Brm transactivates the telomerase reverse transcriptase (TERT) gene and modulates the splicing patterns of its transcripts in concert with p54<sub>nrb</sub>


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We report that a DBHS (Drosophila behaviour, human splicing) family protein, p54<sup>nrb</sup>, binds both BRG1 (Brahma-related gene 1) and Brm (Brahma), catalytic subunits of the SWI/SNF (switch/sucrose non-fermentable) chromatin remodelling complex, and also another core subunit of this complex, BAF60a. The N-terminal region of p54<sup>nrb</sup> is sufficient to pull-down other core subunits of the SWI/SNF complex, suggesting that p54<sup>nrb</sup> binds SWI/SNF-like complexes. PSF (polypyrimidine tract-binding protein-associated splicing factor), another DBHS family protein known to directly bind p54<sup>nrb</sup>, was also found to associate with the SWI/SNF-like complex. When sh (short hairpin) RNAs targeting Brm were retrovirally expressed in a BRG1-deficient human cell line (NCI-H1299), the resulting clones showed down-regulation of the TERT (telomerase reverse transcriptase) gene and an enhancement of ratios of exon-7-and-8-excluded TERT mRNA that encodes a β-site-deleted inactive protein. All of these clones display growth arrest within 2 months of the Brm-knockdown. In NCI-H1299 cells, Brm, p54<sup>nrb</sup>, PSF and RNA polymerase II phosphorylated on CTD (C-terminal domain) Ser<sup>2</sup> specifically co-localize at a region incorporating an alternative splicing acceptor site of TERT exon 7. These findings suggest that, at the TERT gene locus in human tumour cells containing a functional SWI/SNF complex, Brm, and possibly BRG1, in concert with p54<sup>nrb</sup>, would initiate efficient transcription and could be involved in the subsequent splicing of TERT transcripts by accelerating exon-inclusion, which partly contributes to the maintenance of active telomerase.

Key words: Brahma, exon-exclusion, growth arrest, p54<sup>nrb</sup>, polypyrimidine tract-binding protein-associated splicing factor (PSF), telomerase reverse transcriptase (TERT).

INTRODUCTION

The SWI/SNF (switch/sucrose non-fermentable) complex contributes to the regulation of gene expression by altering chromatin structures, and plays many important roles during epigenetic regulation in many organisms [1]. In mammals, this complex is an assembly of at least eight polypeptides, and each complex contains a single molecule of either Brm (Brahma) or BRG1 (Brahma-related gene 1), but not both [2,3]. These two proteins are the catalytic subunits of the complex and have DNA-dependent ATPase activity that drives the remodelling of nucleosomes [4]. Brm and BRG1 show high similarity but seem to regulate a set of target promoters that is not entirely overlapping [5]. Indeed, Brm and BRG1 show clear differences in their biological activities: Brm is essential for the maintenance of MuLV (murine leukaemia virus)-based retroviral gene expression, whereas BRG1 appears to play no role in this process [6].

The expression of Brm has been found to be absent from several human tumour cell lines [6–10]. In these cells, the Brm gene is transcribed efficiently, but the mRNA and protein expression levels of Brm are tightly suppressed, indicating that this gene is regulated at the post-transcriptional level [9,10]. The absence of expression of the BRG1 subunit is also often observed in non-small cell lung carcinomas, and in other cancers and cell lines derived from these tumours [11,12]. For example, the NCI-H1299 cell line established from a human non-small cell lung carcinoma exhibits a 69-base deletion in the genomic sequence of BRG1, which incorporates a splicing donor site and results in a lack of protein expression [12].

The SWI/SNF complex interacts with various proteins, such as DNA-bound transcriptional activators, through many specific and varied associations between its different subunits. The interacting proteins include products of proto-oncogenes such as c-fos and c-jun [13], and tumour-suppressor proteins such as pRb (retinoblastoma protein) [7] and p53 [14]. The SWI/SNF complexes also function as transcriptional repressors through their ability to interact with repressor complexes such as mSin3A/HDAC2 (histone deacetylase 2) co-repressor [15]. These larger complexes are recruited to some promoters by additional transcriptional repressors such as NRSF (neural restrictive silencer factor) [16,17]. In addition to its role in chromatin remodelling at the sites of gene promoters, SWI/SNF has also been reported to be involved in transcriptional elongation [18]. Furthermore, SWI/SNF subunits have been detected in some RNA polymerase II holoenzyme preparations, although in substoichiometric amounts [19].

