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Constitutive expression of the *brg1* gene requires GC-boxes near to the transcriptional start site

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We previously reported that BRG1, an ATPase subunit of SWI/SNF chromatin remodelling complexes, is constitutively expressed and that the alternative ATPase subunit (BRM) is inducibly expressed through differentiation in mammalian cells. In the present study, the regulatory elements that confer constitutive expression on *brg1* were explored. First, we analysed the promoter proximal region surrounding its transcriptional start site. Using computer-aided analysis, a TATA-less, GC-rich promoter containing four putative binding sites for Sp1/3 was predicted. One of the putative Sp1/3-binding sites (from −21 to −15 bp) overlapped with a putative YY1-binding site. A gel-shift assay showed that YY1 but not Sp1/3 bound to this sequence and that Sp3 but not Sp1 bound to the other three predicted binding sites. Furthermore, chromatin immunoprecipitation analysis showed that Sp3 and YY1 bound to the promoter region together with TATA-binding protein *in vivo*. *In vivo* and *in vitro* binding assays showed that Sp3 and YY1 interacted with each other. Together, these results suggest that Sp3 and YY1 recruit general transcription factors and facilitate the assembly of a preinitiation complex.

Keywords: BRG1/GC-rich sequence/Sp3/YY1.

Abbreviations: BRG1, Brm-related gene 1; Sp, specificity protein; YY1, Yin Yang 1.

The SWI/SNF family of chromatin remodelling complexes is evolutionarily conserved, and their orthologues have been identified in yeast, *Drosophila* and mammals. Mammalian SWI/SNF complexes contain two distinct ATPase subunits named BRG1 and BRM, and they are mutually exclusive in the SWI/SNF complex (*1*). Mammalian SWI/SNF complexes are divided into BAF and PBAF complexes; the former contains either BRG1 or BRM, and the latter solely contains BRG1 (*2*). They share most other subunits, which are called BAF (BRM-associated factors), but each complex also contains distinct BAF subunits: PBAF complex invariably contains BAF180 and BAF200, whereas, BAF complex contains BAF250 (*2*, *3*). Although the functional differences between PBAF and BAF complexes remain to be elucidated, it has been proposed that PBAF, but not BAF, complex facilitates ligand-dependent transcriptional activation via several nuclear hormone receptors, such as Vitamin D and the peroxisome proliferator-activated receptor (*4*). Conversely, transcription mediated by the glucocorticoid receptor is promoted by BAF, but not PBAF, complex (*5*). This difference in the activation of nuclear hormone receptors is attributable to the functions of the unique BAF subunits of PBAF and BAF complexes. In addition, BRG1 and BRM also have a certain degree of selectivity for transcription factors (TFs); BRG1 binds to zinc-finger proteins but BRM does not; whereas, the latter physically interacts with ankyrin repeat proteins (*6*). To clarify and understand the functional differences between BRG1 and BRM in relation to the roles of the BAF and PBAF complexes, we studied the functions of BRM and BRG1. To this end, it is particularly interesting that the expression patterns of these two ATPases are different; BRG1 is expressed constitutively, and BRM is expressed specifically in differentiated cells (*7*), which may be a reflection of the functional differences between them.

We recently reported the structures of the promoter regions of the *brg1* and *brm* genes (*8*). A reporter assay using luciferase genes showed that the *brm* gene possesses a control region in the portion of its sequence located up to −1.5 kb, in which C/EBPβ- and GATA3-binding sites were identified. On the other hand, in the *brg1* gene, a short promoter proximal sequence located in the region up to −100 bp showed the maximum luciferase activity. Interestingly, a typical TATA-box was found in the *brm* promoter but not in *brg1*.

The core promoter is essential for the assembly of transcriptional initiation complexes near to the transcriptional start site. In addition to the best-characterized TATA box, several other core promoter sequences have been identified such as the initiator (Inr) sequence, the TFIIB recognition element (BRE), and the downstream promoter element (DPE) (*9*–*12*). TFIID binds to DPE through TATA-binding protein associated factor $(TAF)_{II}60$ (*13*). It is also well known that the promoters of many constitutively expressed housekeeping genes associate with CpG islands (*10*).

The *brg1* promoter proximal region contains four putative GC-boxes within the 150 bp region encompassing the transcriptional start site. According to several previous reports, GC-boxes are targets of TFs such as Sp1/3 (*14*, *15*), which are considered to regulate transcriptional initiation. Sp1, Sp3 and Sp4 are closely related sequence-specific TFs, and their binding to GC-boxes is dependent on the presence of three conserved zinc finger motifs. Sp1 and Sp3 are expressed ubiquitously, and Sp4 is specifically enriched in the brain. In contrast to the transcriptional activators Sp1 and Sp4, the ubiquitously expressed Sp3 protein both activates and represses transcription (*16–18*).

YY1 is a sequence specific TF that binds to a broad range of modulator proteins including p300/CBP, RB and Sp1 (*19–21*). It was reported that the YY1-binding sequence is enriched in TATA-less promoters and around the translational start sites of genes containing short 5′-untranslated regions (UTRs) (*22*).

In the present study, we demonstrate that the binding of Sp3 and YY1 to the GC-rich stretches of DNA encompassing the transcriptional start site is essential for the expression of the *brg1* promoter.

