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Mammalian Chromatin Remodeling Complex SWI/SNF Is Essential for Enhanced Expression of the Albumin Gene during Liver Development

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The chromatin remodeling complex SWI/SNF is known to regulate the transcription of several genes by controlling chromatin structure in an ATP-dependent manner. SWI/SNF contains the Swi2p/Snf2p like ATPases BRG1 or BRM exclusively. We found that the expression of BRM gradually increases and that of BRG1 decreases as liver cells differentiate. Chromatin immunoprecipitation assays revealed that the ATPase subunits of SWI/SNF and tumor suppressor retinoblastoma (RB) family proteins bind to the promoter region of the albumin gene in hepatocytes, and that the replacement of BRG1 with BRM and pRB with p130 at this site occurs over the course of differentiation. Small interfering RNA experiments showed that blocking the expression of BRG1 and BRM in fetal and adult hepatocytes, respectively, causes a reduction in albumin expression. In luciferase reporter assays with a pREP4-based reporter plasmid that forms a chromatin structure, BRG1 showed activity stimulating the expression of the albumin promoter mediated by CCAAT/enhancer-binding protein α (C/EBPα). This enhancement was facilitated by the RB family members pRB and p130. ATPase assays showed that both pRB and C/EBPα proteins directly stimulate the ATPase activity of BRG1. Our findings suggest that the mechanism by which the activity of transcription factors is enhanced by RB family members and SWI/SNF includes an increase in the ATPase activity of the chromatin remodeling complex.

Key words: albumin, CCAAT/enhancer-binding protein α, primary hepatocytes, retinoblastoma protein, SWI/SNF.

Abbreviations: BRG1, Brm-related gene 1; C/EBP, CCAAT/enhancer-binding protein; HNF, hepatocyte nuclear factor; pRB, retinoblastoma protein.

The mechanisms by which genomic DNA is packaged into chromatin, and the specific region that is unwound during the process of cellular differentiation have remained obscure. However, recent studies on histone acetyltransferases and deacetylases, histone methyltransferases, chromatin remodeling factors and several heterochromatin-specific DNA-binding proteins have suggested that cells use various types of cellular machinery to establish and maintain various programs for controlling the state of chromatin and subsequent regulation of gene expression (*1–6*). Among them, chromatin remodeling multisubunit complexes alter the organization of the nucleosome structure by hydrolyzing ATP. Mammalian chromatin remodeling complexes contain various central ATPase subunits that show similarity to yeast SWI2/SNF2. These ATPases can be divided into three subfamilies, SWI2/SNF2, Mi-2/CHD and ISWI, based on their structures (*7*).

Mammalian SWI/SNF complexes consist of ~15 subunits and contain BRG1 or BRM exclusively as the ATPase subunit. Although BRG1-associated factors (BAFs) such as BAF155, BAF170, BAF60 and INI1 are included in all types of complexes studied to date, there are several different complexes containing different combinations of BAFs and/or tissue-specific isoforms of a common subunit (*8–11*). Furthermore, BRM and/or BRG1 complexes containing mSin3A and MeCP2, which are related to gene repression, are also detected (*12–14*).

SWI/SNF has been genetically shown to regulate subsets of inducible genes in yeast (*15*), and to associate with numerous regulators of gene activation in mammalian cells. These regulators include the glucocorticoid receptors, estrogen receptors (*16–19*), the retinoblastoma tumor suppressor protein, pRB (*20*), cyclin E (*21*), EKLF (*22*), CCAAT/enhancer- binding protein β (C/EBPβ) (*23*) and C/EBPα (*24*). The association of these regulators with SWI/SNF directs recruitment of the complex to nucleosomal sites where stable remodeling occurs (*25*). Moreover, it was recently reported that SWI/SNF facilitates the function of the preinitiation complex in several genes (*26*, *27*).

The involvement of a SWI/SNF-related complex in the developmental and tissue-specific regulation of the human β-globin locus has been also demonstrated *in vivo* (*28*, *29*). Moreover, SWI/SNF is critical for adipocyte, enterocyte and muscle differentiation (*26*, *27*, *30*). Taken together,

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these studies show the requirement for or contribution of SWI/SNF to a wide variety of transcriptional programs during development.

We have previously shown that the expression of BRM is upregulated through neural differentiation in mice, although that of BRG1 is constant (*31*). To shed more light on the activity of SWI/SNF complexes in cell differentiation, we examined the mechanism regulating the transcription of the liver-specific albumin gene. We report here that the expression patterns of BRM and BRG1 are different and complementary during liver development, and that SWI/SNF regulates the transcription of the liver-specific albumin gene in cooperation with C/EBPα and possibly with RB family proteins.

MATERIALS AND METHODS

Plasmids—Mouse BRG1 and BRM cDNAs were obtained by reverse transcription (RT)–PCR using mRNA prepared from cultured mouse neural precursor cells (NPCs) and introduced into pcDNA4/TO/*myc*-His (Invitrogen).

The C/EBPα gene was amplified from pMSV-C/EBPα (*32*) and introduced into pcDNA4/TO/*myc*-His. pRB and p130 cDNAs were synthesized by RT-PCR from RNA from human endothelial cells and mouse NPCs, respectively, and introduced into pcDNA4/TO/*myc*-His. To construct a plasmid for the expression of glutathione *S*-transferase (GST)–fused C/EBPα, the full-length cDNA encoding C/EBPα was introduced into pGEX-5X-2.

For *in vitro* transcription-translation, DNA fragments encoding the BRM N-terminal (1–333 aa), BRM charged (334–739 aa), BRM helicase (740–1234 aa) and BRM C-terminal (1235–1587 aa) domains, and BRG1 N-terminal (1–344), BRG1 charged (345–756 aa), BRG1 helicase (757–1262 aa) and BRG1 C-terminal (1253–1614 aa) domains were amplified by PCR and subcloned into pBluescript KS$^-$ (Toyobo) or pETBlue2 (Novagen). Full-length BRG1 and BRM cDNAs were also subcloned into the vectors.

To generate an albumin promoter-luciferase reporter plasmid, 216 bp of the albumin promoter region was amplified from rat genomic DNA by PCR and introduced into pGL3 (Promega). The reporter plasmid was designated pGL3-alb. In addition, the *Sal*I–*Xho*I fragment containing the albumin promoter region and luciferase gene of pGL3-alb was subcloned into pREP4/CAT (Invitrogen). The reporter plasmid was designated pREP4-alb.

We constructed the ATPase mutant of BRG1 by a PCR method. For this purpose, two primers containing a mutated ATP-binding motif (ΔATP reverse primer, 5′-CATAAGCTTCCCAGCCCCATCTCAT-3′ and ΔATP forward 5′-CTAAAGCTTCATCCAGACCATCGCGCTC-3′, *Hin*dIII sites are underlined) were designed. The 5′ side fragment of BRG1 was amplified using the ΔATP reverse primer and BRG1 5′ primer (5′-CCACAAGCTTAGTTCCAGTAAAGATGTCTACTCC-3′, *Hin*dIII site is underlined), and the 3′ side fragment was amplified with the ΔATP forward primer and BRG1 3′ primer (5′-AAAAGCGGCCGCGTCTTCCTCACTGCCACTTCCTG-3′, *Not*I site is underlined). These fragments were then ligated to produce the BRG1 ATPase mutant (BRG1ΔATP). In this mutant, the ATP-binding motif was replaced with a *Hin*dIII site, and the KT sequence (785–786 aa) of the BRG1 ATP-binding motif was replaced with SF.

