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Variations in the Composition of Mammalian SWI/SNF Chromatin Remodelling Complexes

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ABSTRACT

The ATP-dependent chromatin remodelling complexes SWI/SNF alter the chromatin structure in transcriptional regulation. Several classes of mammalian SWI/SNF complex have been isolated biochemically, distinguished by a few specific subunits, such as the BAF-specific BAF250A, BAF250B and BRM, and the PBAF-specific BAF180. We have determined the complex compositions using low stringency immunoprecipitation (IP) and shown that the pattern of subunit interactions was more diverse than previously defined classes had predicted. The subunit association at five gene promoters that depend on the SWI/SNF activity varied and the sequential chromatin immunoprecipitations revealed that different class-specific subunits occupied the promoters at the same time. The low-stringency IP showed that the BAF-specific BAF250A and BAF250B and the PBAF-specific BAF180 co-exist in a subset of SWI/SNF complexes, and fractionation of nuclear extract on size-exclusion chromatography demonstrated that sub-complexes with unorthodox subunit compositions were present in the cell. We propose a model in which the constellations of SWI/SNF complexes are ''tailored'' for each specific chromatin target and depend on the local chromatin environment to which complexes and sub-complexes are recruited. J. Cell. Biochem. 108: 565–576, 2009.

KEY WORDS: BRG1; BRM; BAF PROTEINS; CHROMATIN REMODELLING

Dynamic alterations of the chromatin structure are prerequisites for transcription to occur in eukaryotic cells. These alterations, which regulate DNA accessibility, are caused by both post-translational modification and structural alterations of the smallest unit, the nucleosome. One group of protein complexes that catalyse structural changes is the ATP-dependent chromatin remodelling complexes [reviewed in Mohrmann and Verrijzer, 2005; Hogan and Varga-Weisz, 2007; Wang et al., 2007]. The complexes are defined by their ATPase subunit: SWI/SNF complexes (contain a SWI2/SNF2 ATPase), ISWI-containing complexes, Mi-2 (CHD) containing complexes and INO80 complexes. The different groups of complex are involved in different processes in the nucleus, although all complexes play a role in transcriptional regulation.

Abbreviations used: SWI/SNF, mating type switching/non-sucrose fermenting; BRG1, brahma related gene 1; BRM, brahma; ISWI, imitation of SWI; CHD, chromodomain; BAF, BRG1-associated factor; BRD7, B-cell leukaemia protein protein 7, CSF1, colony stimulating factor 1, CRYAB, α-B crystallin; ECM1, extracellular matrix protein 1; SPARC, secreted protein acidic cystein rich (osteonectin); TAGLN, transgelin (SM22); THBS, thrombospondin 1; PAI1, plaminogen-activator-inhibitor 1; PBAF, polybromo associated factor; BAP, brahma associated protein; PBAP, polybromo associated protein; HAT, histone acetyl transferase; HDAC, histone deacetylase; HMT, histone methyl transferase; IP, immunoprecipitation; ChIP, chromatin immunoprecipitation, SDS; sodium dodecylsulphate; PMSF, phenyl–methyl sulphonylfluoride; DTT, dithiothreitol; TSA, Trichostatin A; 5-AZA-dC, 5-azo-2′-deoxycytidine.Jessica Ryme and Patrik Asp contributed equally to the work.

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The SWI/SNF complexes are mainly associated with transcriptional activation or repression and functions at the promoter. The complexes are present in all eukaryotic species investigated and are highly conserved between species, both in the sequence of individual subunits and in overall subunit composition. The mammalian paralogues brahma-related gene 1 (BRG1) and brahma (BRM), orthologues to the yeast Swi2/Snf2 proteins and the *Drosophila* brahma, provide the ATPase activity, while other subunits act as regulators or modulators of activity, mediating interactions with components of the transcription machinery [Phelan et al., 1999]. A minimal mammalian SWI/SNF complex with the same enzymatic activity as that of the native complex has been reconstituted in vitro using recombinant proteins [Phelan et al., 1999]. The mammalian SWI/SNF complex is thus organised around a central remodelling core that consists of the ATPase BRG1 or BRM and the modulating subunits BAF155 (BRG1-associated factor), BAF170 and INI1/SNF5/BAF47. The constitutive SWI/SNF subunit INI1/SNF5/BAF47, however, is essential neither for the integrity of mammalian SWI/SNF complexes nor for the activation of several genes whose expressions depend on SWI/SNF [Doan et al., 2004]. Similarly, the *Drosophila* orthologue of INI1/SNF5/BAF47, SNR1, is not essential for all functions of the SWI/SNF complexes [Zraly et al., 2003].

SWI/SNF complexes are involved in both the activation and the repression of gene expression [Mohrmann and Verrijzer, 2005; Hogan and Varga-Weisz, 2007; Wang et al., 2007]. The effect varies significantly: some genes are absolutely dependent on SWI/SNF for expression, others require SWI/SNF only for maximal induction, and others require SWI/SNF complexes for repression [Liu et al., 2001]. The functions of each subunit in the SWI/SNF complexes are still unclear, although some subunits favour the recruitment of co-repressors under certain conditions [Nagl et al., 2007; Zhang et al., 2007]. Subunits also have domains that are responsible for the interaction with different nuclear proteins, such as domains in the subunits BAF250A, BAF60A and BAF57 that interact with nuclear receptors [Belandia et al., 2002; Inoue et al., 2002]. The two homologues BAF155 and BAF170 are scaffolding proteins that regulate the protein level of other subunits, such as INI1, BAF60A, BAF57 and BRG1, by protecting them from degradation by the proteasome [Chen and Archer, 2005; Sohn et al., 2007]. Furthermore, Zhang et al. [2007] showed that the BAF155 and BAF170 subunits are differently recruited to genes regulated by nuclear receptors, resulting in different responses. BAF155 is involved in repression, and recruits histone deacetylases (HDACs) and mSin3, whereas both subunits are involved in the recruitment of histone acetyl transferases (HATs), such as p300 [Zhang et al., 2007]. Distinct function other than parts of the SWI/SNF complexes has also been attributed to other core subunits than INI1, such as the BAF57 and BRG1 in thymic development [Chi et al., 2002].

Several subclasses of the SWI/SNF complexes have been described based on biochemical purifications. One variation in mammalian cells, giving at least two separate complexes, is the mutually exclusive presence of the BRG1 and BRM ATPases [Wang et al., 1996; Sif et al., 2001]. The function of BRG1 and BRM are in many cases redundant, but examples of functional differences have been found. Processes early in development are clearly dependent on BRG1 [Griffin et al., 2008], as is the self-renewal in neuron development [Machida et al., 2001; Wu et al., 2007]. BRM is expressed later in development, and is involved in differentiation [Inayoshi et al., 2006; Yan et al., 2008]. Kadam and Emerson [2003] showed that these two proteins interact with different kinds of transcription factor: BRG1 interacts with zinc finger proteins while BRM interacts with proteins having ankryn motif, which may provide specificity to these two complexes. Other variations are the BAF and PBAF constellations, which are evolutionarily conserved, PBAP and BAP in *Drososphila* and SWI/SNF and RSC in yeast [Mohrmann and Verrijzer, 2005]. These subclasses share a common core of proteins, including one ATPase, and are distinguished by specific proteins, ''signature subunits,'' that direct the SWI/SNF core to specific genes. The signature subunits in BAF/dBAP are either BAF250A or B/dOSA, and the subunits BAF180/d-polybromo and BAF200/dBAP170 in the PBAF/dPBAP [Xue et al., 2000; Lemon et al., 2001; Yan et al., 2005]. In *Drosophila*, the ''signature subunits'' of the two subclasses are distributed at partially different sites on polytene chromosomes, and they regulate both distinct and overlapping genes [Moshkin et al., 2007]. The *Drosophila* protein SYAP is a further PBAP-specific subunit, and it is required for the stability of the PBAP complex [Chalkley et al., 2008], while BRD7 is a novel PBAF-specific mammalian subunit [Kaeser et al., 2008]. Most genes and transcription factors do not exhibit a clear preference between the complexes [Mohrmann and Verrijzer, 2005; Hogan and Varga-Weisz, 2007; Wang et al., 2007], but more and more genes are reported to be dependent on one type of subunit or complex. Knock-down of specific subunits in cells or cells from knock out mice show different expression patterns, although a clear BAF and PBAF distinction have not been seen. The PBAF subunit BAF200 regulates interferon-dependent genes, without requiring BAF180 [Yan et al., 2005].