Materials and Methods

Cell culture and plasmids

P19 embryonal carcinoma cells were maintained in αMEM containing 10% fetal bovine serum. The S2 *Drosophila* cells were a kind gift from Dr Kadowaki of Nagoya University. The cells were grown in Schneider's *Drosophila* medium (Invitrogen, Carlsbad, CA, USA). To construct the mouse *brg1* promoter-luciferase reporter plasmid, the mouse *brg1* promoter region (−98 to +226 bp) was amplified from mouse genomic DNA by PCR using the following forward primer: 5′-CATCTCGAGACTAGTACGCGTGCGCACAG-3′ (*Xho*I site is underlined) and reverse primer: 5′-CATAAGCTTGT AACGGCGCCGTAACCTTC-3′ (*Hind*III site is underlined). The amplified DNA fragments were introduced into the pGL3-basic vector (Promega, Madison, WI, USA). Mutants containing deletions in the putative Sp1/3-binding sites were constructed from the mouse *brg1* promoter-luciferase reporter plasmid by PCR using the following forward primers: 5′-CATGAATTCACGAGGCCGGCCGG GC-3′ (*Δ* −57 to −28 bp), 5′-CATGAATTCAGGGCAAGTGGA GCGGGTAG-3′ (*Δ* −27 to −2 bp), 5′-CATGAATTCGCGCGTG CGGTGGGGGG-3′ (*Δ* + 14 to +43 bp) and 5′-CATGAATTCAA GCCCAGCGGGCGCGC-3′ (*Δ* + 44 to +73 bp) and reverse primers: 5′-CATGAATTCCGCGCATGCTCAGCCATTCGC-3′ (*Δ* − 57 to −28 bp), 5′-CATGAATTCGCGCGCGCCAGCCC AC-3′ (*Δ* − 27 to −2 bp), 5′-CATGAATTCCGCTCCACTTGCCC TGCTTCC-3′ (*Δ* + 14 to +43 bp) and 5′-CATGAATTCCCGCCGC GCGCCCCC-3′ (*Δ* + 44 to +73 bp) (*Eco*RI sites are underlined). After amplification, the PCR products with *Eco*RI sites at both ends were cut with *Eco*RI and self-ligated. To generate point mutation in the putative YY1-binding sequence, PCR was performed using the following mutated primers: 5′-GCGCACGAG GCCGATTGGGCAGCGGAAGCA-3′ and 5′-TGCTTCCGCTG CCCAATCGGCCTCGTGCGC-3′ (mutated sequences are underlined), and PCR products were directly introduced into *Escherichia coli* DH5α.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was performed using salmon sperm DNA-conjugated protein A agarose (Upstate, Lake Placid, NY, USA). The mean size of the DNA fragments after sonication was about 300 bp. The immunoprecipitated DNA and the input DNA were analysed by PCR using 5′-CCGAGCCTGAGGAC TTTAAT-3′ (as a forward primer) and 5′-AAACAAAGAAGCCGT CTCCG-3′ (as a reverse primer) to amplify the fragment located in the region from −350 to +140. As a negative control, the coding region (exon 29) of the mouse *brg1* gene was also amplified using 5′-AGGTCCGGCAGAAGAAATCT-3′ and 5′-GATCACAGCAT CCACGATCT-3′ as primers. Anti-Sp4 (sc-645X), -YY1 (sc-281), -TATA-binding protein (TBP) (sc-273) and -RNA polymerase II (PolII) (sc-899) antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). Antibodies to Sp1 (07-645) and Sp3 (07-107) were purchased from Upstate.

Electrophoretic mobility shift assay

Double stranded oligonucleotides with the following sequences were used as probes: 5′-CAGAGAGCGGAAGGGTGGGCTGGCGCG CGC-3′ (probe 1), 5′-ACGAGGCCGGCCGGGCAGCGGAAGC AGGGC-3′ (probe 2), 5′-GGTAGACAGGGAGGCGGGGGCG CGCGGCGG-3′ (probe 3), 5′-GCGCGTGCGGTGGGGGGGG GTGGCCTGGCG-3′ (probe 4) and 5′-ACACCCCGCGCCGCCC CGCCTCTACTCC-3′ (a positive probe for Sp1) (*23*). The oligonucleotides were end-labelled with [γ-^{32}P] ATP. For the production of GST-Sp3 and GST-YY1 fusion proteins, expression plasmids were constructed by inserting mouse Sp3 and human YY1 cDNA in-frame into the pGEX-6P-2 vector, and the proteins were produced in *E. coli* BL21, followed by purification with glutathione sepharose 4B (GE Healthcare, Cardiff, Wales, UK). Purified Sp1 protein was purchased from JENA BIOSCIENCE (JENA, Germany). The binding reaction was carried out in a final volume of 20 μl containing a ^{32}P-labelled DNA probe (∼40,000–50,000 cpm), 1 μg of poly (dI/dC), 1 mM $MgCl_2$, 37.6% glycerol and 5 μg of protein. Protein–DNA complexes were resolved by electrophoresis on a 5% polyacrylamide gel in 22.2 mM Tris, 22.2 mM boric acid and 0.5 mM EDTA. The purity of the GST-fusion proteins was confirmed by SDS–PAGE.

Reverse transcription–PCR analysis

Five micrograms of RNA were subjected to a reverse transcription (RT) reaction. The reverse transcriptase ReverTra Ace (Toyobo, Osaka, Japan) was used to generate cDNA according to the manufacturer's instructions. Primers specific to the luciferase gene (5′-TG AAGCGAAGGTTGTGGATCTGGA-3′ and 5′-TCAGGCGGTC AACGATGAAGAAGT-3′) were used. For *brg1*, primers for exon 29 were used. As a control, PCR using primers specific to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was carried out with the same samples. For quantitative PCR (qPCR), the samples were analysed using Platinum SyBR Green qPCR SuperMix-UDG (Invitrogen) and a LightCycler (Roche, Basel, Switzerland).

Luciferase reporter gene assay

P19 cells and S2 *Drosophila* cells were seeded in 24-well plates and transfected with reporter plasmids using Lipofectamine 2000^{TM} reagent (Invitrogen). Reporter gene assays were performed with a dual luciferase assay kit (Promega).