C-terminal deletion mutants of BRG1 were also constructed by the PCR method using C-terminal primers; 5′-CAAGCGGCCGCCTTCTCAATCTTCTGCCGT-3′ for BRG1ΔC1, 5′-CAAGCGGCCGCTGGGGACAGCTTCTCAGCA-3′ for BRG1Δbromo, 5′-CAAGCGGCCGCCCGGACCTCCTCTTCGAT-3′ for BRG1ΔKR, 5′-CAAGCGGCCGCATCCTCTTCCATCAG-3′ for BRG1ΔE7 and 5′-CAAGCGGCCGCGGCCTGCAGGAAGGCAC-3′ for BRG1ΔC2.

For ATPase assays, DNA fragments coding for BRG1 helicase and the C-terminal regions (714–1614 aa), and BRG1ΔE7 (714–1308 aa) were amplified by PCR with the following primers: BRG1 helicase and C-terminal forward, 5′-CATCCATGGATGCTGTGGCCCATGCAGT-3′, reverse, 5′-ATTGCGGCCGCGTCTTCCTCACTGCCACTT-3′; BRG1ΔE7 forward, 5′-CATCCATGGATGCTGTGGCCCATGCAGT-3′, reverse, 5′-CAAGCGGCCGCATCCTCTTCCATCAG-3′. These fragments were cloned into pETBlue2 (Novagen). For the production of the HSV/His-tagged pRB protein, a DNA fragment coding for full-length pRB was amplified using primers (5′-CATCCATGGCGCCCAAAACCCCCCGAAAAAC-3′ and 5′-CATCTCGAGTTTCTCTTCCTTGTTTGAGGTA-3′) and also cloned into pETBlue2. The integrity of the DNA sequences of all constructs was confirmed.

Antibodies and Western Blot Analysis—Antibodies to BRG1 and BRM were prepared as described previously (*31*). Antibodies against BAF155, C/EBPα, pRB, p130, human actin and normal rabbit IgG were purchased from Santa Cruz Biotechnology. Western blotting was performed by the standard procedure.

Cell Culture—Hepatocytes were isolated from fetal (17 and 19 days), newborn and adult (8 weeks) Sprague-Dawley rats as described previously (*33*). Fetal hepatocytes from the 17-day stage were cultured in William's medium E (WE) (Invitrogen) supplemented with 5% fetal calf serum (FCS), 0.1 μM $CuSO_4$, 25 nM Na_2SO_4, 0.1 μM insulin (Sigma), 1.0 μM dexamethasone (Wako), 48 μg/ml gentamicin sulfate (Sigma) and 100 μg/ml chloramphenicol (Wako). Adult hepatocytes were cultured in WE medium without serum. Hepatocytes were cultured for RNA interference experiment and the chromatin immunoprecipitation (ChIP) assay. C33A and SW-13 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS.

Immunoprecipitation—For immunoprecipitation assays, C33A cells (3×10^6) were seeded into 100-mm dishes the day before transfection and co-transfected with 2.5–5 μg of the expression vectors for BRM or BRG1, C/EBPα and pRB or p130 by the calcium phosphate method (*34*). Hepatocytes and transfected C33A cells (approximately 1×10^7 cells) were used for immunoprecipitation assays as described previously (*35*).

GST Pull Down Assay—GST and GST-C/EBPα were expressed in *Escherichia coli* BL21, purified and coupled to glutathione-Sepharose beads (Amersham Biosciences). [^{35}S] Met-labeled full-length BRM and BRG1, and their domains were prepared using the TNT-coupled transcription-translation rabbit reticulocyte lysate system (Promega).

RT-PCR Analysis of Albumin—Total RNA was isolated using a total RNA isolation kit, EASYPrep (Takara). RNA (5 μg) was subjected to RT-PCR analysis. The reverse transcriptase ReverTra Ace (Toyobo) was used to generate

cDNAs in a total volume of 20 μl using oligo $(dT)_{12–18}$ as primers according to the manufacturer's instructions. One microliter of cDNAs was mixed with 10 pmol of primers specific to the albumin gene (5′-TCTGTTGGAAAAATCCCACTGC-3′ and 5′-TGGTGTAACGAACTAATAGCGCATT-3′), and PCR was conducted. As a control, PCR using primers specific to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was carried out using the same samples.

Small Interfering RNA (siRNA) Gene Silencing—siRNAs were transfected with a transfection reagent (Oligofectamine, Invitrogen) into fetal (17 days) or adult hepatocytes at 30% confluency in 24-well plates. siRNAs for rat BRG1 and BRM were purchased from Dharmacon Inc. The sequences are as follows: sense strand, 5′-UUUGAACUUCUUGAUAGAAdTdT-3′ and antisense strand, 5′-UUCUAUCAAGAAGUUCAAAdTdT-3′ for BRG1; sense strand, 5′-CCUGGGUCCCAGUAUUUCAdTdT-3′ and antisense strand, 5′-UGAAAUACUGGGACCCAGGdTdT-3′ for BRM. The transfected hepatocytes and culture supernatant were harvested 48 or 72 h post-transfection.

ATPase Assays—The hydrolysis of ATP was measured as the release of inorganic phosphate, $^{32}P_i$, from [γ-^{32}P]ATP by TLC. The reaction mixture (21 μl) contained 10 μl of buffer (50 mM Tris-HCl (pH 7.0), 20 mM $MgCl_2$, 2 mM DTT, and 2 mM ATP), 1 μCi of [γ-^{32}P]ATP (1 μl) (Amersham Biosciences), 9 μl (2.5 μg) of purified proteins in phosphate buffered saline, and 1 μl (0.5 μg) of nucleic acid. We used pBluescript KS(+) as nucleic acid. The reaction was performed at 37°C and terminated at various time points by adding 1 μl of 0.5 M EDTA. One microliter of the reaction mixture was spotted onto a poly (ethyleneimine)-cellulose plate (Merck), and the plate was developed in 0.5 M LiCl and 1 M formic acid. Plates were autoradiographed at –70°C for 12–16 h. For the ATPase assays, protein samples were treated with the anti-GroEL antibody, since GroEL is known to be a bacterial ATPase and to be copurified with GST-fused proteins. In this study, this treatment was also done for His-tagged proteins.

RESULTS

Expression Pattern of BRM and BRG1 during Liver Development—To clarify the function of the SWI/SNF complex, especially its ATPase subunits, in the differentiation of hepatocytes, changes in the expression levels of BRG1 and BRM during liver development were studied by Western blotting (Fig. 1A). The expression of BRM increased as the liver cells differentiated and remained at relatively high levels in adult hepatocytes. In contrast, the expression of BRG1 decreased over the course of differentiation, but remained detectable in adult hepatocytes. Among other subunits of SWI/SNF, BAF155 also showed a subtle decrease, but the liver-specific transcription factor C/EBPα showed no remarkable changes in expression during differentiation (Fig. 1A). In fact, immunoprecipitation experiments showed that BAF155 precipitated with the anti-BRG1 antibody in fetal hepatocytes isolated from E17 embryos, and with the anti-BRM antibody in terminally differentiated adult hepatocytes (Fig. 1B). Figure 1C also shows that BRG1 was precipitated with the anti-BAF155 antibody in fetal hepatocytes while BRM was precipitated in adult hepatocytes. These results indicate