In addition to the ''signature subunits,'' isoforms exist of many subunits, such as BAF250A and B, in mammalian cells, and these are present in separate BAF constellations [Nie et al., 2003; Nagl et al., 2007; Yan et al., 2008]. The isoforms are sometimes expressed in a tissue-specific manner or at different stages during development, which gives rise to tissue-specific SWI/SNF complexes. This has lead Lessard et al. [2007] to propose a combinatorial assembly mechanism. This model suggests that stable complexes of the available subunit isoforms are assembled in each cell, resulting in several different SWI/SNF variants. The neuronal-specific SWI/SNF complex (nSWI/SNF), which contains the neuronal-specific BAF53b and BAF45b subunits [Lessard et al., 2007; Wu et al., 2007] and directs the complex to specific neuronal genes, would then be formed as a result of the specific expression of these subunits in post-mitotic neuronal cells. However, some isoforms are co-expressed and have interchangeable functions, calling for additional assembly mechanisms to form functional SWI/SNF complexes. It has also been shown that only core subunits are required for some functions of SWI/SNF complexes [Carrera et al., 2008].

In order to further understand the formation of multiprotein SWI/SNF complexes, we have analysed the protein composition of SWI/SNF complexes in HeLa cells and in the BRG1-deficient cell line SW13 using different purification conditions. We discovered that the plasticity is greater than previously shown, and we have shown

by immunoprecipitation (IP) analysis of SWI/SNF subunit that an association between the specific "signature subunits" BAF250 and BAF180 is present. Chromatin immunoprecipitations (ChIPs) also show that the components recruited to genes do not follow the definition of complexes based on biochemical purifications, but vary according to gene. We propose a model in which part of the heterogeneity in SWI/SNF complex composition is a result of function; complexes and sub-complexes with the subunits required are recruited to the local chromatin environment, which then has a significant impact on the composition and subsequent function of mammalian SWI/SNF complexes.

MATERIALS AND METHODS

CELL CULTURE

HeLa cells and SW13 cells were grown in Dulbecco's Modified Eagle's Medium (Hyclone) supplemented with 5% foetal calf serum and 0.06 mg/ml penicillin/streptamycin under a 7% CO_2 atmosphere.

ANTIBODIES

The following antibodies were used in immunoblots and IPs: Anti-BRG1, two rabbit (against the C- and N-terminals) and mouse [Wang et al., 1996; Östlund Farrants et al., 1997] (EuroMedex), anti-BRM [Sif et al., 1998] (Abcam, Bethyl Laboratories, A301-015A), anti-BAF47/INI1 [Doan et al., 2004] (Santa Cruz sc-16189), anti-Baf250/Osa 1/2 [Inoue et al., 2002] (Bethyl Laboratories A301-046A), anti-ARID1 (Abcam), anti-BAF180 [Xue et al., 2000] (Bethyl Laboratories A301-590A and A301-591A), anti-Polybromo [Nicolas and Goodwin, 1996], anti-BAF170 (Santa Cruz sc-10757), anti-BAF155 (Shanahan et al., 1999), anti-BAF57 [Belandia et al., 2002], anti-RNA polymerase II CTD (Abcam), anti-β-actin (Sigma ac-15), anti-pan-actin (Sigma ac-AC40), anti-pan-actin (Santa Cruz sc-7210) and anti-SNF2h (Abcam).

IMMUNOPRECIPITATION (IP)

HeLa crude nuclear extract was prepared from adherent cells as follows: Cells were harvested and collected by centrifugation at 500*g* for 5 min at 4°C. The cells were then suspended in five pellet volumes of hypotonic homogenising buffer (H-buffer) (20 mM HEPES at pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT supplemented with 0.5 mg/ml each of aprotinin, antipain, chymostatin and leupeptin, 10 μM benzamidine, 1 μg/ml phenantroline, 5 μg/ml pepstatin A and 1 mM PMSF), incubated on ice for 15 min and homogenised using 30 strokes in a Dounce homogeniser, pestle B. Nuclei were collected by centrifugation at 500*g* for 15 min at 4°C, resuspended in NE-buffer (0.42 M KCl) and incubated on ice for 30 min. The extract was centrifuged for 30 min at 16,000*g* in a microfuge. Crude HeLa nuclear extract (200–300 μg) was quickly diluted to 0.15 M KCl with dilution buffer (NE-buffer without KCl, supplemented with 0.5 mg/ml each of aprotinin, antipain, chymostatin and leupeptin, 10 μM benzamidine, 1 μg/ml phenantroline, 5 μg/ml pepstatin A and 1 mM PMSF), adjusted to 0.1 mM ATP, and incubated overnight with the indicated antibodies at 4°C; after which 15 μl of a 50% slurry of protein A/G Sepharose was added, and the samples were incubated for an additional hour at 4°C. The beads were washed six times with 1 ml batches of wash buffer (NE-buffer with 0.2 M KCl, supplemented with 1 mM PMSF), after which the beads were resuspended in 15 μl of sample buffer and separated on a 5–15% SDS–PAGE gradient gel.

PURIFICATION OF SWI/SNF COMPLEXES

Crude nuclear extract was prepared as described above and immediately frozen at −80°C until used. SWI/SNF complexes were purified as described by Lemon et al. [2001] with the following modifications: The POROS columns were substituted with the equivalent MonoQ and Heparin columns (GE Healthcare), and the final MonoS column (GE Healthcare) was excluded. ATP (0.1 mM) was included in all buffers. All buffers also contained 1 mM dithiothreitol (DTT) and 1 mM PMSF, while 0.1 mg/ml bovine serum albumin was added to the buffer in the final Superose 6 HR step.

MONONUCLEOSOME DISRUPTION ASSAY

The mononucleosome disruption assay was performed as previously described [Östlund Farrants et al., 1997] with the following modification: The DNA probe was synthesised by PCR using a 5′ primer that had been labelled with [^{32}P] by T4 polynucleotide kinase according to the manufacturer's instructions (Invitrogen).

RNA PREPARATION AND cDNA FORMATION

RNA was prepared by Trizol (Invitrogen) extraction and converted to cDNA using Superscript III (Invitrogen) with random primers. The primers used for the detection of expressed genes were: CSF1: 5′-GTCATATGTTGAGCCTGTGG, 3′-GGCTACGGAGATGACAGAAT, CRYAB: 5′-ATGGACATCGCCATCCAC, 3′-CTATTTCTTGGGGGCTGC, ECM1: 5′-CCGAATTCTGGGAGGAAG, 3′-CTGGATCCGGCCTTCCA-TGTA, TAGLN: 5′-TTCCCCAGCCCCTTGCCCCTC, 3′-GGCAGGCT-GGGCTGGTTCTTC, SPARC: 5′-AGGGCCTGGATCTTCTTT, 3′-AAG-ATCCTTGTCGATATCCTT, and actin: 5′-GGACTTCGAGCAAGAG-ATGG, 3′-AGCACTGTGTTGGCGTACAG, THBS: 5′-ACAACTGCC-TGCCCTGCCCC, 3′-TGATGCCATTGCCAGCGTAGCC: Nodal: 5′-CTT-CTCCTTCCTGAGCCAACAAGAGG, 3′-GGTGACCTGGGACAAAGT-GACAGTG, p21: CCGAAGTCAGTTCCTTGTGG, 3′-GCCATTAGCG-CATCACAG, PAI1: 5′-ACGAACCGCCAATCGCAAGG, 3′-CTGCGCC-ACCTGCTGAAACA.

