

A human protein with homology to *Saccharomyces cerevisiae* SNF5 interacts with the potential helicase hbrm

Christian Muchardt, Claude Sardet¹, Brigitte Bourachot, Christina Onufryk¹ and Moshe Yaniv*

Unité des Virus Oncogènes, UA1644 du CNRS, Département des Biotechnologies, Institut Pasteur, 75724 Paris cedex 15, France and ¹Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, MA 02142, USA

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ABSTRACT

In yeast, the SNF/SWI complex is involved in transcriptional activation of several inducible promoters, possibly by causing a local modification of the chromatin structure. Recently, two human homologues of the SNF2/SWI2 protein have been isolated, hbrm and BRG-1. In addition, a complex containing one of the SNF2/SWI2 homologues and having an *in vitro* activity similar to the yeast complex has been partially purified from HeLa cells. Here we describe the characterization of a cDNA encoding a human nuclear protein containing a large domain of homology with SNF5, another member of the yeast SNF/SWI complex. This protein can be co-immunoprecipitated with hbrm and the interaction between the two proteins is dependent on the region conserved between the human and the yeast SNF5. These findings suggest that the cDNA we have cloned encodes one of the members of the human SNF/SWI complex.

INTRODUCTION

The *Saccharomyces cerevisiae* proteins SWI1, SNF2/SWI2, SWI3, SNF5 and SNF6 (SWI, mating type switch; SNF, sucrose non-fermenting) have been shown to act as global activators, involved in transcriptional activation of a large set of inducible genes. None of these proteins contains any known site-specific DNA binding motifs. They are believed to activate transcription by assisting some gene-specific activators in opening access to DNA binding sites hidden in nucleosomal structures. This activity is in part dependent on a domain of the SNF2/SWI2 protein sharing homology with known RNA or DNA helicases. This domain has been shown to harbour DNA-dependent ATPase activity (for a review see 1 and 2).

The activities of the SNF/SWI proteins are closely interconnected. For example, mutations in any of the five *snf/swi* genes result in very similar phenotypes (3,4). Besides, three of these

proteins, SNF2/SWI2, SNF5 and SNF6, can activate transcription when tethered to the DNA through a LexA DNA binding domain. However, this activation can only occur at maximum levels in the context of a yeast strain with wild-type *snf/swi* genes (3,5). A possible explanation for these observations is that the SNF/SWI proteins are members of the same protein complex. This hypothesis has recently been supported by the purification from yeast extract of a 2 MDa multiprotein complex containing the known SNF/SWI proteins but also five other proteins, one of which is TFG3/TAF30 (transcription factor g3/TBP associated factor, 30 kDa) (6-9). As expected from the *in vivo* data, this purified complex is able to facilitate the binding of a Gal4 derivative to a DNA probe assembled into nucleosome cores (7).

Several observations suggest that a homologue of the SNF/SWI complex is also present in higher eukaryotes. First, gene-specific activators, like the *Drosophila* bicoid homeoprotein or the rat glucocorticoid receptor ectopically expressed in yeast, are no longer active in strains with mutations in the *snf/swi* genes (10,11). In addition, the SNF2/SWI2 protein has known counterparts in several multicellular organisms. In *Drosophila* this protein is known as brahma or brm (12). It is involved in transcriptional regulation of several homeotic genes and could possibly also act on chromatin, since it was first identified as a suppressor of polycomb (Pc) mutations. The Pc group of genes repress homeotic genes by regionally compacting the chromatin (13,14). Other proteins more distantly related to SNF2/SWI2 have also been identified in *Drosophila* (15). In humans SNF2/SWI2 has two closely related homologues: hbrm and BRG-1 (16-18). These two proteins, encoded by two clearly different genes, are 75% identical and are both able to cooperate with several nuclear receptors in transcriptional activation. For hbrm, this cooperation has been shown to be dependent on the ATP binding site in the conserved helicase domain. Similarly, a mutation in the ATP binding site of BRG-1 transforms this protein into a dominant negative effector that represses transcription from the EF-1 α promoter.

