ras transformation is associated with decreased expression of the brm/SNF2 α ATPase from the mammalian SWI–SNF complex

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The brm and BRG-1 proteins are mutually exclusive subunits of the mammalian SWI–SNF complex. Within this complex, they provide the ATPase activity necessary for transcriptional regulation by nucleosome disruption. Both proteins were recently found to interact with the p105Rb tumor suppressor gene product, suggesting a role for the mammalian SWI–SNF complex in the control of cell growth. We show here that the expression of brm, but not BRG-1, is negatively regulated by mitogenic stimulation, and that growth arrest of mouse fibroblasts leads to increased accumulation of the brm protein. The expression of this protein is also down-regulated upon transformation by the *ras* oncogene. Re-introduction of brm into *ras* transformed cells leads to partial reversion of the transformed phenotype by a mechanism that depends on the ATPase domain of the protein. Our data suggest that increased levels of brm protein favour the withdrawal of the cell from the cycle whereas decreased expression of the brm gene may facilitate cellular transformation by various oncogenes.

Keywords: Brahma/cell cycle/chromatin/transcription

Introduction

The SWI–SNF genes were first characterized in *Saccharomyces cerevisiae*, where their products were defined as transcriptional activators, involved in the regulation of a subset of inducible genes. In this organism, the 11 SWI–SNF proteins identified so far are assembled in a 2 MDa complex that affects transcriptional activity by modifying the chromatin structure in the vicinity of target promoters (for review, see Carlson and Laurent, 1994; Peterson, 1996; Burns and Peterson, 1997a). The molecular mechanism by which the SWI–SNF complex facilitates the assembly of the pre-initiation complex has not yet been fully elucidated. However, it is likely to create stable openings in the nucleosomal array, which help general transcription factors in competing with histones for DNA binding (Owen-Hughes *et al.*, 1996; Burns and Peterson, 1997b). A direct interaction of the SWI–SNF complex with the RNA polymerase II holoenzyme has also been suggested (Wilson *et al.*, 1996).

Several members of the yeast SWI–SNF complex have now identified counterparts in higher eucaryotes. In *Droso-*

phila, the SWI2/SNF2 homologue is known as Brahma or brm (Tamkun *et al.*, 1992). The gene encoding this protein was initially identified as a suppressor of Polycomb mutations. The brm protein resembles SWI2/SNF2 over its entire length and contains, like the yeast protein, a bromodomain and a helicase-type domain with an ATP-binding site. Like in yeast, brm is associated with a large complex, also containing SNR-1, the *Drosophila* homologue of SNF5 (Dingwall *et al.*, 1995; Tamkun, 1995). In mammalian cells, two proteins closely related to SWI2/SNF2 have been identified. These proteins, known as hbrm/hSNF2 α and BRG-1/hSNF2 β in humans, and mbrm and mBRG-1 in mouse, are both associated with the so called mammalian SWI–SNF complex, composed of at least eight other proteins, including an SNF5 homologue (hSNF5/INI1), two SWI3 homologues (BAF155 and BAF170) and an SWP73 homologue (BAF60) (Khavari *et al.*, 1993; Muchardt and Yaniv, 1993; Chiba *et al.*, 1994; Kalpana *et al.*, 1994; Randazzo *et al.*, 1994; Muchardt *et al.*, 1995; Wang *et al.*, 1996a,b). Within this complex, the two SWI2/SNF2 related proteins are mutually exclusive, indicating that at least two versions of the mammalian SWI–SNF complex, associated with either the brm or the BRG-1 subunit, coexist in mammalian cells. In addition, several other proteins are associated with the complex only in certain cell types, adding further complexity to the SWI–SNF complex in higher eucaryotes (Wang *et al.*, 1996a,b).