Prior to the RNA preparation, HeLa cells and SW13 cells were treated with 10 μM Trichostatin A (TSA) for 18 h or with 10 μM 5-aza-2′-deoxycytidine (5-AZA-dC) for 4 days.

CHROMATIN IMMUNOPRECIPITATIONS (ChIP)

ChIP analyses were performed as described by Takahashi et al. [2000] and repeated in five independent experiments. The IP was performed in 1% Triton-100, and 0.1% DOC, to which 15 μl protein A/G was added. The IP was subsequently washed six times with RIPA buffer containing NaCl. The primers for the genes were: CSF1: 5′-GGAGGGAAAGTCCCTTGGG, 3′-TAAGAGAGGACTGGGGGGC, CRYAB: 5′-GGCCCAAGATAGTTGCTGGC, 3′-CTTCAGCTGGAGGT-AGAGCC, ECM1: 5′-GCTGTAAACTCCTTTGTGCCC, 3′-AATTGT-CTGGTGTGATCTCCC, TAGLN: 5′-CAGTGAAGTAGGAGCAGCCG, 3′-GGGTGAGGGGTTTAAAGGGC, SPARC: 5′-TCTCCAGGCCTCA-CTTGCC, 3′-AGAGACAGGCAACAGGAAACC, THBS: 5′-GGAGA-GAGGAGCCCAGACTG, 3′-GGAATGCCTGTGCGTCCGGAG, Nodal:

5′-GGAGACTGGACAGTGAATTGC, 3′-GCCATTGTCTAGTTCTCCTGG, PAI1: 5′-CCTCCAACCTCAGCCAGACAAG, 3′-CCCAGCCCAACAG-CCACAG [Xi et al., 2008], p21: 5′-CCAGCCCTTTGGATGGTTTG; 3′-GCCTCCTTTCTGTGCCTGAAAC, and actin: 5′-CAGAAGGATTCC-TATGTGGG, 3′-TGGATAGCAACGTACATGGC.

Re-ChIP was performed as described by Young et al. [2007]. Briefly, the first antibody-antigen interaction was disrupted by adding 10 mM DTT, final concentration. The DTT was diluted 50-fold prior to addition of the second antibody. qPCR was performed using SYBR-green from KAPA according to the manufacturer's instructions, and analysed in a Corbett Rotor-Gene 6000.

PROTEIN DETERMINATION

Protein concentrations were determined using the Bradford reagent (Bio-Rad) according to the manufacturer's instructions.

RESULTS

VARIATIONS IN ASSOCIATION OF SWI/SNF SUBUNITS WITH PROMOTERS OF SWI/SNF-DEPENDENT GENES

Many genes depend on the SWI/SNF complex for expression in a process that requires binding of the components to the promoter and the subsequent remodelling of nucleosomes. Liu et al. [2001] showed that the mRNA levels of the genes CSF1, TAGLN, CRYAB, SPARC and ECM1 are induced more than twofold when BRG1 is introduced into the SW13 cell line, which is deficient in both BRG1 and BRM. We investigated the binding pattern of the SWI/SNF subunits BAF250A/B, BRG1, BRM, BAF180, BAF170, BAF155 and INI1 at the promoters of these genes in HeLa cells and SW13 cells. ChIP experiments showed that all five genes recruited SWI/SNF components to the promoter in HeLa cells (Fig. 1A). The β-actin promoter was used as a control since it is not dependent on BRG1 [Pal et al., 2003]. BRG1, BAF170 and INI1 were associated with all

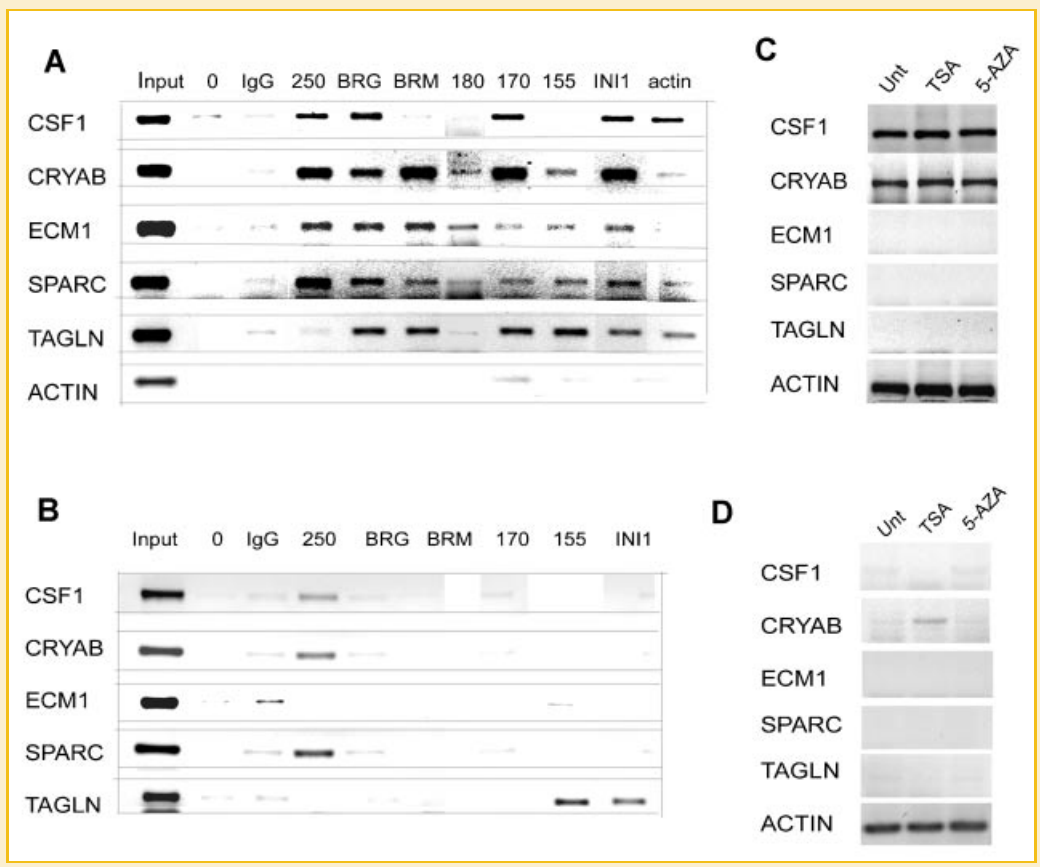

Fig. 1. ChIP analysis of SWI/SNF subunit binding to promoter regions. A: Chromatin was prepared from 10×10^6 growing HeLa cells and sheared by sonication to an average length of 500 base pairs. The antibodies used in the ChIP to precipitate DNA fragment are specified at the top, and the gene promoters detected in the PCR are indicated on the left. Chromatin-bound actin was immunoprecipitated using a pan-actin antibody (sc-7210). Input represents 0.5% of the total chromatin in each IP. Beads without antibodies are labelled NoAb and 2 μg of goat-anti-rabbit IgG antibodies were used in the mock samples, labelled IgG. B: ChIP from growing SW13. The antibodies used in the ChIP to precipitate DNA fragments are specified at the top, and the gene promoters detected in the PCR are indicated on the left. Beads without antibodies are labelled NoAb and 2 μg of goat-anti-rabbit IgG antibodies were used in the mock samples, labelled IgG. Expressions of five selected BRM and BRG1-dependent genes were investigated in cDNA converted from total RNA preparations from HeLa cells (C) and SW13 cells (D). The genes are specified on the left. Untreated cells (Unt) and cells treated with gene-expression activating agents, Trichostatin A (TSA) and 5-AZA-dC (5-AZA) are marked at the top.

five selected promoters, whereas the presence of other subunits varied (Fig. 1A). Only CSF1 displayed a binding pattern in line with a BAF-regulating gene and TAGLN in a pattern in accordance with a gene only requiring core subunits (Fig. 1A). Actin, which has been reported to be a SWI/SNF subunit [Zhao et al., 1998], was also present at these promoters, except at that of ECM1 (Fig. 1A). Furthermore, the binding of BAF155 and BAF170, both of which are regarded as core subunits, varied. This is consistent with the findings of Zhang et al. [2007], who showed that the two proteins could be recruited independently to the promoter, BAF170 with co-activators and BAF155 with both repressors and activators.