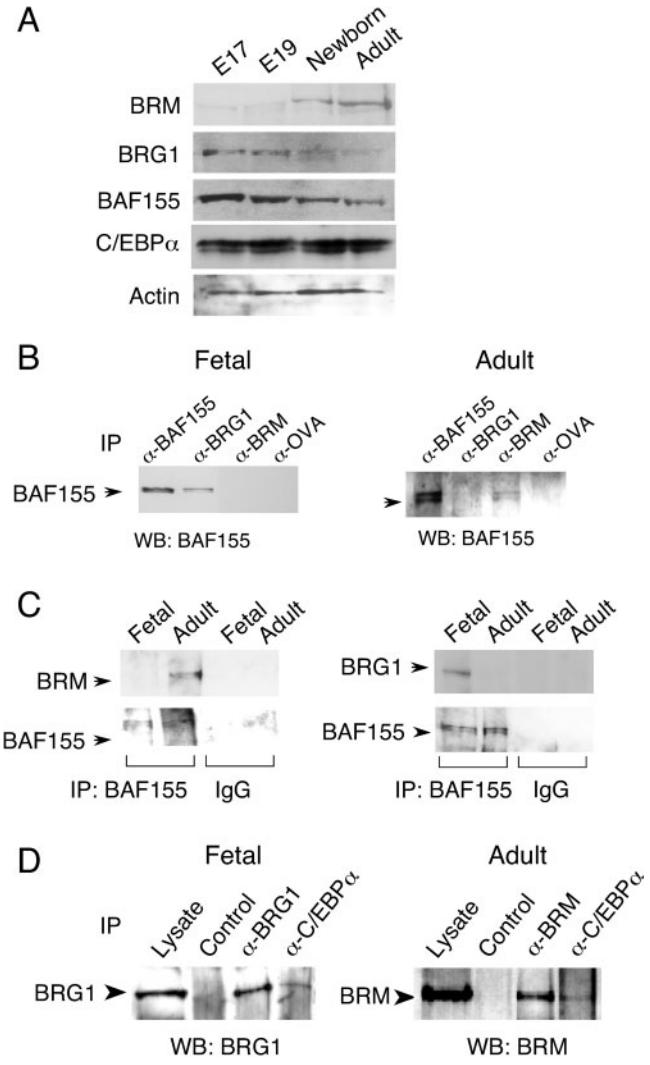

Fig. 1. **The expression levels of ATPase subunits of SWI/SNF change during liver development.** (A) Expression of BRM, BRG1, BAF155 and C/EBPα during liver cell differentiation determined by Western blotting. Equal amounts of protein lysate from freshly isolated hepatocytes were subjected to SDS-PAGE, transferred to a PVDF membrane and detected with each respective antibody. (B) Immunoprecipitation of BAF155 with anti-BRG1 and anti-BRM antibodies from hepatocyte lysates. Anti-ovalbumin (OVA) antibody was used as a control. (C) Immunoprecipitation of BRG1 and BRM with anti-BAF155 antibody. Non-specific goat IgG was used as a control. (D) Cell lysates from adult and fetal hepatocytes were immunoprecipitated with anti-BRM, anti-BRG1, anti-C/EBPα, and anti-OVA antibodies and then subjected to Western blotting with anti-BRM or anti-BRG1 antibodies. Anti-OVA antibody was used as a negative control.

that the main portion of the ATPase subunit of SWI/SNF changes from BRG1 to BRM during the development of the liver. BRG1 and BRM seem to work semi exclusively in dividing-fetal hepatocytes and terminally-differentiated hepatocytes, respectively.

Interaction between the ATPase Subunits of SWI/SNF and C/EBPα in Hepatocytes—Liver-concentrated transcription factors such as HNFs and C/EBPs are involved in liver-specific gene regulation such as that observed with albumin and α-fetoprotein (*36–40*). Since several studies

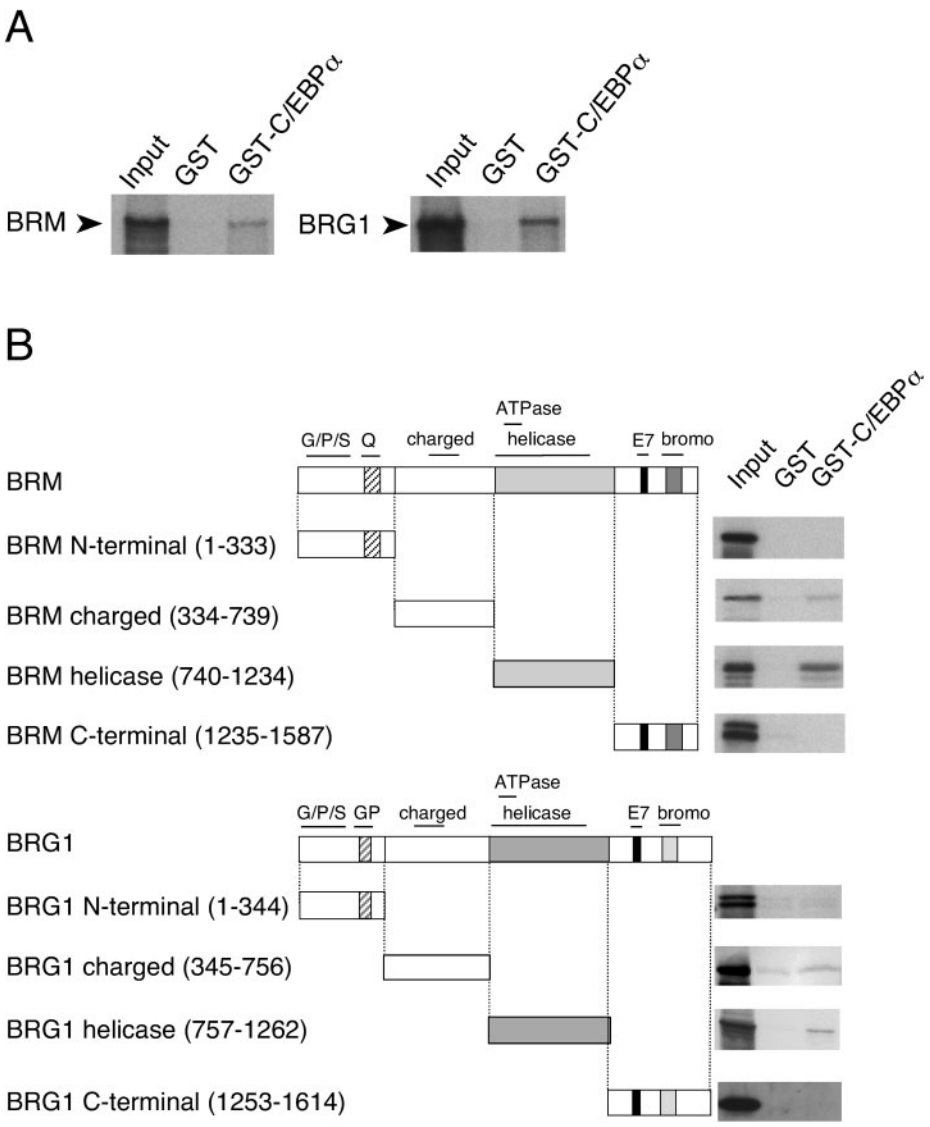

Fig. 2. **BRM and BRG1 are associated with C/EBPα *in vitro*.** (A) Binding of [^{35}S]-labeled *in vitro*–translated BRM and BRG1 to GST (negative control for binding) and GST-C/EBPα. GST-fused C/EBPα or the GST control was immobilized on glutathione-Sepharose resin and incubated with [^{35}S]-labeled *in vitro*-translated BRM and BRG1. The beads were extensively washed, and the bound proteins were subjected to SDS-PAGE and autoradiography. As positive controls, input (4%) was also applied. (B) A schematic representation of the segments of BRG1 and BRM translated *in vitro* (numbering according to the sequence in the database). G/P/S: a region rich in glycine, proline and serine, Q: glutamine-rich region and G/P: glycine- and proline-rich region. These segments were used for GST-pull down assays. Representative results of triplicated experiments are shown.

have shown that C/EBPs bind to BRM and BRG1 using a model system in which these proteins are overexpressed (*23*, *24*), the physical interaction between these chromatin remodeling factors and C/EBPs was first analyzed by conducting co-immunoprecipitation assays with cell extracts from cultured hepatocytes. BRM was found to co-precipitate with C/EBPα from rat adult hepatocytes (Fig. 1D). Instead, BRG1 was associated with C/EBPα in fetal hepatocytes (Fig. 1D). We confirmed these associations by reciprocal experiments using the anti-C/EBPα antibody for detection and the anti-BRM or anti-BRG1 antibody for immunoprecipitation (data not shown). These results indicate that C/EBPα physically interacts with BRM-based SWI/SNF complexes in terminally-differentiated hepatocytes and with BRG1-based complexes in fetal hepatocytes, and suggest that the C/EBPα-SWI/SNF complex contributes to the regulation of cellular differentiation-related genes in hepatocytes.