The SWI/SNF complexes are thus predicted to manifest diverse functions due to their binding capacity for a variety of factors. In the present study, we have isolated such factors using direct nanoflow LC (liquid chromatography)-MS analysis [20]. Among these, we have scrutinized p54<sup>nrb</sup> as well as PSF (polypyrimidine tract-binding protein-associated splicing factor),

Abbreviations used: AP-1, activator protein 1; BRG1, Brahma-related gene 1; Brm, Brahma; ChIP, chromatin immunoprecipitation; CoAA, coactivator activator; CTD, C-terminal domain; DBHS, Drosophila behaviour, human splicing; GST, glutathione transferase; H5, anti-RNA polymerase II CTD phosphorylated on Ser<sup>2</sup>; HA, haemagglutinin; HEK-293T cells, human embryonic kidney cells expressing the large T-antigen of SV40 (simian virus 40); LC, liquid chromatography; MOI, multiplicity of infection; MuLV, murine leukaemia virus; NP40, Nonidet P40; pRB, retinoblastoma protein PSF, polypyrimidine tract-binding protein-associated splicing factor; shRNA, short hairpin RNA; RT–PCR, reverse transcription–PCR; SWI/SNF, switch/sucrose non-fermentable; TERT, telomerase reverse transcriptase; VNTR, variable number of tandem repeats.

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and present evidence that complexes containing Brm or BRG1, in conjunction with p54\textsuperscript{nb} and PSF, function at the splicing step of the endogenous TERT (telomerase reverse transcriptase) gene, which results in growth arrest and senescence.

**EXPERIMENTAL**

**Cell culture and transfection**

HEK-293T cells [human embryonic kidney cells expressing the large T-antigen of SV40 (simian virus 40)], the human cervical adenocarcinoma cell line HeLaS3 and the human non-small cell lung carcinoma cell line NCI-H1299 were cultured in DMEM (Dulbecco’s modified Eagle’s medium) with 10% FCS (fetal calf serum). Approx. 1 × 10\(^8\) HEK-293T cells were transfected with FLAG-tagged BRG1 plasmids (pCAGF1-IG-BRG1, 240 \(\mu\)g) using Lipofectamine\textsuperscript{TM} PLUS reagents according to the manufacturer’s protocol (Invitrogen). At 3 days after transfection, the cells were used for the preparation of nuclear extracts.

**Purification and analysis of FLAG–BRG1-containing complexes**

Approx. 2 × 10\(^8\) HEK-293T cells transfected with FLAG–BRG1 plasmids were harvested and incubated for 10 min at 4°C in buffer AS [10 mM Hepes (pH 7.9), 1.5 mM MgCl\(_2\), 10 mM KCl, 0.05% NP40 (Nonidet P40) and protease inhibitors (1 mM PMSF, 100 \(\mu\)g/ml antipain, 100 \(\mu\)g/ml leupeptin, 100 units/ml aprotonin)]. The nuclei were then pelleted rapidly at 2100 \(\times\) g for 5 min at 4°C and extracted in buffer CS [20 mM Hepes (pH 7.9), 1.5 mM MgCl\(_2\), 0.5 M NaCl, 25% (v/v) glycerol and protease inhibitors] for 30 min at 4°C. The supernatants were then diluted in an equal volume of dilution buffer [25 mM Hepes (pH 7.9), 2.5 mM EDTA (pH 8.0), 0.1% NP40 and protease inhibitors] and ultracentrifuged at 100,000 \(\times\) g for 20 min at 4°C. The resultant nuclear extracts were incubated with anti-FLAG affinity gel (Sigma) at 4°C overnight, and the gel was washed five times with wash buffer [25 mM Hepes (pH 7.9), 250 mM NaCl, 1 mM EDTA and 0.1% NP40] and immunoprecipitates were eluted twice with elution buffer (250 \(\mu\)g/ml FLAG peptide in wash buffer). The eluates were either silver-stained or subjected to direct nanoflow LC-MS analysis as described previously [20].

**Plasmids and antibodies**

To generate BAF60a cDNA with a Kozak consensus sequence, the relevant fragment was excised from pGEX4T-BAF60a and inserted into the pGEM2-475/Met vector (harbouring a fragment 475 sequence for efficient translational initiation) to generate pGEM2-475/BAF60a. The BAF60a cDNA with a Kozak consensus sequence was then excised from pGEM2-475/BAF60a and inserted with a synthetic oligonucleotide DNA fragment encoding a single copy of the HA (haemagglutinin) sequence into pBabe-ires puro [21] to generate pBabe-BAF60a-HA IRES puro, which encodes C-terminally HA-tagged BAF60a. pGEX4T-p54\textsuperscript{nb} full was generated by inserting the p54\textsuperscript{nb} DNA sequence derived from pCMVSPORT6-p54\textsuperscript{nb} in a blunt-ended NcoI (1.0 kb) and Acc65I–NotI fragment (1.3 kb) into the pGEX4T vector respectively. Other deletion mutants such as Δc1, Δc2 and Δc3 were generated by restriction of the following fragments which were then blunt-ended and self-ligated: Δc1, Ncol–NotI fragment (5.5 kb); Δc2, EcoRI–NotI fragment (5.9 kb); and Δc3, Acc65I–NotI fragment (6.1 kb).