The binding patterns of SWI/SNF subunits to the five selected genes in the BRG1 and BRM-deficient SW13 cell line demonstrated that only BAF250, BAF155 and INI1 bound occasionally to the gene promoter (Fig. 1B). This clearly shows that the ATPase is crucial for the association of SWI/SNF subunits and most probably directs the other subunits to chromatin. Zhao et al. [1998] have also demonstrated that BRG1is important for the binding of SWI/SNF subunits to chromatin structures.

EXPRESSION OF SWI/SNF-DEPENDENT GENES DISPLAY CELL TYPE SPECIFICITY

Only two of the genes selected were expressed in HeLa cells: CRYAB and CSF1 (Fig. 1C), despite the fact that HeLa cells express functional SWI/SNF complexes. As expected, none of the genes were expressed in SW13 cells (Fig. 1D). There was no consistent difference in the pattern of subunits recruitment to the active or inactive genes in HeLa cells. Tumour cell lines often have an altered epigenetic state compared to normal cells, and changes in DNA methylation status may alter gene expression patterns [Zhu and Yao, 2009]. We therefore treated HeLa cells with reagents that are known to activate gene expression by changing the epigenic state. HeLa cells were treatment with a methyl-transferase inhibitor (5-AZA-dC) for 4 days, but no alteration in the expression of the genes were observed (Fig. 1C). Nor had the histone deacetylase inhibitor TSA, which leads to hyperacetylated histones, any effect on the expression of the genes (Fig. 1C). Interestingly, the CRYAB gene was expressed after TSA treatment of SW13 cells (Fig. 1D). SWI/SNF component represses genes by recruiting HDACs, but all genes were activated by BRG1 expression in SW13 cells [Liu et al., 2002], suggesting that cell type specific factors are missing in HeLa cells for these genes to be expressed.

VARIATION IN ASSOCIATION WITH PROMOTERS OF SWI/SNF SIGNATURE PROTEINS

The recruitment of the signature subunits to the five promoters selected were studied in more detail by qPCR of ChIP in which different antibodies for BAF250A and B, BRM, BAF180, and actin (Bethyl Laboratories and Abcam, see Materials and Methods Section) were used. An antibody was also added against the PBAF specific BAF200. These results corroborated the initial RT-PCR experiments, except that BAF180 was also bound to the CSF1 promoter (Fig. 2A). In addition, the two BAF250 proteins, BAF250A and BAF250B, displayed distinct binding patterns, except for CRYAB promoter to which both isoforms were associated (Fig. 2A). Only BAF200 of the two PBAF subunits were present at the TAGLN promoter, suggesting that not a complete PBAF complex was recruited. The two PBAF subunits may function separately, which has been reported for certain genes [Yan et al., 2005; Chalkley et al., 2008]. Actin also associated with all five promoters. In addition, we investigated the binding of RNA pol II, and showed that it was present on all genes, even those that were not expressed. It is possible that the binding of SWI/SNF subunits allows for the recruitment of the transcription machinery.

We investigated three other genes that have been reported to depend on specific subunits: THBS (thrombospondin 1) that requires the PBAF subunit Brd7 for repression and Nodal that requires BRG1 for repression in mouse embryonic stem cells [Kaeser et al., 2008] as well as p21 that requires BAF180 for expression [Xia et al., 2008]. The plaminogen-activator-inhibitor 1 (PAI1) gene, which recruits BRG1 at low concentrations upon TFGβ-signalling [Xi et al., 2008] were used as a control for BRG1-association. Nodal was the only gene of these four that was not expressed or exhibited low expression in HeLa cells (Fig. 2B). The association of factors at the promoter did not fully reflect the proposed requirement for expression and repression; BAF250A and B, together with the ATPases BRM and BRG1, and the two PBAF subunits BAF200 and BAF180 associated with THBS promoter (Fig. 2C), in line with a gene dependent on both BAF and PBAF for expression. The ATPases and BAF250B associated with the promoter of Nodal, which require core subunits (and maybe BAF250A) [Kaeser et al., 2008 for repression (Fig. 2C). All subunits tested associated with the p21 promoter (Fig. 2C), despite that BAF250A and B are not required for expression. The PAI1 promoter bound BRG1 as expected. BAF250A and B and BAF180 associated well, but very low levels of BAF200 were bound. This would suggest recruitment of an incomplete PBAF complex. Taken together, the association of subunits with these promoters cannot be explained by the recruitment of PBAF or BAF complexes, and the pattern points to a more complex recruitment mechanism.

SIMULTANEOUS BINDING OF SWI/SNF SUBUNITS TO PROMOTERS

The different associations of subunits with the five BRG1-dependent promoters led us to investigate whether the BAF-specific subunit BAF250 and the PBAF-specific subunit BAF180 were recruited to the promoter simultaneously. We performed Re-ChIPs in which polyclonal BAF180 antibodies [Xue et al., 2000] were first used on cross-linked chromatin, and subsequently antibodies BAF250A/B were used on the chromatin fraction precipitated. A clear signal was obtained on the ECM1 (Fig. 2D) and SPARC promoters (not shown). Similarly, BRG1 and BRM were found to associate with the SPARC (Fig. 2D) and ECM1 promoters. We conclude that BAF250 and BAF180, as well as BRG1 and BRM, are able to associate with the same promoter at the same time.

BAF250 AND BAF180 CO-IMMUNOPRECIPITATE IN LOW-STRINGENCY IPs

The different association patterns of SWI/SNF subunits observed with ChIP and the finding that the subunits BAF250A/B and BAF180, as well as BRG1 and BRM, associated with the same promoter fragment indicated that subunit interactions were more diverse than previously reported. We performed a series of IPs at