The GST-pull down assay also revealed that both BRM and BRG1 physically interact with C/EBPα (Fig. 2A). To examine which part of the BRM and BRG1 protein interacts with C/EBPα, BRM and BRG1 were divided into four regions (N-terminal, charged, helicase and C-terminal domains) (Fig. 2B), each of which was labeled with [^{35}S]methionine by an *in vitro* transcription and translation system, and used for GST-pull down assays. Among the BRM fragments, the helicase-like domain (BRM helicase) showed a significant ability to bind C/EBPα, and the charged domain showed weak interaction (Fig. 2B). The central helicase-like domain of BRG1 also showed a good ability to interact with C/EBPα (Fig. 2B).

To confirm the results of the GST-pull down assay, co-immunoprecipitation assays were performed with C33A cells overexpressing C/EBPα and the BRM charged or BRM helicase domain. Consistent with the GST-pull down assay, it was found that C/EBPα could interact with the charged and helicase-like domains of BRM (data not shown). These results suggest that the interaction is mediated by the charged and helicase-like domains of BRG1 and BRM.

BRM and BRG1 Interact with a Promoter Region of the Albumin Gene in Hepatocytes—The observed association

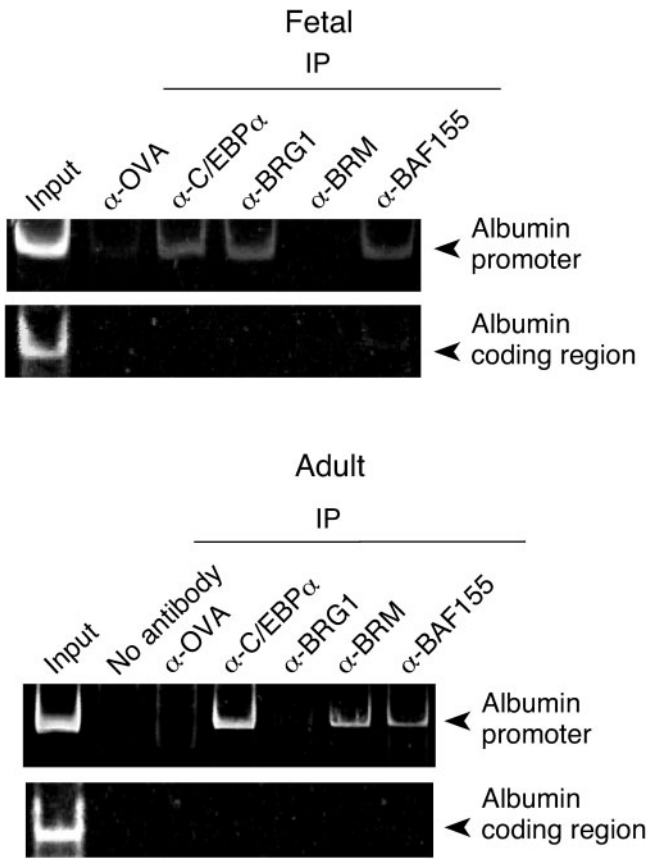

Fig. 3. **Binding of BRG1 and BRM in the albumin promoter during hepatocyte differentiation.** ChIP assays were performed according to the standard procedure with some modifications. Fetal and adult hepatocytes were treated with 1% formaldehyde for 10 min at room temperature. Crosslinking was stopped by the addition of glycine to 0.125 M. The immune complexes were collected by adding salmon sperm DNA/protein A agarose beads (Upstate Cell Signaling Solutions). The albumin promoter region (–303–+24) was amplified by PCR (35 cycles) and analyzed by Tris-borate EDTA–polyacrylamide gel electrophoresis. As negative controls, samples with anti-OVA or without antibody were used. The albumin-coding region (part of exon N) was also amplified as a negative control using primers, 5′-TGTGTTTTCAAGGC-TACCCTGA-3′ and 5′-GATGAAGGGAGGAAACCGAG-3′. Input (2%) was used as a positive control. Representative results of triplicated experiments are shown in the figure.

between either BRG1 or BRM and C/EBPα suggests that SWI/SNF is recruited to C/EBP-binding sites of target genes. The albumin gene is known to have a C/EBPα-binding site in its promoter region (*40*). To clarify whether BRM and BRG1 bind to this promoter region *in vivo*, we performed a ChIP assay that would allow us to detect the presence of various factors in association with the chromatin of target genes *in vivo*. It was observed that the anti-BRG1 antibody apparently precipitated the albumin promoter region in fetal hepatocytes, and that the anti-BRM antibody precipitated the region in adult hepatocytes (Fig. 3). The anti-C/EBPα and anti-BAF155 antibodies detected the promoter region in both fetal and adult hepatocytes. These results suggest that the BRG1- and BRM-based SWI/SNF complexes together with C/EBPα are associated with the albumin promoter region *in vivo*. Thus, the replacement of BRG1 with BRM in the SWI/SNF complex takes place in the albumin promoter during liver development.

Effects of BRG1 and BRM on Transactivation of the Albumin Promoter—Since the ATPase subunit of SWI/SNF changes from BRG1 to BRM during hepatocyte differentiation, we studied whether BRM and BRG1 affect the transactivation of the albumin gene mediated by C/EBPα by conducting luciferase reporter assays. When BRG1 and BRM-negative SW-13 cells were co-transfected with both the C/EBPα and BRM expression vectors and the pGL3-alb reporter plasmid, luciferase activity was slightly enhanced (1.3-fold), but BRG1 did not affect the activity (data not shown). Since these ATPase subunits did not show a clear enhancement of transactivation mediated by C/EBPα, the promoter-luciferase construct was cloned into a pREP4-based episomal vector that is known to form a chromatin structure (*41*). The albumin promoter in the pREP4 vector was activated 8-fold by BRG1 in the presence of C/EBPα, but less so by BRM (Fig. 4A). In this assay, expression vectors for myc-tagged BRG1 and BRM were used to confirm the expression level of these proteins. Similar enhancement by BRG1 and BRG1-myc was observed (compare Fig. 4, A and B). We also obtained similar results with non-tagged and myc-tagged BRM (data not shown). We then performed a reporter assay using a BRG1 mutated in the ATP-binding motif (ΔATP) to clarify whether the activation by BRG1 requires its ATPase activity. The mutant BRG1 lost its ability to enhance C/EBPα-mediated transcription (Fig. 4B). In addition, we confirmed that the ΔATP mutant still bound to C/EBPα and BAF155 by performing co-immunoprecipitation assays (data not shown). Since the apparent activation of the albumin promoter by BRG1 seemed to be dependent on the chromatin structure, we investigated the expression of the endogenous albumin gene in SW-13 cells. In this experiment, an HNF-1α expression plasmid was co-transfected with the C/EBPα and BRG1 or BRM expression plasmids, because this transcription factor is known to be required for the expression of the albumin gene (*40*). BRG1, but not BRM, induced the expression of the albumin gene together with C/EBPα and HNF-1α in non-liver SW-13 cells (Fig. 4C). In the absence of HNF-1α, no albumin expression was detected. Since no direct interaction between HNF-1α and BRG1 was observed in the co-immunoprecipitation and luciferase reporter assays (data not shown), the HNF-1α–dependent albumin expression by C/EBPα and BRG1 seems not to be due to the interaction between HNF-1α and BRG1. We recently reported that HNF-1α promotes the activity of the albumin promoter by recruiting CBP/p300 (*42*). Therefore, it is possible that the function of the HAT complex recruited by HNF-1α is prerequisite for the induction of the albumin gene by C/EBPα and SWI/SNF complex in a genomic environment. Together, these results suggest that BRG1 can induce the expression of the albumin gene in chromatin.