The presence of a human SNF/SWI complex has been further confirmed by its partial purification from HeLa cell nuclear extract. Indeed, using an antibody made against BRG-1, Kwon *et*

* To whom correspondence should be addressed

al. (19) have shown that the human SNF2/SWI2 homologue co-elutes with six other proteins with apparent molecular weights of 43, 45, 50, 60, 150 and 170 kDa. This complex, like its yeast homologue, can facilitate the *in vitro* binding of a Gal4 derivative to nucleosomal DNA in an ATP-dependent manner.

In this paper, we describe the isolation of a human cDNA coding for a 44 kDa nuclear protein with high homology to yeast SNF5 in its C-terminal part. This protein is shown by co-immunoprecipitation assays to interact with the human SNF2/SWI2 homologue hbrm. The interaction between the two proteins is dependent on the conserved SNF5 homology domain of the human SNF5 protein and on the N-terminal region of hbrm.

MATERIALS AND METHODS

Cloning of full-length hSNF5 cDNA and Northern blot analysis

Yeast two hybrid screening was essentially performed as previously described (26), using full-length pRB fused to LexA in pJG202 as a 'bait' to screen a pJG4-5 'prey' library generated from serum-starved WI38 human fibroblasts. A 'prey' clone, G29, with sequence relatedness to the product of the yeast *SNF5* gene was isolated. Full-length cDNA for hSNF5 was isolated from a human HEL cell cDNA library in λ GT11 screened with the initial 'prey' clone G29 as a probe. cDNA was sequenced using sequenase 2.0 (USB). For Northern blot analysis, a Human Multiple Tissue Northern Blot (Clontech) was probed with the G29 clone or human β -actin cDNA.

Serum-starved WI38 human fibroblast cDNA library

Polyadenylated mRNAs were prepared from 8×10^8 serum-starved WI38 cells and *Xho*I/oligo(dT)-primed cDNAs were synthesized using a modification of the procedure described by Gyuris *et al.* (26,27). A *Not*I/*Eco*RI adaptor (Invitrogen) was ligated at the 5'-end of the cDNAs. Half of the *Not*I/*Xho*I restricted cDNAs were size-selected on Sepharose CL4B spin columns (Pharmacia) and the other half on Sephacryl S500 (Pharmacia). Both eluates were combined and ligated into pJG4-5 with *Not*I and *Sal*I. Electrocompetent *Escherichia coli* DH10B (BRL) were transformed, leading to 3×10^6 individual colonies, >80% of which contained a cDNA insert of average size 1.4 kb.

Plasmid constructs

The CMV-hSNF5 expression vector was constructed by inserting an *Xba*I-*Eco*RV fragment from the original pBluescript-hSNF5 isolate into pCG (28) linearized with *Xba*I and *Sma*I. The HA-tagged (29) derivatives of hSNF5 were constructed by inserting an *Eco*RI site after nucleotide 192 for the wild-type, at nucleotide 786 for hSNF5 Δ 1, at nucleotides 192 and 742 for hSNF5 Δ 2 and at nucleotides 192 and 1168 for hSNF5 Δ 3, using standard PCR techniques. All PCR products, after verification by DNA sequencing, were inserted into a pCDNA3 derivative (Invitrogen) containing an in-frame HA epitope. The wild-type CMV-hbrm expression vector, as well as Δ 3 and Δ 4, have been previously described (16). Δ 6 contains a deletion between nucleotide 2557 and the *Afl*III site at nucleotide 4015 in the published hbrm sequence. Δ 8 was constructed by inserting a stop codon at the *Afl*III site in Δ 3.

Immunoprecipitations and Western blotting

The protocol was adapted from Cairns *et al.* (6). In brief, C33A cells were passed 1 day prior to transfection (1.5×10^6 cells/100 mm dish). The medium was changed and after 5 h, cells were transfected with a total of 20 μ g DNA by calcium phosphate precipitation. Cells were washed 14 h later. After an additional 24 h, cells were harvested in IP buffer (20 mM HEPES, pH 7.6, 10% glycerol, 25 mM MgCl₂, 0.1 mM EDTA, 0.2% Nonidet P-40) containing 0.1 M potassium acetate and 2.25 μ g/ml pepstatin, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 2 mM PMSF and 0.1 mM DTT. The cells were then sonicated twice for 5 min, with vigorous vortexing after each sonication. Debris was pelleted and the extracts were pre-cleared for 1 h with 20 μ l 50% protein A-Sepharose suspension (Pharmacia). The pre-cleared extracts were then transferred to another 20 μ l protein A-Sepharose suspension that had been pre-incubated for 1 h with 0.8 μ g purified 12CA5 monoclonal mouse antibody. After 3 h incubation at 4°C, the beads were washed three times in IP buffer with 0.6 M potassium acetate, then once with IP buffer without HEPES. The precipitate was eluted twice with 7 μ l buffer C (50 mM glycine, pH 2.3, 150 mM sodium chloride). The two eluates were pooled, fractionated by SDS-PAGE and transferred to nitrocellulose. This membrane was then blocked with PBS/0.2% Tween 20/10% horse serum and incubated with the various antibodies. Enhanced chemiluminescence reagents (Amersham) were used for detection. The hSNF5 polyclonal rabbit antibody was prepared against a GST fusion of full-length hSNF5. The hbrm antibody has been described previously (16).

