB2 mRNA is highly expressed in unstimulated, resting B cells and is rapidly down-regulated by a variety of stimuli that activate B cells such as the CD40 ligand, anti-IgM antibodies, lipopolysaccharides, and interleukin (IL)-4 (19). In the Burkitt's lymphoma cell line, BL2 cells, the level of BHLHB2 mRNA is induced by surface BCR cross-linking (55). In BHLHB2-overexpressing B lymphocytes, cell cycle progression to the S phase is decreased along with the decrement of FAS-mediated apoptosis and colony formation activity (19). Therefore, it would appear that BHLHB2 is a negative regulator of B lymphocyte activation. In B- and T-lineage cell-specific BHLHB2 transgenic mice, the IL-7 responsiveness of lymphocyte progenitors is limited and the activation and differentiation of mature B cells are also inhibited (56). In natural killer NK92 cells, the expression of BHLHB2 mRNA is induced by IL-2, IL-6, IL-12, IL-15, tumor necrosis factor-□, and interferon-□ (36). The level of BHLHB2 mRNA is also induced by anti-CD3 in T cells (57).

Table 4. Reagents regulating expression of the *BHLHB2* and *BHLHB3* genes

Reagents	Gene	Level	Effect	Cells or Tissues	References
Growth factors					
Nerve Growth Factor	B2, B3	mRNA	up	PC12 cells	13
Transforming Growth Factor-beta	B2	mRNA	up	Colorectal or breast cancer cell lines	27, 43, 54
Bone Morphogenic Protein-2	B2	mRNA	up	ATDC5 cells	43
Platelet-Derived Growth Factor	B2	mRNA	up	3T3 cells	53
Epidermal Growth Factor	B2	Protein	up	HC11 mammary epithelial cells	47
Cytokines					
CD40 ligand, anti IgM antigen IL-4, Lipopolysaccharides	B2	mRNA	down	Unstimulated splenic B cells	19
IL-2, IL-6, IL-12, IL-15 Tumor Necrosis Factor-alpha Interferon-beta	B2	mRNA	up	Natural killer cell line NK92 cells	36
Anti-CD3	B2	mRNA	up	T cells	57
Hormones					
Insulin	B2	mRNA	up	Liver, 3T3-L1 adipocytes, L6 myotube	70, 71
Growth Hormone	B2	mRNA	up	3T3-F442A preadipocytes	45
Glucocorticoids	B2	Protein	down	HC11 mammary epithelial cells	47
Gonadotropins	B2	mRNA	up	Ovary, Leydig cell line MA-10 cells	31
Parathyroid Hormone	B2	mRNA	up	ATDC5 cells, chondrocytes	69
cyclic AMP	B2	mRNA	up	Human embryo chondrocytes, ATDC5 cells MRC5 cells, HeLa cells, T98G cells, MDCK cells, SK-MEL cells, MA-10 cells	11, 31, 69
Others					
Retinoic Acid	B2	mRNA, Protein	up	P19 cells	12
Trichostatin A	B2	mRNA	up	P19 cells	25
Surface B Cell Receptor-crosslinking	B2	mRNA	up	Burkitt's lymphoma BL2 cells	55
Kainic Acid	B2	mRNA	up	Cerebral cortex	13
Prostaglandin E2	B2	mRNA, Protein	up	Podocytes	65
Epigallocatechin Gallate	B2	mRNA	up	H4IIE hepatoma cells	67
Forskolin	B2	mRNA	up	ATDC5 cells	69

Up, up-regulation; down, down-regulation

BHLHB2-gene knockout mice showed lymphoid organ hyperplasia including enlargement of spleen, thymus, and lymph nodes and chronically developed autoimmune disease by aging (57). In the knockout mice, activation and differentiation of T cells are impaired, which is associated with reduction of IL-2 production by the BHLHB2 deficiency. Therefore, BHLHB2 appears to be a key regulator of lymphocyte activation.

4.4.3. Expression in neuronal system

The expressions of both BHLHB2 and BHLHB3 mRNAs are increased by the administration of kainic acid (glutamatergic stimulation) in cortical neurons (13). In the hippocampus, BHLHB2 mRNA level is temporally increased by ischemia-reperfusion in the forebrain (58). The expression of both BHLHB2 and BHLHB3 mRNAs is suppressed shortly after axonal injury and expression returns to normal levels after 14 days (59). In addition, BHLHB2 mRNA level is down-regulated in dorsal root ganglia cells after axonal injury and its level returns to normal levels by day 14 post-injury (59).

4.5. Expression in hypoxic condition

The exposure of cells to hypoxic conditions causes alterations in gene expression. The HIF complex plays a pivotal role in this event (Figure 4) (60). The HIF complex contains a heterodimer of HIF-1α and HIF-1β (also referred to as the aryl hydrocarbon nuclear translocator, ARNT). Whereas HIF-1α is induced by hypoxic conditions, HIF1-β is constitutively expressed. In normoxia, two prolyl residues of the HIF-1α are hydroxylated by prolyl hydroxylase and dioxygen. The hydroxylated protein interacts with the von Hippel-Lindau protein (pVHL) and the ubiquitin E3 ligase complex and is rapidly degraded by the proteasome pathway. In contrast, under hypoxic conditions, the HIF-1α protein becomes stable since prolyl hydroxylation of the protein is poor and is not degraded by the proteasome machinery. Thus, HIF-1α is translocated into the nucleus, forms a heterodimer with HIF-1β, binds to the hypoxia response element (HRE, 5'-RCGTG-3'), and activates gene transcription. Most genes that are involved in angiogenesis and anaerobic energy metabolism, such as VEGF, glucose transporter 1, and glycolytic genes, are up-regulated by HIF-1.