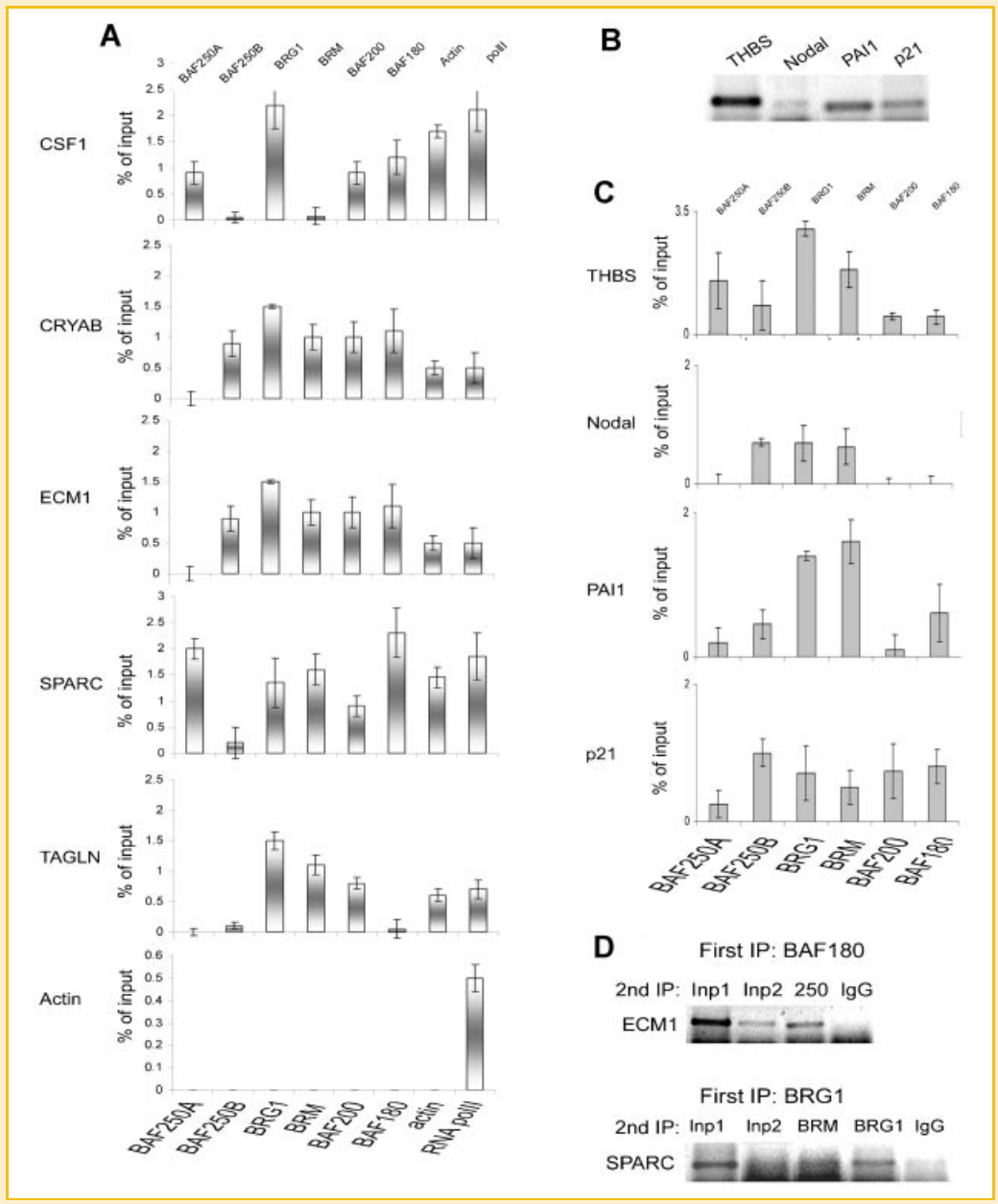

Fig. 2. ChIP analysis and re-ChIP analysis of subunits associating with BAF and PBAF-dependent genes. A: ChIP of the five selected genes analysed by qPCR when using other antibodies than those in B: anti-BAF250A, anti-BAF250B, and anti-BAF180 (Bethyl Laboratories), anti-BAF200 and anti-BRM (Abcam), anti-actin (Sigma-AC40) and anti-RNA polymerase IICTD (Abcam). The results are presented as percent of input sample for each gene. The antibodies are specified below and above the graphs, and the genes defined to the left of each graph. Standard deviation is from triplicates of three separate experiments. B: Expression of the BAF or PBAF specific genes THBS, Nodal, p21 and the control PAI1 from RNA prepared from HeLa cells and converted to cDNA. The genes are specified above the lanes. C: ChIP of the THBS, Nodal, p21 and PAI promoters analysed with qPCR. The results are presented as percent of input sample for each gene. The antibodies are specified below and above the graphs, and the genes defined to the left of each graph. Standard deviation is from triplicates of three separate experiments. D: Re-ChIP of HeLa cell chromatin. The antibody used in the first ChIP is specified at the top, the antibody in the second ChIP specified above the lanes, and the gene amplified is given to the left. Input is 0.05% of total chromatin volume; second input is 5% of the precipitated chromatin fragments by the first antibody. Two micrograms of IgG was used in the control lane marked IgG.

different stringencies using antibodies against several subunits in order to map the interaction patterns further. Nuclear extract were prepared at high protein concentrations at 0.42 M KCl, based on the salt concentrations used in the fractionation experiments (Supplementary Information, Fig. S1). The crude fraction of SWI/SNF subunits from chromatin using increasing KCl concentrations demonstrated that 90% of the BRG1 was released at 0.42 M KCl. The extract was subsequently quickly diluted to 0.15 M KCl prior to IP. The low stringency co-IPs revealed patterns of interaction between subunits that were not consistent with the patterns seen in the separate SWI/SNF complexes: PBAF and the BAF complexes. Anti-BAF250 antibodies precipitated BAF180 (Fig. 3A) when the IP was

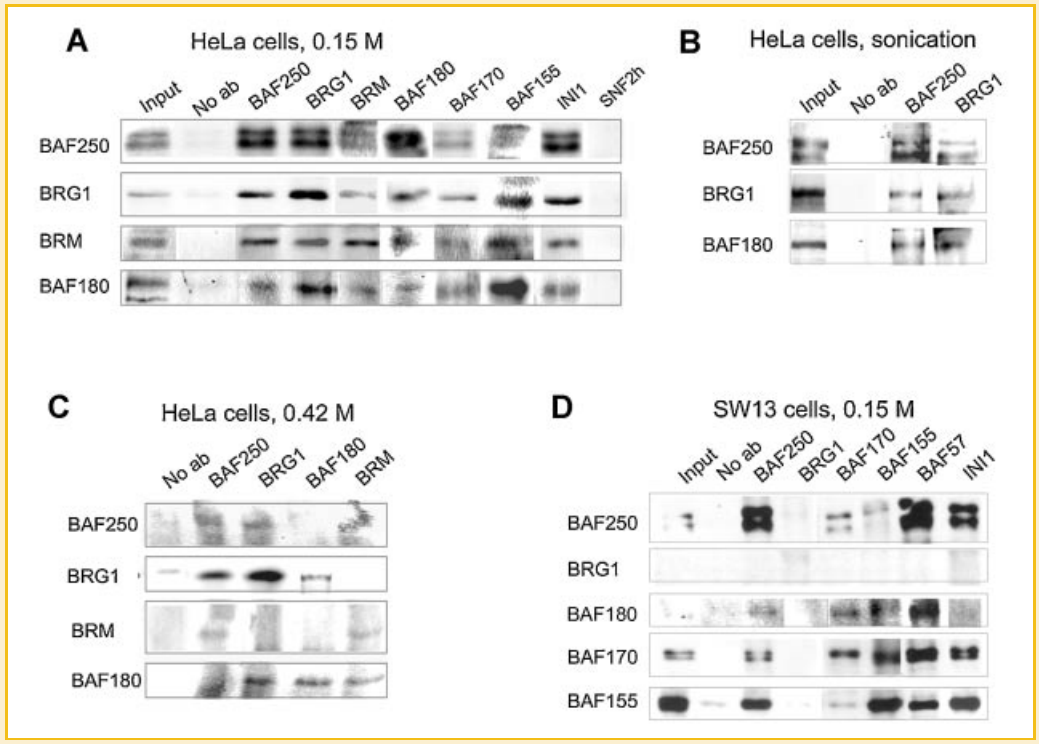

Fig. 3. Immunoprecipitation analysis of SWI/SNF complex composition and simultaneous binding of SWI/SNF subunits to promoters. A: IP performed at 0.15 M KCl, starting with 200–300 μg of HeLa nuclear extract. The antibodies are specified on the top. Precipitated proteins were separated on a 7% SDS–PAGE, immunoblotted and probed with the antibodies marked on the left. NoAb: control beads without antibodies. The input was 30 μg of HeLa nuclear extract. B: IP performed on sonicated nuclear extract prepared in PBS. The antibodies are specified at the top of the lanes and the antibody used to probe the immunoblot indicated to the left. C: IP was performed at 0.42 M KCl and a buffer with higher stringency was used during the washes, 0.42 M KCl. The antibodies used are marked on the top, and the proteins detected are given on the left. Noab is IP with only beads. D: Two hundred micrograms of SW13 nuclear extract was immunoprecipitated with the antibodies specified on the top. Precipitated proteins were separated on a 5–15% SDS–PAGE, immunoblotted and probed with the antibodies indicated on the left. NoAb is control beads without antibodies and the input was 30 μg of SW13 nuclear extract.

