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A human homologue of *Saccharomyces cerevisiae SNF2/SWI2* and *Drosophila brm* genes potentiates transcriptional activation by the glucocorticoid receptor

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Several of the *SNF* and *SWI* genes of *Saccharomyces cerevisiae* code for proteins believed to assist transcriptional activators by relieving nucleosome repression. One of these proteins, SNF2/SWI2, has a homologue in *Drosophila*, a regulator of homeotic genes known as brahma or brm. In this report, we show that a counterpart of SNF2/SWI2 also exists in mice and humans. The human protein, designated hbrm, is a 180 kDa nuclear factor that can function as a transcriptional activator when fused to a heterologous DNA binding domain. The mouse homologue of hbrm is expressed in all mouse organs tested while hbrm was detected in some but not all investigated human cell lines. In cells failing to express the endogenous gene, transfected hbrm co-operates with the glucocorticoid receptor (GR) in transcriptional activation. However, hbrm had no effect on the activity of several other transcription factors, including the homeoprotein HNF-1. The co-operation between hbrm and GR required the DNA binding domain of GR and two separated regions of the hbrm protein, including a domain with homology to known helicases.

Key words: Brahma/glucocorticoid receptor/helicase/transcription

Introduction

Transcription by RNA polymerase II requires two classes of transcription factors. One class, comprising general factors, is responsible for basal promoter activity, while the second comprises modulators, which function either to activate or repress transcription. The general factors, including TFIIA, IIB, IIE, IIF and IIG, form a pre-initiation complex which is bound to the core promoter via the TATA binding protein complex TFIID. The modulators are often tissue-specific and bind to DNA sequences upstream of the core promoter (Johnson and McKnight, 1989; Lewin, 1990; Sawadogo and Sentenac, 1990). These factors appear to regulate transcription by interacting with TFIID or TFIIB to influence the formation of the pre-initiation complex (Sharp, 1992; Hoey *et al.*, 1993; Roberts *et al.*, 1993).

It has been suggested that at least some transcriptional activators function by modulating the access of TFIID to binding sites which are occluded by histones (Kornberg and Lorch, 1991; Felsenfeld, 1992; Travers, 1992; Adams and Workman, 1993). *In vitro*, several transcription factors including TFIID have been shown to prevent the transcriptional inhibition by nucleosomes if they are added to the transcription system prior to the histones (Workman and Roeder, 1987; Van Dyke *et al.*, 1988; Croston *et al.*, 1991; Workman *et al.*, 1991). *In vivo*, however, it was believed that the chromatin structure could only be reprogrammed in a dividing cell during DNA replication. Recent studies on the yeast *PHO5* promoter suggest that this is not the case. Upon *PHO5* induction, nucleosome disruption on the promoter is also observed when DNA replication is prevented (Schmid *et al.*, 1992). Disruption of the nucleosomes at the *PHO5* promoter is apparently due to the binding of transcription factors to the histone-covered DNA in a ternary complex. Such complexes have been observed *in vitro* with the chimeric protein GAL4-VP16, which can bind to its DNA site and relieve nucleosome repression even when the DNA template has been pre-packed with purified histones (Taylor *et al.*, 1991; Workman and Kingston, 1992). The effect of GAL4-VP16 on nucleosome repression can, however, only be observed with a crude *in vitro* transcription system. When GAL4-VP16 is assayed with a system reconstituted from a fractionated yeast extract (functioning on naked DNA templates), no effect on nucleosome repression is apparent (Lorch *et al.*, 1992). This observation suggests that at least in yeast, transcriptional activators require additional components besides the general initiation factors and RNA polymerase II to overcome chromatin repression. Five yeast genes have been identified that may be involved in this mechanism: *SWI1*, *SNF2/SWI2*, *SWI3*, *SNF5* and *SNF6*.

The *SWI* genes were first identified as regulators of *HO*, a gene involved in mating type switching (SWI = switch) (Peterson and Herskowitz, 1992). Later, *SWI2* was found to be identical to *SNF2*, a gene involved in the regulation of the *SUC2* gene (Laurent *et al.*, 1991). In fact, the transcriptional regulation of the *HO* gene is now known to be dependent on two other *SNF* genes, *SNF5* and *SNF6*. Mutations in any of the *SWI1*, *SNF2/SWI2*, *SWI3*, *SNF5* or *SNF6* genes result in a similar phenotype, suggesting that these five genes code for members of the same protein complex. Furthermore, the SNF and the SWI gene products activate transcription of numerous other genes besides *SUC2* and *HO* (Estruch and Carlson, 1990; Laurent *et al.*, 1990; Happel *et al.*, 1991; Yoshimoto and Yamashita, 1991; Peterson and Herskowitz, 1992). All the activated genes are highly inducible and in most cases have a known dedicated activator (GAL4 for *GAL1*, ADR1 for *ADH2* and INO2/INO4 for *INO1*). The *SNF/SWI* genes have also been shown to be required for activation by the transcription factors GAL4 or Bicoid in yeast (Laurent and Carlson, 1992). Hence, it is believed that the SNF/SWI factors form a complex that activates transcription by co-operating with inducible transcription factors.

Several findings suggest that the SNF/SWI protein complex may be implicated in a mechanism of unwinding of the chromatin structure in the vicinity of the promoters

they activate. Indeed, mutations in the *SNF* and *SWI* genes can be compensated by mutations that may affect chromatin structure. For example, the dependence of the *HO* gene on *SWI1*, *2* and *3*, can be relieved by mutations that alter the *SIN1* or the *SIN2* genes. These two genes encode an HMG-1 like protein and the histone H3 respectively (Kruger and Herskowitz, 1991; Travers, 1992). Likewise, deletions of the *HTT1-HTT2* cluster, which encodes one of the two copies of histones H2A and H2B, allows expression of the *SUC2* gene in the absence of SNF2/SWI2 (Hirschhorn *et al.*, 1992). It has also been demonstrated that the activation of the *SUC2* gene by sucrose induction results in a nucleosome displacement on the promoter. This displacement is absent or partial in *snf5* and *snf2* (*swi2*) mutants (Hirschhorn *et al.*, 1992; Matallana *et al.*, 1992). Lastly, the sequence analysis of the SNF2/SWI2 factor has revealed the presence of a domain conserved among a variety of proteins with determined or presumed helicase activity (Laurent *et al.*, 1992; Peterson and Herskowitz, 1992). Helicase activity is known to be involved in chromatin decondensation during DNA replication (Gruss and Sogo, 1992).

A homologue of the yeast SNF2/SWI2 exists in *Drosophila*. This factor, known as brahma or brm, is similar to its yeast counterpart in both size and structure and exhibits 57% amino acid identity within the helicase domain (Tamkun *et al.*, 1992). Like SNF2/SWI2, the brm gene product has been implicated in transcriptional activation and it appears to have a positive effect on the expression of several homeotic genes including Scr and Antp. The *brm* gene was first identified as a suppressor of Polycomb (Pc) mutations, mutations resulting in homeotic transformation by derepression of the Antennapedia and the Bithorax complexes. Interestingly, it has been suggested that the Pc group of genes repress homeotic genes by regionally compacting the chromatin (Franke *et al.*, 1992). Thus, *brm* may be part of a mechanism restoring accessibility of these compacted regions.

In this report, we investigated whether a counterpart of the *SNF2/SWI2* gene was present in cells from higher eukaryotes. Using a fragment from the *brm* gene coding region, we cloned a human nuclear factor closely related to brm and SNF2/SWI2, within the helicase domain but also in flanking regions. The human factor, termed hbrm, is shown to be a strong activator of transcription when tethered to DNA by fusion to a known DNA binding domain. In addition, intact hbrm potentiates transcriptional activation by co-transfected glucocorticoid receptor (GR) and retinoic acid receptor α, in cell lines lacking endogenous hbrm.

Results

Cloning of human and mouse homologues of the Drosophila brm gene

Oligonucleotides complementary to the published sequence of the *Drosophila brm* gene were used to isolate a partial *brm* coding sequence from genomic *Drosophila* DNA by PCR amplification. The 729 nucleotide amplified fragment encoded a region inside the helicase domain of brm and was highly conserved between brm and SNF2/SWI2. This fragment was used to search for a putative human homologue of *Drosophila brm*. Southern analysis carried out under low stringency conditions with genomic DNA from HeLa cells, cleaved with either *Bam*HI or *Eco*RI, revealed for both enzymes a single DNA species which hybridized to the *brm* probe (data not shown). These results suggested that a gene highly homologous to *brm* was present in the human genome.