Immunofluorescent staining

HeLa cells grown on coverslips were fixed in 3% freshly prepared paraformaldehyde in PBS for 10 min. To enhance exposure of the recognized hSNF5 epitopes, cells were extracted and treated with 6 N guanidine hydrochloride as described previously (30). The slides were then incubated for 2 h with anti-hSNF5 antibody in PBS/10% horse serum/0.05% Tween-20. A fluorescein-linked anti-rabbit antibody from Amersham was used for detection. The cellular DNA was labelled with 0.05% DAPI. The cells were observed at 50 \times magnification under a Zeiss microscope.

RESULTS

Isolation of a human homologue of the *S.cerevisiae* SNF5 gene

Our initial purpose was to use the yeast two hybrid system to identify proteins encoded in a growth-arrested human fibroblast (WI38) cDNA library which would interact with the retinoblastoma gene product (pRB). One of the positive clones from this screen was found to code for a polypeptide with clear homology to regions in the *S.cerevisiae* SNF5 protein. Further studies showed that the interaction of this polypeptide with pRB was likely to be non-specific (data not shown).

To investigate whether the identified clone could be a human counterpart of the yeast *snf5* gene, we isolated a full-length cDNA. This 1684 nt long cDNA encodes a putative 385 amino acid protein that we named hSNF5. The protein is rich in charged amino acids (27.5% of total) and has a global negative charge of -6. Screening of the data banks showed that in addition to SNF5, the hSNF5 protein is highly homologous over its total length to a putative protein encoded by a *Caenorhabditis elegans* DNA

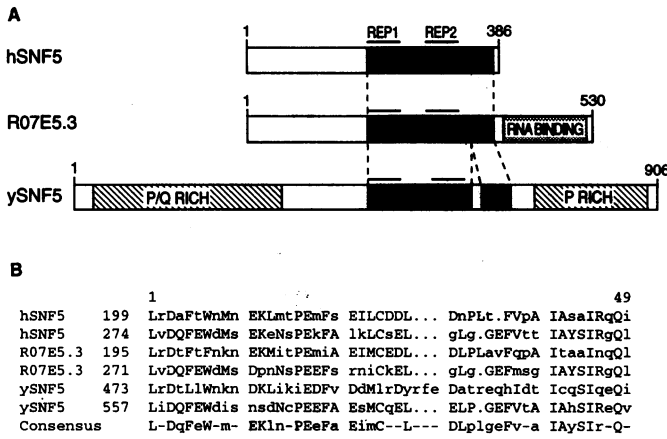


Figure 1. Repeated sequence contained in the conserved SNF5 domain. (A) Schematic representation of the three SNF5 proteins. The conserved SNF5 homology regions are shaded with black, the two repeated sequences are overlined. (B) The repeated sequences from all three proteins were aligned with PileUp and a consensus sequence was calculated with Pretty (GCG package). Numbering corresponds to the first amino acid in each sequence. In the consensus, amino acids conserved in at least four sequences are capitalized

