[Frontiers in Bioscience 12, 1911-1926, January 1, 2007]

BS69, a corepressor interacting with ZHX1, is a bifunctional transcription factor

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1. ABSTRACT

Corepressor BS69 interacts with ZHX1, a member of the ZHX family having zinc-fingers and homeoboxes. In the rat, we have identified four forms of splicing variants, BS69α, BS69β, BS69γ, and BS69δ. Based on the amino acid sequence, BS69α corresponded to the human orthologue. BS69β and BS69γ contain a novel 56 amino acid region encoded by the exon 11b of the rat *BS69* gene. Both BS69γ and BS69δ lacked a region encoded by exon 3 of the gene. Although all four variants were ubiquitously expressed in rats, the transcripts having the exon 11b were detected in mice and rats but not in humans. A common C-terminal MYND domain of BS69 was required for the interaction with PxLxP motif of ZHX1. Although BS69 was originally found as a corepressor interacting with ZHX1, BS69 was also found to function as a transcriptional activator in HEK293 cells, in which the activation required the MYND domain of BS69. Co-transfection of BS69 with a mutant form of ZHX1, which cannot interact with BS69, led to increase the transcriptional activation of BS69, suggesting that transcriptional activation mediated by BS69 is suppressed by ZHX1. In contrast, BS69 showed transcriptional repression in COS-7 and CV-1 cells and the repression domain was mapped to the N-terminus of BS69β. Both the wild type and mutant form of ZHX1 had no effect on the BS69 repression, suggesting that the repression mediated by BS69 in COS-7 and CV-1 cells may require a cofactor other than ZHX1 in the cells. Therefore, our results suggest that BS69 may function either as a transcriptional repressor or as a transcriptional activator depending on its regulatory partner.

2. INTRODUCTION

The zinc-fingers and homeoboxes (ZHX) family consists of three members, ZHX1, ZHX2, and ZHX3 (1-3). These proteins contain two Cys_2-His_2-type zinc-finger motifs and five homeodomains (1-4). They form homo- and hetero-dimers with each other and function as transcriptional repressors that are localized in the nuclei of cells (2, 3, 5-7). Dimerization with ZHX1 is important for repression activity in both ZHX1 and ZHX3 (2, 3, 6). Although the *ZHX* family genes are broadly expressed, their regulation is different among tissues and cells (1-3, 5, 7, 8). For example, while the expression of ZHX1 mRNA is induced in a cell growth-dependent manner by interleukin-2 in mouse cytotoxic CTLL-2 cells, the mRNA levels of ZHX2 remain unchanged and ZHX3 mRNA is not detected in the cells (9).

Nuclear factor-Y (NF-Y), a CCAAT-binding protein and a ubiquitous transcriptional activator, is composed of three subunits, YA, YB, and YC (10). NF-Y is essential for the expression of various cell cycle-regulatory genes, such as *cyclinA, cdc25C, cdc2*, and so on (11, 12). The overexpression of a NF-YA mutant, which can interact with a YB-YC complex but not bind to a cognate DNA sequence, inhibits the transcription of the *cyclinA*, *cdc25C*, and *cdc2* gene promoters, and leads to cell cycle arrest at both the G1 and G2/M phase (13). All the ZHX family proteins interact with the activation domain (AD) of NF-YA (1-4). We previously reported that ZHX2 represses the promoter activity of the *cdc25C* gene stimulated by NF-Y (2). Harousseau *et al.* recently reported a negative correlation between *ZHX2* gene expression and the expression of 30 proliferation-associated genes including genes that are positively regulated by NF-Y (14). These evidences suggest that ZHX family proteins regulate the expression of cell growth-associated genes stimulated by NF-Y in various tissues and may participate in the control of cell growth.

We previously identified the ubiquitous BS69 co-repressor as a ZHX1-interacting protein using a yeast two-hybrid screening system (3). It has been suggested that BS69 may have some properties as tumor suppressor gene (15-18). The BS69 protein is associated with the 13S isoform of adenovirus type 5 E1A and has transcriptional repression activity in U2OS cells (15, 17). BS69 inhibits transactivation by E1A and suppresses the E1A-stimulated transcription of the *retinoic acid receptor* gene promoter in COS-7 cells (15). Co-expression of BS69 and ets-2 results in the repression of the promoter activity of the *BRCA1* gene in SW13 cells although either BS69 or ets-2 alone did not repress (19). Thus far, it is unclear whether the BS69 is a DNA-binding protein or not. It has been reported that two forms of the protein are produced from a single human *BS69* gene. One is human BS69, which consists of 562 amino acid residues, and the other is BRAM1, which consists of 198 amino acid residues. BRAM1 contains the amino acid residues from 377 to 562 of BS69 and an additional twelve amino acid residues at the N-terminal region (20). The MYND domain is a common region between BS69 and BRAM1, which contains a zinc-finger motif and functions as an interaction domain of other proteins including E1A (15-18). It has been suggested that the MYND domain may be involved in repression through N-CoR recruitment (17). However, BS69, but not BRAM1, has transcriptional repression activity, indicating that the N-terminal region of BS69 also plays a crucial role in transcriptional repression (17). In addition to E1A, other oncoproteins, Epstein-Barr virus EBNA2 and the Myc-related cellular protein MGA, also associate with BS69 (15, 18). These three proteins all have a common Pro-x-Leu-x-Pro (PxLxP; where x is any amino acid residue) motif, which is required for an interaction with the MYND domain of BS69 (15, 18).

In this study, we identified and characterized novel forms of rat BS69 and examined the physical and functional interactions of BS69 with ZHX1. The findings indicate that BS69 unexpectedly functions as a transcriptional activator and that an interaction of ZHX1 via a PxLxP motif is required for the suppression of BS69 activity as a transcriptional activator but not a transcriptional repressor.

3. MATERIALS AND METHODS

3.1. Materials

Dulbecco's modified Eagles medium and an anti-FLAG M2 monoclonal antibody-peroxidase conjugate (A8592) were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). The rat brain, mouse liver, human placenta, human testis marathon-ready cDNAs, Advantage PCR kit, human genomic DNA, yeast two-hybrid system, rat Multiple Tissue Northern blot, and ExpressHyb hybridization solution were purchased from CLONTECH (Palo Alto, U.S.A.). The TRIZOL reagent, Superscript III, pcDNA3.1/His C, and LIPOFECTAMINE PLUS reagents were purchased from Invitrogen (Groningen, the Netherlands). The ExTaq DNA polymerase and BcaBest DNA labeling kit were obtained from Takara BIOMEDICALS (Kyoto, Japan). The pGEM-T Easy, pGL3-Control, pRL-CMV, and dual luciferase assay system were purchased from Promega (Madison, U.S.A.). The Big Dye terminator FS cycle sequencing kit was purchased from Applied Biosystems Japan (Tokyo, Japan).

The pCMV-Tag3B, QuikChange site-direct mutagenesis kit, and pCMV-Tag2B were obtained from Stratagene (La Jolla, U.S.A.). α-^{32}P dCTP (110 TBq/ mmol) and the ECL plus kit were purchased from Amersham Biosciences (Cleveland, U.S.A.). The Invisorb plasmid kit was obtained from Invitek (Berlin, Germany). The anti-c-myc monoclonal antibody (11667149) was purchased from Roche Diagnostics Corporation (Indianapolis, U.S.A.). The Immobilon-P PVDF membrane was purchased from Millipore Co. (Bedford, U.S.A.). The Blocking One was purchased from Nakalai tesque (Kyoto, Japan).

3.2. Oligonucleotides

rBS69-5RACE-As1, 5'-CATTG TGGGG ATTTC CTGGC TGG-3'; rBS69-5RACE-As2, 5'-GGAAC TTACT GCTTC CGTTT CTGG-3'; rBS69-5RACE-As3,

5'-GCTGT TCATT ACTGG TGGAG GAG-3'; rBS69-5RACE-As4, 5'-GTTTT CAGAA GGAAT CCAGG CCCTC-3'; S-rBS69-Met, 5'-CCGGG AATTC ATGTC CCGGG TCCAC GGC-3'; As-rBS69-stop, 5'-CCGGT CTAGA TCATC TCTTC CGGCG GCAG-3'; S-rBS69-126, 5'-GGAAG GATAT TGGTT ACCAG GAG-3'; As-rBS69-1350, 5'-CACCC TCTCA ATTGG CTGAG-3'; S-rBS69-157, 5'-GACTG GGAAA CAGAA ACTC-3'; As-rBS69-1268, 5'-GGAGA CTGTG CACTC GCG-3'; S-GAPDH, 5'-GAACG GGAAG CTCAC TGGCA-3'; As-GAPDH, 5'-TCCAC CACCC TGTTG CTGTA-3'; S-hrmBS69-1088, 5'-GTGTG GAGCC CAAAA AGG-3'; As-hrmBS69-1220, 5'-GAAGA GGCAC TTAAC TTCTT-3'; S-rBS69-1548, 5'-GGCCG AATTC TTGCG TTCTG AAATG GAAGA-3'; S-rBS69-1640, 5'-GGCCG AATTC GTGAA GGAAA AGTGC AAGG-3'; S-rBS69-1720, 5'-GGCCG AATTC AAGAA GAAGC AGTGG TGCTA-3'; S-rBS69-1780, 5'-GGCCG AATTC ACGTC CTACT GCTCC ATC-3'; As-rBS69-1782, 5'-CGTGT TCCAG CAGCA GTGG-3'; As-rBS69-1730, 5'-GCTTC TTCTT GGTCT GAGAA-3'; As-rBS69-1548, 5'-CTTTT CCAGC GCTTC CCGC-3'; As-rBS69-1108, 5'-TTCCT TTTTG GGCTC CACAC-3'; S-rBS69-1108, 5'-GGCCG AATTC GTAAG TTGCC CGCCT CACAG-3'; As-rBS69-1275, 5'-GGTTC TGGGG ATGGA GACTG-3'; S-hZHX1-Met, 5'-GGCCG AATTC ATGGC AAGCA GGCGA AAATC-3'; As-hZHX1-815, 5'-CTGCT GAGCA GATAC AGCAG-3'; S-hZHX1-88, 5'-GGCCG AATTC GAAGG TCCTC CTGTG CTTAC-3'; S-hZHX1-88-mut, 5'-GGCCG AATTC GAAGG TCCTC CTGTG GCTAC-3'; S-hZHX1-168, 5'-GGCCG AATTC GTGGA TTCAG ACAAT CAGC-3'; S-hZHX1-PxLxP-oli, 5'-AATTC CCTGT GCTTA CACCT G-3'; As-hZHX1-PxLxP-oli, 5'-GATCC AGGTG TAAGC A CAGG G-3'; S-hBRCA1-1357, 5'-CCGGA GATCT CTTTA TGGCA AACTC AGGTA-3'; As-hBRCA1-1623, 5'-CCGGA AGCTT TCCAG GAAGT CTCAG CG-3'; S-hEts-2-Met, 5'-GGCCG AATTC ATGAA TGATT TCGGA ATCAAG AA-3'; As-hEts2-stop, 5'-GTCCT CCGTG TCGGG CTG-3'; S-hZHX1-PxLxP-mut, 5'-GAAGG TCCTC CTGTG GCTAC ACCTG TAGAA AAC-3', and As-hZHX1-PxLxP-mut, 5'-GTTTT CTACA GGTGT AGCCA ACAGA GGACC TTC-3', were used in this study.