C/EBPα Stimulates the ATPase Activity of BRG1—As indicated above, we found that C/EBPα interacts with the proximal region of the ATP-binding motif of BRG1 and BRM. Therefore, it is possible that the interaction between these proteins affects the ATPase activity of BRG1. To clarify this point, *in vitro* ATPase assays were performed using His-tagged BRG1 (714–1614) and

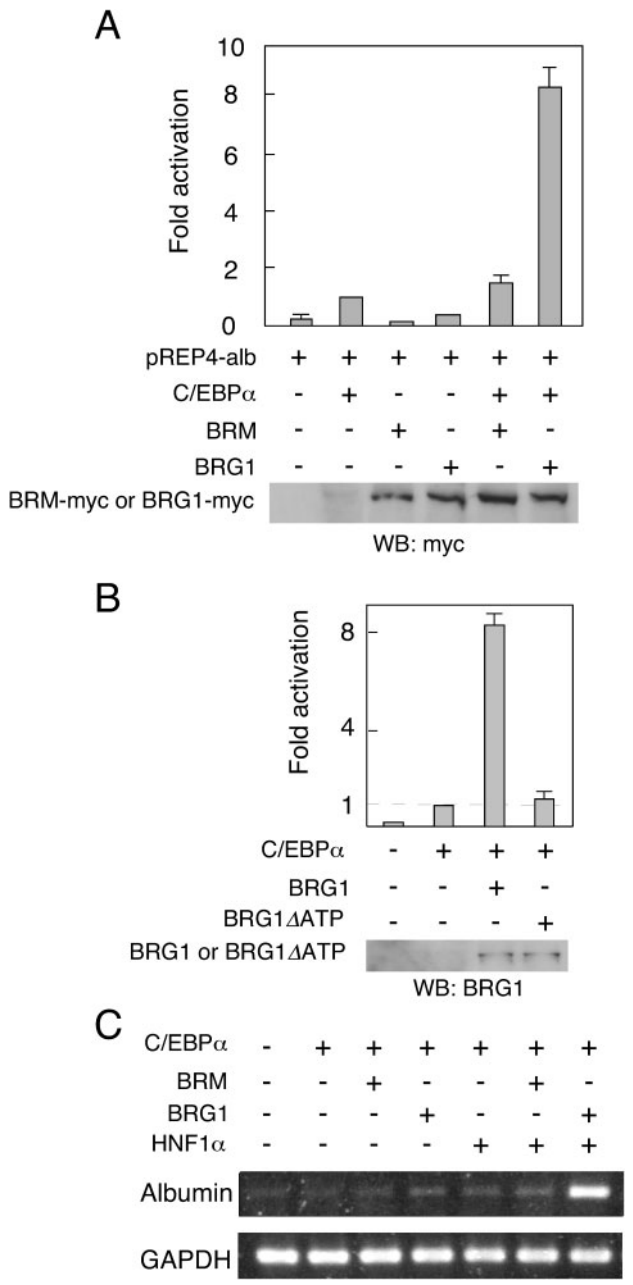

Fig. 4. **BRG1 and BRM stimulate C/EBPα-mediated transcription from the albumin promoter.** (A) The BRM or BRG1 expression vector (0.5 μg) was transiently transfected with LipofectoAMINE 2000 into SW-13 cells together with pREP4-alb luciferase reporter plasmid (0.05 μg) and the C/EBPα expression vector (0.05 μg). Reporter activation by C/EBPα alone is expressed as 1. The total amount of DNA was kept constant by adding empty expression vectors. Reporter assays were performed using the dual luciferase assay kit (Promega) according to the manufacturer's instructions. Luciferase activity was measured with a luminometer (Atto) and normalized with respect to *Renilla* luciferase activity. Expression levels of myc-tagged BRG1 and BRM are also shown. (B) SW-13 cells were co-transfected with the pREP4-alb luciferase reporter plasmid, and expression vectors for C/EBPα and BRG1 ΔATP. (C) Induction of the endogenous albumin gene from the genome by BRG1. SW-13 cells were transfected with expression plasmids as indicated above the figure, and the activation of endogenous albumin expression was monitored by RT-PCR. As a control, GAPDH was amplified from the same samples. Representative results of triplicated experiments are shown.

GST-fused C/EBPα proteins. As shown in Fig. 5A, C/EBPα clearly enhanced the ATPase activity of BRG1. By counting the radioactivity of these spots, the ATPase activity was found to be enhanced by about 2.5-fold (Fig. 5B). The reaction containing only C/EBPα also showed a small amount of ATP hydrolysis that may be caused by natural degradation or a co-purifying contaminant from *E. coli*, although GroEL was removed. This result suggests that C/EBPα not only recruits the SWI/SNF chromatin remodeling complex, but also stimulates its ATPase activity in the albumin promoter region, which may enhance the remodeling activity.

Both BRM and BRG1 Contribute to the Expression of the Albumin Gene during Hepatocyte Differentiation—In order to clarify whether both BRG1 and BRM are important for albumin expression in hepatocytes, an RNA interference experiment was performed. BRM and BRG1 siRNAs were used for adult and fetal hepatocytes, respectively. As shown in Fig. 6A, these siRNAs effectively repressed the expression of BRM and BRG1. Albumin expression was significantly repressed when BRM or BRG1 siRNA was added to adult and fetal hepatocytes (Fig. 6B). This result indicates that BRG1 facilitates the expression of albumin in fetal hepatocytes, and that BRM also plays an important role in adult hepatocytes. In these experiments, hepatocytes were cultured for 3 or 4 days. During that time, the expression levels of albumin and C/EBPα showed certain changes depending on the cell-isolation stage as reported previously (*33*). For instance, albumin secretion from E17 fetal hepatocytes increased 3 to 4 fold during the first 3 days of culture, and then decreased. Therefore, it can not be concluded that the experiments shown in Fig. 6 completely reflect physiological conditions. However, it is reasonable to assume that BRG1 and BRM are required for albumin expression.

Retinoblastoma Protein Enhances the Stimulation by BRG1 on the Albumin Promoter—We next constructed several deletion mutants of BRG1 and examined their effects on transactivation mediated by C/EBPα. These mutants contained deletions of various sizes in the C-terminal region (Fig. 7A). Charged region of BRG1 contains C/EBPα binding site, and the function of the N-terminal region has not been clear except for binding of several transcription factors. On the other hand, C-terminal region contains various functional domains. Therefore, we concentrated on C-terminal region in the deletion analyses. Furthermore, we constructed a chimera BRG1 (BRM 1–529/BRG1 540–1614) in which N-terminal and a part of charged regions were exchanged to those of BRM, and investigated the activity by luciferase reporter assay. Since the chimera BRG1 enhanced C/EBPα-mediated transactivation of the albumin promoter to the same level as wild type BRG1 (data not shown), we assumed that the N-terminal region of BRG1/BRM does not contain functional domain for the enhancement of C/EBPα-mediated transactivation and excluded the deletion of the N-terminal region of BRG1. Luciferase reporter assays showed that BRG1 mutants lacking a bromodomain still had a significant stimulative effect on C/EBPα-mediated transcription. Since the bromodomain is known to mediate the binding of BRG1 to acetylated histones (*43*), this binding is not required for the enhancement by BRG1 in the luciferase reporter assay. Similarly, no reduction in stimulative activity was observed when the