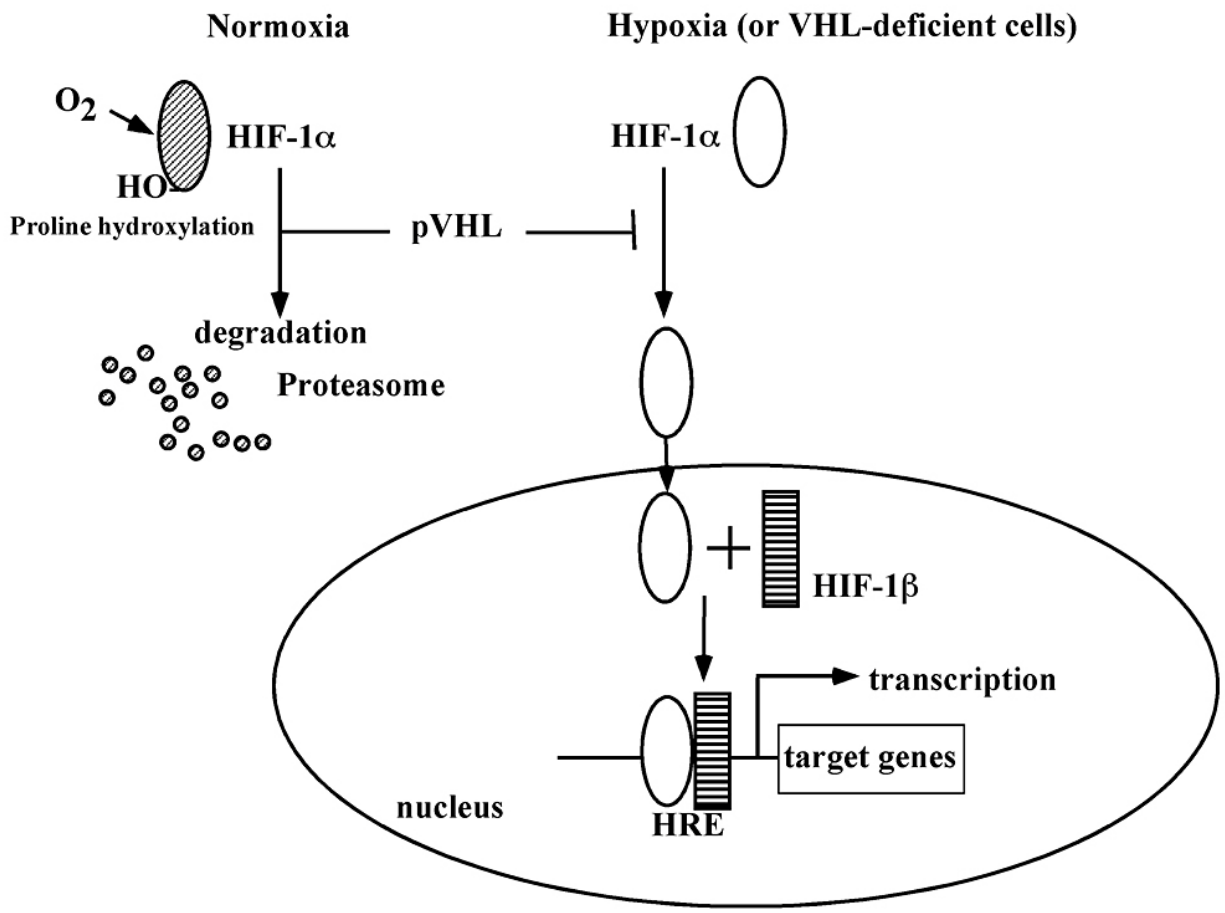

Figure 4. Regulation of HIF-1α stability by normoxia, hypoxia, and in the VHL-deficient cells. In normoxia, HIF-1α is hydroxylated at proline residues. The hydroxylated HIF-1α associates with pVHL and is then degraded by the proteasome pathway. In contrast, under conditions of hypoxia, the level of proline hydroxylation of HIF-1α is decreased and pVHL cannot associate with HIF-1α. In the case of VHL-deficient cells, HIF-1α with or without hydroxylated proline residues is not associated with pVHL. Thus, these HIF-1α escape from the proteasome pathway, become stable, are translocated into the nucleus, form heterodimers with HIF-1β, bind to the hypoxia response element (HRE) of the transcriptional regulatory region of the target gene, and stimulates transcription.

The expression of both BHLHB2 and BHLHB3 mRNAs is up-regulated by hypoxia (33, 48-50, 61). Therefore, these genes function downstream from HIF-1.

The *von Hippel-Lindau* (*VHL*) gene is known to be a tumor suppressor gene. Mutations in the *VHL* gene lead to the development of tumors in the kidney, CNS, retina, pancreas, and adrenal gland (62). In these tumors, the absence of or a mutant form of pVHL leads to the stabilization of HIF-1α and activates the transcription of target genes in oxygen concentration-independent manner. In addition, hypoxic conditions are often present in solid tumors. BHLHB2 mRNA is also up-regulated in VHL-deficient cells and these tumors (48). In contrast, it has been reported that the level of BHLHB3 mRNA is low in colon carcinomas, but not in the adjacent normal tissues (29). These findings raise the possibility that BHLHB2 and BHLHB3 mRNAs are both induced by hypoxia but the expression of BHLHB3 mRNA is predominantly repressed by BHLHB2 in tumor cells. Indeed, it has been reported that the overexpression of BHLHB2 leads to a decrease in the level of endogenous BHLHB3 mRNA (63) and that the up-regulation of hepatic BHLHB3 mRNA occurs in *BHLHB2* gene-knock out mice (64).