Knockdown of Sp1, Sp3 and YY1 by small interfering RNA

The following double-stranded RNA oligonucleotides were used as small interfering RNA (siRNA): 5′-UUGACAGGUAGCAAGGU GAUGUUCC-3′ (siRNA1) and 5′-ACUCAGGGCAGGCAAAUU UCUUCUC-3′ (siRNA2) for Sp1, 5′-CAGAUCAUUCCUGGCUC UAAUCAAA-3′ (siRNA1) and 5′-UGACUACUACUAGUGGGC AAGUCCA-3′ (siRNA2) for Sp3 and 5′-GGCUGCACAAAGAUG UUCAGGGAUA-3′ (siRNA1) and 5′-GCGUUCGUUGAGAGC UCAAAGCUAA-3′ (siRNA2) for YY1. These siRNA were introduced into undifferentiated P19 cells using the Lipofectamine 2000^{TM} reagent, the cells were harvested at 72 h post-transfection, and their BRG1 expression was analysed.

Results

Sp3 and YY1 bind to the brg1 promoter

Our previous study suggested that a DNA fragment located in the region up to around 100 bp upstream of the transcriptional start site plays a crucial role in the expression of the *brg1* gene (*8*), but the region does not contain any previously reported typical core promoter elements such as the TATA-box, downstream core element (DCE), or BRE (*10*). DPE is known to

act in conjunction with Inr, and the core sequence of the DPE is typically located at +28 to +32 relative to the A_{+1} nucleotide in the Inr motif (*11*). In the *brg1* promoter region, the DPE motif (AGACA) was found between +17 and +21 from A_{+1}, but the Inr motif, which is required for the core promoter function of DPE, was not found within the correct distance, suggesting that the motif does not function as a core promoter. One Inr motif, and a DNA sequence containing one mismatch from the Inr consensus sequence were found downstream of the transcriptional start site between +104 and +168 (Fig. 1A), but it was not clear whether these sequences functioned as an promoter.

To investigate the mechanism of the transcription initiation of *brg1*, we analysed from the −100 to +169 region using the TFSEARCH program (http://www.cbrc.jp/research/db/TFSEARCHJ.html) to search for TF-binding sites. In this region, the stretch of DNA between −60 to +100 is rich in G and C, and four putative Sp1/3-binding sites were predicted. Furthermore, one of the four predicted Sp1/3-binding sites overlapped with a putative YY1-binding sequence (Fig. 1A). Sp1 and Sp3, which are ubiquitously expressed in mammalian cells, bind to GC-boxes with similar affinity and regulate the expression of many housekeeping and tissue-specific genes (*10*, *14*, *15*). Thus, we first demonstrated the binding of these TFs *in vivo* using a ChIP assay (Fig. 1B). We have previously shown that the expression of BRM is upregulated, but that of BRG1 remains constant, during the differentiation of P19 embryonal carcinoma cells (*7*). Thus, we examined the regulation of *brg1* transcription using this cell line. In the undifferentiated P19 cells, no Sp1 or Sp4 binding was detected by the ChIP assay. On the other hand, Sp3 did bind to this region. In differentiated cells, Sp3, but not Sp1 or Sp4 bound to this region. In addition, the binding of YY1, TBP and PolII to this region was confirmed in both differentiated and undifferentiated cells. It was reported that TAF4, an essential subunit of the TFIID complex, binds to Sp1 (*24*, *25*) and TBP is a direct target of the glutamine-rich activation domains of Sp1 (*26*). Since the structure of Sp3 is similar to that of Sp1, it was assumed that Sp3 recruited TBP or TAF4 to the promoter region in the present case. To substantiate this assumption, *E. coli*-produced GST-Sp3 and -TBP fusion proteins were used for the binding assay. The GST-portion of the fused Sp3 protein was removed by digesting it with Factor Xa to avoid interactions between GSTs. As shown in Fig. 1C, the *in vitro* binding abilities of TBP and Sp3 were confirmed by immunoprecipitation. We also demonstrated the interaction between TBP and Sp3 in a coimmunoprecipitation assay using P19 cell extract, as shown in Fig. 1C.

We then performed electrophoretic mobility shift assay (EMSA) to provide further evidence for the binding of Sp3 but not Sp1 to the promoter proximal region *in vitro*. We synthesized four DNA probes that each contained one of the four GC-boxes containing Sp1/3--binding sequences. Judging from the band shift patterns, the *E. coli*-produced Sp3 bound to upstream probe 1 (from −57 to −28 bp) and downstream probes 3 (+14 to +43 bp) and 4 (+44 to+73 bp). Furthermore, the formation of the shifted bands was reduced by the addition of oligonucleotides containing a typical Sp3-binding sequence. On the contrary, Sp1 did not bind to any of the probes, while it was confirmed to bind to a typical Sp1-binding sequence (Fig. 2). On the basis of these findings, we postulated that three Sp3 molecules potentially bind to three different sites in the GC-rich promoter proximal region of the *brg1* gene. In this assay, we detected two shifted bands in the case of Sp3. The two close bands may be resulted from partial protein degradation of *E. coli*-produced GST-Sp3.

Effects of Sp3 on the transcriptional activity of the brg1 promoter

EMSA and ChIP assays showed that Sp3, but not Sp1, binds to the GC-rich promoter proximal sequences found in the *brg1* gene. To provide further evidence that Sp1 does not affect *brg1* expression, S2 *Drosophila* cells were transfected with a luciferase reporter plasmid and either an Sp1 or Sp3 expression plasmid, since S2 *Drosophila* cells are devoid of endogenous Sp1. The expression of Sp3 and YY1 in this cell line is obscure, but the effect of Sp3 could be detected in reporter assays (*27*). In the reporter assay, Sp3 increased the luciferase activity of the cells, but Sp1 did not have any effect (Fig. 3). Although the structures of Sp1 and Sp3 are very similar, it was confirmed that Sp3, but not Sp1, binds to the control region of the *brg1* gene and activates its expression.