The antibodies used are as follows: anti-FLAG (mouse, Sigma), anti-BRG1 (rabbit, Santa Cruz Biotechnology), anti-Brm (rabbit, Abcam), anti-Brm/BRG1 (mouse, originally designated as anti-Brm by BD Biosciences), anti-BAF155 (rabbit, Santa Cruz Biotechnology), anti-BAF60a (mouse, BD Biosciences), anti-Ini1 (mouse, BD Biosciences), anti-p54\textsuperscript{nb} (mouse, BD Biosciences), anti-PSF (mouse, Sigma), anti-β-actin (mouse, BD Biosciences), anti-HA (goat, Santa Cruz Biotechnology) and H5 [anti-RNA polymerase II CTD (C-terminal domain) phosphorylated on Ser2] (mouse, Covance).

**Retroviral vectors and transduction**

SV-S (vesicular stomatitis virus G protein)-pseudotyped, MuLV-based replication defective retroviral vectors were prepared using the prepackaging cell line PLAT [22,23]. Viruses expressing the shRNAs (short hairpin RNAs) shBRG1 and three types of shBrm (shBrm-1, shBrm-2 and shBrm-4) were produced as described previously [9,17,24]. HeLaS3 cells were transduced with shBRG1 virus at a MOI (multiplicity of infection) of 3. H1299 cells were transduced with a mixture of shBrm-1, shBrm-2 and shBrm-4 three times every 14 h at a MOI of 1.

**Protein analysis**

GST (glutathione transferase)-pull downs, SDS/PAGE and Western blotting analyses using anti-BRG1, anti-Brm, anti-Brm/BRG1, anti-BAF155, anti-BAF60a, anti-Ini1, anti-p54\textsuperscript{nb}, anti-PSF and anti-β-actin antibodies were performed as described previously [13,25].

**Nuclear extraction and immunoprecipitation**

Nuclear extracts were prepared from HEK-293T cells or HEK-293T cells stably expressing BAF60a–HA essentially as described previously [26,27]. The nuclear extracts of HEK-293T cells were incubated with either normal rabbit IgG, anti-BRG1 or anti-Brm antibodies in buffer D [20 mM Hepes (pH 7.9), 100 mM KCl, 20% (v/v) glycerol, 0.2 mM EDTA, 0.1% NP40 and 0.5 mM PMSF] at 4°C overnight. Protein A/G Plus–agarose beads (30 \(\mu\)l) were then added, and samples were incubated at 4°C for 1 h. In the case of the nuclear extracts prepared from HEK-293T cells stably expressing BAF60a–HA, the samples were incubated with 30 \(\mu\)l of anti-HA beads at 4°C overnight, followed by washing. The beads were then collected and washed three times with buffer D, and bound protein complexes were analysed by SDS/PAGE followed by Western blotting using the relevant antibodies.

**RT–PCR (reverse transcription–PCR)**

RT–PCR was performed as described previously [9,10,13] using the following primer sets: TERT exon 5–12, forward 5′-CTCTGAGCTGTACTTTGTCAAG-3′ and reverse 5′-AGGTTAGACGTGCTCTGAG-3′ and reverse 5′-AGTCTGTTTCTCTAGAAG-3′ and reverse 5′-CCCTGAGCTGTACTTTTGCAG-3′ and reverse 5′-AGTTAGACGTGCTCTGAG-3′ and reverse 5′-AGTCTGTTTCTCTAGAAG-3′ and reverse 5′-CCCTGAGCTGTACTTTTGCAG-3′.