4.6. Expression in oxidative stress

The production of reactive oxygen species (ROS) has been implicated in cancer, diabetes mellitus, atherosclerosis, ischemia, and glomerulonephritis. It has been proposed that prostaglandin E_2 (PGE_2) protects podocytes against cellular injury. When podocytes are treated with PGE_2, the levels of both BHLHB2 mRNA and protein are increased (65). The overexpression of BHLHB2 in podocytes stimulates hemeoxygenase-1 gene expression leading to a decrease in the production of ROS and a decrease in NADPH oxidase activity, lactate dehydrogenase release, and cell death rate (65). When highly-differentiated rat hepatoma H4IIE cells are treated with epigallocatechin gallate, a green tea constituent, an increase in ROS is observed (66). Under this condition, the

level of BHLHB2 mRNA is rapidly induced (67). Therefore, it appears that BHLHB2 plays an important cytoprotective role against oxidative stress by ROS.

4.7. Regulation of expression by hormones

4.7.1. Gonadotropins

Gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), regulate ovarian function including folliculogenesis, ovulation, and luteinization (68). FSH stimulates cell growth and differentiation in the granulosa cells of small antral to preovulatory follicles, thus leading to follicular development. LH induces steroidogenesis in theca interna cells and also acts on preovulatory follicles, resulting in ovulation and luteinization.

The BHLHB2 mRNA is detected in the untreated immature female rat ovary. The level of BHLHB2 mRNA abruptly and temporarily increases as the result of the action of gonadotropins in the rat ovary (31). BHLHB2 mRNA levels rapidly increase in primary cultured rat granulosa cells as the result of FSH treatment and in mouse Leydig MA-10 cells treated with LH or cAMP. Thus, BHLHB2 is a gonadotropin-inducible bHLH transcription factor. This is the first observation of a bHLH protein, the production of which is regulated by gonadotropins in the rat ovary and a mouse Leydig cell line.

4.7.2. Parathyroid hormone

In a rabbit chondrocyte culture and ATDC5 cells, the level of BHLHB2 mRNA is biphasically increased within an hour and after six hours by treatment with parathyroid hormone (69). Treatment of ATDC5 cells with forskolin, an activator of adenylate cyclase, and cAMP, rapidly induces BHLHB2 mRNA level. In addition, in various cell lines, cAMP treatment increases BHLHB2 mRNA levels, indicating that the *BHLHB2* gene expression is regulated by the cAMP-signaling pathway.

4.7.3. Insulin

Insulin is secreted by pancreatic β-cells that sense hyperglycemia after feeding. The secreted insulin binds to receptors in target organs such as the liver, muscle, and adipose tissues, stimulates blood glucose uptake and its metabolism in these tissues, and leads to a lowering in the blood glucose levels.

The levels of hepatic BHLHB2 mRNA increase when a high-carbohydrate diet is fed to normal rats or when insulin is administered to diabetic rats (70). In primary cultured rat hepatocytes, insulin rapidly induces an accumulation of BHLHB2 mRNA level even in the absence of glucose. The increase in BHLHB2 mRNA levels by insulin is blocked by an addition of wortmannin and LY294002, inhibitors of phosphoinositide 3-kinase (PI 3-K) and protein kinase B/Akt. In addition, the induction of BHLHB2 mRNA by insulin is also blocked by treatment with actinomycin D, an inhibitor of RNA polymerase II. Indeed, transcription rate of the rat *BHLHB2* gene is stimulated by insulin. Thus, insulin induces the transcription of the rat *BHLHB2* gene via a PI 3-K pathway in the liver. (70). Insulin also induces the expression of the *BHLHB2* gene in both 3T3-L1 adipocytes and L6 myotubes and gene expression enhanced by insulin is regulated by the cell type-specific pathway: the former requires a PI 3-K pathway and the latter, at least in part, a mitogen-activated protein kinase pathway (71). Interestingly, cAMP increases the levels of BHLHB2 mRNA in both cells in an additive manner. As no increase of the level of BHLHB2 mRNA by cAMP is observed in primary cultured rat hepatocytes, tissue-specific and hormone-specific regulatory mechanisms must be operative.

4.8. Circadian rhythm

A variety of organisms have circadian rhythms, 24 h-cycle rhythms in physiology and behavior, which are controlled by a circadian clock (72-74). The rhythm is reset by a light signal. In mammals, the central clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus. Both the *BHLHB2* and *BHLHB3* genes are rhythmically expressed in the rat SCN, with a higher expression during the day (75, 76). The pattern is not changed under conditions of constant darkness, indicating that these genes have an endogenous rhythm. A brief light pulse induces BHLHB2 but not BHLHB3 expression in an SCN in a phase-dependent manner (75).

In peripheral tissues such as the liver, heart, kidney, lung, and monocytes, BHLHB2 gene expression is also regulated in a circadian fashion (64, 77, 78). Although the level of BHLHB3 gene expression is very low, its hepatic expression is regulated in a circadian-fashion (77). These gene expressions in the peripheral tissues are high during the subjective night, which is different from the SCN (77).

The SCN-pineal system acts as a neuroendocrine transducer of seasonal changes in the photoperiod by regulating melatonin formation. In the rat pineal, the expression of BHLHB3 mRNA but not BHLHB2 mRNA is affected by photoperiod (79). As compared with the short photoperiod, the duration of increased expression under the long photoperiod is shortened for BHLHB3 mRNA.

4.9. Infection

The level of BHLHB2 mRNA is rapidly induced by an infection of live *Staphylococcus aureus* to airway cells (80). The infection causes stabilization of the HIF-1α protein and induces BHLHB2 gene expression as a HIF-1 target gene.

5. STRUCTURE AND TRANSCRIPTIONAL REGULATION OF THE BHLHB2 AND BHLHB3 GENES

5.1. Chromosomal location and organization of the BHLHB2 and BHLHB3 genes

In mammals, the human, rat, and mouse *BHLHB2* gene have been cloned and mapped on chromosomes 3p26, 4q41, and 6E-F1, respectively (Table 5) (25, 81-84). The mammalian *BHLHB2* genes are approximately 6-kb in length and consist of five exons and four introns (82, 83) (Figure 5).