3.3. Cells and cell culture

MH_1C_1 cells, a rat hepatoma cell line, and HEK293 cells, a human embryonic kidney cell line, were purchased from the American Type Culture Collection (Manasses, U.S.A.). CV-1 cells, an African green monkey kidney cell line, and COS-7 cells, a transformant derived from CV-1 cells, were provided by the Japan Cancer Research Resources Bank. Cells were cultured in Dulbecco's modified Eagles medium supplemented with 10 % fetal bovine serum at 37 °C in a 5 % CO2 incubator.

3.4. Rapid amplification of the cDNA ends (RACE), PCR, and reverse transcription (RT)-PCR

To obtain 5'-noncoding and a part of the coding sequence of the rat BS69 cDNA, we employed the 5'-RACE method using rat brain marathon-ready cDNA and the Advantage 2 PCR kit. The rBS69-5RACE-As1, rBS69-5RACE-As2, rBS69-5RACE-As3, and rBS69-5RACE-As4 oligonucleotides, were used as the gene-specific primers. The RACE procedure was carried out according to the manufacturer's recommended protocol.

Total RNA was prepared from MH_1C_1 cells and rat tissues using the TRIZOL reagent according to the manufacture's recommended protocol. RT-PCRs were performed as described previously with minor modifications (1). A combination of S-rBS69-Met and As-rBS69-stop was employed as RT-PCR primers and two amplified products were subcloned into the pGEM-T Easy vector to produce pGEM-T Easy rBS69α (1-562) and pGEM-T Easy rBS69β (1-618), respectively. Oligonucleotides, S-rBS69-126 (Primer 1), As-rBS69-1350 (Primer 2), S-rBS69-157 (Primer 3), and As-rBS69-1268 (Primer 4) and a combination of S-GAPDH and As-GAPDH were also used as RT-PCR primers.

PCR and genomic PCR were performed as described previously with minor modifications (1, 4). Rat brain, mouse liver, human placenta, and human testis marathon-ready cDNAs and human genomic DNA were used as the template. A combination of S-hrmBS69-1088 and As-hrmBS69-1220 was employed as the PCR primers. All of the amplified DNA fragments were subcloned into the pGEM-T Easy vector. The nucleotide sequences of the inserts of these plasmids were determined by sequencing.

3.5. Plasmid constructs

The pSG424, pGEM-T Easy 36B4, pAD-G5, and pAD-L18 have been described previously (3, 21-23). The *Eco*RI fragment of the pGEM-T Easy rBS69β (1-618) or pGEM-T Easy rBS69α (1-562) was subcloned into the *Eco*RI site of the pSG424, pACT2 or pCMV-Tag3B vector to obtain GAL4-rBS69β (1-618), GAL4-rBS69α (1-562), pAD-BS69β (1-618), and myc-BS69β (1-618), respectively. PCRs were carried out using the pGEM-T Easy rBS69β (1-618) as a template with combinations of S-rBS69-1548 and As-rBS69-stop, S-rBS69-1640 and As-rBS69-stop, S-rBS69-1720 and As-rBS69-stop, S-rBS69-1780 and As-rBS69-stop, S-rBS69-1640 and As-rBS69-1782, S-rBS69-1640 and As-rBS69-1730, S-rBS69-Met and As-rBS69-1548, S-rBS69-Met and As-rBS69-1108, S-rBS69-1108 and As-rBS69-stop, and S-rBS69-1108 and As-rBS69-1275, as primers. All amplified DNAs were subcloned into the pGEM-T Easy to produce pGEM-T Easy rBS69β (517-618), pGEM-T Easy rBS69β (547-618), pGEM-T Easy rBS69β (574-618), pGEM-T Easy rBS69β (594-618), pGEM-T Easy rBS69β (547-594), pGEM-T Easy rBS69β (547-576), pGEM-T Easy rBS69β (1-516), pGEM-T Easy rBS69β (1-369), pGEM-T Easy rBS69β (370-618), and pGEM-T Easy rBS69β (370-425), respectively. The *Eco*RI fragments of the resulting plasmids were subcloned into the *Eco*RI site of the pACT2 or pSG424 vector to produce pAD-BS69β (517-618), pAD-BS69β (547-618), pAD-BS69β (574-618), pAD-BS69β (594-618), pAD-BS69β (547-594), pAD-BS69β (547-576), pAD-BS69β (1-516), GAL4-rBS69β (517-618), GAL4-rBS69β (547-618), GAL4-rBS69β (574-618), GAL4-rBS69β (594-618), GAL4-rBS69β (547-594), GAL4-rBS69β (1-516), GAL4-rBS69β (1-369), GAL4-rBS69β

(370-618), and GAL4-rBS69β (370-425), respectively.

pDBD, pDBD-ZHX1 (1-873), pGAL4-ZHX1 (1-873), and pZHX1 (272-873) have all been described previously (3-6). PCRs were also performed using the pGAL4-ZHX1 (1-873) as a template with combinations of S-hZHX1-Met and As-hZHX1-815, S-hZHX1-88 and As-hZHX1-815, S-hZHX1-88-mut and As-hZHX1-815, S-hZHX1-168 and As-hZHX1-815, as primers. PCR products were subcloned into the pGEM-T Easy to produce pGEM-T Easy hZHX1 (1-271), pGEM-T Easy hZHX1 (30-271), pGEM-T Easy hZHX1 (30-271) L35A, and pGEM-T Easy hZHX1 (56-271), respectively. After digestion with *Eco*RI, the fragments were subcloned into the *Eco*RI site of the pGBKT7 to give pDBD-ZHX1 (1-271), pDBD-ZHX1 (30-271), pDBD-ZHX1 (30-271) L35A, and pDBD-ZHX1 (56-271), respectively. A 1.8-kb *Sma*I/*Bam*HI fragment of the pZHX1 (272-873) was subcloned into the *Sma*I/ *Bam*HI sites of the pGBKT7 to obtain pDBD-ZHX1 (272-873). S-hZHX1-PxLxP-oli and As-hZHX1-PxLxP-oli oligonucleotides were annealed, phosphorylated, and inserted into the *Eco*RI/ *Bam*HI sites of the pGBKT7 to give pDBD-ZHX1 (33-37).

The 5 x GAL4-pGL3 Control, 5 x GAL4-E1b, E1b-Luc, pcDNA3.1HisC-ZHX1EX, and pHIS-ZHX1 (1-873) have been described previously (3, 22, 24, 25). Genomic PCR was carried out using human genomic DNA as a template with a combination of oligonucleotides, S-hBRCA1-1357 and As-hBRCA1-1623 as primers. An amplified DNA was digested with *Bgl*II and *Hind*III and the fragment was subcloned into the *Bgl*II/ *Hind*III sites of the pGL3-Basic to give phBRCA1/Luc. PCR was also carried out using human placenta cDNA as a template with a combination of S-hEts-2-Met and As-hEts2-stop as primers. PCR product was subcloned into the pGEM-T Easy to give pGEM-T Easy hEts-2. An *Eco*RI fragment of the resulting plasmid was subcloned into the *Eco*RI site of the pcDNA3.1/His C vector to obtain pcDNA-ets-2.

An *Eco*RI fragment of pGEM-T Easy hZHX1 (1-271) was subcloned into the *Eco*RI site of the pcDNA3.1/His C vector to produce pcDNA-ZHX1 (1-271). Site-direct mutagenesis was carried out using the QuikChange site-direct mutagenesis kit. The reaction was performed using pcDNA-ZHX1 (1-271) as a template and a combination of S-hZHX1-PxLxP-mut and As-hZHX1-PxLxP-mut as primers. The resultant plasmid was digested with *Eco*RI and a 810-bp fragment was subcloned into the *Eco*RI site of the pcDNA3.1HisC-ZHX1EX to obtain pHIS-ZHX1 (1-873) L35A. A *Bam*HI fragment of the pHIS-ZHX1 (1-873) or pHIS-ZHX1 (1-873) L35A was subcloned into the *Bam*HI site of the pCMV-Tag2B vector to obtain FLAG-ZHX1 (1-873) and FLAG-ZHX1 (1-873) L35A, respectively. The nucleotide sequences of the inserts of these plasmids were determined by sequencing.

3.6. Poly A^+- RNA blot analysis

A 330-bp of *Hinc*II/ *Nhe*I fragment of GAL4-rBS69β (1-618) and a 954-bp *Eco*RI fragment of pGEM-T Easy 36B4 were employed as probes (3). Rat Multiple Tissue Northern blot was hybridized with probes labeled with α-^{32}P dCTP using the BcaBest DNA labeling kit. For prehybridization and hybridization, the ExpressHyb hybridization solution was used. The conditions for the prehybridization, hybridization, and washing were according to the manufacture's recommended protocol. The blot was exposed to a FUJIX imaging plate (Kanagawa, Japan). Hybridization signals were detected with the FUJIX BAS-2000 image analyzing system.

3.7. Yeast two-hybrid system

For analysis of the interaction domain of ZHX1 with BS69, SFY526 yeast strains harboring the pACT2, pAD-G5, or pAD-BS69β (1-618) were transformed with pDBD or various truncated forms of ZHX1 fused to the GAL4 DNA-binding domain (DBD). For mapping the interaction domain of BS69 with ZHX1, a SFY526 yeast strain harboring the pDBD or pDBD-ZHX1 (1-873) was transformed with pACT2 or various truncated forms of BS69 fused to the GAL4 AD. These β-galactosidase activities were determined from at least three different experiments, as described previously (1, 4, 5).

3.8. DNA transfection

HEK293 cells and COS-7 cells (5 x 10^4) and CV-1 cells (2 x 10^5) were seeded per well in a 24 well plate 24 hours before transfection. All DNA transfection was carried out using the LIPOFECTAMINE PLUS reagents following to the protocol provided by the supplier.

For the determination of transcriptional activity of BS69 in HEK293 cells, the 5 x GAL4-pGL3 Control, pGL3-Control, 5 x GAL4-E1b Luc, and E1b Luc were used as reporter plasmids. One hundred ng of the reporter plasmid, 1 ng of the pRL-CMV, and the indicated amount of GAL4-rBS69β (1-618) or 200 ng of the GAL4-rBS69α (1-562) expression plasmids were transfected. The total amount of plasmid (301 ng) was adjusted by the addition of pSG424, if necessary. In both COS-7 and CV-1 cells, 5 x GAL4-E1b Luc and E1b Luc were used as the reporter plasmid. Two hundreds ng of a reporter plasmid, 0.1 ng of the pRL-CMV, and the indicated amount of GAL4-rBS69β (1-618) expression plasmid were transfected. The total amount of plasmid (300.1 ng) was adjusted by the addition of pSG424, if necessary.