**Southern blotting**

Genomic DNA was prepared as described previously [22], digested with HinI and EcoRI and analysed by Southern blotting with a telomeric sequence probe using Gene Images 3′-oligolabelling and CDP-Star detection reagent (GE Healthcare).
ChIP (chromatin immunoprecipitation) assay

ChIP assays were performed essentially according to the manufacturer’s protocol (ChIP Assay Kit, Upstate) [6,17]. Using anti-Brm, anti-BRG1 and anti-p54nrb antibodies, salmon sperm DNA/Protein G–agarose was incorporated into the method. In the case of anti-PSF and H5, salmon sperm DNA/Protein A–agarose was used, and rabbit anti-mouse immunoglobulins reactive to mouse IgGs and IgM was added for 2 h at 4°C before capture of the immune complexes with the agarose. The specific antibodies used for immunoprecipitation were rabbit anti-BRG1 and anti-Brm, mouse anti-p54nrb, anti-PSF and H5, and normal rabbit or mouse IgG. After the protein–DNA cross-links in the immunoprecipitates were reversed, DNA was extracted for PCR using the following primers: TERT promoter, forward 5′-TCTGTGCCCTTCGTCACTGC-3′ and reverse 5′-TCTGTGCCCTTCGTCACTGC-3′; TERT exon 1, forward 5′-GTCCTGCCCCCTTCTCACTTC-3′ and reverse 5′-GCACGCTGCTGAACACTC-3′; TERT exon 4, forward 5′-AGGGGTCTGA ATGTGGTAG-3′ and reverse 5′-GCCACACGCA TA TCACAG-3′; TERT exon 7, forward 5′-AGGGGTCTGA ATGTGGTAG-3′ and reverse 5′-GCCACACGCA TA TCACAG-3′; TERT exon 16, forward 5′-TCTCAAGGACCAGTCCACCC-3′ and reverse 5′-GTGCAGGGCACACCTTTG-3′.

RESULTS

p54nrb binds SWI/SNF-like complexes through its interaction with BRG1 or Brm

To elucidate the diverse functions of the SWI/SNF complex, we intended to analyse a series of proteins which substoichiometrically bind and confer novel functions on this complex. Nuclear extracts of HEK-293T cells transfected with FLAG–BRG1-expressing plasmids were prepared, from which FLAG–BRG1-containing complexes were isolated using an anti-FLAG affinity resin (Figure 1). Western blotting analyses using anti-Brm, anti-BAF155, anti-BAF60a and anti-Ini1 antibodies show that the components of the BRG1-type SWI/SNF complex are immunoprecipitated with FLAG–BRG1 as expected (results not shown). Significantly, silver staining shows that these isolated complexes contain components other than those of the SWI/SNF complex (Figure 1), and we therefore subjected the eluates to direct nanoflow LC-MS. The resulting mass spectra led to the identification of almost all of the known components of the SWI/SNF complex [2,3], except for Brm, BAF250b, BAF180 and BAF60c (Supplemental Table 1, at http://www.BiochemJ.org/bj/411/bj4110201add.htm).

Among the proteins that are not components of the SWI/SNF complex, we found p54nrb and PSF (Supplemental Table 1). We also confirmed the presence of these proteins in the isolated complexes by Western blotting (results not shown). p54nrb was originally identified as an abundant and highly conserved nuclear protein with RNA-binding activity, harbouring a DBHS (Drosophila behaviour, human splicing) domain [28] and is thought to be involved in regulating diverse pathways at the level of pre-mRNA splicing. p54nrb has also been implicated in transcriptional control and in the nuclear retention of ‘A to 1’-edited RNAs. PSF also belongs to the same family as p54nrb and has been shown to have the ability to bind p54nrb. PSF is implicated in not only pre-mRNA splicing, but also transcriptional control, nuclear retention and DNA recombination.

Thus we examined the possible diverse functions of the SWI/SNF complex that would be conferred through its binding to p54nrb. Since both the BRG1- and Brm-type SWI/SNF complexes include BAF60a and Ini1 as core subunits, we tested whether p54nrb binds these subunits, expressed in rabbit reticulocyte lysates, using a GST-pull-down assay. We found that BRG1, Brm and BAF60a bind recombinant GST–p54nrb fusion protein in vitro with similar binding efficiency, but no interaction was detected between Ini1 and GST–p54nrb (results not shown). These results suggest that p54nrb could bind both Brm- and BRG1-type complexes through multiple interfaces, although this experiment cannot exclude the possibility that these interactions are not direct. We then examined whether BAF60a binds both p54nrb and other components of the SWI/SNF complex such as BRG1, Brm and Ini1 in vivo. When nuclear extracts of HEK-293T cells stably expressing retrovirally transduced BAF60a–HA were subjected to immunoprecipitation by anti-HA antibodies, p54nrb and PSF, as well as the components of the SWI/SNF complex, were specifically co-immunoprecipitated with BAF60a–HA (Figure 2A).