An intriguing question was raised in this study as to which Sp3-binding site(s) is important for *brg1* gene expression *in vivo*. To elucidate this, we performed assays using luciferase reporter plasmids containing a series of truncated *brg1* promoters, in which one of the putative Sp3-binding sequences had been deleted. As shown in Fig. 4A, the *brg1* promoter proximal region lacking the upstream Sp3-binding site caused a reduction in luciferase activity in P19 cells. We cannot rule out the possibility of other TFs directly binding to the deleted region (Δ−57 to −28). However, this seems unlikely because TFSEARCH did not show any binding sites for other TFs. On the other hand, deletion of the second or third Sp3-binding sequences, which are located downstream of the transcriptional start site, augmented gene expression by a currently unknown mechanism. The fact that the deletion in the 5′-UTRs increased the gene expression leads us further confirmation that the results of the reporter assay reflect transcription but not translation. The results of the RT–PCR analyses were roughly consistent with those of the reporter assay, and the deletion of the most upstream Sp3-binding site significantly reduced the transcription level analysed by RT–PCR (Fig. 4B). These results indicate that the binding of Sp3 to these sites is important for transcriptional activation of the *brg1* gene.

YY1 binds to the second GC-Box near to the transcriptional start site and may interact with Sp3 in the promoter region of the brg1 gene

Since the ChIP assay showed that YY1 binds to from −350 to +140 region of the *brg1* gene and