For the analysis of the effects of ets-2 and BS69 on the promoter activity of the human *BRCA1* gene in HEK293, the phBRCA1/Luc and pGL3-Basic were used as the reporter plasmid. One hundred ng of a reporter plasmid, 1 ng of the pRL-CMV, 100 ng of pcDNA3.1/His C or pcDNA-ets-2, and 100 ng of pCMV-Tag3B or myc-BS69β (1-618) were transfected.

For the domain mapping of the transcriptional activity of BS69 in HEK293 cells, one hundred ng of 5 x GAL4-pGL3 Control, 1 ng of pRL-CMV, and 100 ng of various GAL4-rBS69 fusion protein expression plasmids were transfected. The total amount of plasmid (201 ng) was adjusted by the addition of pSG424, if necessary. In COS-7 cells, 200 ng of the 5 x GAL4-E1b Luc, 0.1 ng of the pRL-CMV, and 100 ng of various GAL4-rBS69 fusion protein expression plasmids were transfected. The total amount of

plasmid (300.1 ng) was adjusted by the addition of pSG424, if necessary.

For the analysis of effects of ZHX1 on the transcriptional activity of BS69, the 5 x GAL4-pGL3 Control in HEK293 cells or 5 x GAL4-E1b Luc in COS-7 cells was used as the reporter plasmid, respectively. One hundred ng of the 5 x GAL4-pGL3 Control or two hundreds ng of the 5 x GAL4-E1b Luc, 1 or 0.1 ng of the pRL-CMV, 100 ng of the GAL4-rBS69β (1-618) fusion protein expression plasmid, and 200 ng of the pCMV-Tag2B, FLAG-ZHX1 (1-873), or FLAG-ZHX1 (1-873) L35A plasmids were transfected. In all transfection experiments, the medium was replaced with fresh medium three hours after transfection. After 48 hours, luciferase activities in the cells were determined. All plasmids used for the transfection were prepared using an Invisorb plasmid kit, followed by CsCl density gradient ultracentrifugation.

3.9. Immunoprecipitation assay and Western blot analysis

HEK293 cells (1.5 x 10^6) were seeded in a 10 cm dish. Combinations of plasmids, pCMV-Tag2B and pCMV-Tag3B, FLAG-ZHX1 (1-873) and myc-BS69β (1-618), or FLAG-ZHX1 (1-873) L35A and myc-BS69β (1-618) were employed. Each one microgram of plasmids were transfected into HEK293 cells using the LIPOFECTAMINE PLUS reagents. Nuclear extracts from HEK293 cells were prepared as described previously (26). Immunoprecipitation procedures followed the manufacture's recommended protocols. Five hundred microgram of nuclear extract was incubated with 4 microgram of anti-c-myc antibody at 4 °C for 1 hour. The precipitated proteins were separated by 7.5 % SDS-PAGE and transferred onto a PVDF membrane. The membrane was immersed in Blocking One at 4 °C for overnight and washed with 0.1 % (v/v) Tween 20/ phosphate buffered saline. The FLAG-ZHX1 proteins were detected and visualized using an anti-FLAG M2 antibody-peroxidase conjugate, at a dilution of 1: 1000 in 0.1 % (v/v) Tween 20/ phosphate buffered saline, and the ECL plus kit.

3.10. Luciferase assay

Firefly and sea pansy luciferase assays were carried out using the dual luciferase assay system. These procedures followed the manufacture's recommended protocol. Luciferase activities were determined using a Berthold Lumat model LB 9501 (Wildbad, Germany). Firefly luciferase activities (relative light units) were normalized by sea pansy luciferase activities. Data are expressed as the mean and standard error from at least four different experiments. Statistical differences were determined by a two-tailed Student's *t*-test.

4. RESULTS

4.1. Molecular cloning of novel rat BS69 cDNAs

In our previous study, nine clones encoding the BS69 co-repressor were isolated as a ZHX1-interacting protein from rat cDNA libraries (3). The longest clone pAD-G5 containing a 633-bp of coding region and a 3' non-coding region encoded unreported 18 amino acid residues followed by the amino acid sequence corresponding to the residues from 370 to 562 of human BS69. Since all clone obtained were partial cDNAs, we attempted to obtain full-length clones.

To isolate the 5'-noncoding sequence and the remaining coding region of the rat BS69 cDNA, we employed 5'-RACE using combinations of gene-specific primers and adaptor primers and RT-PCR using the total RNA from MH_1C_1 cells. We obtained four forms of rat BS69 cDNAs, BS69α, BS69β, BS69γ and BS69δ, respectively. The schematic representation of these forms are shown in Figure 1A. The sizes of their entire coding sequences of BS69α, BS69β, BS69γ, and BS69δ were determined to be 1,686-, 1,854-, 1,692-, and 1,524-bp, respectively (submitted to the DNA Data Bank of Japan Accession No. AB162617, AB162618, AB162619, and AB162620). BS69α was found to correspond to human BS69 and contained an open reading frame of 562 amino acids, being a predicted molecular mass of 61.8 kDa. In comparison with human BS69, the similarities of the nucleotide sequences in the coding region and the deduced amino acid sequences were 91 % and 90 %, respectively. As seen in Figure 1A, BS69β and BS69γ contained novel 56 amino acid residues derived from exon 11b. Both the BS69γ and BS69δ proteins lacked a 54 amino acid region encoded by exon 3 that corresponds to the amino acid sequence between 53 and 106 of human BS69. As a result, the BS69β, BS69γ, and BS69δ proteins consisted of 618, 564, and 508 amino acid residues, respectively. These proteins had a predicted molecular mass of 68.0, 62.0, and 55.9 kDa, respectively. These results indicate that rat BS69 cDNAs exist in four forms; BS69α is a human BS69 orthologue and the other three are novel forms.

To examine whether corresponding transcripts of BS69 having the exon 11b sequence are expressed in humans and mice, PCR was performed using cDNAs from mouse liver, rat brain, human placenta, and human testis as templates and homologous primer sets that located in exon 11a and 12. Two products were obtained from both rat and mouse; one was a 301-bp product that contains a novel 168-bp nucleotide sequence derived from exon 11b and the other was a 133-bp product (Figure 1B). In contrast, only the 133-bp product was detected in human tissues (Figure 1B). These results indicate that both the rat and mouse, but not the human, express BS69 mRNA containing the novel sequence. To understand the reason why the difference occurs in these species, we performed genomic PCR in this region and the nucleotide sequences of the human, rat, and mouse *BS69* genes were compared. As shown in Figure 1C, both rat and mouse *BS69* genes were found to have a splice donor site "GT" just downstream of the novel sequence although human *BS69* gene lacks this splice donor site. The results indicate that the *BS69* gene in rats and mice has alternative splicing sites in exon 11 and produces two types of transcripts, one of which was newly found in this study.

On the Northern blot analysis using rat mRNA (Figure 1D), a 4.4-kb BS69 transcript was expressed in all tissues examined although the expression level was very