The function of the mammalian SWI–SNF complex has so far only been investigated by *in vitro* assays or transfection studies. Like its yeast counterpart, purified human SWI–SNF complex contains ATP-dependent nucleosome disruption activity that leads to facilitated binding of TBP and Gal4 derivatives to synthetic promoters (Imbalzano *et al.*, 1994; Kwon *et al.*, 1994). In transient transfections, hbrm and BRG-1 are both able to stimulate glucocorticoid receptor activated transcription from an MMTV promoter. A stimulating effect has also been reported for other nuclear receptors, including the retinoic acid receptor and the estrogen receptor (Muchardt and Yaniv, 1993; Chiba *et al.*, 1994). Interestingly, the effect of the SWI2/SNF2 proteins on the glucocorticoid receptor appears to depend on the presence of the retinoblastoma p105Rb protein, and several studies have now shown that both hbrm and BRG-1 are able to interact with members of the pRb family (Dunaief *et al.*, 1994; Singh *et al.*, 1995; Strober *et al.*, 1996). This interaction has been mapped to an LXCXE sequence that is present in the two proteins and resembles a motif that allows interaction between the pRb proteins and several viral oncoproteins, including the simian virus large T antigen, the papillomavirus E7 and the adenovirus E1a. The association between p105Rb and hbrm or BRG-1 can only occur when p105Rb is hypophosphorylated, suggesting that the

SWI2/SNF2 proteins may be necessary for the tumor suppressing activity of p105Rb. Besides, in SW13 cells that contain wild type p105Rb but low levels of endogenous SWI2/SNF2 proteins, transiently transfected hbrm or BRG-1 expression vectors induce the formation of flat, growth-arrested cells. This flat cell inducing activity is reduced either by the deletion of the pRb interaction domain of the two proteins or by the co-transfection of E1a (Dunaief *et al.*, 1994; Strober *et al.*, 1996). Finally, co-transfection of hbrm and p105Rb expression vectors leads to increased E2F repression as compared with p105Rb alone (Trouche *et al.*, 1997). These observations raise the possibility that the mammalian SWI-SNF complex may co-operate with p105Rb to induce growth-arrest.

In an earlier study, we have shown that in human cells, hbrm and BRG-1 are phosphorylated at the G₂/M transition, leading to decreased affinity of these proteins for their nuclear attachment sites, and resulting in their chromosomal exclusion upon mitosis. During this phase of the cell cycle, the hbrm protein is also partially degraded, only to be resynthesized in a dephosphorylated form after cytokinesis (Muchardt *et al.*, 1996). These observations suggest that the mammalian SWI-SNF complex is re-activated during the G₁-phase, at a time when the cell can either exit from the cycle or continue into S phase. This prompted us to investigate the effect of growth conditions on the cellular pools of mammalian brm and BRG-1. We will show that in mouse fibroblasts, mbrm but not mBRG-1, accumulates to high levels in growth-arrested cells, but is down regulated upon serum induced re-entry into the cell cycle. In a similar way, transformation of fibroblasts with a constitutively activated *ras* leads to strongly decreased levels of mbrm, as compared with the parental non-transformed cells. Again, the level of mBRG-1 is not affected by *ras* transformation. Re-introduction of a constitutively expressed hbrm cDNA into *ras* transformed cells leads to partial reversion of the *ras* transformed phenotype. This effect of hbrm was abolished by a mutation in the ATP-binding site of its helicase-like domain. Screening of other rodent cell lines revealed that reduced levels of mbrm were frequently observed in transformed cells of various origins. Taken together, our observations suggest that high levels of mbrm is one of the factors that will favour entry into G₀, and that down-regulation of mbrm is an obligatory event in some of the pathways leading to fibroblast transformation.

Results

Mitogenic stimulation of NIH3T3 cells leads to decreased expression of the mbrm protein

To determine the effect of growth factors on brm and BRG-1 expression, we cultured cells in medium containing increasing serum concentrations. For this experiment, we used NIH3T3 mouse fibroblasts, because their growth is highly dependent on the presence of mitogens in the medium and they can be efficiently arrested in G₀ by serum starvation. The mouse mbrm protein present in NIH3T3 cells appeared to be regulated in a way very similar to the human hbrm present in Hela cells (Muchardt *et al.*, 1996). As determined by Western blotting and immunofluorescent staining, the mbrm protein was phosphorylated during mitosis and excluded from the con-