The *Drosophila brm* probe was then used to screen a human liver cDNA library. Six phages with different inserts were isolated. The inserts were subcloned and by restriction mapping shown to contain fragments of the same gene. The longest cDNA (5 kb) was sequenced and found to contain an open reading frame of 1587 codons defined by an in-frame ATG preceded by stop codons in all three reading frames. A fourth isolate was found to extend 800 nucleotides further toward the 3′ end of the cDNA. Sequence analysis of this clone revealed the presence of a poly(A) tail. The composite sequence of this human homologue of *brm* is shown in Figure 1. This factor will be referred to hereafter as hbrm.

A murine λZAP lung cDNA library was also screened by probing with a fragment from the helicase domain of hbrm. Eight recombinant phages containing different inserts were isolated. DNA sequencing analysis of several of these partial cDNAs revealed that they coded for parts of a protein, 90–95% homologous to hbrm at the amino acid level inside the sequenced regions. This mouse homologue of *brm* was termed mbrm. Oligonucleotides specific for DNA sequences of this mouse gene were used to determine the pattern of expression of mbrm in the adult mouse, using a quantitative RT-PCR technique. mbrm messenger RNA was detected at similar levels in heart, brain, lung, liver, thymus, kidney and spleen (data not shown).

Structure of the predicted hbrm protein, the human homologue of brm

The predicted hbrm protein has a calculated molecular mass of 180 649 Da. Analysis of the amino acid sequence of the protein revealed that hbrm is 56% identical and 72% similar to *Drosophila* brm. Three regions are particularly conserved from *Drosophila* to human, including the potential helicase domain encoded by the probe used for the cloning, a bromodomain and a third amino-terminal region of yet unknown function (Figure 2A and B). The potential helicase domain contains seven regions which are strongly conserved among a variety of known or putative helicases from different organisms including *Escherichia coli*, RNA and DNA viruses, *Saccharomyces cerevisiae*, *Drosophila melanogaster*, mice and humans (Gorbalenya *et al.*, 1988, 1989; Hodgman, 1988; Linder *et al.*, 1989; Okabe *et al.*, 1992; Soininen *et al.*, 1992; Troelstra *et al.*, 1992; Delmas *et al.*, 1993; Schaeffer *et al.*, 1993). Two of these seven regions match the consensus sequence of the bipartite NTP binding motif common to ATP or GTP requiring enzymes (Walker *et al.*, 1982; Pai *et al.*, 1989; Wittinghofer and Pai, 1991). In SNF2/SWI2, these two regions have been shown to hydrolyse ATP actively and are required for transcriptional activation (Laurent *et al.*, 1993).

The second homology domain between hbrm and brm is located at the carboxy-terminal end. This domain has been defined as a bromodomain and is conserved in several transcriptional regulators including the yeast factors SNF2/SWI2 and SPT7, the *Drosophila* fsh protein and the human $TAF_{II}250$/CCG1 protein (Hisatake *et al.*, 1993; Ruppert *et al.*, 1993). It has been suggested that the bromodomain may mediate protein–protein interactions among transcriptional activators (Haynes *et al.*, 1992; Tamkun *et al.*, 1992). The hydrophobicity plot (Figure 2C) shows that hbrm is a very hydrophilic protein except for the

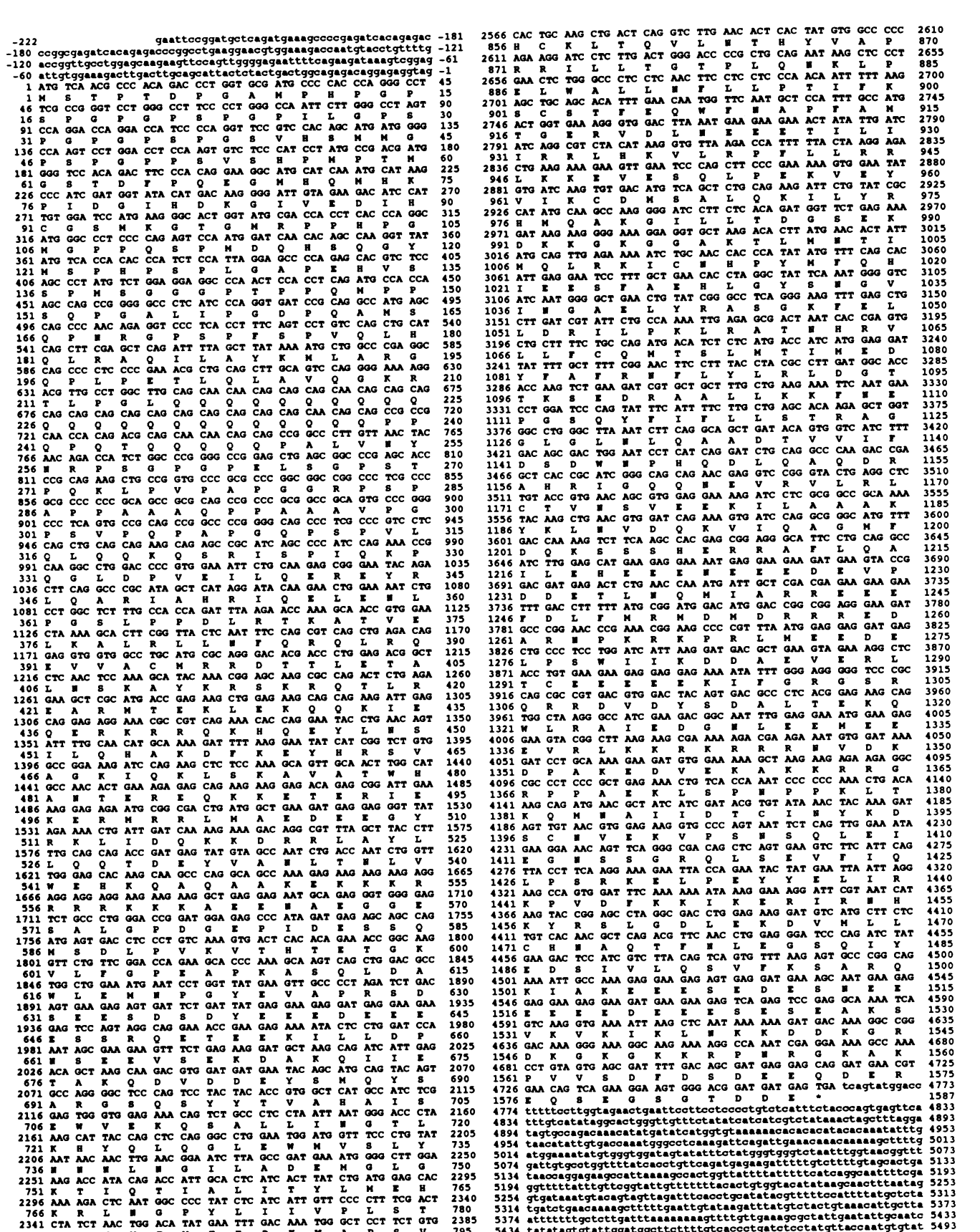

Fig. 1. Complete nucleotide and amino acid sequence of the hbrm cDNA. EMBL Data Library accession number of the hbrm sequence: X72889.