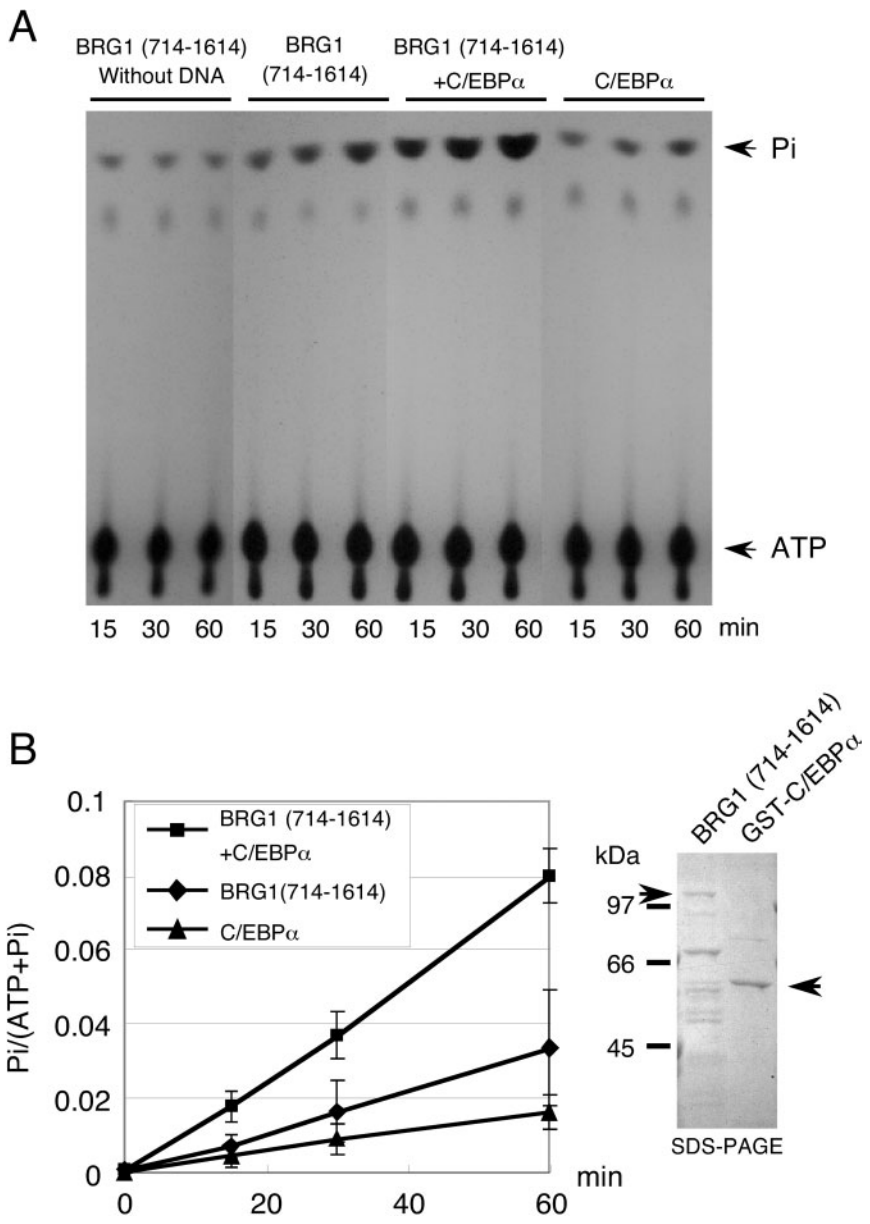

Fig. 5. **C/EBPα stimulates the ATPase activity of BRG1.** (A) *In vitro* ATPase assay of BRG1 expressed in *E. coli*. Purified histidine and HSV-tagged BRG1 (714–1614) protein were mixed with [γ-^{32}P]ATP in the presence or absence of GST-fused C/EBPα protein, and the reaction products were separated on PEI-cellulose. Arrowheads show ATP and released phosphate. The reaction was stopped by the addition of 0.5 M EDTA at 0, 15, 30 and 60 min. (B) ATPase activity was estimated as the ratio of released phosphate to ATP plus phosphate. The spots on the TLC plate were cut out and the radioactivity was measured in a liquid scintillation counter. SDS-PAGE of the purified proteins is shown to the right of the graph.

KR domain was deleted. Since it has been reported that HMGI/Y binds to this domain (*44*), the binding of HMGI/Y through this domain seems not to be prerequisite for the stimulation by BRG1. On the other hand, a mutant lacking the E7 domain in which aa1309–1614, including an LXCXE motif, were deleted could not activate the albumin promoter (Fig. 7B).

Since the LXCXE motif is a putative pRB-binding site (*20*, *45*), the transcriptional regulation is possibly mediated by the tumor suppressor protein. Alternatively, the mutant may have lost its ATPase activity. To distinguish between these possibilities, we performed ATPase assays using purified His-tagged pRB and BRG1 (714–1614). Purified BRG1 (714–1614) showed DNA-dependent ATPase activity, but BRG1ΔE7 (714–1308) had weak or negligible activity (Fig. 8A). This suggests that the deletion in ΔE7 (306 aa from C-terminal) caused a conformational change that resulted in inactivation. Alternatively, it is also possible that aa1309–1372 play an essential role in ATPase activity in addition to pRB binding. To confirm this, a structural analysis of this ATPase is required.

As shown in Fig. 8A, pRB enhanced the ATPase activity more than two-fold. A similar enhancement was observed when the GST-fused pRB pocket domain was used (data not shown). Moreover, we also observed that the ATPase activity of BRM was stimulated by the pRB protein (data not shown). In the ATPase assay, the purity of BRG1 proteins seemed not to be enough. However, the band patterns of contaminated proteins were similar between BRG1 (714–1614) and BRG1ΔE7 (714–1308) (Fig. 8A). Since the ΔE7 mutant containing the similar contaminants did not show ATPase activity, the ATPase activity measured in this study seemed to be come from the BRG1 protein.

We then investigated the effects of RB family proteins on C/EBPα-mediated transactivation and its enhancement by

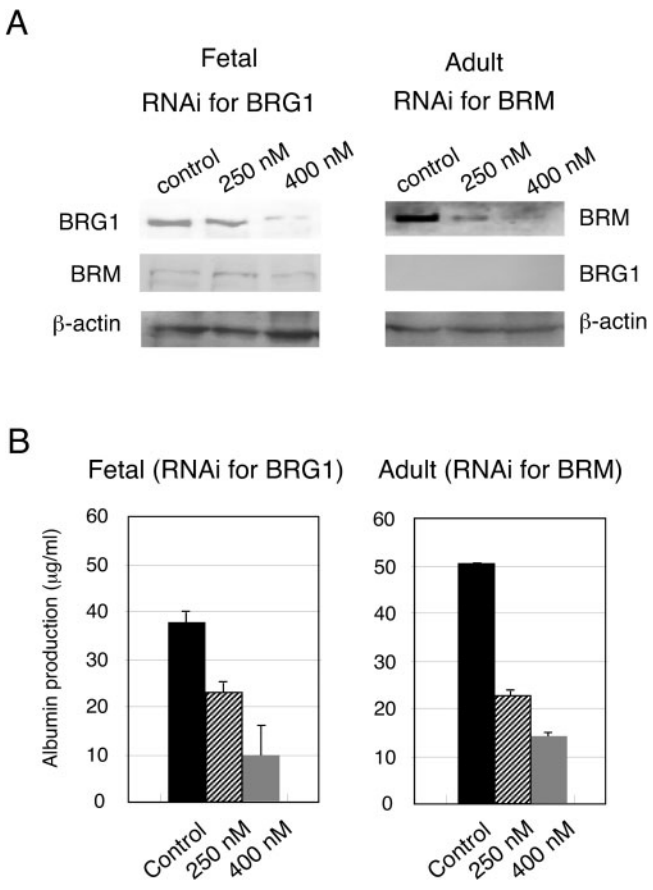

Fig. 6. **Knock-down of BRG1 and BRM by siRNA represses albumin expression in cultured hepatocytes.** Hepatocytes were cultured without serum. (A) Effects of siRNA duplexes on BRM and BRG1 expression. siRNAs were transfected into hepatocytes, and BRM and BRG1 were analyzed by Western blotting. As a control, scrambled siRNA was used at 400 nM. The blotting membrane was re-probed with anti-actin antibody to confirm the equal loading of total protein. (B) Production of albumin by hepatocytes treated with siRNA duplexes. Culture supernatants were collected and albumin production was measured by ELISA (*33*).