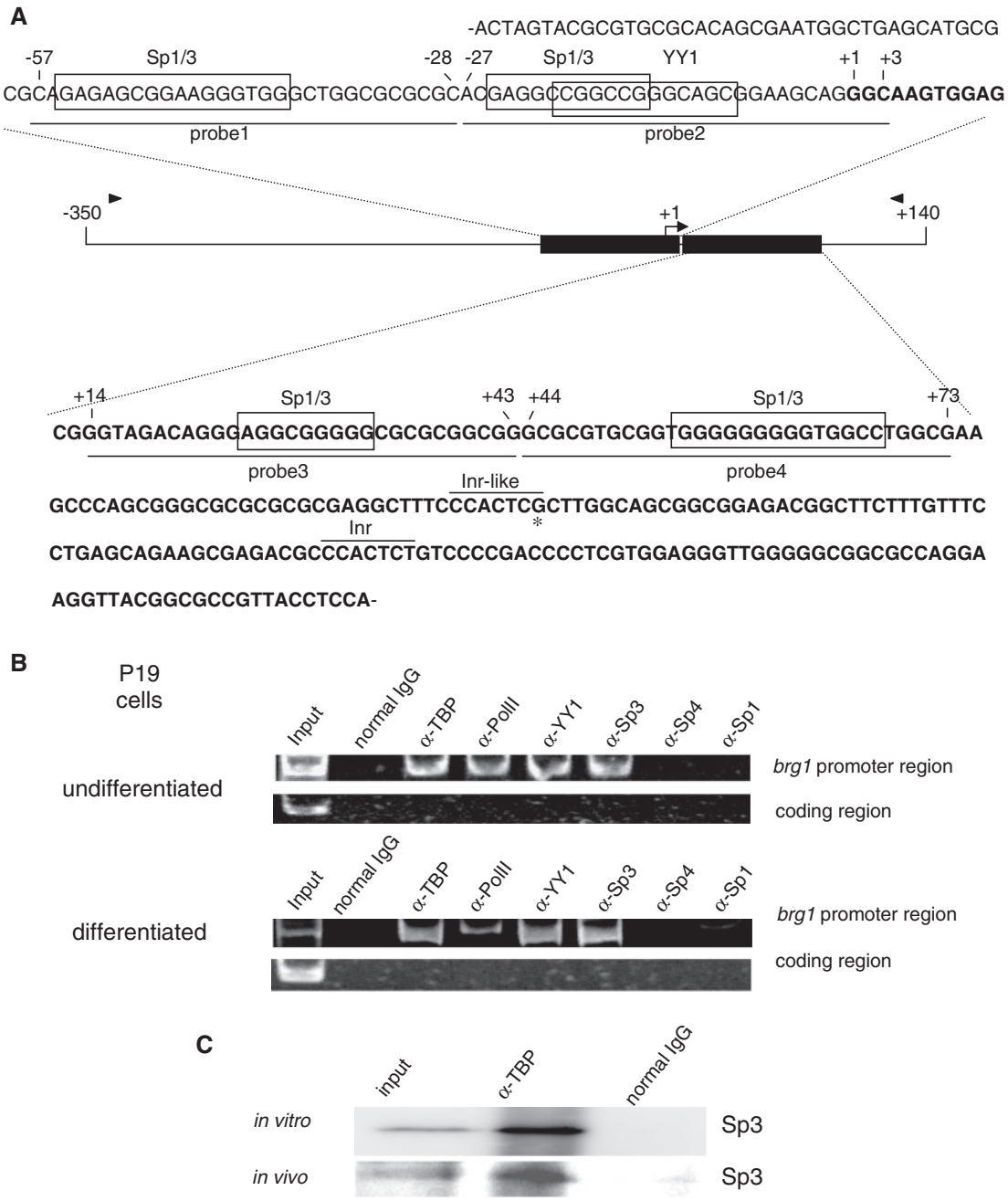

Fig. 1 The binding of TFs and PolII in the *brg1* regulatory region of P19 cells. (A) Schematic drawing of the promoter proximal sequence containing the Sp1/3- and YY1-binding sites. The arrow indicates the transcriptional start site. The arrowheads indicate the primers used for ChIP analysis. The underlined sequences are those of the probes used in EMSA. The first exon of the *brg1* gene is indicated by boldface type. The asterisk in the Inr-like sequence indicates a base that is different from the typical Inr sequence. (B) ChIP analysis of the *brg1* promoter in undifferentiated and differentiated P19 cells using anti-Sp1, 3 and 4; YY1; TBP; and PolII antibodies. Normal rabbit IgG was used as a negative control for immunoprecipitation. The coding region of *brg1* (exon 29) was amplified as a negative control for PCR. Input DNA (2%) was used as a positive control. Representative results of triplicated experiments are shown. (C) The binding between Sp3 and TBP *in vitro* and *in vivo*. For the *in vitro* experiment, purified GST–TBP fusion protein (Santa Cruz) (10 μg/ml) and Sp3 protein (10 μg/ml) were mixed and immunoprecipitated with anti-TBP antibody. To prepare the Sp3 protein, purified GST–Sp3 was incubated with Factor Xa (15 μg/ml) at room temperature for 36 h to remove the GST portion. The Sp3 was separated from the GST using glutathione Sepharose. For the *in vivo* experiment, crude extracts were prepared from undifferentiated P19 cells and subjected to immunoprecipitation with anti-TBP antibody. Before immunoprecipitation, the crude extract was sonicated and treated with DNaseI (100 U/ml), Benzonase (200 U/ml) and RNase (10 μg/ml) for 30 min to digest the nucleic acids. Western blotting was performed using anti-Sp3 antibody. Normal rabbit IgG was used as a negative control. Input (4%) was used as a positive control. Representative results of triplicated experiments are shown.

TFSEARCH detected a potential YY1-binding site in the second GC-box, we analysed whether YY1 binds to the GC-rich region using EMSA. As shown in Fig. 5A, probe 2 (−27 to +3) bound to *E. coli*-produced YY1 *in vitro*; whereas, probes 1, 3 and 4 did not (data not shown), suggesting that YY1 bound to the GC-box juxtaposed to the Sp3-binding site. In this assay, two shifted bands were observed, as was found in our previous report (*28*). We also confirmed the importance of the YY1 binding sequence for the

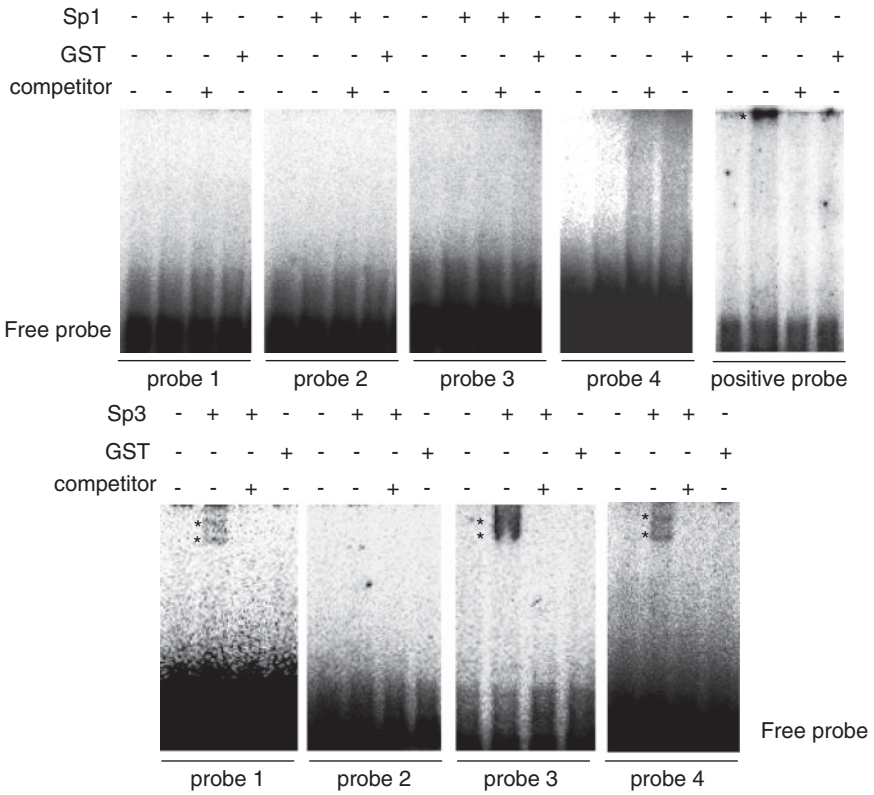

Fig. 2 EMSA for detecting Sp3 binding to the *brg1* promoter. EMSA was performed with purified GST-Sp1 and -Sp3 proteins (5 μg). The asterisks indicate specific shifted bands. GST protein was used as a control. Non-labelled oligonucleotides were used as competitors at about a 200-fold higher concentration than that of the labelled probes. Representative results of triplicated experiments are shown.

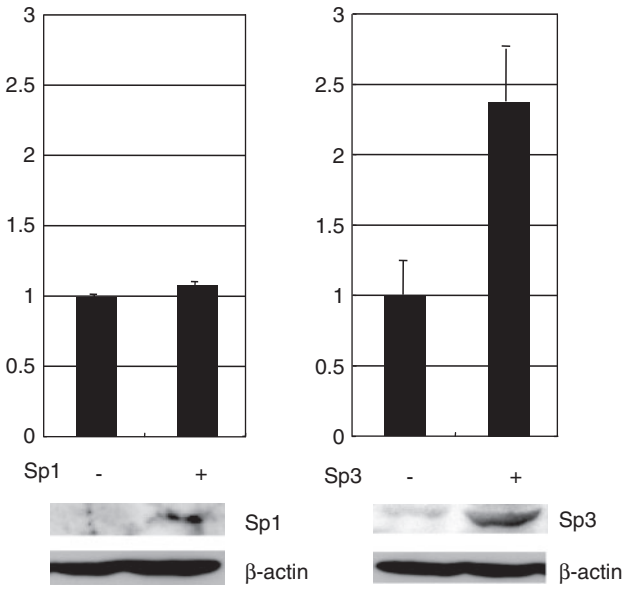

Fig. 3 Transcriptional activation of the *brg1* gene by Sp3. The pGL3 luciferase reporter plasmid containing the *brg1* regulatory region (30 ng) was introduced into S2 *Drosophila* cells together with the expression vector for Sp1 or Sp3 (100 ng), and then the luciferase activity of the cells was determined. The pGL3-basic vector was used as a control, and the luciferase activity produced by this vector was expressed as 1. The error bars represent the standard error of the mean of three independent experiments. The expression of Sp1/3 was analysed by western blotting. The expression of β-actin was also analysed as a control.