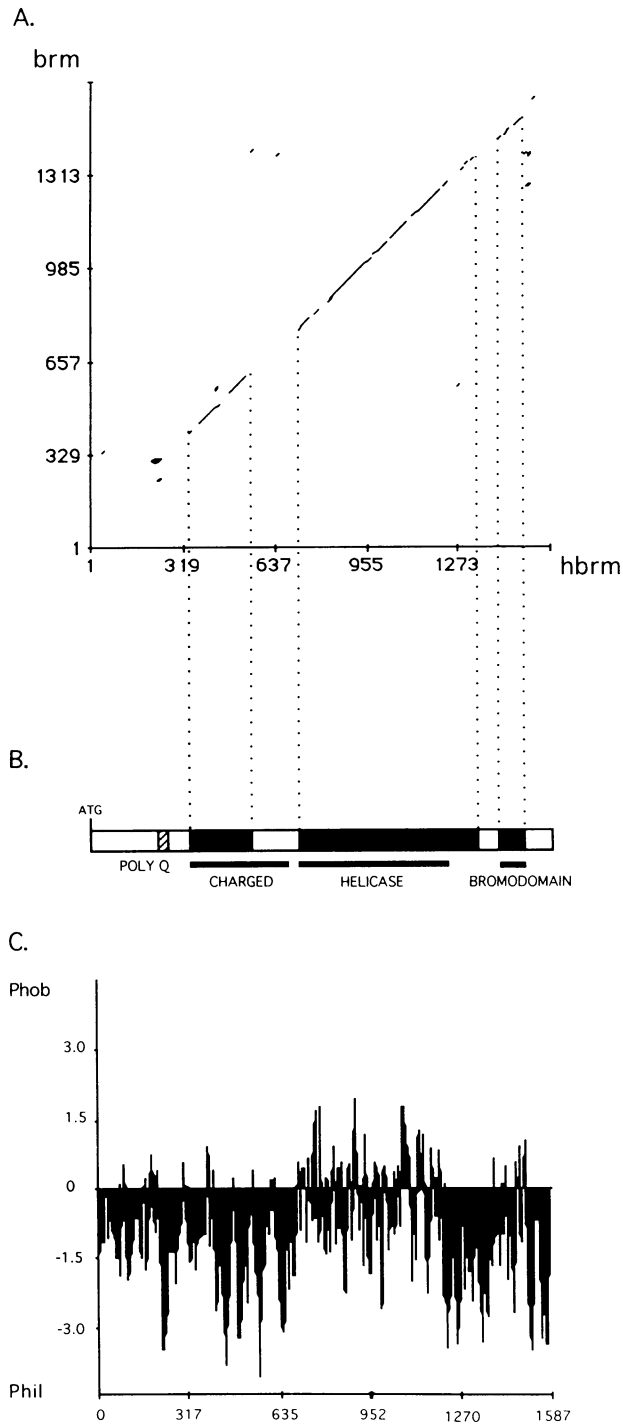

Fig. 2. Structural comparison between brm and hbrm proteins and hydrophobicity plot of hbrm. (**A**) The complete amino acid sequences of brm and hbrm were compared by a Pustell matrix analysis using 'Mac Molly's Heart' software (Soft Gene, Berlin). The minimal window size was eight nucleotides with a maximum number of mismatches of two. (**B**) Localization on the hbrm protein of the regions with the highest degree of conservation between brm and hbrm (shaded regions). The charged region, the helicase domain and the bromodomain are underlined. The polyglutamine track is indicated by POLY Q. (C) Kyte and Doolittle hydrophobicity plot of hbrm using 'DNA Strider'. The plot is aligned on schematic B.

helicase domain and the bromodomain. These two domains may constitute the only structured regions in an otherwise very flexible protein.

The last region of homology between hbrm and brm is located in the amino-terminal end of the proteins. Only ~200 amino acids of the two proteins are actually conserved in the primary sequence. However, both in the *Drosophila* and in the human protein, the amino-terminal end is characterized by a highly proline- and glutamine-rich region overlapping a charged region. The proline/glutamine-rich region of hbrm (extending from amino acids 1 to 366) contains 22% prolines and 16% glutamines, including a glutamine stretch of 23 residues. Such regions have been characterized as important for transcriptional activation in various factors including AP-2 (Williams and Tjian, 1991) and Oct-2 (Clerc *et al.*, 1988; Muller *et al.*, 1988; Scheidereit *et al.*, 1988). The charged region of hbrm, which contains the actual sequence of homology with brm, comprises a succession of positively and negatively charged stretches very similar to those found in Rap74 (Finkelstein *et al.*, 1992). The region extends from amino acids 320 to 684 and contains 20% basic residues and 21% acidic residues. Charged clusters are also a common feature among nuclear transcription factors (Brendel and Karlin, 1989).

The proline/glutamine-rich and charged regions of hbrm (termed P/Q-charged hereafter) is also present in brm and SNF2/SWI2. However, this region is either partial or missing in the yeast STH1 and the human hSNF2L, two proteins otherwise very similar to brm and SNF2/SWI2 (Laurent *et al.*, 1992; Okabe *et al.*, 1992). Interestingly, it has not been possible to show transcriptional activity for STH1 and hSNF2L, whereas SNF2/SWI2 is an activator. This observation favours an implication of the P/Q-charged region in transcriptional activation.

Finally, the structure of hbrm is characterized by highly charged regions located in the hinge region between the helicase domain and the bromodomain and at the complete carboxy-terminal end of the protein.

hbrm activates transcription when tethered to the DNA

The proline- and glutamine-rich region of hbrm is reminiscent of regions found in transcriptional activators. In addition, immunofluorescent staining of the human cervical epithelium cell line C33, transfected with a CMV expression vector containing the hbrm coding region, shows that the hbrm protein localizes to the nucleus (data not shown). However, the analysis of the primary sequence of the hbrm protein does not reveal the presence of any canonical DNA binding domain. Therefore, to assay hbrm for potential transcriptional activity, the entire open reading frame was linked at its carboxy-terminal end to the DNA binding domain of the BPV-1 E2 protein, which by itself does not activate transcription. This fusion protein, together with various promoter constructs cloned upstream of the bacterial chloramphenicol acetyl transferase (CAT) gene (Figure 3A), was used in co-transfection assays in C33 cells. In each experiment, the effect on transcriptional activity of the hbrm−E2 fusion was compared with the effect of native BPV-1 E2. The strongest transcriptional effect of the hbrm−E2 fusion was observed with a construct containing six E2 sites upstream of the HSV-1 thymidine kinase (TK) promoter. With this promoter construct, hbrm−E2 yielded a 15-fold activation above basal transcription, ~5-fold less than the activity obtained with wild-type E2 (Figure 3A, line 1). A similar promoter construct with only two E2 sites resulted in a reduced activation by both hbrm−E2 and wild-type E2 (2-fold activation instead of 15-fold for hbrm−E2