performed at 0.15 M KCl. The reciprocal IP, using anti-BAF180 antibody, showed that both BAF250A and BAF250B were precipitated (Fig. 3A). Two different BAF180 antibodies were used in IP, the anti-BAF180 described in Xue et al. [2000] and a polyclonal anti-BAF180 (Bethyl Laboratories), and both precipitated BAF250 subunits. BAF180 was detected in immunoblots also by two different antibodies; polybromo described in [Nicolas and Goodwin, 1996] and anti-BAF180 (Bethyl Laboratories). As a control we used an anti-SNF2h polyclonal antibody (Abcam), and we could not detect any signals. We also tested another method for preparing the nuclear extract, without using high salt. Anti-BAF250 antibodies precipitated BAF180 in nuclear extracts prepared by low-intensity sonication in PBS (0.15 M NaCl), supplemented with protease inhibitors (Fig. 3B). The results suggest that the BAF250 and the BAF180 subunits can be present in the same complex in HeLa cells.

The BAF180 and the BAF250 proteins were not co-immunoprecipitated when IPs were conducted at 0.42 M KCl (Fig. 3C). This indicated that the interaction found in 0.15 M KCl was less stable and most probably different from those in the BAF and PBAF complexes between core subunits and "signature subunits."

The interaction patterns between SWI/SNF subunits in the IP performed at 0.15 M KCl also showed that antibodies against BAF180, in contrast to the reported configuration of PBAF, precipitated the BRM protein at a low level (Fig. 3A). Anti-BRM also precipitated BAF180 at a low level (Fig. 3A), indicating that complexes containing both BRM and BAF180 were formed. Furthermore, low levels of the BRG1 and BRM proteins co-precipitated in HeLa cells under physiological salt conditions. Several different antibodies were used giving the same result (see Materials and Methods Section for source of antibodies). The weak association between BRG1 and BRM observed in 0.15 M KCl IPs (Fig. 3A) disappeared when using higher salt (Fig. 3C), but not that between BAF180 and BRM, when precipitating with an anti-BRM antibody.

Next, we investigated the association pattern of SWI/SNF subunits at a physiological salt concentration in SW13 cells. Antibodies against BAF250, BAF170, BAF155 and BAF57 precipitated the other subunits: BAF250, BAF170, BAF155 and BAF57 (Fig. 3D). The level of BAF180 was low in SW13 cells (Fig. 3D, the input lane), which is consistent with the results of DeCristofaro et al. [2001] and Wang et al. [1996]. Despite the low level of BAF180 in the nuclear extract, the protein was sufficiently enriched in the precipitate by antibodies against the core subunits to be detected. Interestingly, the anti-BAF250 antibodies precipitated BAF180, suggesting that these proteins interact even without BRG1 (Fig. 3D). The level of BAF155 varied, which could be due to sensitivity to degradation [Zhang et al., 2007], which also affects the level of other subunits. In summary, we conclude that most SWI/SNF subunits

interact when isolated at 0.15 M KCl and that subunits interact also in the absence of BRG1 and BRM.

BAF250 AND BAF180 CO-IPs IN LOW-SALT FRACTIONS FROM SUPEROSE 6HR SIZE EXCLUSION

The results from the low-stringency IPs strongly suggested that SWI/SNF complexes harbouring both BAF250 and BAF180 were present in cells. We performed low-stringency IPs of fractions from a Superose 6HR size-exclusion column run in PBS to address whether sub-complexes with different compositions were present in nuclear extract. BRG1 from nuclear extract eluted in a broad peak ranging from 2 MDa to 800 kDa (Fig. 4A). IPs of fractions with anti-BAF250 and anti-BAF180 showed that these proteins co-precipitated in a fractions eluting at a high Mr, corresponding to 1 MDa (Fig. 4B,C). BAF250 and BRM also co-precipitated, but the peak eluted in a separate fraction from that in which BAF180 and BAF250 co-eluted (Fig. 4B). Anti-BAF180 immunoprecipitated the BRM protein in fraction no 21, the same fraction as BAF180 and BAF250 were found (Fig. 4C). This fraction also contained BAF155. This indicates that several SWI/SNF complexes exist, and some of these contain both BAF250 and BAF180.

PURIFICATION OF MAMMALIAN SWI/SNF COMPLEXES

The distinction between PBAF and BAF-complexes is based on the purification of separate complexes from Jurkat and HeLa cells [Xue et al., 2000; Lemon et al., 2001; Yan et al., 2005]. Two separate SWI/SNF complexes have also been isolated from *Drosophila* S2 cells [Moshkin et al., 2007]. Since our results from low stringency IPs did not support the existence of only two separate complexes, we decided to examine how the BRG1, the BRM and the BAF proteins co-fractionated over four chromatographic columns. The purification scheme is outlined in Figure 5A. A total of 400 mg of HeLa crude nuclear extract, extracted at 0.42 M KCl, was fractionated on a phosphocellulose 11 (P-11) column into two fractions, 0.3–0.5 M KCl and 0.5–1.0 KCl. The 0.5 M KCl eluate from the P-11 column eluted from the MonoQ column at 0.42 M KCl, and was finally fractionated into Complex 1 with a Mr of 2 MDa (Fig. 5B). Complex 1 most closely resembled a classical BAF complex in that it contained all BAFs investigated except BAF180, and both BRG1 and BRM were present (Fig. 5C).

The 1 M KCl fraction from the initial P-11 column was subsequently separated on a MonoQ and two BRG1 peaks were eluted, one at 0.4 M KCl and one at 0.55 M KCl. These peaks were further purified on a Heparin column separately before being fractionated on a Superose 6HR gel filtration column into Complex 2 with a Mr of 1 MDa and Complex 3 with Mr of 0.7 MDa (Fig. 5B). These complexes did not contain BAF250; Complex 2 only contained core subunits, whereas Complex 3 contained BRG1, BAF180, BAF170 and BRM. The fact that these complexes had no BAF250 suggests that they are variants of PBAF [Xue et al., 2000; Lemon et al., 2001]. However, Complex 3 differed from PBAF in that it did contain BRM (Fig. 5C).

Actin was present only in the 1.0 M KCl elute from the initial P-11 column. This complex contained no detectable levels of actin after the final Superose 6 HR column (Fig. 3C), despite our efforts to stabilise the interaction with actin by including 0.1 mM ATP in the buffers. We suggest that it associates with such complexes transiently.