Table 5. Genetic loci of the *BHLHB2* and *BHLHB3* genes

Gene	Species	Chromosome	Accession number	References
BHLHB2 gene	Human	3p26	AB043885, ENSG00000134107	81, 82
	Rat	4q41	AB096137, ENSRNOG00000006059	83
	Mouse	6E-F1	ENSMUSG00000030103	84
BHLHB3 gene	Human	12p11.23-12p.1	ENSG00000123095	14
	Rat	4q43-q4	Not registered	14
	Mouse	6G2-G3	AB126167, ENSMUSG00000030256	14
	Canine	27	AY204567	23

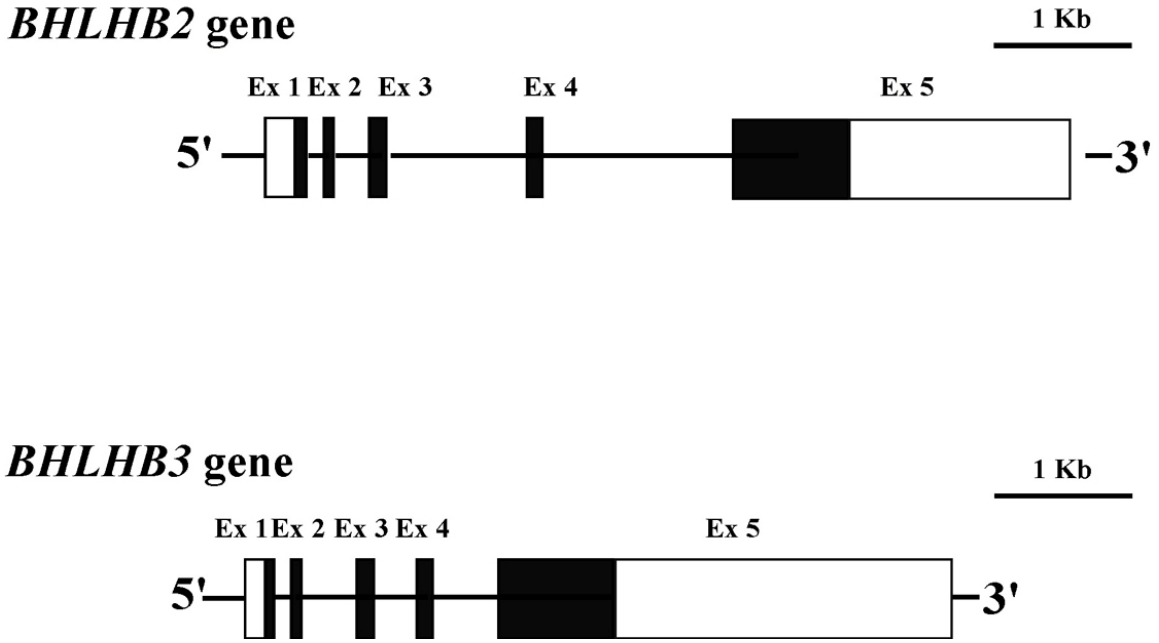

Figure 5. Schematic representations of genomic organization of the *BHLHB2* (top) and *BHLHB3* (bottom) genes. Exons are depicted by *boxes*. *Open* and *closed boxes* represent non-coding and coding sequences, respectively. The scale for 1 kb is indicated. The *BHLHB2* and *BHLHB3* genes in length are 5.65-kb and 4.89-kb, respectively. Both genes consist of five exons and four introns.

The human, rat, mouse, and canine *BHLHB3* gene have been mapped on chromosomes 12p11.23-p12.1, 4q43-q4, 6G2-G3, and 27, respectively (Table 5) (14, 23). Searches of the databases from Genome projects revealed that the mammalian *BHLHB3* genes are approximately 5-kb in length and consist of five exons and four introns, as well as the *BHLHB2* gene (Figure 3). As shown in Figure 3, the coding regions of both proteins are intervened by introns. The positions of the intervened amino acid residues are identical to or very close to each other (82, 83). Thus, it appears that a common ancestral gene has been duplicated in two genes.

5.2. Structure of promoter and transcriptional regulation of the BHLHB2 and BHLHB3 genes

Transcriptional regulatory mechanisms of both the *BHLHB2* and *BHLHB3* genes are not fully known although their gene expressions are altered by multiple stimuli.

5.2.1. Basal transcription

The *BHLHB2* gene belongs to the house-keeping genes since its expression is ubiquitous and the promoter region lacks a canonical TATA box and transcription is initiated from multiple sites (82, 83). The basal transcription of the *BHLHB2* gene is controlled by multiple E box sequences and GC or GT boxes which have not been precisely determined (22, 61, 63, 64, 83). These transcriptional regulatory elements and their binding proteins are shown in Figure 6. A CLOCK/BMAL1(or 2) heterodimer complex binds to the E4, E5, and E6 of several E box sequences and then activates transcription (63, 64). In addition, the co-transfection of Sp1, a GC box- or GT box-binding factor, stimulates the promoter activity of the *BHLHB2* gene (22). Homo- and hetero-dimers of the USF proteins bind to E3 and E4 *in vitro*, respectively (83).