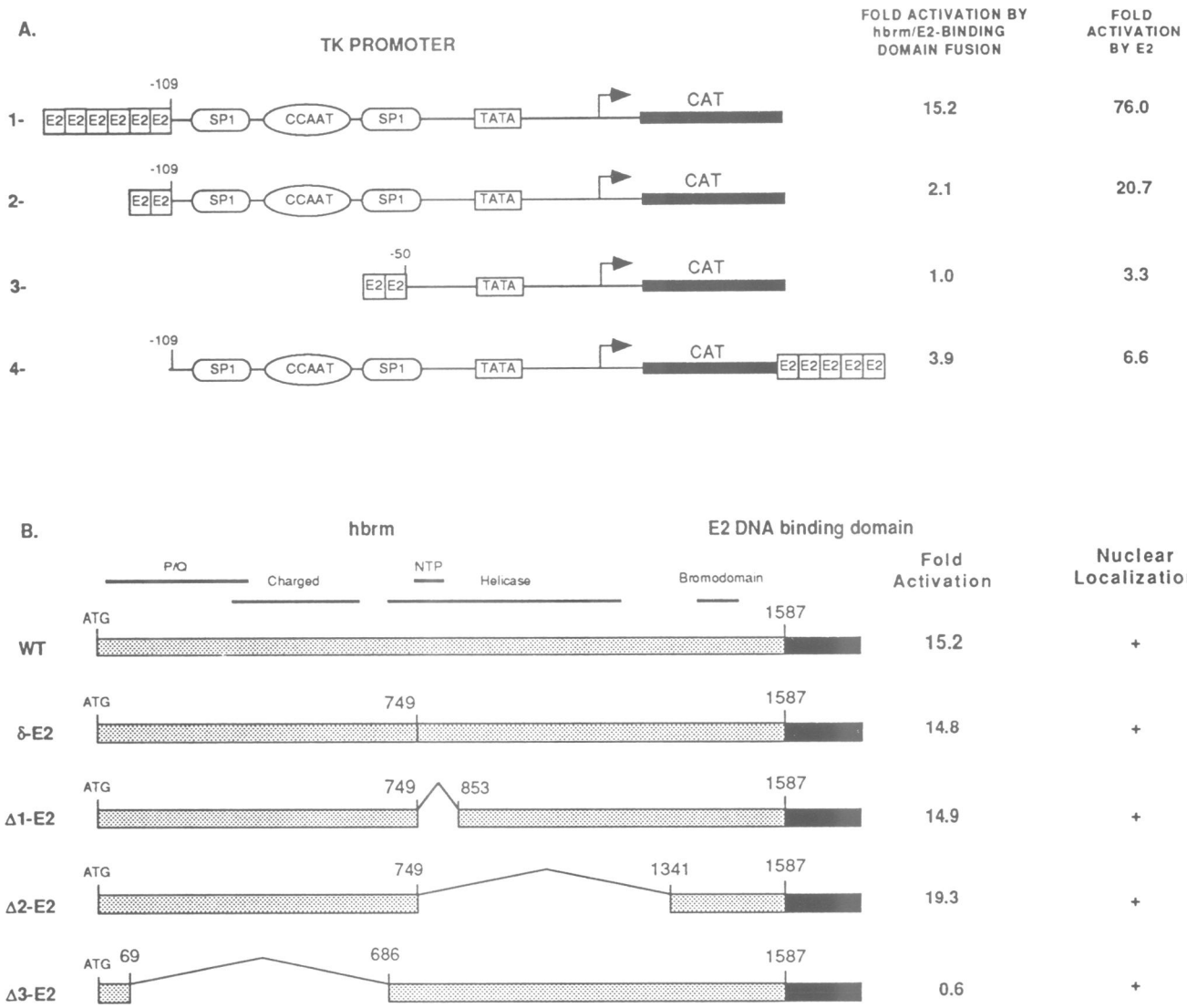

Fig. 3. hbrm activates transcription as a fusion protein with the BPV-1 E2 DNA binding domain. (**A**) Either 2 μg of hbrm−E2 fusion expression vector or 100 ng of wild-type BPV-1 E2 expression vector were co-transfected into C33 cells with 1 μg of one of the shown TK−CAT reporter constructs. Basal level was estimated by transfecting either 2 μg (for hbrm−E2) or 100 ng (for wild-type E2) CMV expression vector without insert. CAT activity was determined 48 h post-transfection. (**B**) Two micrograms of either hbrm−E2 or shown mutation constructs or CMV expression vector without insert were co-transfected into C33 cells with 1 μg of reporter construct #1 from panel A. CAT activity was determined 48 h post-transfection. Transfected cells were also submitted to immunofluorescent staining using an antibody recognizing the amino-terminal end of hbrm. The cellular localization of the different mutants as determined by this technique, is indicated. On the schematic of the mutants, the amino acid position of the mutations or deletions is indicated. The proline/glutamine-rich region (P/Q), the charged region, the NTP binding site (NTP), the helicase domain and the bromodomain are overlined. The results given in (A) and (B) are calculated from at least three independent experiments.

and 20-fold instead of 76-fold for wild-type E2) (Figure 3A, line 2). Activity of the hbrm−E2 fusion was also tested on a minimal promoter containing two E2 sites and a canonical TATA box. This construct was only activated 2- to 3-fold by wild-type E2 and was not activated by hbrm−E2 (Figure 3A, line 3). Ultimately, we assayed a construct containing five E2 sites downstream of the TK promoter and the CAT gene. On this construct, hbrm−E2 fusion gave a 4-fold activation which is very similar to the activation obtained with wild-type E2 (Figure 3A, line 4). None of the above promoter constructs were activated by native hbrm. These experiments show that hbrm, when tethered to the DNA through a DNA binding domain, is a potent transcriptional activator, although weaker than the strong viral E2 activator. The hbrm−E2 fusion protein also shares several classical properties with DNA binding transcriptional activators, including co-operative activation when several binding sites are present, the requirement of the presence of other promoter elements for maximal activation and the ability to activate transcription from a distance.

Regions responsible for transactivation by the hbrm−E2 fusion protein

To identify the domains of hbrm responsible for its transcriptional activity, several mutants were constructed and assayed by co-transfection in C33 cells, together with the 6xE2-TK promoter−CAT construct (schematically shown in Figure 3A, line 1). All of the mutants described were found to conserve a nuclear localization and to be expressed at similar levels when detected by immunofluorescence in transfected cells. The first region targeted for mutations was the putative NTP binding site located in the helicase domain, an essential region for helicase activity. Surprisingly, neither a mutation in the GKT consensus sequence of the NTP

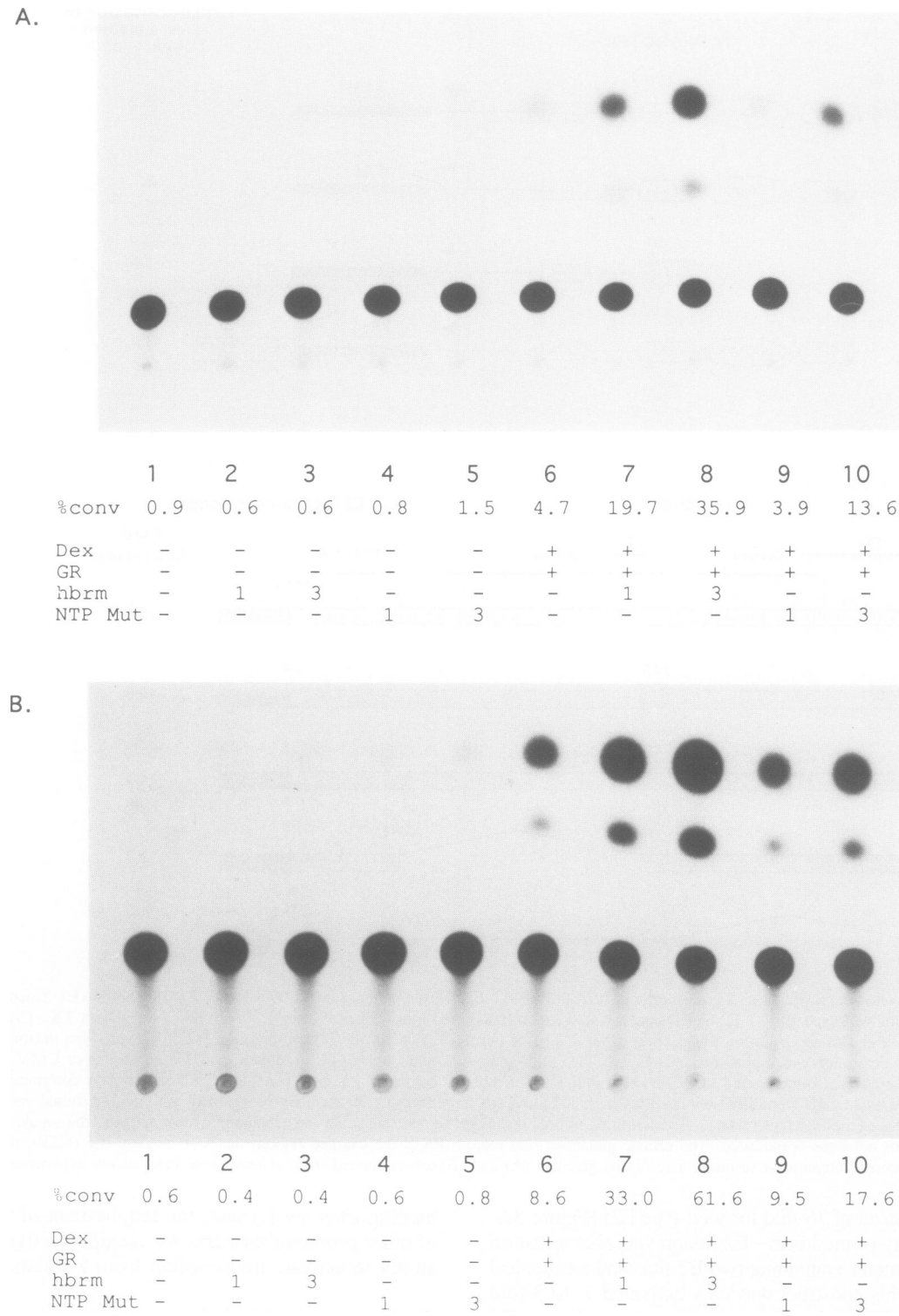

Fig. 4. hbrm can co-operate with GR for transcriptional activation. As indicated, 1 μg of MMTV/CAT (**A**) or GRE_2-TK−CAT (**B**) reporter construct and either 3 μg of CMV expression vector without insert, 1 or 3 μg wild-type hbrm expression vector (hbrm) or 1 or 3 μg NTP site mutated hbrm expression vector (NTP mut) were co-transfected into C33 cells in the absence or in the presence of dexamethasone (Dex) and 50 ng of a CMV GR expression vector. CAT activity was determined 48 h post-transfection. The CAT assays shown are representative of at least three independent experiments. Percent conversion is indicated under each lane.