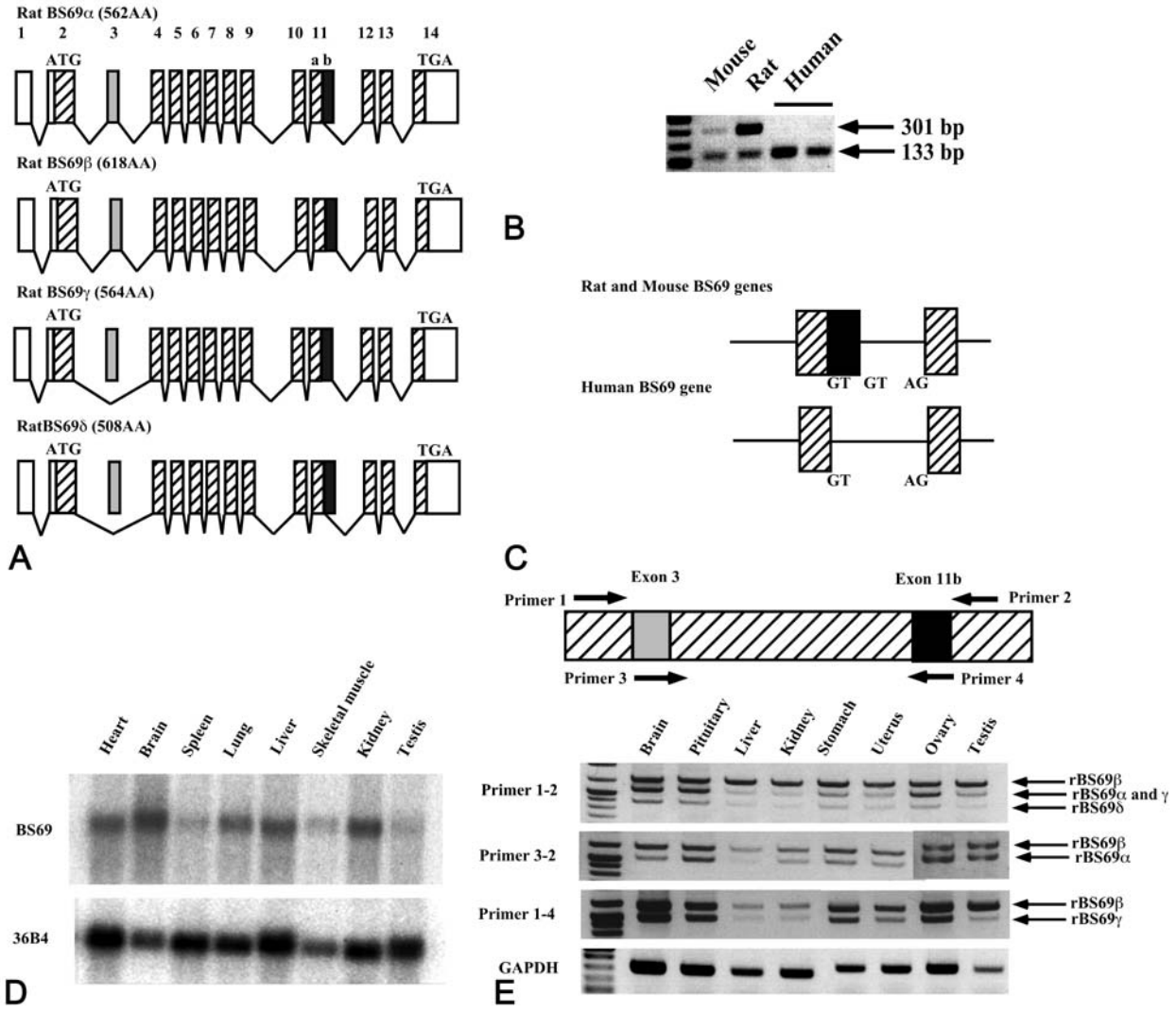

Figure 1. Four forms of rat BS69 cDNA. (A) Schematic representation of the splicing variants of the rat *BS69* gene. The number indicates each exon of the rat *BS69* gene. The open box represents the non-coding region and other boxes represent coding regions, respectively. The closed box is the novel exon contained by both rat BS69β and BS69γ cDNAs. Both BS69γ and BS69δ cDNAs do not contain nucleotide sequence of exon 3. (B) Differential expressions of the novel region of BS69 among mouse, rat, and human. A 301-bp but not a 133-bp of the RT-PCR product contains 168-bp of the novel region of BS69. (C) Comparison of the genomic structures of the rat, mouse, and human *BS69* genes. Nucleotide sequences downstream of the novel region shown in a closed box of the rat and mouse *BS69* genes but not the human gene were "GT", which corresponds to the splicing donor site. (D) Northern blot analysis of expression of rat BS69 mRNA. A rat Multiple Tissue Northern blot was hybridized with ^{32}P-labeled rat BS69 and 36B4 cDNAs, respectively. Prehybridization and hybridization procedures are described in "*Materials and methods*". Each lane contains 2 microgram of poly A^+-RNA isolated from indicated tissues. (E) RT-PCR analysis of tissue distribution of mRNA of rat BS69 splicing variants. Oligonucleotides, S-rBS69-126 (Primer 1), As-rBS69-1350 (Primer 2), S-rBS69-157 (Primer 3), and As-rBS69-1268 (Primer 4), were used as primers to distinguish each form. The levels of glyceraldehyde 3-phosphate dehydrogenase were shown as GAPDH at the bottom.

low in the spleen, skeletal muscle, and testis. Using different combination of primers on RT-PCR (shown in Figure 1E), we next examined the tissue distribution of each type of the rat BS69 splicing variants. Template RNAs were obtained from various rat tissues, such as the brain, pituitary, liver, kidney, stomach, uterus, ovary, and testis. All splicing variants of BS69 were ubiquitously expressed in the rat although the pattern of expression was different in each tissue (Figure 1E). A major transcript of the rat *BS69* gene was the β type containing both the exon 3 and exon 11b regions.

4.2. Mapping of the interaction domains between ZHX1 and BS69

To determine the domain of ZHX1 that interacts with BS69, yeast two-hybrid experiment was carried out

using yeast SFY526. As seen in Figure 2B, a series of rat BS69β deletion mutants were fused to GAL4 AD (GAL4 activation domain) using pACT2 plasmid having GAL4 AD alone. A series of ZHX1 deletion mutants were also fused to GAL4 DBD (GAL4 DNA-binding domain) as prey plasmids (Figure 2A).

The β-galactosidase activity was very low in the yeasts cotransformed with pACT2 and a series of pDBD plasmids. When a reporter yeast harboring the pAD-G5 or pAD-BS69β (1-618) was transformed with pDBD, pDBD-ZHX1 (272-873), or pDBD-ZHX1 (56-271), β-galactosidase activity was also very low. In contrast, yeasts transformed with the pDBD-ZHX1 (1-873), pDBD-ZHX1 (1-271), or pDBD-ZHX1 (30-271) expressed high β-galactosidase activities (Figure 2A). The pAD-G5 contains the MYND domain of BS69β, which encodes the amino acids between residues 578 and 618 (Figure 2B). It has been reported that the MYND domain associates with the PxLxP motif of the target protein (18). Since there is a PVLTP motif between residues 33 and 37 of human ZHX1 (1), we examined whether the PVLTP motif in ZHX1 is required for an interaction with BS69. We prepared two other prey plasmids, pDBD-ZHX1 (30-271) L35A, in which the central 35 th leucine residue is replaced with alanine in the motif, and pDBD-ZHX1 (33-37), which contains the only PxLxP motif of ZHX1. When these plasmids were transformed into reporter yeasts harboring the pAD-G5 or pAD-BS69β (1-618), the yeasts exhibited low β-galactosidase activities (Figure 2A). These results indicate that the amino acid sequence from 30 to 271 of ZHX1 is enough for the functional interaction with BS69, and that the PxLxP motif in ZHX1 is essential for interaction with BS69 but the PxLxP motif alone is not sufficient for the functional binding. To examine whether the MYND domain of BS69 is required for the interaction with ZHX1, we used a reporter yeast strain harboring pDBD-ZHX1 (1-873) with various plasmids that express truncated forms of BS69β fused to the GAL4 AD (Figure 2B). The yeasts harboring pAD-BS69β (1-618), pAD-G5, pAD-BS69β (517-618), or pAD-BS69β (547-618) showed a high level of β-galactosidase activities while control yeasts having pDBD with the pAD-BS69 plasmids showed very low β-galactosidase activities. These results showed that the MYND domain from 547 to 618 amino acids of BS69β is required to interact with ZHX1 but the MYND domain alone is not sufficient for the functional interaction.

To examine possible interactions between ZHX1 and BS69 in mammalian cells, we used five expression plasmids, pCMV-Tag2B, FLAG-ZHX1 (1-873), FLAG-ZHX1 (1-873) L35A, pCMV-Tag3B, and myc-BS69β (1-618). FLAG-ZHX1 (1-873) expresses a full-length of ZHX1 fused to the FLAG-tag and FLAG-ZHX1 (1-873) L35A expresses its L35A mutant. The myc-BS69β (1-618) expresses a full-length of BS69β fused to the c-myc-tag. The pCMV-Tag2B and pCMV-Tag3B are parent vectors for expression of FLAG- and myc-tagged protein, respectively. Combinations of plasmids, pCMV-Tag2B and pCMV-Tag3B, FLAG-ZHX1 (1-873) and myc-BS69β (1-618), or FLAG-ZHX1 (1-873) L35A and myc-BS69β (1-618) were introduced into HEK293 cells. Immunoprecipitation of nuclear extracts was carried out with anti-c-myc antibody to precipitate myc-BS69β-interacting proteins, and FLAG-tagged ZHX1 proteins were detected with anti-FLAG M2 antibody (Figure 2C, lanes 1-6). When the nuclear extract from cells co-transfected the FLAG-ZHX1 (1-873) and myc-BS69β (1-618) plasmids were immunoprecipitated with the anti-c-myc antibody, FLAG-tagged ZHX1 (1-873) was detected in the precipitated complex (Figure 2C, lane 5). However, when combinations of pCMV-Tag2B and pCMV-Tag3B, and FLAG-ZHX1 (1-873) L35A and myc-BS69β (1-618) were co-transfected into HEK293 cells, FLAG-tagged ZHX1 could not be detected in the precipitants (Figure 2C, lanes 4 and 6). These results strongly suggest that the PxLxP motif of ZHX1 is required for the interaction with BS69 in mammalian cells as was in yeast cells.

4.3. BS69 is a transcriptional activator

The human BS69 has been reported to have a transcriptional repression activity in U2OS cells (17). To determine the transcriptional role of α and β types of rat BS69, we used a mammalian one-hybrid system. The 5 x GAL4-pGL3 Control reporter plasmid consists of five copies of the GAL4-binding site that are fused to the SV40 enhancer/ promoter followed by the coding sequence of luciferase as a reporter gene (24). Three effector plasmids, pSG424, which expresses GAL4 DBD alone, and GAL4-rBS69β (1-618) and GAL4-rBS69α (1-562), which express the entire coding region of rat BS69β and BS69α fused to the C-terminal of the GAL4 DBD, respectively, were prepared. As shown in Figures 3A and B, when the 5 x GAL4-pGL3 Control and various amounts of GAL4-rBS69β (1-618) or GAL4-rBS69α (1-562) were co-transfected into HEK293 cells, the luciferase activities were unexpectedly increased in a dose-dependent manner. In contrast, when the pGL3-Control lacking five copies of the GAL4-binding sites was transfected with the pSG424, and GAL4-rBS69β (1-618) or GAL4-rBS69α (1-562), the luciferase activities remained unchanged (Figures 3A and B). These results show that the GAL4-rBS69 fusion proteins activate luciferase expression by specifically binding to GAL4-binding sites.

To examine promoter dependence of the activation mediated by rBS69, E1b minimal promoter (22) was used instead of SV40 enhancer/ promoter in 5 x GAL4-pGL3 Control reporter plasmid. As shown in Figures 3C and D, the luciferase activities were also increased in a dose-dependent manner, suggesting that the activation mediated by rBS69α and rBS69β is not enhancer/promoter-dependent. This activation was not observed when 5 x GAL4 binding site was not available. These findings indicate that both BS69α and BS69β function as transcriptional activator in HEK293 cells.

In order to confirm the function of BS69 in HEK293 cells, we employed a natural promoter context-based reporter system. It has been reported that the co-expression of ets-2 and BS69 but not either ets-2 or BS69 alone in SW13 cells results in the repression of the