The *BHLHB3* gene acts in a tissue-restricted manner compared than the *BHLHB2* gene. However, the issue of whether the gene belongs to house-keeping genes remains to be determined since transcription initiation sites are currently unclear. Furthermore, only the E box sequences have been characterized as transcriptional regulatory elements. One or two upstream E box

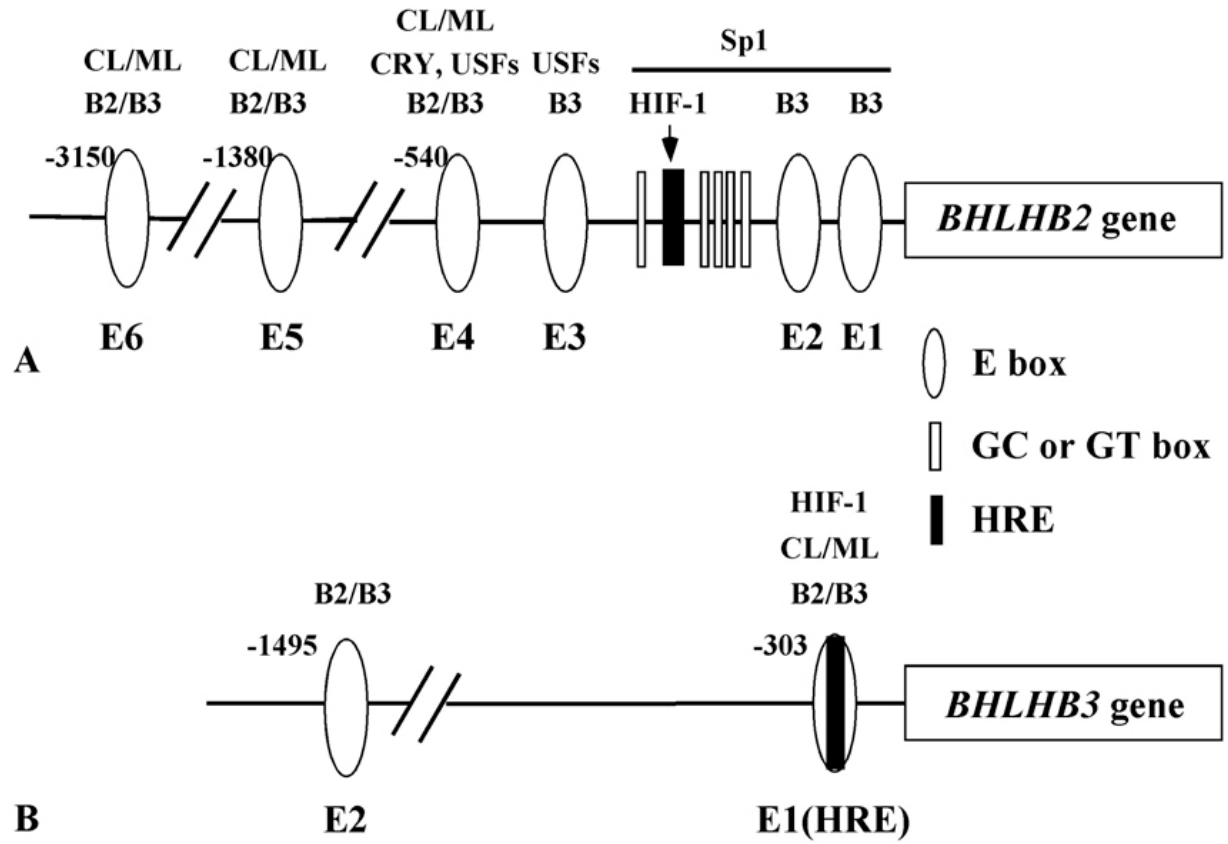

Figure 6. Schematic representations of the regulatory elements and their binding proteins of the (A) *BHLHB2* and (B) *BHLHB3* genes. Nucleotide residues are numbered negatively from the start site of translation. E box sequence, GC or GT box, and HRE are shown as open circle, open box, and closed box, respectively. Proteins binding to the each regulatory element are described at the *top*. Sp1 affects on the region indicated by a thick line and stimulates *BHLHB2* gene transcription. HRE, hypoxia response element; HIF-1, Hypoxia-inducible factor-1; CL/ML, CLOCK/BMAL heterodimer; B2, BHLHB2; B3, BHLHB3; USFs, upstream stimulatory factor 1 and 2.

sequences regulate the transcription of the *BHLHB3* gene (29, 61, 85). A CLOCK/BMAL1 heterodimer complex binds to the E box sequences and activates transcription (85).

As well as many other bHLH transcription factors, transcription of both the *BHLHB2* and *BHLHB3* genes is auto-regulated *per se* or by each other between family members (22, 25, 29, 63, 64, 85). The transcription of the *BHLHB2* gene is repressed via E box sequences by both BHLHB2 and BHLHB3, and that of the *BHLHB3* gene is repressed by the same mechanism as for BHLHB2 and BHLHB3 (Figure 6). Interestingly, BHLHB3 represses the transcription of the *BHLHB2* gene in an E box sequence-independent manner by physically and functionally interacting with Sp1, a transcriptional activator of the gene (22).

Many investigators have reported that the level of BHLHB2 mRNA is rapidly induced by many hormones and factors. However, transcriptional regulatory mechanism of the *BHLHB2* gene has not been characterized. For example, the identification of the cAMP responsive element (CRE) of the *BHLHB2* gene promoter or involvement of CRE-binding protein in the *BHLHB2* gene transcription has not been reported. To account for regulation of BHLHB2 gene transcription by multiple extracellular signals, there are important questions to be answered. The first involves the transcriptional regulatory element, as to whether there is an element responsible for a stimulus (for example, retinoic acid response element (RARE) for retinoic acid) or a unique element responsible for various stimuli is operative. The second one is whether transcription factors exist for each stimulus (retinoic acid receptor/retinoid X receptor complex for RARE) or whether there is a common transcription factor activated by various stimuli. The final question involves whether the mechanism of interaction of *cis*-acting element(s) with transcription factor(s) or protein-protein interactions integrate the regulation of the *BHLHB2* gene transcription by multiple stimuli.