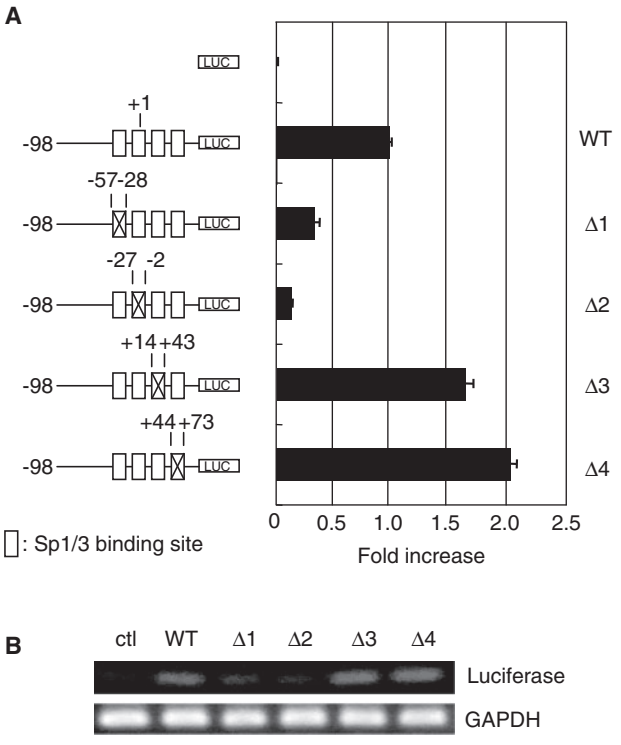

Fig. 4 Transcriptional activity of mutated *brg1* promoters lacking one of the Sp3-binding sites in undifferentiated P19 cells. (A) P19 cells were transiently transfected with pGL3-truncated *brg1* promoter luciferase reporter plasmids. The pGL3-basic vector was used as a negative control. The luciferase activity of the wild-type (WT) promoter was expressed as 1. The error bars represent the standard error of the mean of three independent experiments. (B) P19 cells were analysed for their luciferase expression by RT–PCR. GAPDH expression was shown as a control.