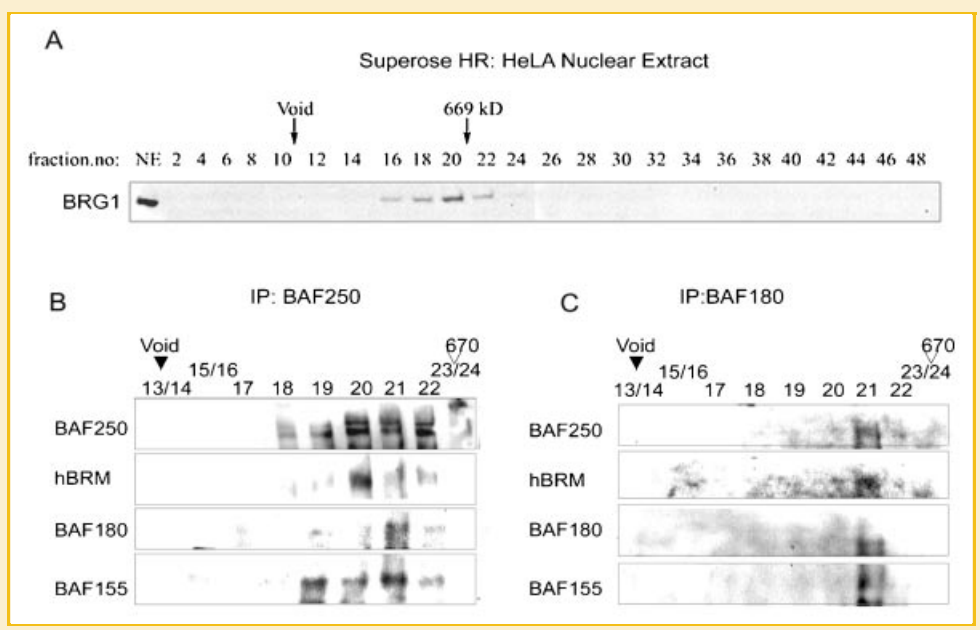

Fig. 4. IP of BAF250 and BAF180 subunits from size fractionation of HeLa cell nuclear extract. A: Immunoblot showing the size distribution of BRG1 in nuclear extracts. Fractions were precipitated and proteins separated on SDS–PAGE. The positions of the void and the size marker 669 kDa are indicated at the top. B: Immunoblots of fractions from a Superose size exclusion column immunoprecipitated with BAF250 antibodies. C: Immunoblots of Superose 6HR fractions subsequently immunoprecipitated by BAF180 antibody (Bethyl Laboratories). The fraction number (the fraction size was 0.6 ml) is given above the lanes, the void and Mr marker at 667 kDa is indicated above the lanes. The detected protein is marked on the left.

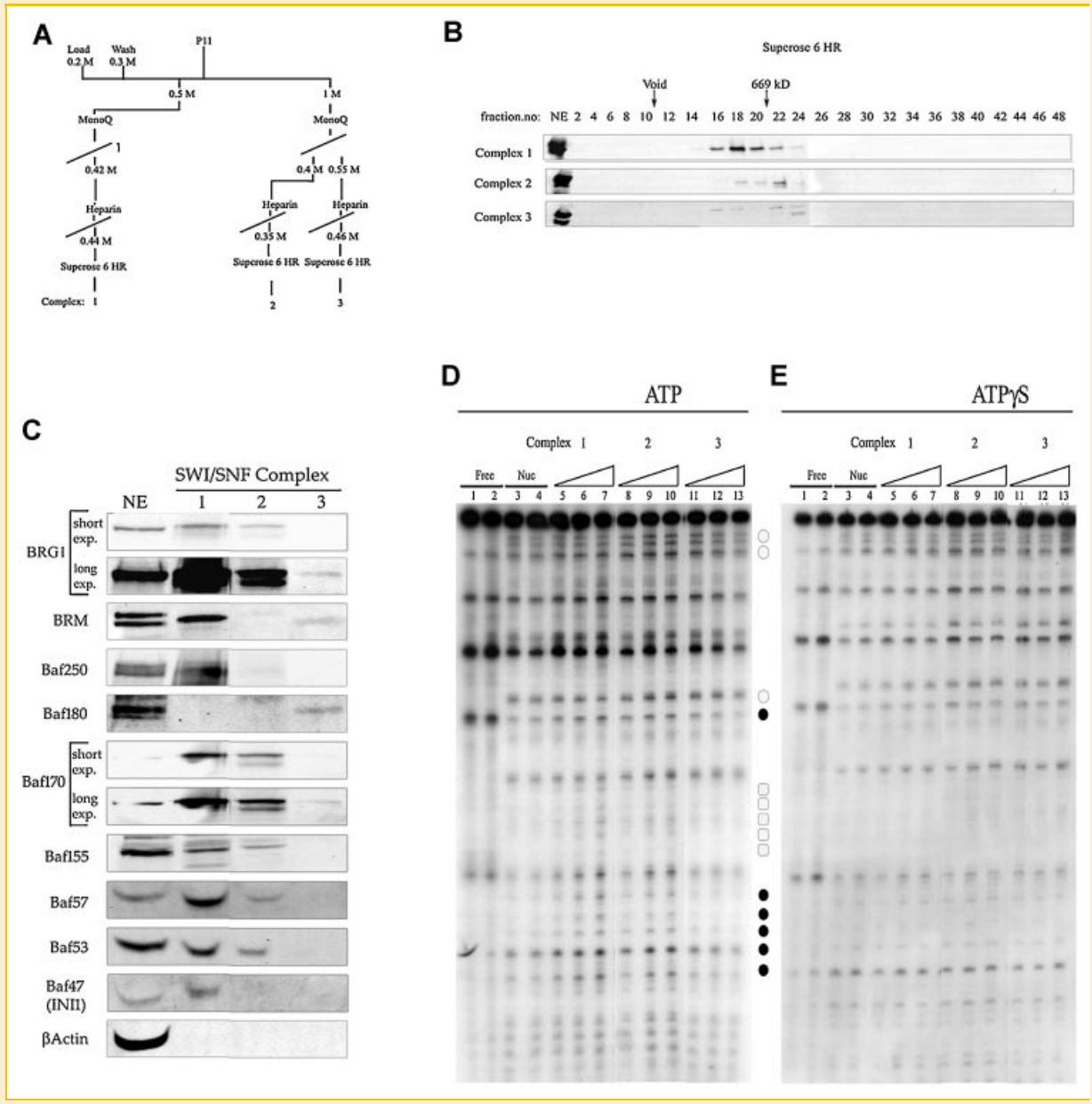

Fig. 5. Purification of mammalian SWI/SNF complexes from HeLa cells. A: Schematic representation of the purification of BRG1-containing fractions. B: Size distribution on a Superose 6 HR column of the three BRG1-containing complexes obtained in the purification. The positions of the void and the size marker 669 kDa are indicated at the top. C: Immunoblot of the three purified BRG1 complexes. Equal volumes of each complex were separated on a 10% SDS–PAGE with 30 μg of HeLa nuclear extract loaded as input: "NE." Two exposures of the BRG1 and BAF170 signals are shown in order to show clear signals both from complexes with low amounts and from complexes with high amounts of proteins. D: Mononucleosome disruption assay of purified complexes. Radioactively labelled nucleosomes were incubated with the three purified complexes, digested with DNase1 and the cut DNA was separated on a 6% denaturing urea gel. Increasing volumes of 3.25, 6.5, and 13 μl of each complex were used in the reactions. ATP (4 mM) was added to the reactions. Filled circles and empty squares mark the increase in accessibility caused by Complex 1 and Complex 2, respectively. Empty circles mark the decrease in accessibility caused by Complex 3. E: Mononucleosome disruption assay of purified complexes in the presence of 4 mM ATP-γS in the reactions to block completely any activity that may have resulted from the 0.1 mM ATP present during the purification of the complexes.

MONONUCLEOSOME DIGESTION ASSAY

We tested the ability of the purified SWI/SNF complexes to remodel nucleosomes in vitro in the presence of ATP or ATP-γS, to see whether the differences in subunit composition affected the activity. Complex 1 and Complex 2 were active in the mononucleosome disruption assay (Fig. 5D, compare lanes 5–10 with lanes 3–4), while Complex 3 consistently caused an overall reduction in DNase1 digestion (Fig. 5D, lanes 11–13). This reduction increased in proportion to the amount of Complex 3 in the reaction. Complex 1 and Complex 2 gave similar DNase cutting patterns, and increased the accessibility of the internal regions of the nucleosome to DNase1, but had a weak effect on the DNA located in the outer edges

(Fig. 5D, hollow squares and filled circles, respectively). The remodelling activities of the complexes depended on ATP, and identical reactions performed with equal concentrations of the non-hydrolysable ATP-γS did not result in any detectable changes of the DNase1 digestion pattern from that observed on nucleosomes (Fig. 5E). Our results supports the findings by Phelan et al. [1999] that the SWI/SNF subunits affect the activity, and together with the finding presented here that the configuration of SWI/SNF complexes is wider than previously believed, this opens for larger outcomes of the remodelling activity of SWI/SNF configuration.