sequence present in GenBank (accession number Z32683). The human and the *C.elegans* proteins are 71.1% similar and 53.1% identical. They are, however, distinguished by the presence, in the C-terminal end of the *C.elegans* protein, of a region with homology to an RNA binding domain found in several snRNP proteins. This RNP motif is absent from the human protein. In both the human and the *C.elegans* protein, the homology with the yeast SNF5 is restricted to a 209 amino acid region. In the human protein, this region spans from amino acid 184 to 377 and is 62.9% homologous to the equivalent region in the yeast protein. In all three proteins, the conserved domain contains a high proportion of charged amino acids and is globally acidic (a net charge of -17 in the human and the yeast and -16 in the *C.elegans* protein). A schematic representation and a sequence alignment of the three proteins are respectively shown in Figures 1a and 2. Interestingly, in all three proteins the conserved SNF5 domain is composed, in part, of two 45 amino acid long imperfect repeats (Fig. 1a and b). Screening the SWISSPROT protein data bank with the consensus sequence of the repeats did not reveal any other proteins with the same motif. No other canonical protein domains were detected in the hSNF5 sequence. In particular, we found no homology to any known DNA binding domain.

Intracellular localization and tissue distribution of hSNF5

The intracellular localization of hSNF5 was determined by fluorescent staining of HeLa cells with DAPI and a polyclonal rabbit antibody prepared against bacterially expressed hSNF5 protein. As shown in Figure 3a, the immunofluorescent signal obtained with the antibody was co-localized with the DAPI DNA staining, indicating that hSNF5 is a nuclear protein. To estimate the tissue distribution of hSNF5, we performed Northern blot analyses using human poly(A⁺) RNA isolated from brain, heart, kidney, liver, lung, pancreas, placenta and skeletal muscle. An hSNF5 probe detected a species of ~2 kb in all tested organs (Fig. 3b). A second less intense species of 4 kb was also visible, but only after a longer exposure. We also used RT-PCR to analyse RNA from

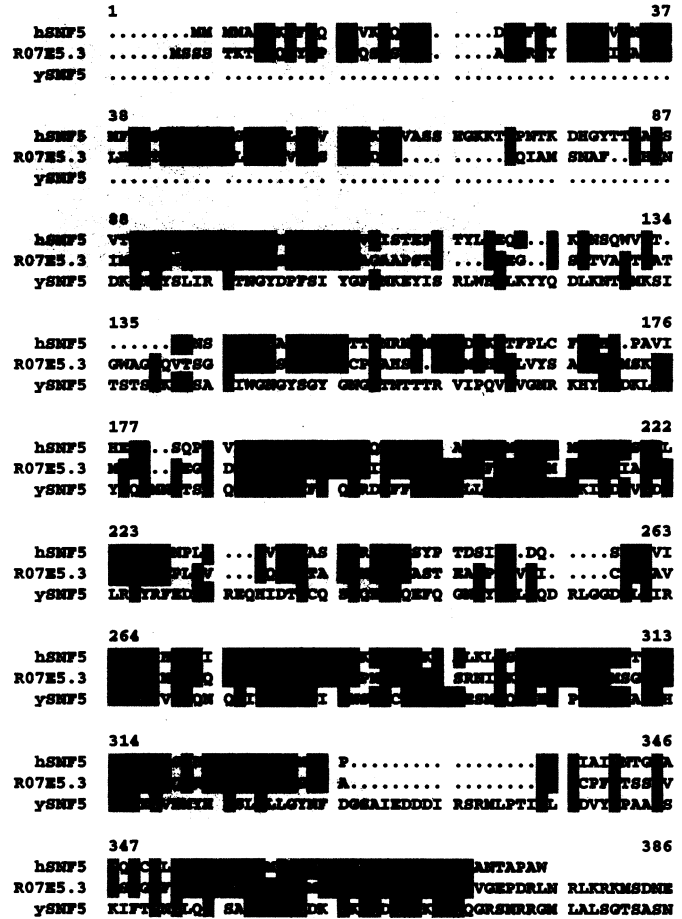


Figure 2. Alignment of the amino acid sequence of yeast SNF5 and its homologues in human and *C.elegans*. Protein sequences deduced from the isolated hSNF5 cDNA, the published yeast SNF5 and the *C.elegans* sequence present in GenBank (R07E5.3) were aligned using BestFit from the GCG package. The conserved amino acids are highlighted. The numbering corresponds to the human sequence. The yeast protein is listed from amino acid 346 to 696, the *C.elegans* protein from amino acid 1 to 393.