binding site, nor the complete deletion of the bipartite NTP binding motif had any effect on transcriptional activation by the hbrm−E2 fusion protein (Figure 3B, mutants δ-E2 and Δ1-E2). In fact, a deletion mutant containing no helicase domain at all was slightly more active than the wild-type hbrm−E2 (Figure 3B, mutant Δ2-E2). However, a construct lacking the P/Q-charged region, had no transcriptional activity (Figure 3B, mutant Δ3-E2). These experiments define the P/Q-charged region as an activating domain of hbrm.

hbrm increases transcriptional activation by steroid/vitamin receptors

Having shown that hbrm could increase gene expression when fused to a DNA binding domain, we wished to determine whether a similar effect could be observed with native hbrm. The factor was assayed in transient transfection with several constitutive or activated promoters including the SV40 early (SVe) and CMV promoters and the HTLV-I and HIV-1 LTRs. hbrm was not found to activate transcription from any of these promoters. Nor did hbrm have an effect on transcriptional activation by AP-2 or the liver enriched, highly diverged homeoprotein HNF-1 (Chouard *et al.*, 1990) at their respective target promoters.

It has recently been demonstrated that mammalian steroid receptors require SWI1, 2 and 3 for ectopic transcriptional activation in yeast. It was found that the rat estrogen and glucocorticoid receptors were unable to stimulate transcription from a reporter construct carrying proper responsive elements when the receptors were transformed into *swi*$^-$ yeast mutants (Yoshinaga *et al.*, 1992). These observations raised the possibility that hbrm, the human homologue of SNF2/SWI2, is implicated in steroid receptor activation in mammalian cells. To investigate this possibility, we assayed the effect of an hbrm expression vector on the activation of the MMTV promoter by the glucocorticoid receptor (GR). The experiments were performed in C33 cells stimulated with dexamethasone. In these cells, either in the absence or presence of the hbrm expression vector, dexamethasone had no visible effect on the basal level of transcription, suggesting that C33 cells may express low levels of endogenous GR. Co-transfection of 50 ng of CMV/GR expression vector resulted in a 5-fold stimulation of transcription from the MMTV promoter (Figure 4A, lanes 1 and 6). Further addition of 1 μg of an hbrm expression vector increased the stimulation to >20-fold (a net 4-fold effect). At 3 μg of hbrm expression vector, the stimulation peaked at ~40-fold (a net 8-fold effect) (Figure 4A, lanes 7 and 8). To determine whether the co-operativity of hbrm with GR was dependent on the putative helicase activity of hbrm, we used an NTP binding site hbrm mutant instead of wild-type hbrm. With this mutant, no synergy with GR was observed with 1 μg of expression vector. Co-transfection of 3 μg of NTP mutant expression vector with GR expression vector resulted in a weak increase in activity compared with GR alone (2- to 3-fold compared with 8-fold with intact hbrm) (Figure 4A, lanes 9 and 10). To confirm that the stimulation by hbrm was solely dependent on the glucocorticoid response elements (GRE) in the MMTV promoter, we tested a construct containing two GRE sites upstream of the minimal TK promoter (GRE_2-TK−CAT). This promoter construct was activated more efficiently by the GR expression vector (14-fold) than the MMTV promoter (Figure 4B, lanes 1 and 6). Again, hbrm was found to enhance receptor mediated activation (Figure 4B, lanes 7 and 8) and this enhancement decreased upon mutation of the putative nucleotide binding site of hbrm (Figure 4B, lanes 9 and 10).

The stimulating effect of hbrm on GR transcriptional activation was unlikely to be due to increased expression from the CMV/GR vector since hbrm was not found to activate a CMV promoter. Furthermore, we assayed the effect of hbrm in the presence of increasing amounts of GR expression vector (Figure 5). Maximal activation of the

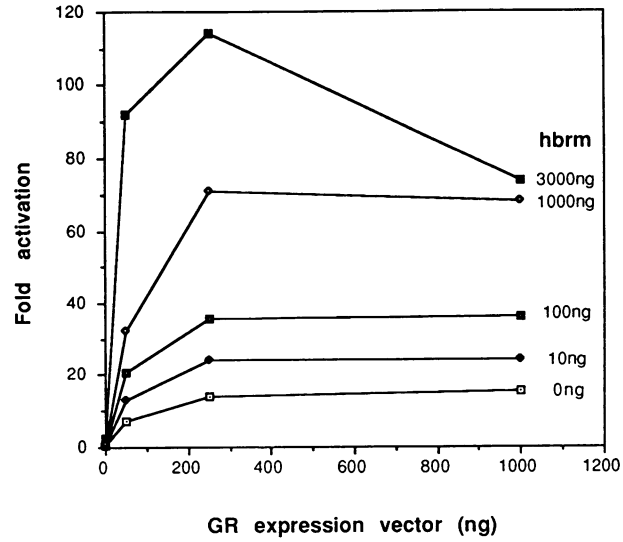

Fig. 5. Effect of hbrm on increasing amounts of transfected GR. 1 μg of GRE_2-TK−CAT reporter construct was co-transfected into C33 cells in the presence of dexamethasone with either 0, 50, 250 or 1000 ng of CMV GR expression vector together with either 0, 10, 100, 1000 or 3000 of CMV/hbrm expression vector. CAT activity was determined 48 h post-transfection. Fold activation above basal level (in the absence of dexamethasone) is indicated on the graph. Values are the mean of two independent experiments. For symbols see key in figure.

GRE_2-TK promoter by GR alone was reached with 250 ng of GR expression vector (~14-fold activation above basal) and this activity did not increase when 1000 ng of GR expression were used. In the presence of these saturating amounts of GR expression vector, GR activation could still be stimulated by co-transfection of increasing amounts of hbrm expression vector. This strongly suggests that hbrm potentiates the activity of GR rather than increasing its level of expression.

The effect of hbrm on activation by the RARα was also investigated. The experiment was performed using a CAT construct containing the retinoic acid responsive promoter from the retinoic acid β gene, transfected into retinoic acid stimulated C33 cells. Under these conditions, RARα activation increased ~3-fold in the presence of an hbrm expression vector. This activation was not observed when the wild-type hbrm was replaced by the NTP binding site mutant (data not shown). The magnitude of the effect of hbrm on RARα activation was lower than on GR activation. However, this result suggests that hbrm may be implicated in the regulation by nuclear receptors other than GR.

C33 and SW13 cell lines do not express endogenous hbrm

To determine whether the stimulating effect of hbrm on GR activation could be observed in cell lines other than C33, transfections were performed in the human hepathelial cell line HepG2 and breast cancer cell line MCF-7. Co-transfection of 50 ng of GR expression vector in the presence of dexamethasone resulted in a 10-fold activation of the GRE_2-TK promoter in HepG2 and in a 230-fold activation in MCF-7. No further activation was observed in the presence of hbrm in HepG2 cells. In MCF-7, addition of hbrm resulted in a slight repression (<2-fold) of GR activation (data not shown).