densed chromosomes. However, the decreased level of the protein during mitosis was less prominent in NIH3T3 than in HeLa cells (data not shown). The NIH3T3 cells were cultured for 72 h in medium containing either 5, 10, 15 or 20% fetal calf serum (FCS). Phase contrast microscopy showed that the cells grown in 5% FCS were flat, whereas in 20% FCS, they were rounder and more refringent, somewhat resembling transformed cells. Western blot analysis of total extracts revealed that the level of mbrm was lowest in cells grown in 20% FCS, and highest in cells grown in 5% FCS (Figure 1A, lanes 1 and 4). Quantitative RT-PCR showed that decreased levels of mbrm protein could be correlated with decreased levels of mbrm mRNA (Figure 1B). On the contrary, the level of mBRG-1 mRNA or protein was either unchanged or moderately increased when cells were grown at 20% FCS, as compared with 5% FCS (Figure 1C and D). To further characterize the effect of the serum concentration on cellular levels of mbrm and mBRG-1, NIH3T3 cells grown in medium containing 20% FCS were shifted to medium containing 0.25% FCS for 24 h. Western blot analysis of extracts from these serum-starved cells revealed a strong increase in the level of mbrm, as compared with the cells grown in 20% FCS (Figure 1E). Again, serum starvation had no effect on the level of mBRG-1 (Figure 1F). The reciprocal experiment was performed by serum starving cells for 48 h and then stimulating them with medium containing 20% FCS. As shown Figure 1G, Western blot analysis of cellular extracts, performed at different time points after stimulation, showed a marked decrease in the level of mbrm protein, first visible 10 h after the addition of serum. Quantitative RT-PCR showed that the decreased level of mbrm protein could be correlated with a decrease in the level of mbrm messenger RNA (Figure 1H). At the same time, we did not observe any significant variations in the level of mBRG-1 protein that apparently was not affected by the serum stimulation (Figures 1I and 1J). To compare the decrease in mbrm expression with the induction of an immediate early gene, we followed the levels of Jun-B protein in the serum stimulated cells (Figure 1K). The level of this protein was already high 1 h after serum stimulation and then decreased between 10 and 20 h, in agreement with previous publications (Lallemand *et al.*, 1997). Since the decrease in mbrm levels is relatively late compared with Jun-B induction, it may be considered as a delayed early or late event in the G₀/G₁ transition. This prompted us to verify that the decrease in mbrm expression occurred at the G₀/G₁ and not at the G₁/S transition or later, and we performed flow cytometric analysis of exponentially growing NIH3T3 cells indirectly labelled with anti-brm antibodies. In this experiment, we observed no variation in the level of mbrm protein between cells in G₁ and S phase, confirming that the expression of mbrm was primarily regulated at the exit from quiescence (data not shown).

The mbrm protein expression is down-regulated in NIH3T3 cells transformed by *ras*

Cellular transformation by the *ras* oncogene depends on the constitutive activation of the *ras*-dependent mitogenic signalling pathway. We therefore compared the levels of mbrm and mBRG-1 present in NIH3T3 cells and in DT cells, a well characterized NIH3T3-derived cell line

Fig. 1. The level of mbrm in NIH3T3 cells is regulated by the growth conditions. (A–D) Protein extracts and total RNA were prepared from 70% confluent NIH3T3 cells grown in medium containing either 5, 10, 15 or 20% FCS. (E and F) NIH3T3 cells grown in 20% FCS were shifted to 0.25% FCS. Protein extracts were prepared after 24 h. (G–K) Serum-starved NIH3T3 cells at 50% confluency were stimulated with medium containing 20% FCS. Protein extracts and total RNA were prepared at the indicated times. All protein extracts were resolved by SDS–PAGE and analyzed by Western blotting with either anti-hbrm (A, E and G), anti-BRG-1 (C, F and I) or anti-Jun-B antibodies (K). RNA samples from the serum stimulation experiment were used to synthesize cDNA either in the absence (– lanes) or in the presence (+ lanes) of reverse-transcriptase. The products of these reactions were then amplified by PCR for 13 cycles with either mbrm or mBRG-1 specific primers (B, D, H and J).