several available human cell lines. Analysis of the PCR products in a linear phase of the amplification (after 13 cycles) showed that hSNF5 message was present in all tested cell lines (data not shown). These cell lines included the immortalized keratinocytes HaCat and HK-40, the cervical carcinoma-derived C33A, HeLa, SiHa and HT-3, the laryngeal carcinoma-derived HEP2, the mammary carcinoma-derived MCF7 and MCF7-KO, the hepatoma-derived HepG2, the acute promyelocytic leukaemia-derived NB4, the colonic adenocarcinoma-derived HT29, the embryonic kidney-derived 293 and the adrenal cortex carcinoma-derived SW13. Among these cell lines, two, C33A and SW13, have previously been shown to have low or undetectable levels of hbrm and BRG-1, the two human homologues of SNF2/SWI2 (16,20).

hSNF5 is co-immunoprecipitated with hbrm

As mentioned above, the yeast SNF2/SWI2 and SNF5 proteins have been shown to be members of the same complex. To determine if their human homologues, hbrm and hSNF5, associate in a similar way, we performed co-immunoprecipitation assays on extracts from cells transfected with CMV-hSNF5 and HA-tagged

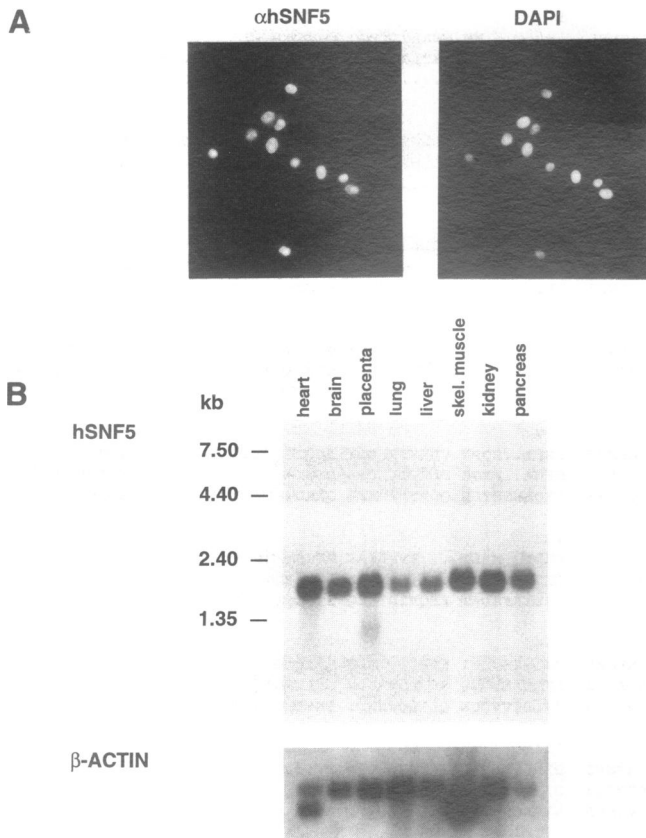


Figure 3. hSNF5 is a ubiquitously expressed nuclear protein. (A) HeLa cells grown on coverslips were fixed and processed as described in the Materials and Methods. The cells were then stained with DAPI and indirectly fluorescein-linked anti-hSNF5 antibody. The left panel shows a fluorescein image and the right panel a DAPI image. (B) The top panel shows a Northern blot probed with a fragment of the hSNF5 cDNA. Each lane contains 2 μ g poly(A⁺) selected RNA from the indicated tissues. The same blot stripped and reprobed with β -actin is shown in the bottom panel.

CMV-hbrm expression plasmids. Several deletion mutants of the CMV-hbrm construct were also included in the experiment to localize the protein domains involved in such an interaction ($\Delta 3$, $\Delta 6$, $\Delta 4$ or $\Delta 8$; Fig. 4a). A CMV expression vector without insert was used as negative control. The transfection was performed in the hbrm-deficient cell line C33A. Using the rabbit polyclonal hSNF5 antibody, we first checked that all the extracts from the different transfected plates contained similar amounts of hSNF5 (Fig. 4b). The extracts were then used to perform immunoprecipitations using the HA-tag-specific mouse monoclonal antibody 12CA5, recognizing the wild-type and mutant hbrm proteins. The precipitate was fractionated by SDS-PAGE and analysed by Western blot using a mixture of both hSNF5 and hbrm polyclonal antibodies. As expected, all hbrm constructs were precipitated with the anti-HA antibody (Fig. 4c). In addition, when the expression vector contained the wild-type hbrm, hSNF5 could also be detected in the precipitate. hSNF5 was similarly detected when the $\Delta 6$ and $\Delta 4$ mutants were used. However, the hSNF5 protein could not be detected when cells had been transfected either with vector lacking insert or with the $\Delta 3$ or the $\Delta 8$ mutants. These results show that hSNF5 and hbrm associate in a complex and that the N-terminal domain of hbrm is necessary for this interaction.