DISCUSSION

We have analysed the composition of mammalian SWI/SNF complexes and have discovered a number of novel variants, in which subunits that are not normally found in the same complex interact. We base this conclusion on our results obtained by analysis of protein–protein interactions by IP of various subunits under different conditions. The analysis of the chromatin binding of individual subunits to endogenous SWI/SNF chromatin targets suggested that other constellations than BAF and PBAF exist in cells. The Re-ChIP also suggested that signature subunit associate very closely with the same promoter. Flowers et al. [2009] recently showed that BRG1 and BRM were associating to the promoter at the same time, but these authors concluded that several different complexes associate to the promoter simultaneously. Our finding that these subunits interact, although sometimes weakly, under physiological salt conditions, raises the possibility that they are present in the same complex. Our results presented here from IPs from size exclusion chromatography suggest that different sub-complexes co-exist, smaller complexes together with larger ones that contain most subunits. SWI/SNF complexes may exist in preferred constellations, such as the BAF and PBAF complexes, since these interactions are stable in high salt, but our results show that unorthodox constellations also are present in cells.

Purifications of SWI/SNF complexes from several sources have revealed several types of complex, which differ in subunit composition. Early biochemical purifications of SWI/SNF complexes showed heterogeneity of complex formation, suggesting that several complexes with different composition exist [Wang et al., 1996]. Two different forms, SWI/SNF A and B were isolated, and it was shown that the mammalian ATPases, BRG1 and BRM, exist in two separate complexes but share many of the subunits [Wang et al., 1996]. The two distinct complexes, SWI/SNF A and SWI/SNF B, were later identified as the PBAF and BAF-complexes. PBAF and BAF are not the only SWI/SNF complexes isolated; other constellations, such as two separate BRG1-containing complexes, which both contain BAF250, in addition to a BRM-containing complex have been purified [Sif et al., 2001]. These BRG1-containing complexes only differ in the association of BAF57, BAF60 and a further protein denoted p202, and display different in vitro chromatin remodelling activity [Sif et al., 2001].

Isolated SWI/SNF complexes often include other nuclear proteins, such as HDACs and mSin3A [Armstrong et al., 1998; Underhill et al., 2000; Sif et al., 2001]. Other examples are the SWI/SNF complexes EBAFa and EBAFb that contain either BAF250A or B and the ENL-protein [Nie et al., 2003]. Proteins or protein complexes that have SWI/SNF subunits associated are EKLF [Armstrong et al., 1998], Ikaros [O'Neill et al., 2000], N-CoR (with a few splice factors) [Underhill et al., 2000] and the histone methyl transferase (HMT) CARM1 [Xu et al., 2004]. The SWI/SNF subunits that co-purify with these complexes, all at salt concentrations above 0.3 M KCl, either constitute the BAF complex or belong to the defined core subunits. It is tempting to speculate that the subunits isolated with these nuclear proteins are those that are tailored for these factors, and whose interactions are stable under the purification conditions. That purification conditions affect the outcome of complex composition has been shown for the mediator complex. When nuclear extract was loaded onto a gel filtration column immediately after extraction with relatively low salt (0.3 M), several of the reported complex variants did not appear [Wang et al., 2001]. The authors suggested that the previously reported complexes might be a result of the purification procedures applied. Similarly, we show that SWI/SNF complexes purified at different conditions display different subunit compositions, and we propose that this reflects a dynamic network of subunit interactions.

Lessard et al. [2007] have proposed that SWI/SNF complexes are formed combinatorially with tissue specific subunits available in each cell. The combinatorial effect can also be seen in the formation of SWI/SNF complexes during differentiation of ES cells, where BAF170, BRM and BAF250B are not expressed, or are expressed at low levels [Yan et al., 2008]. The expressions of these subunits increase during differentiation, whereas that of BAF250A decreases [Kaeser et al., 2008; Yan et al., 2008]. Despite these variations in expression, many subunit isoforms are present simultaneously in cells. We have not specifically addressed the interactions of the ''isoforms,'' such as BAF250A and B, but studied the interaction pattern of core and ''signature'' subunits, where some can be regarded as different forms, (such as BAF155 and BAF170, BRG1 and BRM). We have shown that signature subunits can interact with each other, even in the absence of BRG1 and BRM. This leads us to propose that the composition of the SWI/SNF assembly at individual sites results from the properties of the local chromatin environment. Different SWI/SNF subunits interact with specific transcription factors, which confer specificity to and target the SWI/SNF complexes or sub-complexes to specific sites. This could explain the function of SWI/SNF subunits, which exhibit both specific and redundant functions, such as the PBAF subunits BAF200 and BAF180 [Yan et al., 2005; Chalkley et al., 2008], and the variation in recruitment observed at SWI/SNF-dependent promoters. Furthermore, two core subunits, BAF170 and BAF155, are independently recruited to genes depending on the state of expression of the gene [Zhang et al., 2007]. Mernedula and Belmont [2003] showed that the recruitment of BRG1 or BRM and BAF155 and BAF170 to genes after induction occurs at different times; the ATPases are detected immediately after gene induction, while the BAFs arrive up to 90 min later. The independent recruitment mechanism supports our idea that sub-complexes, not only pre-assembled complexes, are recruited to the target genes as a result of functional requirements posed on the assembly at the specific site in chromatin. However,

studies have also shown that subunits are recruited that is not needed for expression. The p21 gene is dependent on BAF180 for expression in human cells, but both BAF250A and B are recruited under different expression states; BAF250A to repress expression during proliferation and BAF250B is present during the active state in quiescent cells, but it is not required for the expression of p21 [Nagl et al., 2005]. The p21 gene in growing HeLa cells recruited both BAF and PBAF subunits, suggesting that BAF250B are recruited as part of complexes harbouring the subunits required at the site or that it is required for a not yet known function.

Only two of the genes that were up-regulated in response to BRG1 in SW13 cells were expressed in HeLa cells, showing that the presence of SWI/SNF is not sufficient for the activation of the genes. These genes were not repressed due to DNA methylation, a common mechanism in transformed cells. The recruitment of SWI/SNF factors, however, may reflect the requirement of chromatin remodelling for proper expression, but further tissue-specific factors are needed for the actual expression. The promoters also bound RNA polymerase II, resembling the state during stalled or paused transcription. SWI/SNF factors are already present on the promoter before activation of interferon regulated genes [Liu et al., 2002] and CSF1 [Liu et al., 2002]. Certain SWI/SNF subunits also repress gene expression, depending on other factors at the promoter.

The different SWI/SNF constellation may display subtle functional differences depending on the interacting subunits. The complexes that we isolated biochemically exhibited differences in their DNase cutting patterns of mononucleosomes in vitro, and this supports a model in which the nature of the change is determined by the BAFs [Phelan et al., 1999]. Similar to our high-stringency IP, BRM co-precipitates with BAF180 in Complex 3, further supporting that unorthodox complexes are present in cells. Interestingly, the activity of Complex 3, which did not affect the DNA at the dyad, resembles that of the BRG1-containing complex II isolated by Sif et al. [2001]. Modifying the association of different subunits in SWI/SNF complexes could form the basis for regulating nucleosome remodelling activity, where small changes in composition lead to specific changes in DNA accessibility. The dynamic complex formation suggested by our model allows for varieties in subunit composition in different isolated SWI/SNF complexes. Our model is particularly interesting since many human tumour cell lines vary significantly in their expressions of SWI/SNF subunits other than BRG1 and BRM [DeCristofaro et al., 2001]. The loss of specific subunits would limit the range of possible SWI/SNF constellations, and this limitation would affect the biological activity.

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