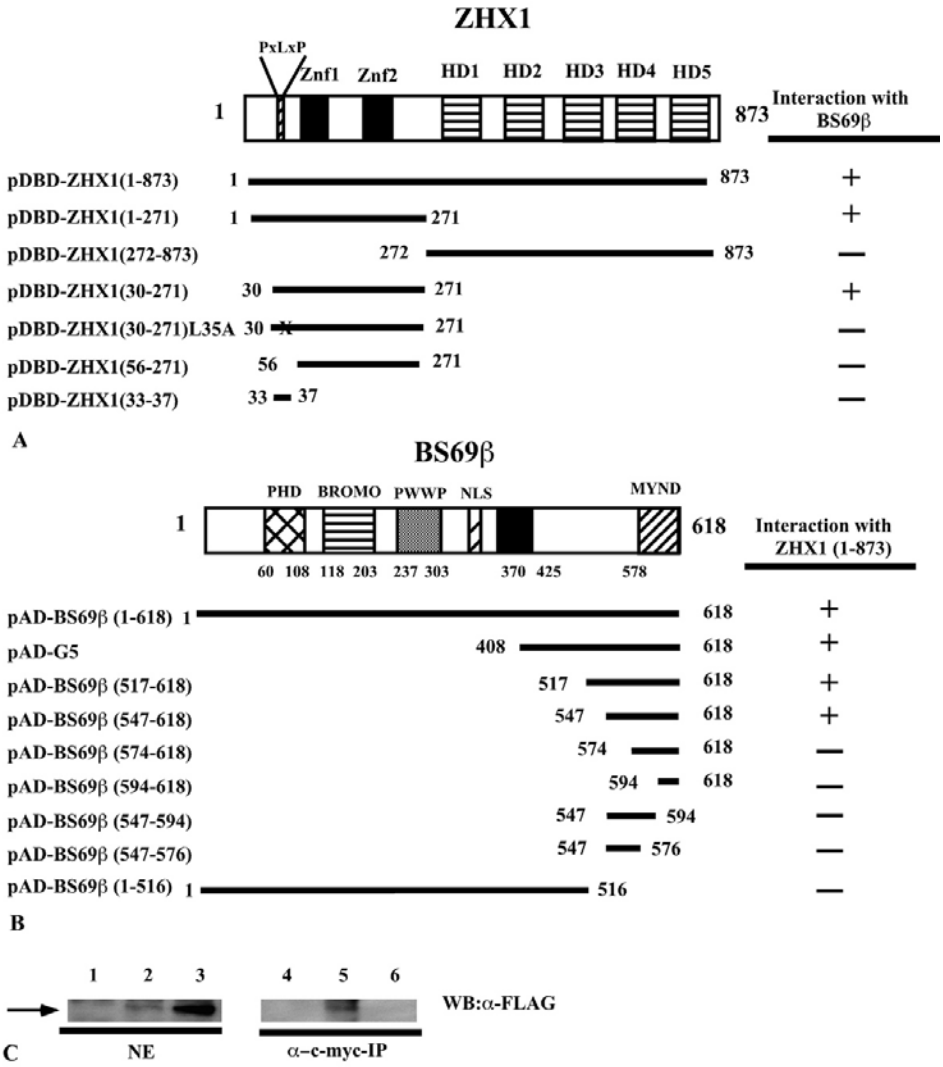

Figure 2. Determination of the interaction domains between ZHX1 and BS69. Using a yeast two-hybrid system, each interaction domain between ZHX1 and BS69 was determined. (A) A schematic representation of human ZHX1 and the GAL4 DBD-ZHX1 fusion constructs are depicted on the *left*. Znf and HD indicate the zinc-finger motif and homeodomain, respectively. The PxLxP motif is located in the amino acid sequences between residues 33 and 37 of human ZHX1. The + and – symbols indicate increased and unchanged levels of β-galactosidase activity, respectively, compared with yeasts harboring a combination of pAD-G5 or pAD-BS69β (1-618) expressing amino acids 408-618 or 1-618 of rat BS69β fused to the C-terminal of the GAL4 AD, and pDBD. (B) A schematic diagram of rat BS69β and its deletion mutants fused to the GAL4 AD are illustrated at the *left*. PHD, BROMO, PWWP, NLS, and MYND indicate the PHD finger motif, bromodomain, PWWP domain, nuclear localization signal, and MYND domain, respectively. The amino acid sequence between residues 370 and 425 of BS69β is a novel region, which is expressed in the rat and mouse but not by the human. The + and – symbols indicate increased and unchanged levels of β-galactosidase activity, respectively, compared with a yeast harboring a combination of pDBD-ZHX1 (1-873) expressing amino acid 1-873 of human ZHX1 fused to the C-terminal of the GAL4 DBD, and pACT2. (C) Immunoprecipitation assay of FLAG-tagged ZHX1 and c-myc-tagged BS69β proteins. HEK293 cells were co-transfected with 2 microgram of combinations of the CMV promoter-directed expression vectors, pCMV-Tag2B and pCMV-Tag3B (Lanes 1 and 4), FLAG-ZHX1 (1-873) and myc-BS69β (1-618) (Lanes 2 and 5), and FLAG-ZHX1 (1-873) L35A and myc-BS69β (1-618) (Lanes 3 and 6), respectively. The FLAG-ZHX1 (1-873) expresses the entire coding sequence of human ZHX1 fused to the FLAG-tag. The FLAG-ZHX1 (1-873) L35A, expresses a mutant protein with the 35 th leucine residue of the FLAG-tagged ZHX1 (1-873) replaced with an alanine residue and cannot interact with BS69. The myc-BS69β (1-618) expresses the entire coding sequence of rat BS69β fused to the c-myc-tag. Forty-eight hours after transfection, nuclear extracts from the cells were prepared. Immunoprecipitation was carried out using an anti-c-myc monoclonal antibody. The nuclear extracts (20 microgram: Lanes 1-3) and immunoprecipitated-proteins (Lanes 4-6) were separated by 7.5 % SDS-PAGE and subjected to Western blot assay using anti-FLAG M2 antibody-peroxidase conjugate and the ECL plus kit. NE, nuclear extract; IP, immunoprecipitation; WB, Western blot assay.

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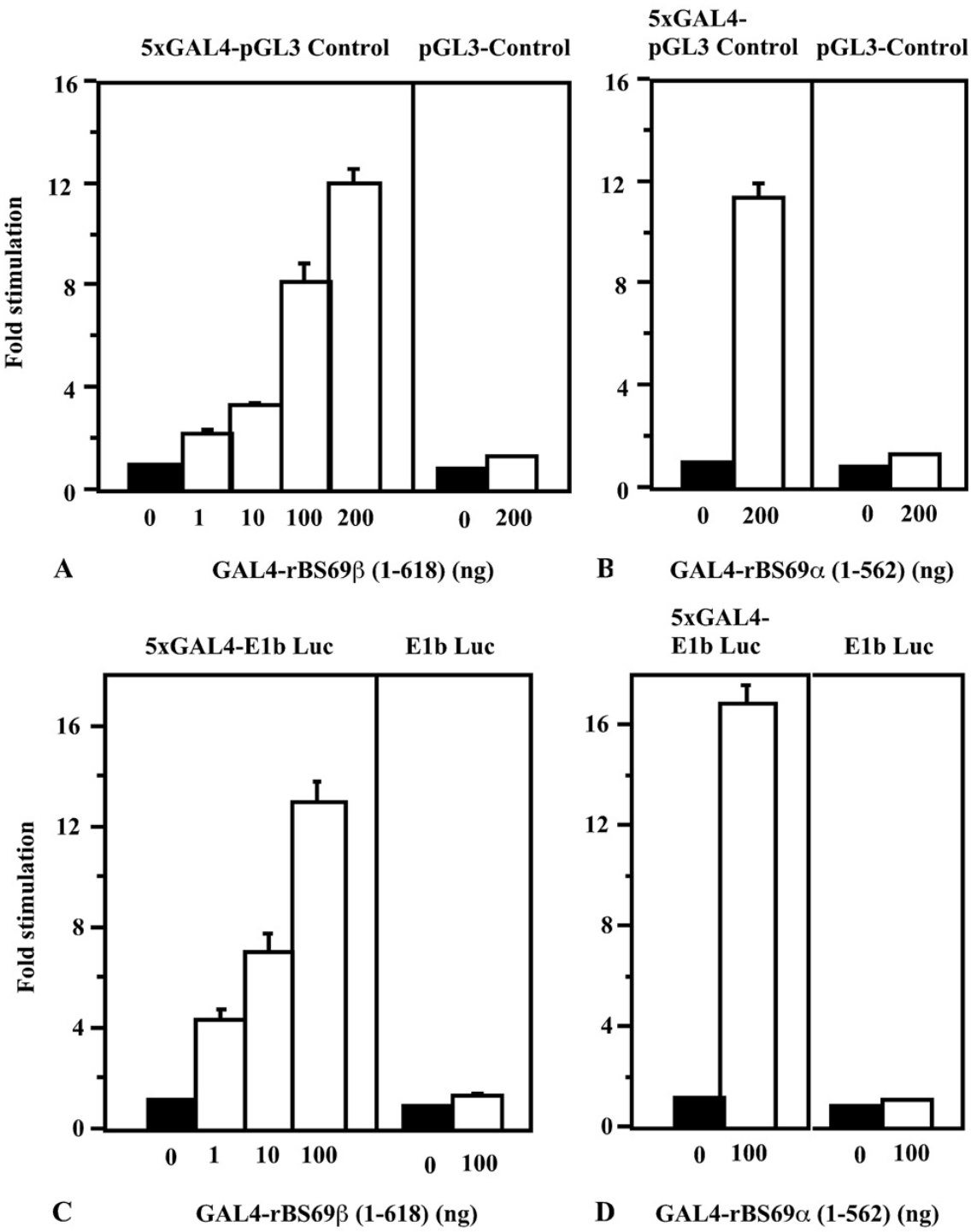

Figure 3. BS69 is a transcriptional activator. HEK293 cells were co-transfected with 1 ng of pRL-CMV, the indicated amounts of SV40 promoter-directed expression vectors (GAL4-BS69β (1-618), and GAL4-BS69α (1-562)), and 100 ng of the 5 x GAL4-pGL3 Control or pGL3-Control reporter plasmid (A and B), or the 5 x GAL4-E1b Luc or E1b Luc reporter plasmid (C and D). The total amount of plasmid (301 ng) was adjusted by the addition of pSG424. GAL4-BS69β (1-618) expresses the entire coding sequence of rat BS69β fused to GAL4 DBD (A and C). The GAL4-BS69α (1-562) expresses the entire coding sequence of rat BS69α fused to the GAL4 DBD (B and D). Cells were harvested forty-eight hours after transfection, and both firefly and sea pansy luciferase activities were determined. Firefly luciferase activities were normalized by sea pansy luciferase activities in all experiments. A value of 1 was assigned to the promoter activity of the reporter plasmid in the presence of 200 ng of the pSG424, respectively. Each column and bar represents the mean and standard error of at least four transfection experiments.

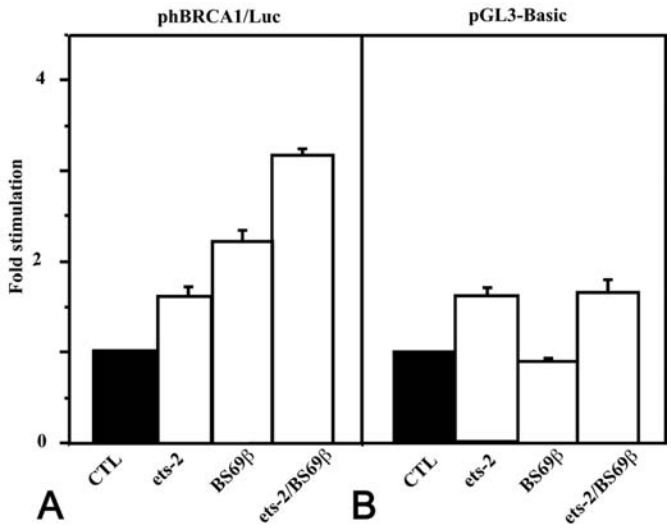

Figure 4. BS69 activates promoter activity of the human *BRCA1* gene. HEK293 cells were co-transfected with 100 ng of the phBRCA1/Luc (A) or pGL3-Basic (B), 1 ng of the pRL-CMV, 100 ng of the pcDNA3.1/His C or pcDNA-ets-2 and 100 ng of the pCMV-Tag3B or pCMV-BS69β (1-618). A value of 1 was assigned to the promoter activity of each reporter plasmid in the presence of 100 ng of the pcDNA3.1/His C, and 100 ng of the pCMV-Tag3B. Each column and error bar represents the mean and standard error of at least three independent experiments. CTL, control.

promoter activity of the *BRCA1* gene (19). Therefore, the human BRCA1 proximal promoter region, located in the nucleotide sequence between 1357 and 1623 of the human *BRCA1* gene (GenBank™ number U37574) (27), was used as a natural promoter fused to luciferase reporter gene. As shown in Figure 4A, the luciferase activities mediated by the proximal promoter of the *BRCA1* gene were increased by the expression of myc-BS69β (1-618) or ets-2 into HEK293 cells, and further increased with the coexpression of myc-BS69β (1-618) and ets-2, whereas the luciferase activities remained unchanged with the pGL3-Basic and myc-BS69β (1-618) into HEK293 cells (Figure 4B). These results indicate that BS69 activates the transcription of luciferase gene through the proximal promoter of the *BRCA1* gene, at least, in HEK293 cells, and that the activation by BS69 may be enhanced in the presence of ets-2.