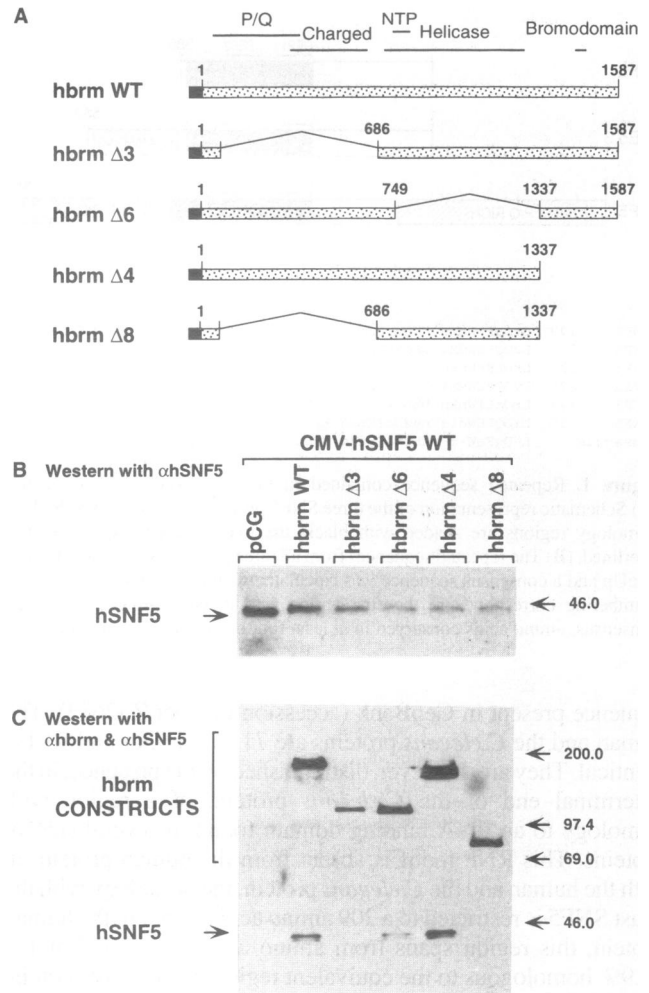


Figure 4. hSNF5 co-immunoprecipitates with hbrm. C33A cells were transfected with a CMV-hSNF5 expression vector and either the CMV vector without insert or CMV-HA-hbrm expression vectors containing either the wild-type sequence or the derived deletion mutants $\Delta 3$, $\Delta 4$, $\Delta 6$ or $\Delta 8$ (A) (black boxes represent the N-terminal HA tag). After 48 h, total extracts were tested for the presence of hSNF5 by Western blotting using the hSNF5 polyclonal rabbit antibody (B). The extracts were then used for immunoprecipitation with the HA-specific monoclonal antibody 12CA5. Precipitated fractions were resolved by SDS-PAGE and proteins visualized by Western blotting with a mixture of hSNF5 and hbrm antibodies (C).

hSNF5 interacts with hbrm through its conserved SNF5 domain

To determine the region of hSNF5 responsible for the interaction with hbrm, we constructed several deletion mutants of hSNF5 all containing an N-terminal HA epitope tag (Fig. 5a). These constructs were transfected into C33A cells together with the wild-type CMV-hbrm expression vector. As in the previous experiment, total extracts were prepared from the transfected cells. These extracts all contained comparable amounts of hbrm, as shown by Western blot analysis with the hbrm polyclonal antibody (Fig. 5b). After immunoprecipitation using the mouse monoclonal 12CA5 antibody, all the hSNF5 constructs could be detected in the precipitate. For this detection we used the 12CA5 antibody to avoid revealing endogenous hSNF5 (Fig. 5c). It