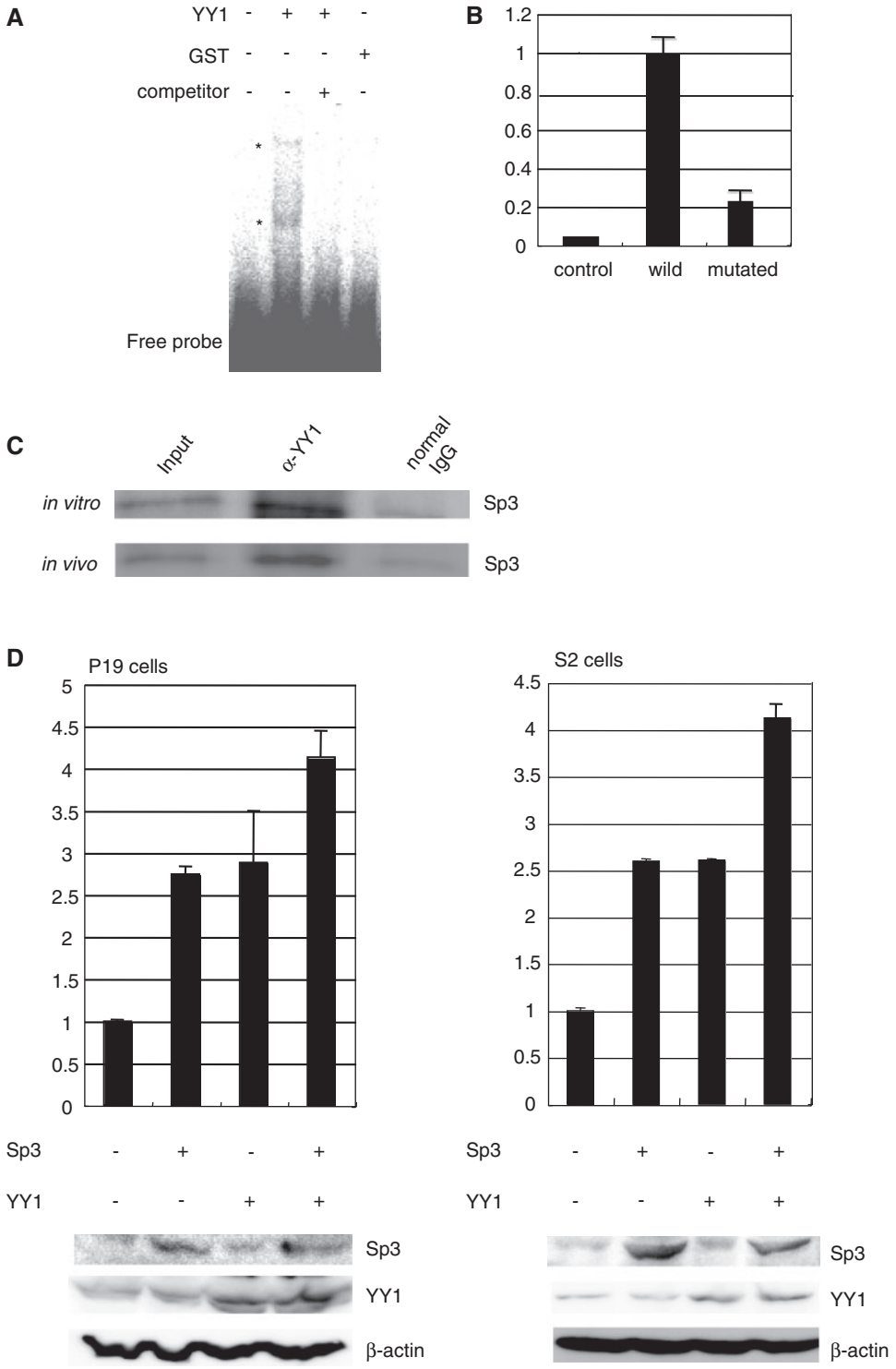

Fig. 5 The binding of YY1 to the *brg1* promoter region. (A) EMSA for detecting YY1 binding to the promoter. EMSA was performed with purified GST–YY1 protein (5 μg). The asterisks indicate specific shifted bands. GST protein was used as a control. Non-labelled oligonucleotide was used as the competitor at about a 200-fold higher concentration than that of the labelled probe. Representative results of triplicated experiments are shown. (B) Luciferase reporter assay of point-mutated promoter. P19 cells were transiently transfected with *brg1* promoter luciferase reporter plasmid containing a point mutation in the YY1-binding site, and then the luciferase activity was measured. The pGL3-basic vector was used as a negative control. The luciferase activity of the WT promoter was expressed as 1. The error bars represent the standard error of the mean of three independent experiments. (C) Physical interaction between Sp3 and YY1. For the *in vitro* experiment, purified His-YY1 fusion protein (Santa Cruz) (10 μg/ml) and GST-Sp3 fusion protein (10 μg/ml) were mixed and immunoprecipitated with anti-YY1 antibody. For the *in vivo* experiment, crude extracts were prepared from undifferentiated P19 cells and subjected to immunoprecipitation with anti-YY1 antibody. Before the immunoprecipitation, the crude extract was sonicated and treated with DNaseI (100 U/ml), Benzonase (200 U/ml) and RNase (10 μg/ml) for 30 min to digest the nucleic acids. Western blotting was performed using anti-Sp3 antibody. Normal rabbit IgG was used as a negative control. Input (4%) was used as a positive control. Representative results of triplicated experiments are shown. (D) Luciferase reporter assays for the *brg1* promoter in P19 and S2 cells. These cells were transiently transfected with the *brg1* reporter plasmid and expression vectors for Sp3 and YY1. The expression levels of these proteins are indicated below the graphs. The error bars represent the standard error of the mean of three independent experiments.

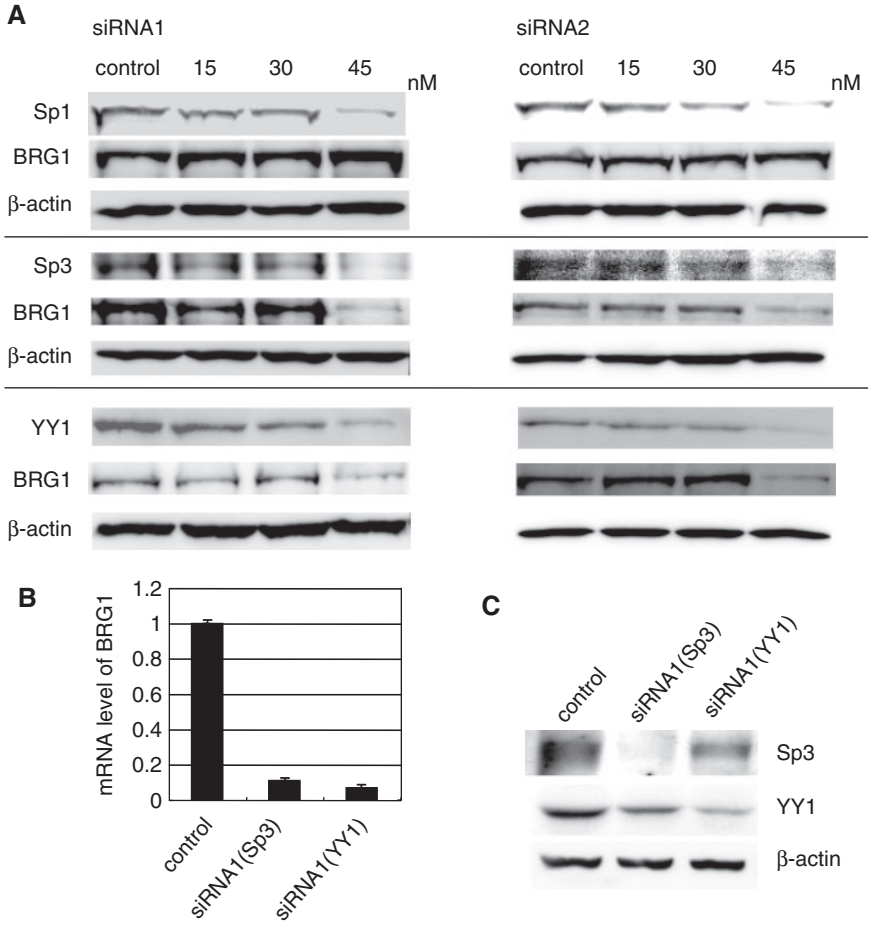

Fig. 6 Knockdown of Sp3 and YY1 by siRNAs decreases BRG1 expression in undifferentiated P19 cells. (A) siRNAs for Sp1, Sp3 and YY1 were introduced into undifferentiated P19 cells, and the expression levels of these proteins and BRG1 were analysed by western blotting. Two kinds of siRNA were used to inhibit the expression of Sp1, Sp3 and YY1. The expression of β-actin was analysed as a control. Representative results of triplicated experiments are shown. (B) The expression of *brg1* in P19 cells in which Sp3 and YY1-expressions were inhibited was also analysed by RT-qPCR. The error bars represent the standard error of the mean of three independent experiments. (C) The effects of Sp3 knockdown on the expression level of YY1 and vice versa were also analysed. The expression of β-actin was analysed as a control. Representative results of triplicated experiments are shown.