5.2.2. Transcriptional regulatory mechanism in hypoxia

As described above, the expressions of both the *BHLHB2* and *BHLHB3* genes are regulated by hypoxia. A functional HRE is located at the nucleotide sequences between -462 and -446 (5'-GGCCAGACGTGCCTGGA-3') in the transcriptional regulatory region of the

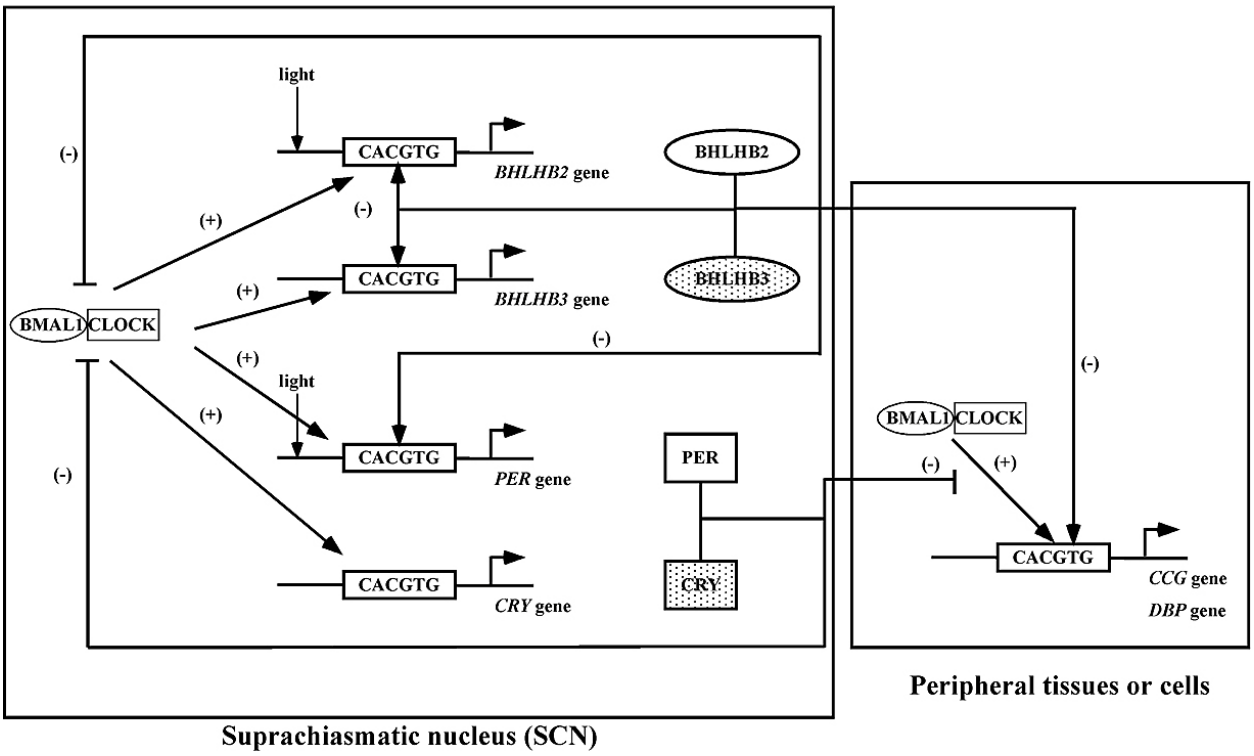

Figure 7. Multiple feedback loops in circadian rhythm. A heterodimer of CLOCK and BMAL1 binds to the E box sequence (CACGTG) and stimulates the expression of the clock or clock-related genes, *BHLHB2*, *BHLHB3*, *PER*, *CRY*, some *clock-controlled genes (CCG)* and *DBP*. Increased levels of BHLHB2 or BHLHB3 suppress the promoter activity of their own genes by autofeedback by binding to the E box sequence, these proteins also repress the expression of the *PER* or *CRY* gene through an interaction with CLOCK/BMAL1 or by binding to the E box sequences of these gene promoters. In addition, increased PER and CRY physically interact with CLOCK/BMAL1 heterodimer and interfere with their activities, then lead to suppression of the promoter activity of their own, *BHLHB2*, and *BHLHB3* genes. The elevated expression of the *CCG* and *DBP* genes by CLOCK/BMAL1 heterodimer is also repressed by same mechanisms. Light induces the expression of both the *BHLHB2* and *PER* genes in the suprachiasmatic nucleus. Stimulatory and inhibitory effects are indicated by + and -, respectively.

human *BHLHB2* gene (Figure 6) (61). A HRE has also been identified at the nucleotide sequences between -311 and -295 (5'-GTTCCGCACGTGAGCTGG-3') in the transcriptional regulatory region of the human *BHLHB3* gene (61). Indeed, HIF-1 binds to these elements and stimulates the transcription of both genes (61). Interestingly, the HRE of the *BHLHB3* gene is a common element involved in transcriptional stimulation by CLOCK/BMAL1 and transcriptional repression by BHLHB2 (29, 61, 85). In these cases, both CLCOK/BMAL1 and BHLHB2 bind to the element. Thus, this E box sequence of the *BHLHB3* gene is a transcriptional regulatory element in which transcription factors from multiple signaling pathways are able to engage in crosstalk.