We then tested whether endogenous Brm and BRG1 form a complex with p54nrb and PSF in HEK-293T cells. Immunoprecipitation with anti-BRG1 or anti-Brm antibodies followed by Western blotting showed that Brm indeed co-immunoprecipitates p54nrb and PSF, and that BRG1 co-immunoprecipitates p54nrb and PSF.
Figure 2  p54\textsuperscript{nb} and PSF interact with SWI/SNF-like complexes

(A) Nuclear extracts of HEK-293T cells stably expressing mock or BAF60a–HA were incubated overnight at 4°C with anti-HA agarose (α HA). Immunoprecipitated complexes were analysed by Western blotting using anti-BRG1, anti-Brm, anti-Ini1, anti-p54\textsuperscript{nb} or anti-PSF antibodies. (B) Nuclear extracts of HEK-293T cells (293T NE) were incubated overnight at 4°C with control normal rabbit IgG, anti-BRG1 or anti-Brm, and the immune complexes were captured by the addition of Protein A/G Plus–agarose. The immunoprecipitated complexes were then analysed by Western blotting using anti-p54nrb, anti-PSF, anti-Brm/BRG1 or anti-Ini1 antibodies. The percentages of the immunoprecipitated proteins to total input are indicated. The asterisk indicates a cross-reacting immunoglobulin heavy chain. (C) Schematic diagram of the motifs found in p54nrb. QH, a glutamine and histidine-rich region. HTH, a sequence predicted to form a helix–turn–helix structure. +/−, a highly charged region. P, a proline-rich region. (D) Approx. 0.5 μg of GST, GST–p54nrb or the GST–p54nrb deletion series was incubated with HEK-293T total cellular extracts, and the indicated proteins were detected by Western blotting. IP, immunoprecipitation; contl., control.

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The lack of BRG1 and Brm alters the splicing patterns of TERT transcripts

The direct involvement of p54\textsuperscript{nb} and PSF in pre-mRNA splicing had been demonstrated. Next, we tested the possibility that Brm and BRG1 also function as regulators of alternative splicing. For this purpose, we adopted a knockdown strategy that used an shRNA (short hairpin RNA)-expressing retroviral vector as the analyses can then be performed under nearly physiological conditions. First, we transduced a shBRG1 retroviral vector in HeLaS3 cells (which is competent for both BRG1-type and Brm-type SWI/SNF complexes) and performed selection with puromycin. Although a mixed population of puromycin-resistant cells was successfully isolated and BRG1 expression was efficiently reduced when tested by Western blotting, we observed attenuation of the knockdown effect as the cell cultures...
Figure 3  Brm-knockdown clones show enhancement of the exclusion of exon 7 and exon 8 of the TERT gene, the transcription of which is dependent upon the catalytic subunit of the SWI/SNF complex

(A) NCI-H1299 cells transduced with shGFP or shBrm viruses were cloned, and within 2 months of shBrm transduction, almost all of the clones displayed growth arrest. After shBrm transduction and selection by puromycin resistance, total cellular extracts of these clones as well as shGFP control clones were prepared and analysed by Western blotting with anti-Brm or anti-β-actin antibodies. (B) Total RNA of each mock- or Brm-knockdown H1299 clone was subjected to RT–PCR amplification of alternative spliced regions of the indicated genes. Open triangles represent exon-excluded RNAs and closed triangles indicate exon-included RNAs. (C) Schematic representation of the genomic region of the TERT gene. The full-length (f), β-site (corresponding to exon 7 and 8) deletion (Δβ) and α-site deletion (Δα) mRNAs are indicated. The 37 VNTRs located 5′-adjacent to the alternative splicing acceptor site are also indicated. ex, exon. (D) Total RNAs of untransduced, shGFP or shBrm-transduced H1299 cell clones were analysed by RT–PCR with the primers shown in (C). Each band intensity was measured in three independent experiments, and the mean ratio of band 1 to band 2 was calculated. (E) Total RNAs from an shGFP or shBrm/BRG1-transduced HeLaS3 mixed population were analysed by RT–PCR as described in (D). Each band intensity was measured in three independent experiments, and the mean ratio of band 1 to band 2 was calculated. (F) Genomic DNAs prepared from untransduced cells, shGFP-transduced H1299 cells or the Brm-knockdown clones described in (A) were digested and analysed by Southern blotting using a non-radioisotopic system. Sizes are indicated in kb.

were continually passaged. We therefore tried to isolate BRG1-knockdown clones that would sustain the knockdown effect, but failed due to growth arrest after approx. 10 days of transduction with the shBRG1 retrovirus. A similar phenotype was observed for the knockdown of BRG1 in MDA-MB435 breast carcinoma cells (which also express both of these subunits). Hence, we tested whether clones lacking the expression of BRG1 and Brm could be established through a Brm-knockdown in originally BRG1-defective cell lines. We were finally able to establish Brm-knockdown clones of NCI-H1299, a cell line deficient in endogenous BRG1, by transduction of shBrm retroviral vectors. Brm expression was found to be efficiently reduced in almost all of the clones tested (Figure 3A), but these clones also displayed growth arrest within 2 months of the knockdown.