promoter activity in luciferase reporter assays using a YY1 site-deleted sequence (Δ2 in Figs 4A and B). Since the nucleotide just beside the transcriptional start site was deleted in this mutant, we also constructed luciferase reporter plasmid with a point-mutated YY1 binding sequence. The mutation also abolished the promoter activity, indicating that YY1 is essential for *brg1* expression (Fig. 5B). These results that one YY1 and three Sp3 molecules bind to the promoter proximal region located from −57 to +73 led to the hypothesis that YY1 and Sp3 may form a physical complex, as has been demonstrated for Sp1 and YY1 (*21*, *29*). To evaluate this possibility, interaction between purified GST-fused Sp3 and His-tagged YY1 was studied by co-immunoprecipitation. Overexpressed Sp3 and YY1 in P19 cells were also subjected to co-immunoprecipitation for *in vivo* assay. As shown in Fig. 5C, these two proteins physically interacted with each other both *in vitro* and *in vivo*.

We then performed a luciferase assay by co-transfecting Sp3 and YY1 expression plasmids into undifferentiated P19 cells and S2 *Drosophila* cells. Sp3 and YY1 additively activated the *brg1* promoter in P19 cells and S2 *Drosophila* cells (Fig. 5D).

Contribution of Sp3 and YY1 to the expression of the brg1 gene in vivo

Sp3 and YY1 were partly knocked down by siRNA in undifferentiated P19 cells to verify their *in vivo* roles in the transactivation of *brg1* expression. As shown in Fig. 6, the expression levels of Sp1, Sp3 and YY1 were decreased to 15–30% of their original levels under suitable conditions, and the knockdown of either Sp3 or YY1 resulted in reduced *brg1* expression in undifferentiated P19 cells, which again demonstrated that they both participate in the expression of *brg1* (Fig. 6A and B). As expected, the knockdown of Sp1 did not have any effect on *brg1* expression (Fig. 6A). We also studied whether the knockdown of one TF; *i.e.* Sp3 or YY1, affects the expression level of the other factor. The knockdown of Sp3 resulted in a moderate reduction in YY1 expression and vice versa in undifferentiated P19 cells (Fig. 6C). This suggested that the mechanism behind the results of the knockdown experiments might not be simple; it is possible that the

knockdown of YY1 reduced Sp3 expression, which in turn would have partly reduced *brg1* expression. A similar mechanism may also have affected the Sp3 knockdown results.

Discussion

We have previously indicated that BRG1 is constitutively expressed in many cell types (*7*), and in this study, we tried to elucidate the mechanism behind its constitutive expression. The promoter of the *brg1* gene does not contain TATA-boxes. Instead, GC-rich stretches of DNA of 160 bp in length encompass the putative transcriptional start site. We showed that two GC-boxes that reside between −57 and +3 are essential for *brg1* gene expression. According to previous studies, CpG islands are associated with approximately 30% of promoters for protein encoding genes and contain multiple Sp1-binding sites (*30*). Furthermore, it was reported that CREB- and YY1-binding sequences are enriched in TATA-less promoters (*22*). Thus, the structure of the *brg1* promoter seems to be in good agreement with previous reports about TATA-less promoters. Furthermore, Sp1 binds to TBP (*16*, *26*) and TAFs (*24*, *25*), and YY1 binds to both TFIIB and TBP (*31*). Therefore, it is reasonable to assume that at least one of these TFs recruits the general TFs that allow assembly of the PolII complex. In addition, the Sp3- and YY1-binding sequences are located in juxtaposed positions, with a distance of 18 bp between them, and YY1 interacts with Sp3 (Fig. 5C). Therefore, it is possible that Sp3 and YY1 may physically interact with each other and work synergistically by forming a physical complex, further facilitating the formation of the PolII initiation complex. Similar cooperative activation was reported for Sp1 and YY1 (*21*). However, as much as we had studied, a synergistic effect of these proteins could not be observed as shown in Fig. 5D.

Truncation of the downstream Sp3-binding sites augmented the expression of *brg1* according to a reporter assay and RT–PCR, suggesting that the binding of Sp3 to these sites hampered the transcription of the gene by an unknown mechanism. It is known that dispersed transcriptional initiation occurs in CpG islands, possibly due to the presence of multiple weak core promoters (*12*). Since the transcriptional initiation site of the *brg1* gene was proposed based on EST analysis, we cannot rule out the possibility that weak transcription occurs from the third and the fourth GC-boxes as well as from Inr, which resides further downstream (Fig. 1) and that the interaction between several transcriptional start sites might reduce the total transcriptional activity, as shown in Fig. 4. Furthermore, several previous reports revealed that TF binding sites including Sp1 sites in 5′-UTR upregulate transcriptional activity (*27*, *32*). Thus, it is also possible that these Sp3 sites in the 5′-UTR of the *brg1* gene might downregulate the promoter activity by unknown mechanism. Knowledge regarding TATA-less promoters is still limited, and the precise mechanisms and functions of downstream Sp3-binding sites remain to be clarified.

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Conflict of interest

None declared.

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