To investigate the origin of the differences we observed between C33, HepG2 and MCF-7, we decided to monitor the levels of endogenous hbrm in human cell lines available in the laboratory. RT-PCR with human hbrm-specific or GPDH-specific primers was performed on total RNA from C33, HepG2 and MCF-7 but also two other cervical carcinoma cell lines HT-3 and HeLa, the laryngeal carcinoma HEp2 and the adrenal cortex carcinoma-derived cell line SW13. After maximal amplification (40 cycles), GPDH message could be detected in all cell lines. On the other hand, hbrm message was not detected in two cell lines, C33 and SW13 (Figure 6A, lanes 5–8). These results prompted us to test whether hbrm may co-operate with GR only in cell lines lacking the endogenous gene product. Consequently, we transfected SW13 cells with the GRE_2-TK–CAT reporter construct. Dexamethasone alone had no effect on this promoter in these cells (Figure 6B, lane 4), while dexamethasone and 50 ng of CMV/GR expression vector resulted in an 18-fold activation above basal level (Figure 6B, lane 7). Addition of 1 or 3 μg of CMV/hbrm expression vector further increased this activation ~5-fold (Figure 6B, lanes 8 and 9). These results clearly show that in the cells we tested, transfected hbrm enhances GR activation when the endogenous gene is silent but has no activating effect when it is expressed.

Co-operation between hbrm and GR requires the DNA binding domain of GR and the helicase domain and the P/Q-charged domain of hbrm

To try to elucidate the mechanism of the GR/hbrm co-operation, we co-transfected C33 cells with the hbrm expression vector and two GR deletion mutants lacking either the amino- or the carboxy-terminal end of the nuclear receptor. The carboxy-terminal end of GR contains the hormone binding domain and the deletion of this region generates a hormone-independent activator. In our experiments, this deletion did not affect the activation of the GRE_2-TK promoter (~10-fold above basal) and the effect of this GR mutant could still be stimulated 7- to 8-fold in the presence of 3 μg of hbrm expression vector (Figure 7, line 2). Deletion of the amino-terminal end of GR, containing the activating domain enh2, resulted in a moderate decrease in GR activation (~7-fold above basal). As with the previous mutant, the activation by this construct was still stimulated by hbrm expression vector. However, this stimulation was only ~4-fold (Figure 7, line 3). These results suggested that the DNA binding domain of GR is critical for the GR/hbrm co-operation since it is the only common domain in the two GR mutants. To investigate this possibility further, we used two constructs containing either a GAL4 DNA binding domain (GAL4 BD) followed by the GR hormone binding domain 'GAL-GR(EF)' or a full GR amino-terminal end followed by the GAL4 BD 'GR(AB)-GAL'. Both constructs were transfected into C33 cells with a reporter construct containing five GAL4 binding sites upstream of an E1b minimal promoter. GAL-GR(EF) was found to activate this reporter construct ~7-fold but co-transfected hbrm expression vector did not increase this activation (Figure 7, line 4). GR(AB)-GAL did not activate expression from our reporter plasmid in the cells we used in either the absence or the presence of the hbrm expression vector (Figure 7, line 5). These results further support the conclusion that the GR DNA binding domain is required for GR/hbrm co-operation.

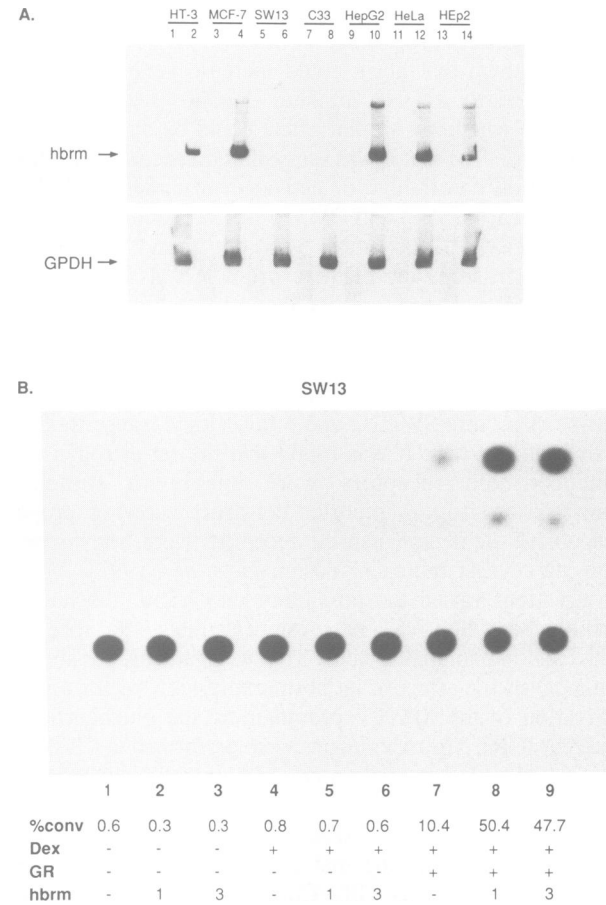

Fig. 6. Endogenous expression of hbrm in various human cell lines. (**A**) cDNA reactions without (odd lanes) or with (even lanes) RT were used for PCR amplification using either hbrm or GPDH specific oligonucleotides. A two-thousandth of the reaction was separated on a native polyacrylamide gel, transferred to nylon membrane and probed with either a ^{32}P-labelled hbrm or GPDH probe as indicated. cDNA was prepared from either HT-3 (lanes 1 and 2), MCF-7 (lanes 3 and 4), SW13 (lanes 5 and 6), C33 (lanes 7 and 8), HepG2 (lanes 9 and 10), HeLa (lanes 11 and 12) or HEp2 (lanes 13 and 14) cell lines. (**B**) As indicated, 1 μg GRE_2-TK–CAT reporter construct and either 3 μg of CMV expression vector without insert or 1 μg or 3 μg of wild-type hbrm expression vector (hbrm) were co-transfected into SW13 cells in the absence or in the presence of dexamethasone (Dex) and 50 ng of a CMV/GR expression vector. CAT activity was determined 48 h post-transfection. The CAT assays shown are representative of three independent experiments. Percent conversion is indicated under each lane.

We also wished to undertake a more detailed analysis of the domains of hbrm involved in the GR/hbrm co-operation. The experiments described earlier showed that an intact helicase domain is necessary for full co-operation between hbrm and GR (Figures 4 and 8). However, the results obtained with the hbrm–E2 fusion protein suggested that other domains of the hbrm protein might be important for its transcriptional activity. To investigate the effect of the P/Q-charged region and the bromodomain on GR/hbrm co-operation, two additional hbrm mutants were tested in transfection into dexamethasone stimulated C33 cells, together with the GRE_2-TK–CAT reporter and a GR expression vector. A deletion of the P/Q-charged domain resulted in a 3-fold decrease of the GR/hbrm co-operation (Figure 8A, Δ3). A deletion of the carboxy-terminal end, including the bromodomain, did not appear to modify the effect of hbrm on GR activation when 1 μg of expression