5.2.3. Transcriptional regulatory mechanism of the molecular clock

Detailed reviews of circadian rhythm are available (72-74). In the SCN, in addition to a core loop by clock-related genes such as *CLOCK*, *Period (PER), Cryptochrome (CRY),* and *BMAL1*, multiple feedback loops containing both BHLHB2 and BHLHB3 regulate circadian rhythm (Figure 7). Both CLOCK and BMAL1 belong to the bHLH-PAS transcription factor family. The PAS domain is a common domain for PER, ARNT, and Single-minded and one of the domains that are transcription factors in most organisms. BMAL1 and CLOCK form a heterodimer, bind to E box sequences that are located in the transcriptional regulatory region of the *PER*, *CRY*, *BHLHB2*, and *BHLHB3* genes, and activate the transcription of these genes (32, 35, 63, 64, 85-87). The PER protein forms a heterodimer with the CRY protein, is translocated into the nucleus, interferes with the activity of the CLOCK/BMAL1 complex by a protein-protein interaction, and represses transcription of the CLOCK/BMAL1-induced *PER*, *CRY*, *BHLHB2*, and *BHLHB3* genes (63, 85, 88, 89). In addition, BHLHB2 and BHLHB3 form a homo- or hetero-dimer, repress the CLOCK/BMAL1-induced transactivation of the *PER*, *CRY, BHLHB2*, and *BHLHB3* genes through an interaction with CLOCK/BMAL1 or by binding to the E box sequences of the *PER*, *BHLHB2*, and *BHLHB3* gene promoters (32, 35, 63, 64, 75, 85). Transcriptional stimulation by CLOCK/BMAL1 is restarted by the degradation of these inhibitory proteins. The cycle is regulated precisely for 24 hours. The expression of both the *PER* and *BHLHB2* genes are induced by light, a key for resetting the biological clock (75, 90).

In peripheral tissues, hepatic expressions of the albumin *D-site binding protein (DBP)* transcription factor gene and *cytochrome P-450* genes such as *Cyp2c70, Cyp2a4 (steroid 15α-hydroxylase), Cyp7a, Cyp8b (sterol 12α-hydroxylase),* and *Cyp51 (lanosterol 14-demethylase)* genes are regulated in a circadian fashion (91, 92). BHLHB3 decreases the promoter activities of the *Cyp7a, Cyp8b*, and *Cyp51* genes (77). While BHLHB3 represses DBP-induced transcription of the *Cyp7a* gene, it decreases basal transcription of the *Cyp8b* and *Cyp51* genes in a DBP-independent manner. In contrast, BHLHB2 does not affect these gene expressions. This is a very important observation. Even though both BHLHB2 and BHLHB3 show a 97 % similarity in their bHLH regions, the possibility that the target genes are different in both genes cannot be excluded.

In the *CLOCK*-mutant mice where CLOCK forms a heterodimer with BMAL1 but does not have transcriptional activity, the expressions of both the *BHLHB2* and *BHLHB3* genes are decreased in the SCN (93). In mice, the *BHLHB2* gene is not expressed in a circadian fashion. The *BHLHB3* gene is expressed in a weak circadian fashion but the rhythm disappears under conditions of constant darkness. In these mice, the hepatic expression of the *BHLHB2* gene is decreased but still maintained in a weak circadian fashion (94). In contrast, the overexpression of BMAL1 and CLOCK in human umbilical vein endothelial cells led to an increase in both endogenous BHLHB2 and BHLHB3 mRNAs (63, 85). These findings indicate that CLOCK/BMAL1 stimulate the expression of both genes both *in vivo* and *in vitro*. In the liver of *BHLHB2* gene-knock out mice, although the expression of the *CLOCK* gene is not altered, the expression of forty-two genes containing the *Cyp2c70* and *Cyp2a4* genes in which the expression is regulated by CLOCK, are significantly altered (Table 1) (64). Furthermore, the overexpression of BHLHB2 in bone marrow-derived mesenchymal cells decreases the level of endogenous BHLHB3, PER2, and DBP mRNAs (63). Therefore, both BHLHB2 and BHLHB3 have a role as a circadian output regulator in peripheral tissues or cells (63, 64).

6. BHLHB2- AND BHLHB3-INTERACTING PROTEINS

Some proteins have been shown to function as BHLHB2- and BHLHB3-interacting proteins (Table 2).

BHLHB2 interacts with bHLH proteins, E47, E12, and MASH1 (10, 12). However, a detailed mechanism and biological role of these interactions remain to be determined. To repress gene transcription, the entire BHLHB2 interacts with components of the basic transcriptional machinery, TBP and TFIIB, and the C-terminal region of BHLHB2 interacts with the co-repressors HDAC1, NCoR, and mSin3A (12, 25). These interactions are a common mechanism in other transcriptional repressors. In contrast, a C-terminal region (135-373) of BHLHB2 interacts with the basic region of USF and represses E box sequence-dependent and USF-dependent transcription (95). The basic region of BHLHB2 interacts with a C-terminal region of BMAL1 including the PAS-B domain, represses BMAL1-dependent transcription, and regulates circadian rhythm (32, 75). Transcriptional repression by these interactions is a specific function of BHLHB2.

There are two interesting reports on analyses of BHLHB2-interacting proteins (20, 36). One is that both the HLH and C-terminal regions of BHLHB2 interact with STAT3 and function as a transcriptional activator (36). In particular, BHLHB2 preferentially interacts with the active (phosphorylated) form of STAT3 and stimulates transcription from STAT-binding sequences with STAT3. Furthermore, BHLHB2 induces apoptosis by stimulating the promoter activity of the *FAS* gene. Although the N-terminal region of BHLHB2, including a bHLH motif, is required for transcriptional activation, the issue of whether BHLHB2 binds directly to the regulatory element of the *FAS* gene remains to be clarified. Another is that the activity of BHLHB2 is regulated by phosphorylation (20). The 58-kDa microspherule protein (MSP58, also referred to as P78 and MCRS1) has been implicated in the regulation of proliferation and apoptosis as a part of the transcriptional repressor complex with p120 and Daxx (96, 97). The basic region of BHLHB2 interacts with the forkhead-associated domain of MSP58 and synergistically represses gene transcription. For this interaction, the phosphorylation of serine/threonine residues within the amino acid sequences between residues 123 and 299 of BHLHB2 are required (20). BHLHB2 interacts with UBC9 via the C-terminal region (293-412) and is degraded by the 26 S proteasome pathway (50). A phosphorylated BHLHB2/MSP58 complex, located in the nucleus, protected from proteasome-mediated degradation, extends their half-lives to a considerable extent. This, therefore, indicates that the phosphorylated form of BHLHB2 is stable.