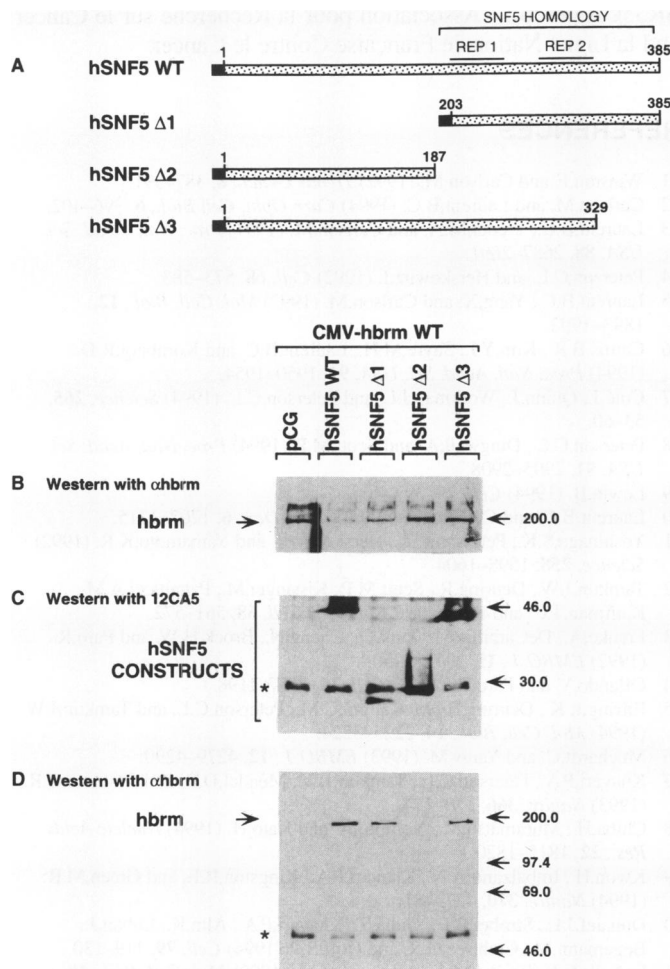


Figure 5. hbrm co-precipitates with hSNF5. C33A cells were transfected with a CMV-hbrm expression vector and either the CMV vector without insert or containing one of the hSNF5 constructs shown (A) (black boxes represent the HA tag, REP 1 and REP 2 indicate the tandem repeats). As in Figure 4, total extracts were prepared and tested by Western blotting for the presence of equal amounts of hbrm (B). The extracts were then used for immunoprecipitation with the 12CA5 antibody. The precipitated fraction was resolved by PAGE and proteins were detected first with 12CA5 antibody (C) then with hbrm antibody (D). (C and D) Background bands corresponding to the heavy and the light chain of the antibody are visible in all lanes (labelled with *).

should be noted that hSNF5 $\Delta 2$ co-migrates with the IgG light chain that appears as a contaminating species in all lanes. As expected, when the same Western membrane was incubated with the hbrm polyclonal rabbit antibody, the hbrm signal could be detected in the precipitated fraction obtained with wild-type hSNF5 and was absent when vector without insert was used in the transfection (Fig. 5d, hSNF5 WT and pCG). In addition, we observed that the hbrm signal was present with all mutants containing the repeats of the SNF5 homology region (Fig. 5d, hSNF5 $\Delta 1$ and $\Delta 3$), but was absent when the repeats were deleted (Fig. 5d, hSNF5 $\Delta 2$). These results show that hSNF5 interacts with hbrm through the conserved SNF5 homology domain and more precisely within the region containing the tandem repeat.

hSNF5 does not activate transcription in human cells when tethered to DNA

The SNF5 domain conserved in the human protein is highly acidic and could be responsible for the transcriptional activity of yeast SNF5 observed when this protein is tethered to DNA through a LexA DNA binding domain (21). To determine whether the human hSNF5 had transcriptional activity, we co-transfected an SV40 expression plasmid encoding a Gal4-hSNF5 fusion protein and a 5 \times Gal-E1b-CAT reporter construct in C33A or MCF-7 cells. In these experiments we were unable to detect any activation by Gal4-hSNF5 in either of the two cell lines. Besides, we have previously shown that hbrm can stimulate activation by the glucocorticoid receptor (GR) of a cognate promoter (16). Co-transfection of an hSNF5 expression vector with hbrm and GR did not appear to modulate this activation, as compared with hbrm and GR alone. These negative results suggest that hSNF5 may have other functions than simple transcriptional activation in human cells. However, when assayed in yeast, we found that a LexA-hSNF5 $\Delta 1$ construct yielded significant activation of a LexA-LacZ reporter construct (results not shown). This observation suggests that the human protein shares some functional properties with the yeast SNF5 protein.