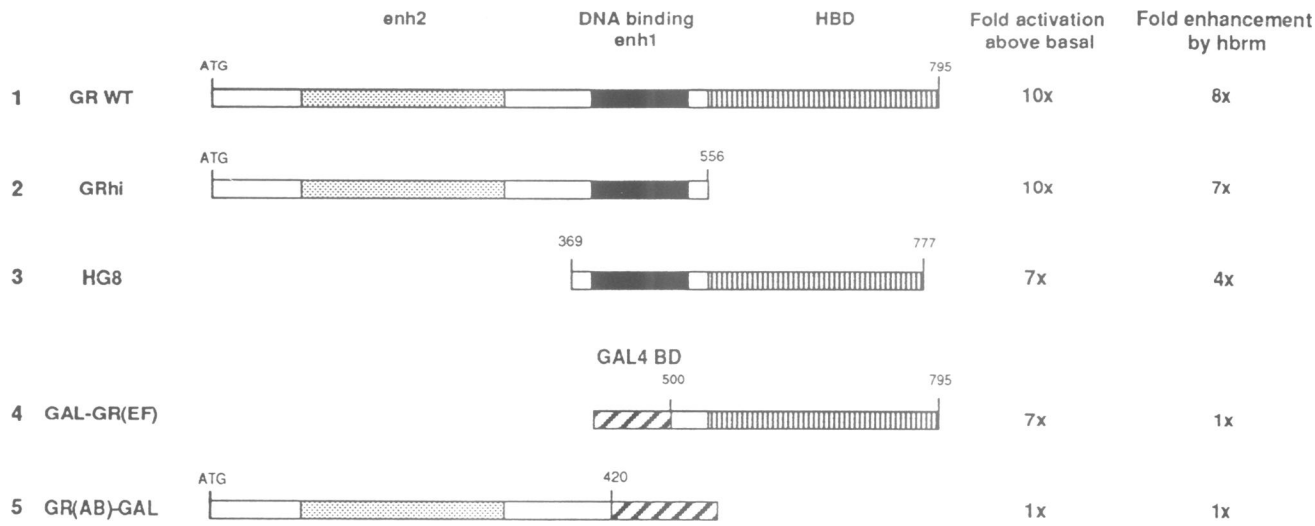

Fig. 7. The GR DNA binding domain is essential for co-operation with hbrm. 1 μg of GRE_2-TK−CAT (lines 1, 2 and 3) or 5xGAL4−E1b/CAT (lines 4 and 5) reporter constructs were co-transfected into C33 cells in the absence or in the presence of 50 ng of GR WT (line 1), hormone-independent GR (GRhi) (line 2), HG8 (line 3) or 1 μg of GAL-GR(EF) (line 4) or GR(AB)-GAL (line 5) expression vectors and either 3 μg of CMV expression vector without insert or 3 μg wild-type hbrm expression vector. Dexamethasone was added for lines 1, 3 and 4. Fold activation above basal level for each GR construct and fold increase of this activation in the presence of the hbrm expression vector are indicated. Result shown are calculated from three independent experiments. The GR DNA binding domain is indicated by a black box and the GAL4 DNA binding domain (GAL4 BD) by a striated box. The amino-terminal activation domain of GR (enh2) and the hormone binding domain (HBD) are also indicated.

Fig. 8. Regions responsible for GR/hbrm co-operation. (**A**) 1 μg of GRE_2-TK−CAT reporter construct and 1 or 3 μg of either wild-type hbrm (hbrm), NTP site mutated hbrm (NTP Mut), Δ3 or Δ4 expression vector were co-transfected into C33 cells in the presence of dexamethasone and 50 ng of a CMV GR expression vector. CAT activity was determined 48 h post-transfection. The fold enhancement of GR activation by the various hbrm expression constructs was calculated from four independent experiments. (**B**) Extracts were prepared from C33 cells transfected with 20 μg of either pUC19 (lane 1), hbrm (lane 2), NTP Mut (lane 3), Δ3 (lane 4) or Δ4 (lane 5), separated by SDS−PAGE and transferred to nitrocellulose. The blot was then incubated with affinity-purified hbrm antibodies and developed by ECL. Size markers in kDa are indicated.

vector was used. However, with 3 μg expression vector, this mutant was ~2-fold more active than hbrm WT in its co-operation with GR (Figure 8A, Δ4).

To determine whether these mutations affected the activity of hbrm or its level of expression, Western blotting was performed on the total extract from C33 cells transfected with the various mutants. Using a purified polyclonal antibody prepared against the first 178 amino acids of hbrm, the hbrm WT, NTP Mut and Δ3 appeared to be present at similar levels in the cells (Figure 8B, lanes 2−4). However, it must be noticed that Δ3 lacks part of the sequence used to prepare the antibody and may be expressed at a higher level than it appears. The last mutant, Δ4, was overexpressed ~6-fold compared with the other constructs (Figure 8B, lane 5). Immunofluorescent staining of C33 cells transfected with each of the four mutants revealed that NTP Mut and Δ3 remained localized to the nucleus like WT hbrm, whereas Δ4 was evenly distributed in the cell (data not shown). We concluded from these experiments that the helicase domain and the P/Q-charged domain are directly implicated in the GR/hbrm synergy and both are necessary for maximal activity of hbrm. On the other hand, the bromodomain may not be involved in transcriptional activation but could control protein stability and nuclear transport.

Discussion

We describe here the cloning of a cDNA coding for hbrm, a human protein homologous to the yeast SNF2/SWI2 and the *Drosophila* brm gene products. The hbrm protein has a modular structure comprising a proline/glutamine-rich region followed by a charged region, a domain with

homology to known helicases, and a bromodomain. When tested as a fusion protein with the BPV-1 E2 DNA binding domain, hbrm functioned as a strong transcriptional activator. In addition, when transfected into cell lines which do not express the endogenous gene, hbrm co-operates with GR and to a lesser extent with RARα, in transcriptional activation of their respective target promoters. This co-operation requires the helicase domain of hbrm.

Domains of hbrm involved in transcriptional activity

The activity of hbrm has been assayed in two different systems, either as an E2 fusion protein or by co-transfection of the intact protein with GR or RARα. When assayed as a fusion protein, hbrm−E2 acted as a classical transcriptional activator, highly dependent on the number of E2 binding sites present in the promoter and able to activate from binding sites located even downstream of the CAT gene. The P/Q-charged domain appeared to be solely responsible for the transcriptional activity of the hbrm−E2 fusion protein. On the other hand, when hbrm was co-transfected with the GR expression plasmid, we observed a significant drop in activity not only when the P/Q-charged domain was deleted but also when the helicase domain was mutated. These observations suggest that the P/Q-charged domain and the putative helicase domain are both necessary for native hbrm activity but may be involved in two different steps of the transactivation. For example, the P/Q-charged domain could interact with the transcription machinery, histones or HMG proteins while the putative helicase domain may affect the dynamic process preceding or following this interaction. This could explain why both the P/Q-charged mutant and the helicase mutant retain some residual activity. Alternatively, the remaining activity of these mutants may be due to other activating regions of hbrm not yet localized. The lack of requirement for the helicase domain in the hbrm−E2 fusion protein contrasts with the observation made in yeast where a LexA−SNF2/SWI2 fusion protein could only activate transcription with an intact NTP binding site (Laurent *et al.*, 1993). Possibly, the position of the fused DNA binding domain which is amino-terminal in the yeast experiments and carboxy-terminal in our experiments, could account for this difference. Alternatively, the mechanism of activation by SNF2/SWI2 and hbrm may not be strictly conserved from yeast to human. Testing the ability of hbrm to complement a *snf2*$^-$ mutation in yeast will provide valuable information on this matter.

The bromodomain, the third conserved region of hbrm, does not appear to be involved in the co-operation between hbrm and GR. Actually, the deletion of this region increases the activating effect of hbrm. In parallel, we observed that the bromodomain mutant had a higher steady state level in the transfected cells compared with WT hbrm and that this mutant is no longer strictly localized to the nucleus. The higher stability of the bromodomain mutant is likely to account for its enhanced activity and suggests that the bromodomain may be implicated in protein turnover.

Mechanism of the co-operation between hbrm and GR

Our studies performed in C33 and SW13 cells show that GR can activate transcription in the absence of hbrm. However, this activation is weak and increases as a function of the amount of hbrm transfected into the cells. Thus, hbrm appears to facilitate GR activation without being essential. Studies made on the MMTV promoter have shown that upon glucocorticoid receptor binding, the nucleosomes on this promoter are disrupted or displaced, allowing other transcription factors like NF-1 and TFIID to access their binding sites (Beato, 1989; Bresnick *et al.*, 1991). Experiments using deletion mutants and GAL4 fusions with receptor activating domains suggest that hbrm enhancement requires the genuine DNA binding domain of the receptor. Hence, it is possible that hbrm is part of a mechanism that facilitates binding of GR to its target sequence and the disruption of the nucleosomes in the vicinity of the promoter. This mechanism could implicate the putative helicase activity of hbrm which may be involved in chromatin decondensation as suggested earlier (Travers, 1992).

Experiments in *S.cerevisiae* suggest that SNF2/SWI2 functions as a complex with other SNF and SWI proteins (Laurent and Carlson, 1992). At least two of these proteins are likely to have counterparts in higher eukaryotes, as was found for SNF2/SWI2. Indeed, the cloning of a *Drosophila* homologue of SWI3 has been announced (Karin, 1992) and antibodies directed against SWI1 immunoprecipitate a protein of similar size in nuclear extract from *Drosophila* embryos (Yoshinaga *et al.*, 1992). It is therefore possible that like SNF2/SWI2, hbrm acts as a part of a multi-protein complex similar to the SNF/SWI complex and this complex may in turn interact with GR. Co-immunoprecipitations in yeast have shown that SWI3 and rat GR can interact in solution (Yoshinaga *et al.*, 1992). In a similar way, a putative human homologue of SWI3 may form a link between hbrm and GR. Preparation of wild-type and mutated purified hbrm proteins and possibly the isolation of human SWI3 will be necessary to investigate this issue.