4.4. Determination of the minimal activation domain of BS69

To determine the minimal activation domain of BS69, 5 x GAL4-pGL3 Control was transfected with plasmids having GAL4 DBD fused to a series of truncated BS69 as seen in Figure 5. The N-terminal truncation up to amino acid 547 of rBS69β showed an activation of the reporter gene expression (Figure 5). The GAL4-rBS69β (517-618) and GAL4-rBS69β (547-594) enhanced the luciferase activity greater than the full-length GAL4-rBS69β (1-618). Further deletion of rBS69β from the N-terminus eliminated the enhancement of luciferase activity. The deletion of rBS69β from the C-terminus, GAL4-rBS69β (1-369) in Figure 5, also showed a basal luciferase activity. These results indicate that the amino acid sequence from 547 to 618 of BS69β (corresponding to the amino acids from 491 to 562 of BS69α), which contains the MYND domain, is essential for the activation.

4.5. BS69 functions as a transcriptional repressor

In COS-7 and CV-1 cells, however, the transfection of 5 x GAL4-E1b Luc plasmid with GAL4-rBS69β (1-618) decreased luciferase activities in a dose-dependent manner (Figures 6A and C). GAL4-rBS69β (1-618) did not decrease luciferase activities on the transfection with the pE1b Luc plasmid lacking five copies of the GAL4-binding sites (Figures 6B and D). These results suggest that BS69 may function as a transcriptional repressor in COS-7 and CV-1 cells.

To determine the minimal repression domain of BS69, COS-7 cells were co-transfected with the 5 x GAL4-E1b Luc and various truncated plasmids (Figure 7). The C-terminal deletion of rBS69β up to the amino acid 516 reduced luciferase activities as the wild type GAL4-rBS69β (1-618), suggesting that the region from 516 to 618 amino acids may not be important for the repressive function of rBS69β. Further truncated constructs, GAL4-rBS69β (1-369), GAL4-rBS69β (370-425), and GAL4-rBS69β (370-618), showed repression activity although the level was decreased compared with GAL4-rBS69β (1-516). In addition, when GAL4-rBS69α (1-562) was co-transfected, the luciferase activity was decreased. Therefore, we concluded that BS69α as well as BS69β function as transcriptional repressors, and that the amino acid sequence between residues 1 and 516 of BS69β (corresponding to the amino acid sequence between residues 1 and 460 of BS69α), might be important for full repressor activity.

4.6. Effects of ZHX1 on transcriptional activities of BS69

As shown in Figure 8A, we examined the effect of ZHX1 on the transcriptional activation of BS69 in HEK293 cells using 5 x GAL4-pGL3 Control and GAL4-rBS69β (1-

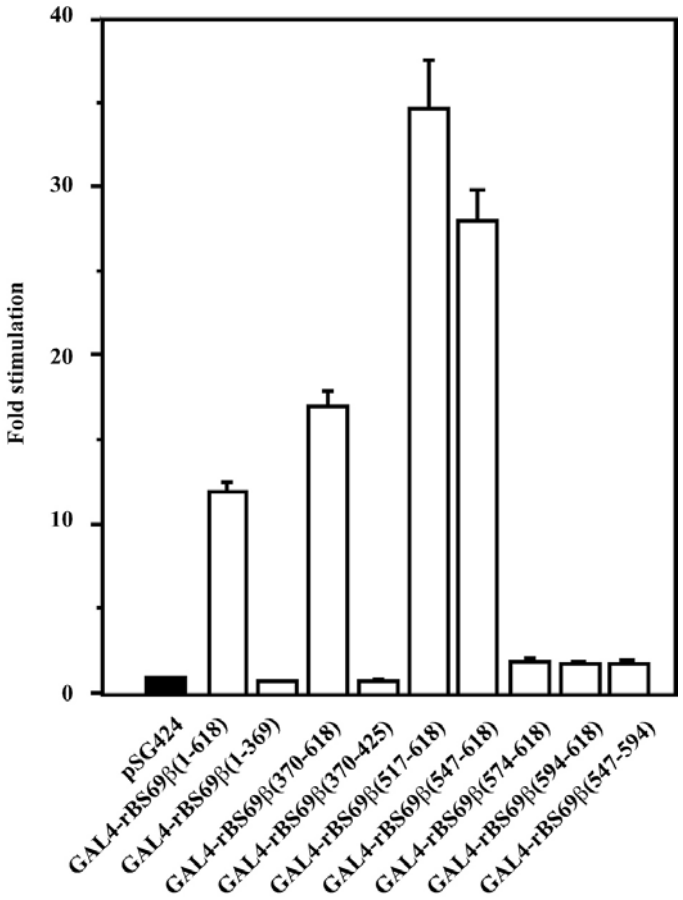

Figure 5. Determination of the minimal activation domain of BS69. HEK293 cells were co-transfected with 1 ng of the pRL-CMV, 100 ng of the SV40 promoter-directed expression vectors (pSG424 and GAL4-BS69β deletion constructs), and 100 ng of the 5 x GAL4-pGL3 Control. The pSG424 and various GAL4-rBS69β construct express GAL4 DBD alone and various deletion mutants of rat BS69β fused to the GAL4 DBD, respectively. Other conditions are the same as described in the legend for Figure 3. Each column and error bar represents the mean and standard error of at least four transfection experiments.

18) plasmids. The FLAG-ZHX1 (1-873) expression did not affect the luciferase activity enhanced by GAL4-rBS69β (1-618). When the level of endogenous transcription factor was high, the activity of exogenously introduced protein frequently could not be detected. A mutant form of ZHX1 expression plasmid, FLAG-ZHX1 (1-873) L35A, which cannot interact with BS69 (Figure 2), was therefore employed. When the FLAG-ZHX1 (1-873) L35A plasmid was co-transfected, the luciferase activity induced by GAL4-rBS69β (1-618) was further increased (Figure 8A).

Finally, we determined whether ZHX1 affects the transcriptional repression of BS69 in COS-7 cells. When FLAG-ZHX1 (1-873) or FLAG-ZHX1 (1-873) L35A plasmids were co-transfected in the assay system, these plasmids had no effect on the repression of BS69 (Figure 8B). These results suggest that ZHX1 may not participate the transcriptional repression of BS69.

5. DISCUSSION

We previously reported the screening of ZHX1-interacting proteins from rat cDNA libraries (3). The BS69 co-repressor, which is associated with some cancer-related proteins such as E1A, EBNA2, MGA, and c-Myb, has been cloned (3, 15, 18, 28). In this study, physical and functional interactions between ZHX1 and BS69 and the transcriptional activities and functional domains of rat BS69 were characterized. We first reported on four forms of rat BS69 orthologues, BS69α, BS69β, BS69γ, and BS69δ, which are different from BRAM1 (Figure 1A). The rat BS69α corresponded to the human form. Both BS69β and BS69γ contained a unique region consisting of 56 amino acid residues, which is encoded by the exon 11b of the rat *BS69* gene (Figures 1A-C and 2A). Based on database searches, no putative specific functional motifs were included in the unique region, and the human *BS69* gene does not have the exon corresponding to exon 11b of rat (Figures 1B and C). Although the mouse *BS69* gene had the corresponding exon, the expression level was lower than that of the rat *BS69* gene (Figures 1B and C). Both the BS69γ and BS69δ proteins lacked a region encoded by exon 3 of the rat *BS69* gene, which corresponds to the amino acid sequence between residues 53 and 106 of the human form (Figure 1A). The region encoded by the exon 3 is a putative PHD finger motif, a plant homeodomain,

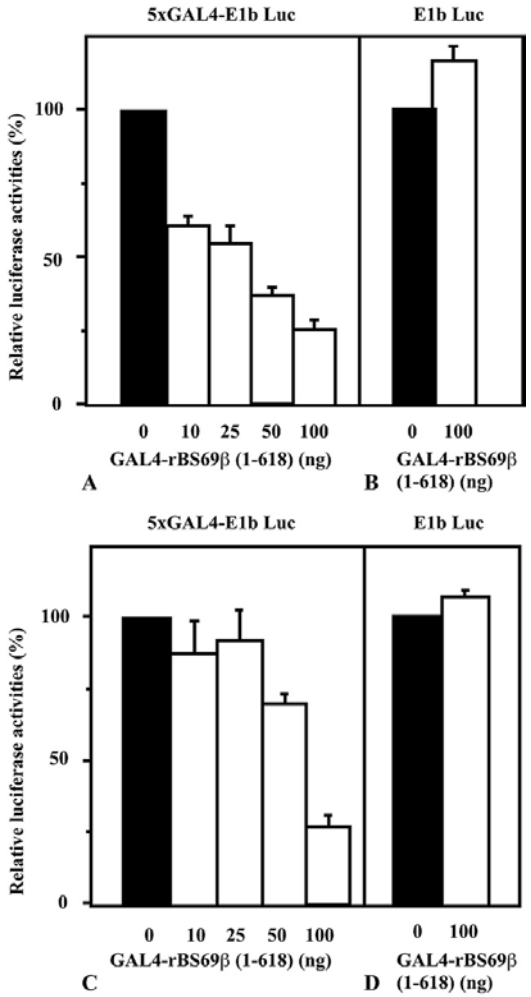

Figure 6. BS69 can function as a transcriptional repressor in both COS-7 and CV-1 cells. COS-7 (A and B) and CV-1 cells (C and D) were co-transfected with 0.1 ng of the pRL-CMV, the indicated amounts of the GAL4-BS69β (1-618), and 200 ng of the 5 x GAL4-E1b Luc (A and C) or E1b Luc (B and D) reporter plasmid. The total amount of plasmid (300.1 ng) was adjusted by the addition of the pSG424. A value of 100 % was assigned to the promoter activity of the reporter plasmid in the presence of 100 ng of the pSG424, respectively. The other conditions are the same as described in the legend for Figure 3. Each column and error bar represents the mean and standard error of at least four transfection experiments.

which is present in many genes in chromatin-mediated transcriptional regulation (29). The transcriptional role of the motif in BS69 remains to be determined.