transformed by the incorporation of two copies of a *Ki-ras* gene (Noda *et al.*, 1983). Western blot analysis showed that DT cells contained normal levels of mBRG-1, but undetectable levels of mbrm (Figure 2A and B, lane 4). To determine if mbrm expression was reproducibly down-regulated upon *ras* transformation, NIH3T3 cells were transfected with an activated *Ha-ras* gene and a plasmid conferring neomycin resistance. After neomycin selection, several resistant clones were isolated and expanded for further analysis. Among these clones, some were morphologically similar to the initial NIH3T3 cells, while others resembled the highly tumorigenic DT cells mentioned above, with a characteristic rounded shape. The extent of transformation of these clones was evaluated by scoring colony formation in soft agar. The ability of these clones to form colonies could be roughly correlated with the level of accumulated *ras* protein, as estimated by Western blotting. These clones were further described elsewhere (Pfarr *et al.*, 1994; Mechta *et al.*, 1997). Two clones, named R2 and R3, were selected as representative of high and low degrees of transformation. R2 had a high level of *ras* expression and a 50% plating efficiency in soft agar, whereas R3 had a lower level of *ras* expression and only ~30% plating efficiency. Western blot analysis

showed that mbrm was not expressed in the R2 clone, but could still be detected in the R3 clone, albeit at a reduced level (Figure 2A, lanes 2 and 3). Again, mBRG-1 accumulation was not affected. These observations suggested that, as in serum stimulated NIH3T3 cells, the level of mbrm, but not mBRG-1, is dependent on the level of mitogenic signal received by the cell. Southern blot analysis of genomic DNA from the various *ras* transformed clones showed no obvious rearrangement of the mbrm gene in the *ras* transformed cell lines (data not shown). Examination of the mRNA levels by quantitative RT–PCR on RNA from DT, R2 or R3 cells showed a direct correlation between the level of RNA and the amount of protein detected in the different cell lines (Figure 2, panels C and D), indicating that transformation, like serum stimulation, down-regulates mbrm levels by affecting transcription efficiency or mRNA stability.

Expression of hbrm leads to partial reversion of the *ras* transformed phenotype

To evaluate the significance of reduced mbrm expression in the *ras* transformation process, we re-introduced a constitutively expressed hbrm cDNA into DT cells. As a full-length mouse brm cDNA has not been cloned, we

Fig. 2. *ras* transformation correlates with down-regulation of endogenous mbrm. Extracts from normal NIH3T3 cells (lane 1), HA-*ras* transformed NIH3T3 cells (lanes 2 and 3) and DT cells (lane 4) were resolved by SDS-PAGE and analyzed by Western blotting with either anti-hbrm (A) or anti-BRG-1 (B) antibodies. Total cellular RNA from the same cell lines was prepared and cDNA was synthesized either in the absence (C and D, - lanes) or in the presence (C and D, + lanes) of reverse-transcriptase. The products of these reactions were then amplified by PCR for 13 cycles with either mbrm (C) or mBRG-1 (D) specific primers. Each PCR sample also contained GPDH specific primers as an internal control of the reaction.

used a construct of human origin containing an HA-tagged version of the hbrm protein, expressed from an MoMuLV LTR (schematically shown in Figure 3A). The function of the human and the murine proteins is likely to be conserved, since fragments sequenced in both species have revealed an overall identity of about 98%. Two of these clones (DT19 and DT21) contained levels of hbrm similar to, or moderately higher than, the normal NIH3T3 cells, while two others (DT24 and DT27) contained lower than normal levels of the protein. The original DT cells, which are flat when first plated, rapidly become round as confluency increases. At a density of $\sim 2 \times 10^6$ cells per 60 mm plate, the cells lose adherence to the plate, most probably because of accumulation in the medium of matrix degrading proteinases (Spinucci *et al.*, 1988). The cells then die within 24 h if they are not replated in fresh medium (Pfarr *et al.*, 1994). Several of these growth characteristics were modified in the DT derived clones expressing hbrm. These cells remained flat, even at high confluency (compare panels E and F in Figure 3) and had a reduced growth rate compared with the parental DT cells (Figure 3G). These modified growth characteristics of the DT clones expressing hbrm could not be related to decreased *ras* expression, since DT cells and the derived clones all accumulated similar levels of *ras* protein (Figure 3B and C). Still, these observations suggested that the hbrm-expressing DT cells might be affected in their degree of transformation, as compared with the original DT cells. Therefore, we subjected the different clones expressing