DISCUSSION

In this work we describe the characterization of a human protein related to the yeast global activator SNF5. Our data show that this protein interacts with hbrm, a human homologue of SNF2/SWI2. This interaction requires the conserved SNF5 homology domain of hSNF5. Our observations suggest that both hbrm and hSNF5 are constituents of the human SNF/SWI complex.

The homology between yeast SNF5 and human hSNF5 is restricted to a 209 amino acid long domain. Yeast SNF5 contains, in addition to this region, a long glutamine- and proline-rich N-terminal region. This region is missing in the human protein. hSNF5 is also highly related to a *C.elegans* putative protein. The similarities between these two proteins extend beyond the SNF5 homology domain. However, the *C.elegans* protein contains an RNA binding domain known as an RNP motif. This domain is essentially found in U snRNP proteins, hnRNP proteins and nucleolin (22–24). Again, this domain is absent in the human hSNF5 we isolated. The differences between the three proteins suggest that their functions could be divergent. Their common SNF5 homology domain may then reflect their ability to interact with the SNF/SWI complex of their respective species. Possibly, within a given species, several proteins may contain SNF5 homology domains.

The function of hSNF5 has yet to be established. A LexA-hSNF5 $\Delta 1$ construct was found to activate transcription from a LexA reporter construct in yeast. A similar result has been described previously for a LexA-SNF5 construct (21). However, we failed to detect any transcriptional activity of a Gal4-hSNF5 construct in human cells. It is possible that cofactors necessary for hSNF5 activation are missing in the cell lines we have tested. Alternatively, hSNF5 may be involved in transcriptional activation in a more indirect way, for example by functioning as a target for the hbrm chromatin decondensing activity. *In vitro* assays using reconstituted chromatin and purified hSNF5 and hbrm will be necessary to confirm this model.

The interaction between hSNF5 and hbrm is dependent on the N-terminal region of hbrm. This region is by itself able to activate transcription when tethered to DNA. It has also been shown to be important for cooperation between hbrm and the glucocorticoid receptor (16). This region is composed of two overlapping domains. The first domain is rich in prolines and glutamines. It is present in all SNF2/SWI2-related proteins, but the exact positioning of the amino acids varies from protein to protein. The second domain is composed of a succession of negatively and positively charged stretches. Inside this region, hbrm and BRG-1 sequences are close to identity. Further analysis will be required to determine which of these two regions is responsible for the hSNF5 contact. In both yeast and human, the SNF2/SWI2-related proteins appear to behave differently from the other members of the SNF/SWI complex. In gel filtration of yeast extracts from strains with a mutation in one of the *snf/swi* genes, SNF2/SWI2 migrates with an apparent molecular weight of 700 kDa, instead of the 2 MDa observed with wild-type yeast. This molecular weight of 700 kDa does not vary, whether the mutation is in the *swi1*, *swi3*, *snf5* or *snf6* gene. This suggests that SNF2/SWI2 is included in an independent sub-complex that does not interact directly with any of the SWI1, SWI3, SNF5 or SNF6 proteins taken individually (6). Besides, during purification of the human SNF/SWI complex, two complexes with different compositions were shown to contain a human SNF2/SWI2 homologue (19). These observations suggest that in human cells, as in yeast, hbrm or BRG-1 are included in a sub-complex that may not contain all the other human SNF/SWI proteins. This sub-complex may, among other proteins, include pRB, which has recently been shown to interact with BRG-1 in a region where this protein is identical to hbrm (20). hbrm and BRG-1 may then interact with hSNF5 only transiently during transcriptional activation.

While this manuscript was in preparation, Kalpana *et al.* (25) reported the isolation of the same cDNA using the HIV-1 integrase as a bait in the yeast two hybrid system. They further showed that the protein, which they called Ini1, stimulated integrase activity *in vitro* and they described experiments suggesting that hSNF5/Ini1 interacts with BRG-1.

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