We next screened candidates for changes in their transcription and alternative splicing patterns through lack of Brm and BRG1 using the H1299-shBrm clone #33 which showed efficient knockdown (Figure 3B and Supplemental Figure 1 at http://www.BiochemJ.org/bj/411/bj4110201add.htm). We have shown previously that SWI/SNF complexes are recruited by a specific subset of Fos/Jun heterodimers, such as c-Fos/c-Jun, through their subunit BAF60a to AP-1 (activator protein 1)-binding sites, to transactivate target genes such as collagenase, c-met, CD44 and vimentin [10,13]. Among the genes reported to be under the control of AP-1, we picked up candidate genes such as CD44, E-cadherin and TERT, because they have been reported to be alternatively spliced [31–33]. In addition, several components of the SWI/SNF complex, including Brm, BRG1 and Ini1, were also picked up, as their pre-mRNA are also known to be alternatively spliced [34,35]. As shown in Figures 3(A) and 3(B), the expression of Brm RNA is efficiently reduced in the shBrm clone. However, Brm, BRG1 and Ini1 display no significant changes in their alternative-splicing patterns in clone #33. We found that CD44 is expressed only as mRNA in which the variant exons are excluded in mock-transduced H1299, and shows no changes in its transcription and alternative-splicing patterns in clone #33, and E-cadherin mRNA is not expressed in H1299 cell line (results not shown). However, we identified the TERT gene, a known AP-1 target, as a positive candidate (Figure 3B).

The TERT transcript has two major alternative-splicing variants, and variants containing both or either of the two deletion sites are present during development as well as in a panel of cancer cell lines. One deletion (β site, 182 bp) causes a premature translational termination, whereas the other deletion (α site, 36 bp) lies within the reverse transcriptase motif. Both splicing variants are known to encode inactive proteins. In the case of the variants lacking the α site, it has been reported to also function as dominant-negative inhibitors of telomerase [32]. RT–PCR experiments for total RNA extracted from NCI-H1299 cells, using the TERT primer sets shown in Figure 3(C), produced two bands. Sequence analysis subsequently showed that band 1 was a full-length transcript, and that band 2 corresponds to the
In H1299 cells, Brm and p54<sup>nb</sup> localize together specifically to the promoter, the region around exon 7 including an alternative splicing acceptor site and the 3′-UTR (untranslated region) of the TERT gene; RNA polymerase II phosphorylated (pol II) on Ser2 in CTD specifically accumulates also at this exon 7 splicing acceptor site and 3′-UTR.

(A) Anti-BRG1, anti-Brm or anti-p54<sup>nb</sup>, and (B) anti-PSF or H5 antibodies were used in the ChIP analysis of bulk chromatin from untransduced H1299 cells. (C) Graphic representation of the recruitment of the proteins assayed in (A) and (B) at the indicated positions of the TERT gene relative to their input values. ex, exon.

β-site deletion; which is an exclusion of exons 7 and 8. Two independent H1299 clones (#21 and #33), showing an efficient Brm-knockdown, exhibit a reduction in TERT transcription and an enhancement of the ratio of TERT mRNAs encoding the β-site-deleted protein (Figure 3D). Similar results were obtained using total RNA from a mixed population of HeLaS3 cells that were transduced with shRNA targeting both Brm and BRG1 and then selected by puromycin (Figure 3E).

In the absence of telomerase, or when this enzyme is expressed at very low levels, DNA synthesis during cell division is known to result in the progressive shortening of telomeric DNA. This shortening eventually compromises telomere integrity and triggers senescence [36]. To examine whether the remaining small quantities of TERT mRNAs encoding inactive protein result in the shortening of telomeres, Southern blotting of genomic DNA extracted from the Brm-knockdown, growth-arrested H1299 cell clones described above was performed with a telomere-repeat-specific probe. All seven Brm-knockdown clones displayed telomere shortening (Figure 3F).