Lastly, our experiments show that among the limited set of cell lines we tested, hbrm co-operates with GR only in those that do not express the corresponding mRNA. In other cell lines tested that express their endogenous hbrm gene, GR activation is either insensitive to the addition of excess hbrm (HepG2) or is slightly repressed (MCF-7). This repression may result from interference of incomplete SNF/SWI complexes formed in the presence of excess hbrm. Taken together, these results suggest that hbrm is essential for optimal function of GR *in vivo*. In its absence, GR may function at low efficiency or use factors resembling hbrm, perhaps other nuclear protein complexes containing helicase activity. The SNF/SWI system may be unique in yeast but redundant in higher eukaryotes. Furthermore, different nuclear receptors may depend to a different degree on such complexes. This may explain the quantitative differences observed in enhancement of GR and RARα activities.

It is noteworthy that among the seven human tumour cell lines that we studied, two lacked detectable hbrm mRNA. This variation in hbrm expression is not likely to be tissue-specific since among the tested cervical carcinomas, only one was lacking hbrm expression. It was suggested in several cases that impairment of hormone responsiveness may be the cause for a blockade in cellular differentiation. Such a blockade, together with autocrine growth stimulatory signal may result in cell transformation (Goldberg *et al.*, 1989; Sande *et al.*, 1993). It is tempting to propose that inactivation of hbrm function may be a cause of blockade in hormonal dependent differentiation and that the absence of hbrm in some tumour cell lines may merit further investigation.

Materials and methods

Cloning of a partial brm cDNA

Oligonucleotides with the sequence ATCATGGTTAATGGTACGCTCAAGG and GTCGGGCAGCTGGTGCTCTACCTCC were synthesized. 400 ng of each oligonucleotide were used in PCR reactions with 1 μg of *D.melanogaster* genomic DNA. PCR reactions were prepared under conditions recommended by Taq polymerase manufacturer (Promega) and amplification was carried out for 40 cycles (1 min 95°C, 1 min 55°C and 1 min 72°C). The 729-nucleotide fragment was phosphorylated, subcloned and checked by dideoxynucleotide sequencing.

Cloning of full-length human hbrm cDNA and a partial mouse homologue

The isolated *brm* fragment was ^{32}P-labelled by random priming and used as a probe on a human oligo(dT) primed liver cDNA library in λGT10 kindly provided by D.Lamy. Hybridization was carried out at 55°C overnight in Amersham RapidHybe buffer. Filters were washed twice in 2 × SSPE/0.1% SDS at room temperature then three times in 1 × SSPE/0.1% SDS at 60°C for 10 min and autoradiographed overnight at −80°C. Positive phages were purified and the inserts were subcloned in pUC19 for dideoxy DNA sequencing. A fragment spanning nucleotides 2221−3446 was amplified by PCR from the human cDNA under conditions previously described. This fragment was then subcloned in pUC19, ^{32}P-labelled and used as probe on a random and oligo(dT) primed mouse lung λZAP cDNA library from Stratagene. pBluescript subclones were excised according to the manufacturer and analyzed by dideoxy DNA sequencing.

Plasmid constructs

An *Eco*RI fragment from the hbrm cDNA was inserted in pGEM7 and in the CMV expression vector pCG (Tanaka and Herr, 1990). Oligonucleotides with the following sequence: CTTAAGAAGCGAAAAAGACG (starting at nucleotide 4016) and GGCCATGGGTACCTCATCATCCGTCCC (overlapping the stop codon) were used in PCR mutagenesis to insert, in place of the hbrm stop codon in the pGEM7 clone, an *Asp*718 restriction site in frame with the *Asp*718 site in BPV-1 E2. The amplified fragment was verified by DNA sequencing then cut with *Afl*II and *Asp*718 and inserted in the hbrm/pGEM7 clone digested with *Afl*II and *Asp*718. The mutated hbrm cDNA was then excised with *Xba*I and *Asp*718, ligated to an *Asp*718 fragment coding for the E2 DNA binding domain and inserted into pCG opened with *Xba*I and *Asp*718.

The mutation of the NTP binding site (mutant δ-E2 and NTP Mut) was done by inserting, by PCR-directed mutagenesis, a *Hin*dIII site at position 2249 using the following oligonucleotides: GTGAAGCTTCAAGCCCCATTTCATCGGCTAAG and CCGAAGCTTCAATACAGACCATTGCACTCATC. Mutant Δ1-E2 was constructed by inserting an additional *Hin*dIII site at position 2557 using a third oligonucleotide (GCCAAGCTTGCAAGAATCACCACTGCAAGCTG). The two *Hin*dIII sites were ultimately ligated together. Mutant Δ2-E2 was constructed by ligating S1 treated *Hin*dIII site at position 2249 from mutant δ-E2 to S1 treated *Afl*II site at position 4016. Mutants Δ3-E2 and Δ3 are deletions from the *Sph*I site at position 206 to the *Sph*I site at position 2057. Mutant Δ4 is a deletion from the *Afl*II site at position 4016 to the stop codon at the end of the hbrm ORF. The T7-CMV-GR, GRhi, HG8, GAL-GR(EF), GR(AB)-GAL and SVe RARα expression vectors and the MMTV, RARβ, 5xGAL-E1b/CAT and TK promoter constructs have previously been described (Godowski *et al.*, 1987; Petkovich *et al.*, 1987; Cato *et al.*, 1988; Sadowski *et al.*, 1988; Tasset *et al.*, 1990; Thierry *et al.*, 1990; de Thé *et al.*, 1991).

Transient transfections and CAT assays

C33 or SW13 cells were split on the day prior to transfection (400 000 cells per 60 mm dish). The medium was changed and after 5 h, cells were transfected with a total of 10 μg of DNA by calcium phosphate precipitation. Cells were washed after 14 h and harvested after a further 24−30 h incubation. CAT assays were performed as described by Gorman *et al.* (1982) using one-third of the extract in a 1 h incubation when the hbrm−E2 fusion protein were assayed and one-sixth of the extract in a 30 min incubation in co-transfections of hbrm and steroid hormone receptors. Transfection efficiency was monitored by β-galactosidase activity assays performed as described by Herbomel *et al.* (1984). When cells were submitted to induction, a final concentration of 10^{-6} M of either retinoic acid or dexamethasone was added to the cells just after the transfection and when the medium was changed. All transfections were repeated at least three times. The results presented in the figures are autoradiograms from representative experiments.

Polymerase chain reaction

For detection of hbrm message, total RNA was purified on a CsCl cushion as previously described by Sambrook *et al.* (1989). Two micrograms of this RNA were then used for cDNA synthesis using 200 ng of random hexamer primer and 200 U of M-MLV RT in 20 μl under conditions recommended by the RT manufacturer (BRL). A negative control was made under the same conditions but without RT. One-tenth of the reaction or the negative control was amplified for 40 cycles in a two step PCR reaction (1 min at 95°C and 1 min at 60°C) using Taq polymerase from Promega and hbrm- or GPDH-specific primers.

Western blotting

C33 cells were transfected as described above with 20 μg of either pUC19 or CMV wild-type or mutated hbrm expression vectors. After 24 h, cells were harvested in lysis buffer (10 mM Tris pH 7.6, 1 mM EDTA, 0.5 mM $MgCl_2$, 1% SDS, 2.25 μg/ml pepstatin, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 2 mM PMSF), heated for 5 min at 95°C and sheared through a 20G needle. Protein concentration was determined with the Bradford protein assay using a dilution of extract that prevented the SDS of the lysis buffer from interfering with the assay. Fifteen micrograms of total protein were then mixed with 2 × loading dye (Sambrook *et al.*, 1989) and separated by SDS−PAGE on a 5% gel. After transfer to nitrocellulose membrane, the samples were incubated overnight with an affinity-purified polyclonal rabbit anti-hbrm antibody recognizing the amino-terminal end of the protein. This antibody was prepared against a GST−hbrm fusion containing amino acids 1−178 expressed in *E.coli* and purified on a glutathione−Sepharose column. The Western blot was first developed using ECL (Amersham), followed by incubation with a ^{35}S-labelled donkey anti-rabbit antibody for quantification using a PhosphorImager. For the quantification, the experiment was repeated four times.

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