BRG1 with luciferase reporter assays. For this, expression plasmids for BRG1, BRM, C/EBPα, and RB family members including pRB and p130 were co-transfected into C33A pRB-deficient cells together with a luciferase reporter plasmid containing the albumin promoter. As shown in Fig. 8B, transactivation of the albumin promoter by C/EBPα and BRG1 in C33A cells was lower than that in SW-13 cells (compare Figs 4A and 8B). This phenomenon is probably caused by the absence of pRB. In fact, pRB and p130 promoted the enhancement of C/EBPα-mediated transcription by BRG1 in C33A cells. pRB alone slightly activated C/EBPα-mediated transactivation. A similar enhancement was also observed with BRM and p130. Consequently, the pRB protein may directly affect the remodeling activity of BRG1 and upregulate the transcription of the albumin promoter.

As shown in Fig. 8C, the expression pattern of pRB during liver development is similar to that of BRG1, and the expression level of p130 increases gradually. No p107, another member of the RB family, was detected throughout liver development. Co-immunoprecipitation assays using C33A cells transfected with expression plasmids for BRG1, BRM and pRB or p130 revealed that both BRG1 and BRM can bind to pRB and p130 (data not shown) as previously described (*46*, *47*). Therefore, it is suggested that SWI/SNF (BRG1)-pRB/p130 and SWI/SNF (BRM)-p130 activate or maintain the expression of albumin in fetal and adult hepatocytes, respectively. In fact, ChIP assays revealed that pRB and p130 bind to the albumin promoter in fetal and adult hepatocytes, respectively (Fig. 8D). These results suggest that the SWI/SNF complex containing BRG1 and pRB plays an important role in albumin gene expression at an early stage of liver development, and that the BRM complex and p130 may take over this role at a later stage.

DISCUSSION

To date it has been reported that C/EBPβ and C/EBPα form a complex with BRM and activate myeloid and adipocyte-specific genes, respectively (*23*, *24*). In this study, we first examined the interaction between C/EBPα and BRG1 or BRM in primary hepatocytes. Indeed, co-immunoprecipitation assays showed interaction between the C/EBPα and BRM or BRG1 under physiological conditions. This observation indicates that BRM and BRG1 may play an important role in C/EBPα-mediated gene regulation in liver cells, as reported in myeloid cells and adipocytes. Previous studies had demonstrated the parts of the C/EBPs (TEIII in C/EBPα and CR1 in C/EBPβ) essential for the interaction with BRM (*23*, *24*), but did not show essential binding domains in BRM and BRG1. It is important to identify which domain of BRM and BRG1 is needed for the interaction between the proteins since BRG1 and BRM interact physically with a wide variety of proteins. GST-pull down assays using BRG1 and BRM domains translated *in vitro* and GST-fused C/EBPα, and co-immunoprecipitation assays using cells overexpressing C/EBPα and a portion of BRM revealed that the central helicase domain containing the ATP-binding motif and the charged domain interacted specifically with C/EBPα. We also observed the same result for BRG1.

Many transcription factors, such as zinc finger proteins, interact with the unique N-terminal region of BRG1 (*48*), and several nuclear proteins, such as pRB and HMGI/Y, bind to the C-terminal regions of BRG1 and BRM (*20*, *44*). In this regard, C/EBPα is unique. If C/EBPα binds near the ATP-binding motif, the interaction might affect the ATPase activity of BRM and BRG1. To test this hypothesis, we measured ATPase activity in the presence or absence of C/EBPα, and found the ATPase activity of BRG1 to be obviously enhanced by C/EBPα. C/EBPα probably interacts with the proximal region of the ATP-binding motif of BRG1 or BRM to facilitate the ATPase activity by inducing a conformational change. Recently, the histone methylation enzyme CARM1 was reported to stimulate the ATPase activity of BRG1 (*49*). Since this protein also binds to the helicase regions of BRG1, including central helicase domain, a similar mechanism may cause the ATPase-stimulating effects of these proteins. The interaction of BRG1 with these proteins through the central helicase

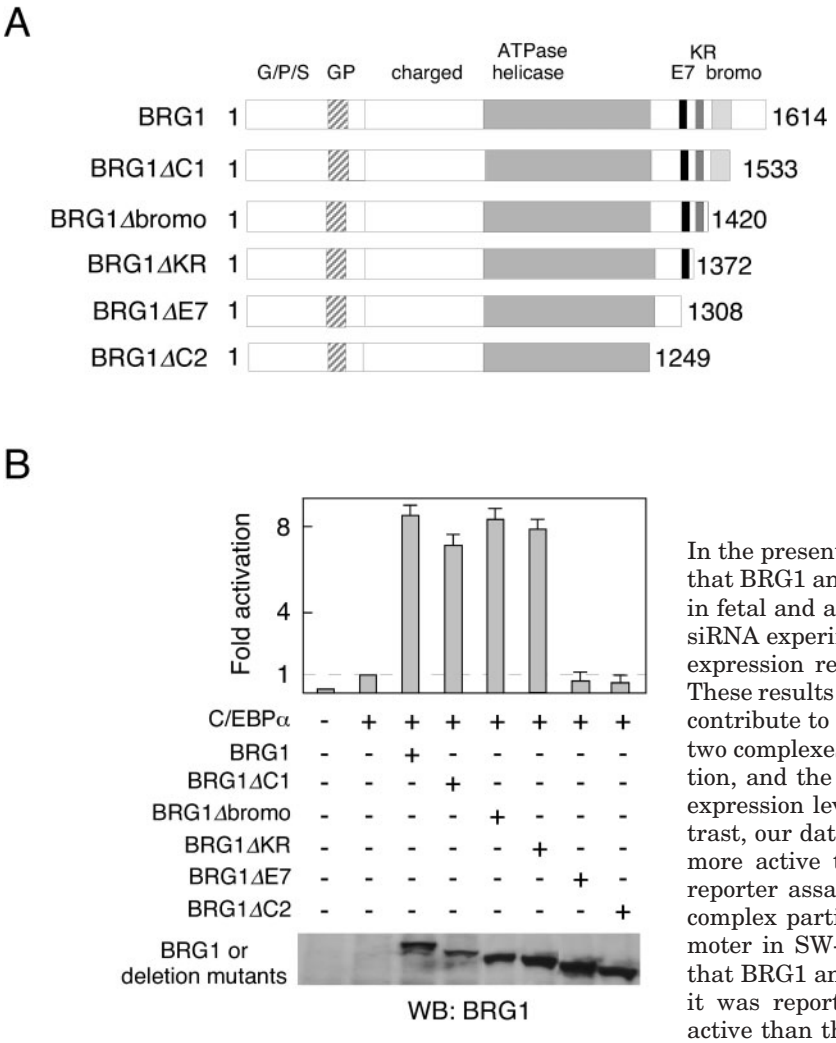

Fig. 7. **BRG1 requires its C-terminal LXCXE region to facilitate the C/EBPα-mediated transcription of the albumin promoter.** (A) A schematic representation of the BRG1 derivatives used in the luciferase assays. (B) SW-13 cells were co-transfected with pREP4-alb luciferase reporter plasmid and expression vectors for C/EBPα, BRG1 and the BRG1 deletion mutants. The expression levels of BRG1 and its derivatives are also shown.

regions presumably provides the physical basis for the modulation of its ATPase activity.