Although rat BS69 mRNAs are expressed ubiquitously, BS69β was a major form in all tissues examined (Figures 1B, D and E). In the pituitary and ovary, the level of BS69α mRNA was almost same as that of BS69β (Figure 1E). While the level of BS69γ mRNA was high in brain, pituitary, stomach, and ovary, the level of BS69δ mRNA was low in all tissue examined (Figure 1E). These results raise the possibility that tissue-specific transcriptional regulation by BS69 occurs in the rat, and that the each splicing variant may have a tissue-specific role.

The physical interaction of ZHX1 with BS69 was dependent on the N-terminal PxLxP motif of ZHX1 although the PxLxP motif alone failed to interact with BS69 (Figures 2A and C). On the other hand, 70 amino acid residues of the C-terminal region of BS69 containing the MYND domain was required for the interaction with ZHX1, but the MYND domain alone failed to interact with ZHX1 (Figure 2B and Figure 9). It has been reported that the MYND domain of BS69 associates with the PxLxP motif of E1A, EBNA2, and MGA (15, 17, 18). However, the TATA-box-binding protein, another PxLxP motif-containing protein, failed to bind to BS69 (18). In addition, a member of the ZHX family, human ZHX2 also contains a PxLxP motif, but an interaction of ZHX2 with BS69 was not observed using a yeast two-hybrid system (data not shown) (2). Thus, these findings suggest that the PxLxP motif of partner proteins is required but not sufficient for a specific protein-protein interaction with the MYND domain of BS69.

Using three cell lines, HEK293, COS-7, and CV-1 cells, we showed that BS69 functions as a transcriptional

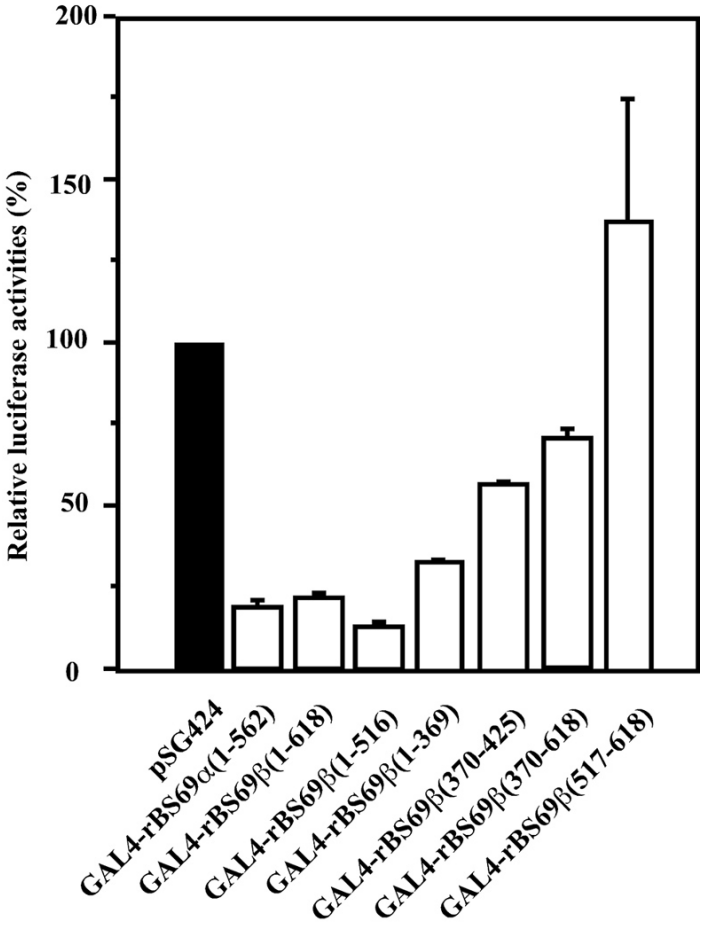

Figure 7. Determination of the minimal repression domain of BS69. COS-7 cells were co-transfected with 0.1 ng of the pRL-CMV, 100 ng of the SV40 promoter-directed expression vectors (pSG424 and GAL4-BS69β deletion constructs), and 200 ng of the 5 x GAL4-E1b Luc. The pSG424 and various GAL4-rBS69β construct express GAL4 DBD alone and various deletion mutants of rat BS69β fused to the GAL4 DBD, respectively. The other conditions are the same as described in the legend for Figure 6. Each column and error bar represents the mean and standard error of at least four transfection experiments.

activator and repressor in a cell-type dependent manner (Figures 3-8). Both BS69α and BS69β functioned as a transcriptional activator in HEK293 cells (Figure 3). The activation domain was mapped to the C-terminal region of BS69 (Figure 5). The MYND domain of BS69 was necessary but not sufficient for the transcriptional activation (Figure 5). The activation domain coincided with the ZHX1-interaction domain (Figures 2B and 5), suggesting that an interaction with ZHX1 might be involved in the regulation of the activation mediated by BS69. Indeed, the overexpression of a mutant form of ZHX1 (1-873) L35A, which cannot interact with BS69, further increased the activation activity of BS69 (Figure 8A). Since ZHX1 forms a homodimer and functions as a transcriptional repressor (6), the mutant form of ZHX1 should form a homodimer with endogenous ZHX1 and interferes with its binding to BS69. Thus, these results suggest that ZHX1 might function as a suppressor of transcriptional activation mediated by BS69.

In COS-7 and CV-1 cells, both BS69α and BS69β were transcriptional repressors as previously reported (Figures 6 and 7) (15, 17, 19). The full-length of both BS69α and BS69β caused about 20-25 % repression in comparison with the control in both cells (the fold repression was 4-5 folds) (Figures 6 and 7). The N-terminal 1-516 region of BS69β is required for full repression activity (Figure 7), which is consistent with a previous report (17). Both a wild type and a mutant form of ZHX1 had no effect on the repressor activity of BS69 (Figure 8B). In addition, ZHX1 did not bind to the N-terminal region of BS69 (Figure 2B). These results indicate that ZHX1 is not involved in the repression mediated by BS69. The repressive activity of BS69 might be associated with three putative domains involved in transcriptional regulation; a putative PHD finger motif located in the amino acid sequence between 60 and 108, a putative bromodomain located in the sequence between 118 and 203, and a putative PWWP domain in the sequence between 237 and 303 (Figures 2 and 9). The bromodomain is present in many proteins that are involved in transcriptional regulation and chromatin remodeling. The PWWP domain containing a Pro-Trp-Trp-Pro motif found in proteins of nuclear origin plays a role in cell growth and differentiation.

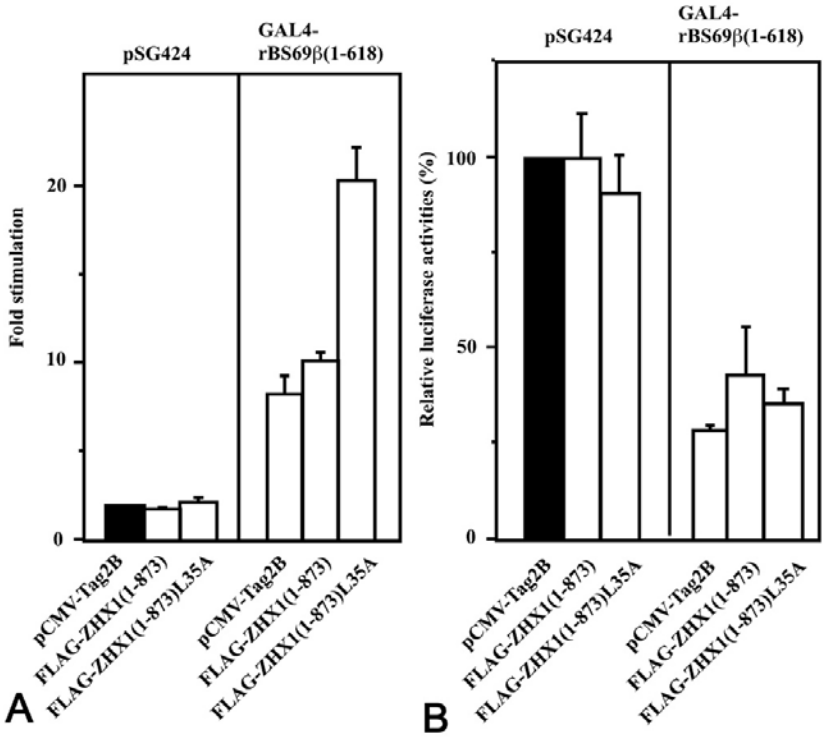

Figure 8. ZHX1 suppresses the transcriptional activation activity of BS69. (A) HEK293 cells were co-transfected with 1 ng of the pRL-CMV, 100 ng of the pSG424 or GAL4-BS69β (1-618), 100 ng of the 5 x GAL4-pGL3 Control, and 200 ng of the CMV promoter-directed expression vectors (pCMV-Tag2B, FLAG-ZHX1 (1-873), and FLAG-ZHX1 (1-873) L35A). A value of 1 was assigned to the promoter activity of the reporter plasmid in the presence of 100 ng of pSG424, and 200 ng of pCMV-Tag2B. (B) COS-7 cells were co-transfected with 0.1 ng of pRL-CMV, 100 ng of pSG424 or GAL4-BS69β (1-618), 200 ng of the 5 x GAL4-E1b Luc, and 200 ng of the CMV promoter-directed expression vectors described in (A). A value of 100 % was assigned to the promoter activity of the reporter plasmid in the presence of 100 ng of the pSG424, and 200 ng of pCMV-Tag2B. The other conditions are the same as described in the legend for Figure 3. Each column and error bar represents the mean and standard error of at least four transfection experiments.

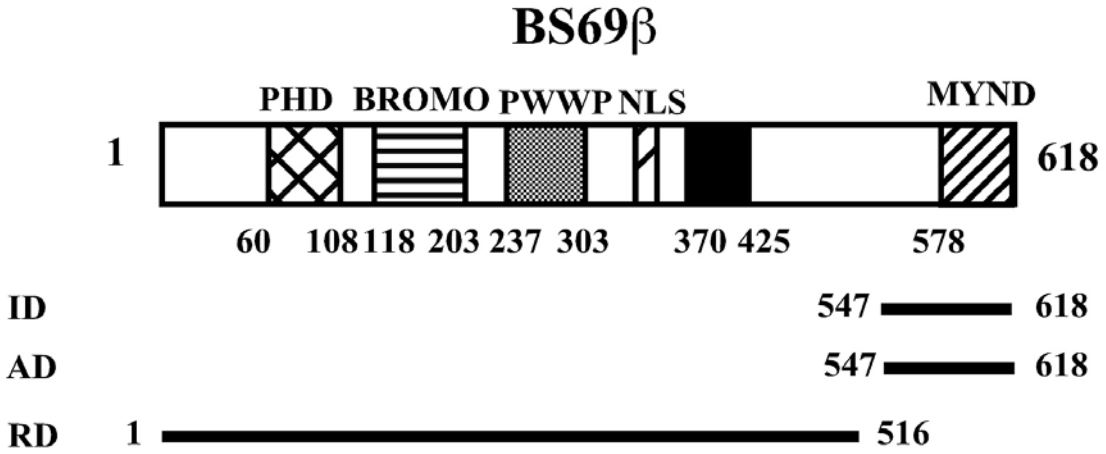

Figure 9. Schematic diagram of the structural and functional domains of rat BS69. PHD, plant homeodomain; BROMO, bromodomain; PWWP, PWWP domain; NLS, nuclear localization signal; MYND, MYND domain; ID, interaction domain with human ZHX1; AD, activation domain; RD, repression domain. The amino acid sequence between residues 370 and 425 of BS69β is a novel region, which is expressed in the rat and mouse but not in the human.