BMAL1, MyoD, E47, BHLHB2, BHLHB3, and Sp1 have been reported to be BHLHB3-interacting proteins (21, 22, 32, 35, 46, 75). However, a detailed domain mapping involved in their interactions has not all been reported. As the biological roles of these interactions, an interaction of BHLHB3 with Sp1or MyoD causes an inhibition of the Sp1-dependent transcriptional stimulation of the *BHLHB2* gene or myoblast differentiation by the inhibition of MyoD activity in C2C12 and 10T1/2 cells, respectively (22, 35, 46). A N-terminal region of BHLHB3 also interacts with BMAL1, represses BMAL1-dependent transcription, and regulates circadian rhythm (75).

7. PERSPECTIVE

As discussed above, the gene expressions of both BHLHB2 and BHLHB3 are induced by multiple stimuli, and these proteins play a variety of biological roles.

In most cases, the expression of both the *BHLHB2* and *BHLHB3* genes are induced by various stimuli. For these stimuli, these genes are rapidly induced as immediate early genes or are slowly induced, or

biphasically induced by a combination of early and late responses. The overexpression of BHLHB2 and BHLHB3 causes the promotion or inhibition of cell differentiation/growth. This delay in time course or controversial events are dependent on the cell-types that are stimulated or overexpressed, indicating that the events are dependent on which genes are expressed in these cells. In addition, these findings suggest that there are strict regulatory mechanisms by which BHLHB2 and/or BHLHB3 repress target gene transcription not only via binding to the E box sequences in a cell type-specific manner, but also through interactions of BHLHB2 and BHLHB3 with other transcription factors and proteins in a cell type-specific manner. Therefore, deregulation of the *BHLHB2* and *BHLHB3* genes cause alterations in homeostasis in all tissues and cells. Moreover, this leads to cell growth and death in cell type-dependent manner, and the subsequent development of diseases including cancer, abnormal immune responses, and abnormal circadian rhythms.

Although both BHLHB2 and BHLHB3 have HDAC-dependent and HDAC-independent transcriptional repression mechanisms, the C-terminal structure of these are clearly different. This indicates that these proteins function via interactions with common factors, and each interacts with distinct factors, recruits unique partners to the target gene promoters, and exhibits unique biological roles. In addition, both BHLHB2 and BHLHB3 repress the gene transcription of each other *in vitro*. This raises some issues as to whether both BHLHB2 and BHLHB3 are expressed in the same cells of most normal tissues, whether they are expressed in a mutually exclusive manner as mammary gland and tumor cells, whether they actually form a heterodimer *in vivo* if they are co-expressed in the same cells, and whether the balance of the protein level between BHLHB2 and BHLHB3 is exactly regulated. Studies of not only mRNA levels, such as *in situ* hybridization and Northern blot analysis but also protein levels, such as immunohistochemistry and Western blot analysis, are required to answer these questions. For a comprehensive understanding of the biological roles of both BHLHB2 and BHLHB3, the screening of cell type-specific target genes and cell type-specific interacting proteins is important. In addition, an analysis of BHLHB2- and BHLHB3-conditional knockout mice and an analysis of BHLHB2/BHLHB3 double knock out mice as well as the identification of both BHLHB2- and BHLHB3-interacting proteins in various tissues are required. These interesting results are ongoing in the world.

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Abbreviations: bHLH: basic helix-loop-helix; USF: upstream stimulatory factor; Id: inhibitors of DNA binding/differentiation; HLH: helix-loop-helix; HES: Hairy and Enhancer of Split; BHLHB: bHLH domain-containing protein, class B; HEY: Hairy/E (spl)-related with YRPW motif; HESR: hairy and enhancer of split related; Eip1: E47 interaction protein 1; SHARP-2: enhancer of split- and hairy-related protein-2; Stra13: stimulated with retinoic acid 13; RA: retinoic acid; SHARP-1: enhancer of split- and hairy-related protein-1; TSA: trichostatin A; HDAC: histone deacetylase; STAT: signal transducers and activators of transcription; VEGF: vascular endothelial cell growth factor; CNS: central nervous system; TGF-β: transforming growth factor-β; PPAR: peroxisome proliferator-activated receptor; HIF: Hypoxia-inducible factor; IL: interleukin; ARNT: aryl hydrocarbon nuclear translocater; pVHL: von Hippel-Lindau protein; HRE: hypoxia response element; VHL: Von Hippel-Lindau: ROS: reactive oxygen species; PGE_2: Prostaglandin E_2; FSH: follicle stimulating hormone; LH: luteinizing hormone; PI 3-K: phosphoinositide 3-kinase; SCN: suprachiasmatic nucleus; CRE: cAMP responsive element; RARE: retinoic acid response element; PER: Period; CRY: Cryptochrome; DBP: D-site binding protein; Cyp2a4: steroid 15α-hydroxylase; Cyp8b: sterol 12α-hydroxylase; Cyp51: lanosterol 14-demethylase; MSP58: 58-kDa microspherule protein.

Key Words: Basic Helix-Loop-Helix Protein, Orange Domain, BHLHB2, BHLHB3, Transcriptional Repressor, cDNA Cloning, Genomic Cloning, Transcription, Promoter, Development, Cell Growth, Cell Differentiation, Hypoxia, Circadian Rhythm, Cancer, Hormone, Insulin, cAMP, Oxidative Stress, EGCG, Infection, HIF-1, Ubiquitination, Protein-Protein Interaction, DNA-Protein Interaction

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