In this study, we found that the main portion of the BRG1-based SWI/SNF complex was replaced by a BRM-based complex during liver development. We previously reported a similar change in the ATPase subunit of SWI/SNF during neural development (*31*). Studies on the expression of these two ATPase subunits under growth-inhibiting or growth-inducing conditions indicated that the level of BRM increased in cells that had withdrawn from the cell cycle and decreased in cells that were actively dividing. Based on these results, it was proposed that BRM negatively regulates cell growth (*50*, *51*). Our results with hepatocytes are in good agreement with the previous observations, since fetal hepatocytes grew actively and adult hepatocytes merely grew under our culture conditions.

However, at present, it is still not clear why there are two distinct ATPase subunits, BRG1 and BRM, in the SWI/SNF complex, and whether the replacement of BRG1 with BRM is physiologically important for the expression of liver-specific genes. In particular, a previous study has indicated that BRG1 and BRM bind to different classes of transcription factors and can be present on different promoters (*48*), supporting the idea of different functions. In the present study, however, the ChIP analysis revealed that BRG1 and BRM are present on the albumin promoter in fetal and adult hepatocytes, respectively. Furthermore, siRNA experiments showed that blocking BRG1 and BRM expression resulted in a reduced production of albumin. These results suggest that both BRG1 and BRM complexes contribute to the production of albumin; alternatively, the two complexes could be redundant, at least partly, in function, and the switch in two complexes simply reflects the expression levels of the BRM and BRG1 proteins. In contrast, our data also show that the BRG1-based complex is more active than the BRM-based complex in luciferase reporter assays and that the BRG1- but not BRM-based complex partially activates the endogenous albumin promoter in SW-13 non-hepatic cells, supporting the notion that BRG1 and BRM have different functions. Previously, it was reported that the BRG1-based complex is more active than the BRM-based complex in nucleosome remodeling (*12*). The data presented here seem to be consistent with such results.

The role of SWI/SNF in mediating pRB-dependent cell cycle inhibition has been elucidated (*20*). For instance, pRB-SWI/SNF suppresses growth-related genes such as *cyclin E*, *cyclin A* and c-*fos* (*52*, *53*). Furthermore, C/EBPα has been reported to require the SWI/SNF complex for proliferation arrest (*54*). In addition, it was recently reported that the C/EBPα-pRB-E2F4-BRM complex represses the proliferative response in livers of aged animals after partial hepatectomy (*55*). In contrast, it is known that pRB activates the transcription of several differentiation-related genes (*56*, *57*). Furthermore, the literature reveals that BRG1 and/or BRM can interact physically with a number of transcriptional regulatory proteins and activate the transcription of cellular differentiation genes (*16*, *23*, *25–27*). In the present study, we found that pRB family proteins, especially pRB and p130, stimulate the function of SWI/SNF in the C/EBPα-mediated expression of the albumin gene. The ATPase assays conducted here indicate that pRB enhances the ATPase activity of BRG1. The enhancement of the ATPase activity, which may lead to an increase in remodeling activity, is likely to be a major mechanism behind the cooperation between BRG1 and pRB for the efficient expression of the albumin gene.

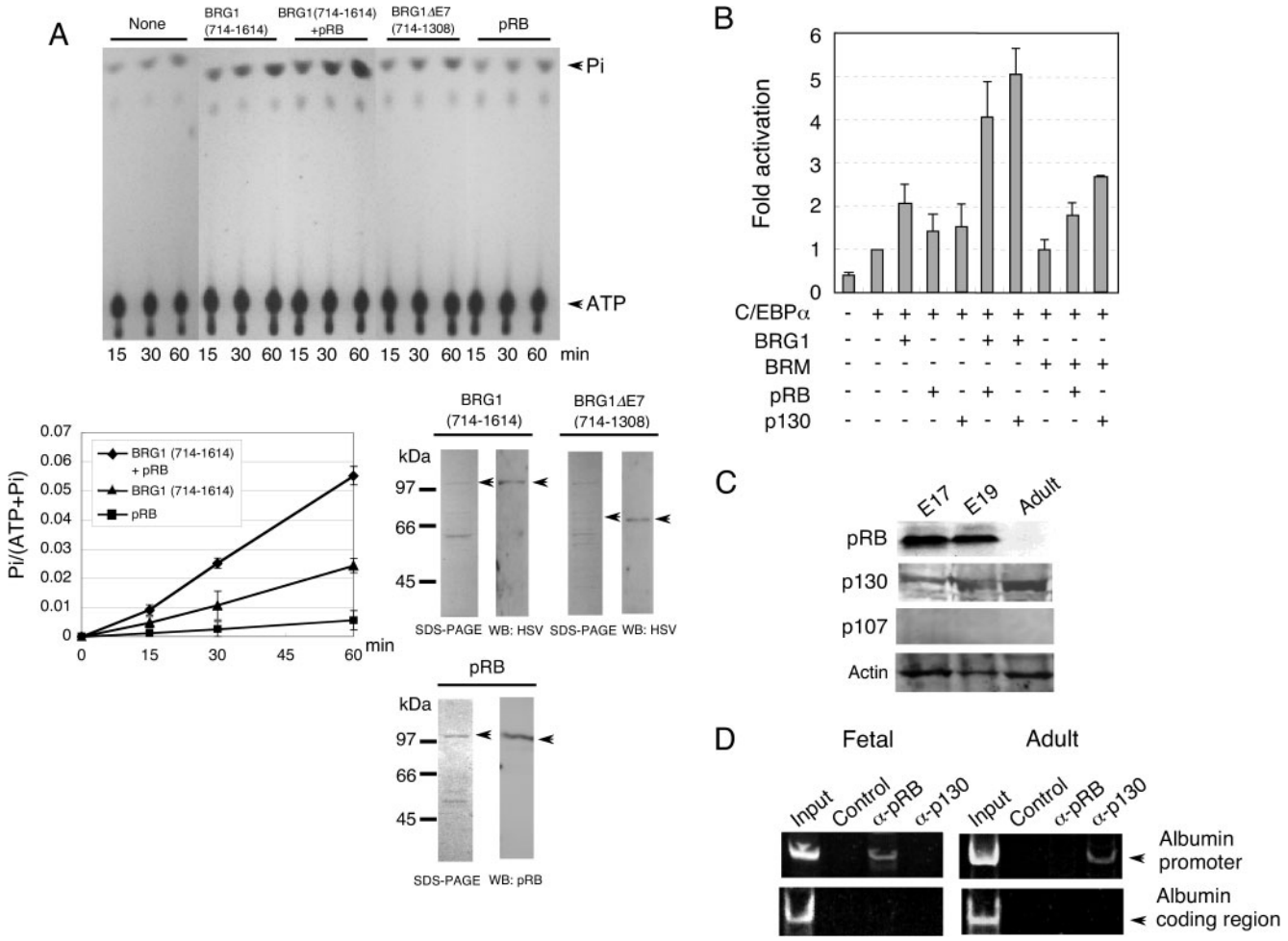

Fig. 8. **RB family proteins enhance the stimulating effect of BRG1 on C/EBPα-mediated transcription of the albumin promoter.** (A) ATPase activity of BRG1 in the presence and absence of pRB. SDS-PAGE and Western blotting using anti-pRB antibody for pRB and anti-HSV antibody for His-HSV–tagged BRG1 (714–1614) were performed to confirm the identities of the purified proteins. Arrowheads show purified proteins. (B) C33A cells were co-transfected with the pREP4-alb luciferase reporter plasmid (0.05 μg), and expression vectors for C/EBPα (0.05 μg), BRG1 (0.2 μg) or BRM (0.2 μg) and RB family proteins (pRB, p130) (0.4 μg) in various combinations. (C) Changes in expression levels of RB family proteins during liver development. (D) ChIP analysis of the albumin promoter in fetal and adult hepatocytes with anti-pRB and anti-p130 antibodies. Input (2%) was used as a positive control. Representative results of triplicated experiments are shown.

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