We tested whether the Brm-knockdown clones showed a senescent phenotype. The Brm-knockdown clones displayed significantly elevated SA-β-gal (senescence-associated β-galactosidase) activities with formation of SAHF (senescence-associated heterochromatic foci) [37] compared with either untransduced or mock-shRNA-transduced cells (results not shown).

p54<sup>nb</sup> and Brm localize at a region including splicing acceptor site of exon 7 of the TERT gene

Next, we examined the distribution of Brm, p54<sup>nb</sup> and PSF on the TERT gene. ChIP analysis with antibodies against Brm, p54<sup>nb</sup> and PSF shows that these proteins co-localize at a region around exon 7 of the TERT gene, which incorporates the alternative splicing acceptor site, and that Brm and p54<sup>nb</sup> also localize at the TERT promoter and at the region adjacent to the polyadenylation signal (Figure 4A). The accumulation of RNA polymerase II phosphorylated on CTD Ser2 was detected by the specific antibody H5 at the region around the acceptor site of the TERT gene, where p54<sup>nb</sup>, PSF and Brm also co-localize, and on the region adjacent to the polyadenylation signal (Figures 4B and 4C). Taken together, these results indicate that Brm functions as a positive regulator of TERT exon inclusion in concert with p54<sup>nb</sup> and PSF, accompanied by the reduction of the elongation rate around the acceptor site of this gene.

DISCUSSION

In the present study, we have identified two DBHS family proteins, p54<sup>nb</sup> and PSF, in isolated FLAG–BRG1-containing complexes by proteomics approaches using direct nanoflow LC-MS. Furthermore, we have shown that p54<sup>nb</sup> binds BRG1 and Brm, both catalytic subunits of the SWI/SNF complex, and also binds an additional core subunit of this complex, BAF60a, both in vitro and in vivo. We scrutinized the functions of Brm and BRG1 as regulators of alternative splicing in concert with p54<sup>nb</sup>. We have demonstrated from our analyses that Brm, and possibly also BRG1, in concert with p54<sup>nb</sup>, functions as a transactivator of TERT in human tumour cell lines and positively regulates exon-inclusion of TERT transcripts, and therefore, at least in part, contributes to the maintenance of an active telomerase.

Either BRG1 [38] or Brm [9,39] can function as tumour-suppressor genes. In the later stage of carcinogenesis, even...
concomitant loss of both BRG1 and Brm would be often observed as is the case with SW13 cell line [11,12]. In such cases, as suggested by our results using the Brm-knockdown clones of H1299 cell line, tumour cells would become senescent by the loss of both catalytic subunits of the SWI/SNF complex. Therefore some genetic or epigenetic changes would also be required for these tumour cells to overcome the senescence and become more malignant. In the case of SW13, TERT expression was found to be marginal and telomerase activity is undetectable, but the telomere lengths were nonetheless observed to be maintained [40]. Interestingly, SW13 uses an ALT (alternative lengthening of telomeres) pathway, which is independent of telomerase, to maintain telomere length. So we expect these mechanisms would be operating in tumour cells that stopped expression of both Brm and BRG1.

The correlation between Brm and alternative splicing has been suggested previously [41]. Muchardt and co-workers recently demonstrated that the overexpression of Brm leads to the variant exon-inclusion of CD44 through the interaction of several components of the splicing machinery, such as PRP6 and Sam68 [41]. In the present study, we designed a retroviral system to enable the stable and long-term expression of shBrm and shBRG1, which we contend creates a more physiological cellular system. These physiological conditions allowed us to not only observe the growth arrest phenotype in cells lacking the expression of Brm and BRG1, but also to confirm the activities of Brm in accelerating exon-inclusion, as also suggested by the study of Muchardt and co-workers [41]. Although Muchardt and co-workers also indicated in their study that the overexpression of Brm accelerates exon-inclusion of E-cadherin as well as CD44 [41], we found in the present study that neither CD44 variants nor E-cadherin are expressed in H1299 cells, and thus could not examine whether Brm also accelerates exon-inclusion of CD44 and E-cadherin in this cell line.

It is well established that high-level expression of BRG1 in tumour cells deficient in BRG1 expression such as SW13 causes rapid growth arrest through the pRb pathway. So it is true that either a SWI/SNF defect or overexpression of the catalytic subunit apparently induces similar phenotypes of senescence. However, in the present study, observations of shBrm-expressing H1299 show the growth arrest was observed after a continuous growth of 2 months, whereas ectopic BRG1 expression in SW13 caused a rather prompt growth arrest (within 12 days of the transfection) [7,42]. Considering that SW13 does not express TERT at all and uses an alternative elongation mechanism of telomeres [40], we expect that molecular mechanisms involved in the senescence would be different.