It has been reported that Sp3, a member of the Sp family, contains independent activation and repression domains, and that transcriptional activities are dependent on both promoter sequences and cellular contexts (30). Rgt1p, a key regulator of glucose-induced genes, is also a bifunctional regulator that functions as a repressor in the absence of glucose and as an activator in the presence of high levels of glucose (31). These findings indicate that the conversion of activity of the transcription factor may be dependent on the presence of other factors and/or complexes in the cells. BS69 acted as a transcriptional activator in HEK293 cells and as a transcriptional repressor in COS-7 and CV-1 cells (Figures 3-8). Thus, the transcriptional activity of BS69 may also depend on the cellular contexts as well as other bifunctional transcription factors. Whereas the C-terminal activation domain of BS69 might interact with an unidentified transcriptional activator(s) in HEK293 cells, the N-terminal repression domain might interact with an unidentified transcriptional repressor(s). Therefore, it would be important to determine specific molecules associate with BS69 in various cell-types.

In summary, we conclude that BS69 is a bifunctional transcription factor. The transcriptional activation by BS69 may be suppressed as the result of interaction with ZHX1. Here, we provide the first demonstration of a novel function of BS69 as a transcriptional activator and a physical and functional linkage between ZHX1 and BS69.

6. ACKNOWLDGMENTS

We are grateful to Ms. Kaoru Matsuura and members of the Miyamoto laboratory for helpful discussions and technical assistance. This investigation was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan, the Ichiro Kanehara Foundation, and CREST of Japan Science and Technology Agency.

7. REFERENCES

1. Yamada K., R. L. Printz, H. Osawa & D. K. Granner: Human ZHX1: cloning, chromosomal location, and interaction with transcription factor NF-Y. *Biochem. Biophys. Res. Commun.* 261,614-621 (1999)

2. Kawata H., K. Yamada, Z. Shou, T. Mizutani, T. Yazawa, M. Yoshino, T. Sekiguchi, T. Kajitani & K. Miyamoto: Zinc-fingers and homeoboxes (ZHX) 2, a novel member of the ZHX family, functions as a transcriptional repressor. *Biochem. J.* 373,747-757 (2003)

3. Yamada K., H. Kawata, Z. Shou, S. Hirano, T. Mizutani, T. Yazawa, T. Sekiguchi, M. Yoshino, T. Kajitani & K. Miyamoto: Analysis of zinc-fingers and homeoboxes (ZHX) 1-interacting proteins: molecular cloning and characterization of member of the ZHX family, ZHX3. *Biochem. J.* 373,167-178 (2003)

4. Yamada K., H. Osawa & D. K. Granner: Identification of proteins that interact with NF-YA. *FEBS Lett.* 460,41-45 (1999)

5. Hirano S., K. Yamada, H. Kawata, Z. Shou, T. Mizutani, T. Yazawa, T. Kajitani, T. Sekiguchi, M. Yoshino, Y. Shigematsu, M. Mayumi & K. Miyamoto: Rat zinc-fingers and homeoboxes 1 (ZHX1), a nuclear factor-YA-interacting nuclear protein, forms a homodimer. *Gene* 290,107-114 (2002)

6. Yamada K., H. Kawata, K. Matsuura, Z. Shou, S. Hirano, T. Mizutani, T. Yazawa, T. Sekiguchi, M. Yoshino, T. Kajitani & K. Miyamoto: Functional analysis and the molecular dissection of zinc-fingers and homeoboxes 1 (ZHX1). *Biochem. Biophys. Res. Commun.* 297,368-374 (2002)

7. Kawata H., K. Yamada, Z. Shou, T. Mizutani & K. Miyamoto: The mouse zinc-fingers and homeoboxes (ZHX) family; ZHX2 forms a heterodimer with ZHX3. *Gene* 323,133-140 (2003)

8. Barthelemy I., L. Carramolino, J. Gutierrez, J. L. Barbero, G. Marquez & A. Zaballos: zhx-1: a novel mouse homeodomain protein containing two zinc-fingers and five homeodomains. *Biochem. Biophys. Res. Commun.* 224,870-876 (1996)

9. Shou Z., K. Yamada, H. Kawata, O. Yokoyama & K. Miyamoto: A mechanism of induction of the mouse zinc-fingers and homeoboxes 1 (ZHX1) gene expression by interleukin-2. *Biochem. Biophys. Res. Commun.* 314,885-890 (2004)

10. Mantovani R.: The molecular biology of the CCAAT-binding factor NF-Y. *Gene* 239,15-27 (1999)

11. Zwicker J., F. C. Lucibello, L. A. Wolfraim, C. Gross, M. Truss, K. Engeland & R. Muller: Cell cycle regulation of the cyclin A, cdc25C and cdc2 genes is based on a common mechanism of transcriptional repression. *EMBO J.* 14,4514-4522 (1995)

12. Mantovani R.: A survey of 178 NF-Y binding CCAAT boxes. *Nucleic Acids Res.* 26,1135-1143 (1998)

13. Chae H. D., J. Yun, Y. J. Bang & D. Y. Shin: Cdk2-dependent phosphorylation of the NF-Y transcription factor is essential for the expression of the cell cycle-regulatory genes and cell cycle G1/S and G2/M transitions. *Oncogene* 23,4084-4088 (2004)

14. Harousseau J. L., J. J. Shaughnessy & P. Richardson: Multiple myeloma. *Hematology (Am. Soc. Hematol. Educ. Program)* 237-256 (2004)

15. Hateboer G., A. Gennissen, Y. F. Ramos, R. M. Kerkhoven, V. Sonntag-Buck, H. G. Stunnenberg & R. Bernards: BS69, a novel adenovirus E1A-associated protein that inhibits E1A transactivation. *EMBO J.* 14,3159-3169 (1995)

16. Wang J., T. Hoshino, R. L. Redner, S. Kajigaya & J. M. Liu: ETO, fusion partner in t(8;21) acute myeloid leukemia, represses transcription by interaction with the human N-CoR/mSin3/HDAC1 complex. *Proc. Natl. Acad. Sci. U.S.A.* 95,10860-10865 (1998)

17. Masselink H. & R. Bernards: The adenovirus E1A binding protein BS69 is a corepressor of transcription through recruitment of N-CoR. *Oncogene* 19,1538-1546 (2000)

18. Ansieau S. & A. Leutz: The conserved Mynd domain of BS69 binds cellular and oncoviral proteins through a common PXLXP motif. *J. Biol. Chem.* 277,4906-4910 (2002)

19. Wei G., A. E. Schaffner, K. M. Baker, K. C. Mansky & M. C. Ostrowski: Ets-2 interacts with co-repressor BS69 to repress target gene expression. *Anticancer Res.* 23,2173-2178 (2003)

20. Kurozumi K., M. Nishita, K. Yamaguchi, T. Fujita, N. Ueno & H. Shibuya: BRAM1, a BMP receptor-associated molecule involved in BMP signalling. *Genes Cells* 3,257-264 (1998)

21. Sadowski I. & M. Ptashne: A vector for expressing GAL4(1-147) fusions in mammalian cells. *Nucleic Acids Res.* 17,7539 (1989)

22. Yamada K., T. Tanaka, K. Miyamoto & T. Noguchi: Sp family members and nuclear factor-Y cooperatively stimulate transcription from the rat pyruvate kinase M gene distal promoter region via their direct interactions. *J. Biol. Chem.* 275,18129-18137 (2000)

23. Yamada K., H. Kawata, Z. Shou, T. Mizutani, T. Noguchi & K. Miyamoto: Insulin induces the expression of the *SHARP-2/Stra13/DEC1* gene via a phosphoinositide 3-kinase pathway. *J. Biol. Chem.* 278,30719-30724 (2003)

24. Tanaka T., T. Inazu, K. Yamada, Z. Myint, V. W. Keng, Y. Inoue, N. Taniguchi & T. Noguchi: cDNA cloning and expression of rat homeobox gene, *Hex*, and functional characterization of the protein. *Biochem. J.* 339,111-117 (1999)

25. Kajitani T., T. Mizutani, K. Yamada, T. Yazawa, T. Sekiguchi, M. Yoshino, H. Kawata & K. Miyamoto: Cloning and characterization of granulosa cell high-mobility group (HMG)-box protein-1, a novel HMG-box transcriptional regulator strongly expressed in rat ovarian granulosa cells. *Endocrinology* 145,2307-2318 (2004)

26. Yamada K., T. Tanaka & T. Noguchi: Members of the nuclear factor 1 family and hepatocyte nuclear factor 4 bind to overlapping sequences of the L-II element on the rat pyruvate kinase L gene promoter and regulate its expression. *Biochem. J* 324,917-925 (1997)

27. Xu C. F., J. A. Chambers & E. Solomon: Complex regulation of the BRCA1 gene. *J. Biol. Chem.* 272,20994-20997 (1997)

28. Ladendorff N. E., S. Wu & J. S. Lipsick: BS69, an adenovirus E1A-associated protein, inhibits the transcriptional activity of c-Myb. *Oncogene* 20,125-132 (2001)

29. Aasland R., T. J. Gibson & A. F. Stewart: The PHD finger: implications for chromatin-mediated transcriptional regulation. *Trends Biochem. Sci.* 20,56-59 (1995)

30. Majello B., P. De Luca & L. Lania: Sp3 is a bifunctional transcription regulator with modular independent activation and repression domains. *J. Biol. Chem.* 272,4021-4026 (1997)

31. Ozcan S., T. Leong & M. Johnston: Rgt1p of Saccharomyces cerevisiae, a key regulator of glucose-induced genes, is both an activator and a repressor of transcription. *Mol. Cell Biol.* 16,6419-6426 (1996)

Abbreviations: AD, activation domain; DBD, DNA-binding domain; NF-Y, nuclear factor-Y; RACE, rapid amplification of the cDNA ends; RT-PCR, reverse transcription-PCR; ZHX, zinc-fingers and homeoboxes.

Key Words: BS69 co-repressor, MYND domain, protein-protein interaction, PxLxP motif, transcriptional activator, zinc-fingers, homeoboxes 1, ZHX1

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