Our ChIP analyses demonstrate that p54<sup>vrb</sup> co-localizes with Brm at the promoter region of the TERT gene, but that PSF does not localize here (Figure 4). p54<sup>vrb</sup> has been reported to act as both a transcriptional activator and a repressor through its interaction with various transcription factors such as thyroid hormone receptor, steroidogenic factor 1 and androgen receptor [43,44]. It has also been shown to directly bind to octamer motifs (Oct DNA-binding site) in the IgH promoter in B-cell leukaemia cells, and to an enhancer element in the long terminal repeats of murine intracisternal A particles, to activate transcription. There are several binding sites for known transcription factors, including Oct, c-Myc, SP-1 (specificity protein 1), NF-κB (nuclear factor κB) and AP-1 in upstream sequences of the TERT promoter [45]. It is thus highly possible that a SWI/SNF-like complex molecule containing Brm and p54<sup>vrb</sup> might transactivate the TERT gene, then subsequently modulate its exon-inclusion. Indeed, in several genes, alternative splicing has been shown to be coupled with transcriptional regulation. For example, in several steroid hormone receptor-stimulated genes, CoAA (co-activator activator) stimulates transcriptional activation mediated by nuclear receptors and transcription factors, and also regulates alternative splicing [46,47]. The CoAA protein is a major component of nuclear paraspeckles and is often found to be co-localized with p54<sup>vrb</sup> [48].

We also find that Brm, p54<sup>vrb</sup>, PSF and RNA polymerase II CTD phosphorylated on Ser<sup>2</sup> specifically accumulate at a region that includes the splicing acceptor site of exon 7 in the TERT gene. The accumulation of these proteins at a region including an alternative splicing donor site of TERT exon 6 was not observed. A previous study showing that p54<sup>vrb</sup> binds PSF [30], and our current observations that these proteins bind Brm-containing complexes, prompted us to speculate that the larger complex would localize for a longer time at splicing acceptor sites owing to the reduced elongation rate, and that Brm functions as a positive regulator of TERT exon-inclusion in concert with p54<sup>vrb</sup> and PSF.

An important issue is the clarification of the mechanisms that underlie the specific targeting of p54<sup>vrb</sup> and Brm to the TERT exon 7 acceptor site. p54<sup>vrb</sup> and PSF are thought to regulate general splicing processes, and Konarska and co-workers have shown that both p54<sup>vrb</sup> and PSF, which bind the CTD of RNA polymerase II, can interact directly with the 5′-splicing site and may mediate contacts between RNA polymerase II and snRNP (small nuclear ribonucleoprotein) during the coupled transcription–splicing process [49]. However, since various cell lines which lack expression of BRG1 and Brm do not show any deficiency in general splicing, a functional SWI/SNF complex must not be essential for these processes. These observations and our present findings prompt us to speculate therefore that the Brm and p54<sup>vrb</sup> proteins that are already loaded on the TERT promoter might slide with RNA polymerase II or loop toward the alternative splicing acceptor site, and then recognize specific RNA or DNA cis-elements at this site together with PSF. We think this specific recognition via the assembly of Brm, p54<sup>vrb</sup> and PSF is essential for determining exon-inclusion. Intron 6 of the TERT pre-mRNA has both a splicing donor and an acceptor site that follows the ‘GU-AG’ rule. As a possible candidate cis-element for specific accumulation of these assemblies, we have found that 37 VNTRs (variable number of tandem repeats; each repeat unit consists of 17–36 bp) are present 5′-adjacent to the acceptor site of exon 7 (Figure 3C and Supplemental Figure 2 at http://www.BiochemJ.org/bj411/bj4110201add.htm). Similar VNTR sequences were not found in other regions of the TERT gene.

VNTRs may therefore be one of the elements specifically recognized by the Brm, p54<sup>vrb</sup> and PSF assembly. There are also several possible mechanisms that may enable recognition of these elements, p54<sup>vrb</sup> and/or PSF might alter their preferences for binding to RNA or DNA when interacting with Brm. It is also possible that this assembly might recognize DNA sequences corresponding to the alternative splicing acceptor site through Brm or other components of SWI/SNF complex. In this regard, several subunits of the SWI/SNF complex, such as BRG1, Brm, BAF250α, BAF250β and BAF57, have been shown to have DNA-binding activity [50]. The larger complex would also recruit other regulators of alternative splicing such as SR (serine/arginine-rich) proteins and hnRNP (heterogeneous nuclear ribonucleoproteins) to accelerate exon-inclusion. To further elucidate these possibilities, we attempted to perform a ChIP assay using our Brm-knockdown clones from the H1299 cell line, but it is not yet possible to do this because a sufficient number of cells could not be obtained due to their growth arrest phenotypes.

Taken together, we think that during the regulation of many genes, SWI/SNF (or SWI/SNF-like) complexes function
throughout their transcriptional activation and the subsequent processing of their transcripts, during which the components of this complex such as Brm (or BRG1) dynamically alter their binding partners. Furthermore, the accumulation of Brm (or SWI/SNF complexes) to the TERT gene promoter might dictate the efficiency of transcription and also exon inclusion.

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