hbrm to standard assays to monitor cellular transformation. Three out of four clones were unable to grow in medium containing a reduced serum concentration (0.25% serum; Figure 4A). Flow cytometric analysis showed that in this medium, cells remained viable but were arrested in the G_0/G_1 phase of the cell cycle (data not shown). The clones were also tested for their ability to form colonies in soft agar. All were found to have a strongly reduced plating efficiency, as compared with the original DT cells (Figure 4B). Two colonies, obtained after plating the DT21 cells in soft agar (DT21.1 and DT21.2), were expanded and re-assayed for hbrm expression. These clones were both found to be revertants containing undetectable levels of hbrm protein (Figure 4C, lane 8 and 9). To further determine the effect of hbrm on DT cell growth, the DT19 and DT21 cells were tested for their ability to form tumors in nude mice. After 10 days, no tumors could be detected in mice injected with DT21 cells. Injection of DT19 cells produced tumors with an average mass of one fifth of the mass of the DT or DT plus neo tumors (Figure 4D). The modified growth characteristics of the DT cells upon re-introduction of an exogenous hbrm gene, suggest that the loss of normal mbrm expression upon transformation by *ras* is at least partially responsible for the transformed phenotype of these cells.

To determine if the effect of hbrm on DT cell growth required the DNA dependent ATPase activity, which has been associated with this protein (Wang *et al.*, 1996a), we constructed DT cell lines constitutively expressing an

Fig. 3. Re-introduction of hbrm modifies the growth characteristics of DT cells. (A) Schematic representation of the two hbrm derived constructs expressed in DT cells. The black box represents the HA tag. (B, C and D) Extracts from DT cells (lane 1) or DT-derived cells expressing either hbrm WT (B, lanes 2–5) or ATPmut (D, lanes 2 and 3) were resolved by SDS-PAGE and analyzed by Western blotting with either anti-hbrm (B and D) or anti-ras (C) antibodies. (E and F) DT cells and a representative hbrm-expressing DT-derived clone (DT21) shown at a density of about 10^6 cells per 60 mm plate. (G) 10^4 DT or DT21 cells were plated on 60 mm plates then cells were counted on days 2, 3 and 4.

hbrm protein, mutated in the ATP-binding site of the helicase-like domain (ATPmut; Figure 3A and D). A similar mutation has previously been shown to convert BRG-1 into a dominant, negative transcriptional activator (Khavari *et al.*, 1993). As shown in Figure 4B, the plating efficiency in soft agar of DT clones expressing ATPmut protein (A2 and A26), was similar to that of the initial DT cells. Again, we checked if the colonies growing in soft agar would still express the transgene. As shown in

Figure 4C, the tested clones derived from A2 (A2.1 and A2.2) expressed similar or higher levels of ATPmut protein, as compared with the initial A2 cells. Thus, expression of an hbrm protein, mutated in its ATPase domain, did not appear to affect DT cell growth.

Down-regulation of mbrm expression is observed in rodent fibroblasts transformed by other oncogenes

The possibility that down-regulation of mbrm could be part of the mechanism leading to cellular transformation, prompted us to investigate the levels of mbrm and mBRG-1 expression in cell lines transformed by oncogenes other than *ras*. First, we tested EHneo cells that are derived from NIH3T3 cells by infection with a retrovirus carrying the *raf* oncogene, acting downstream of *ras*. Like the DT cells, these cells were found to have normal levels of mBRG-1 protein, but levels of mbrm significantly lower than those observed in NIH3T3 cells (Figure 5, lane 2). This observation clearly confirms that the signal transduction pathway activated by *ras* and *raf* is responsible for the down-modulation of mbrm synthesis. We next tested a rat fibroblast cell line, PYF 3.2, transformed by Polyoma middle T (Gorga *et al.*, 1990). This oncogene activates several growth promoting signals, including the *ras* pathway. Again, we found that the PYF 3.2 cell line showed reduced mbrm expression, as compared with the parental immortalized rat fibroblast cell line F111 (compare lanes 3 and 4 of Figure 5). This observation indicates that down-regulation of mbrm expression can also be induced by viral oncogenes. To verify that reduced levels of mbrm were not specific for *in vitro* transformation, we tested a cell line derived from a methylcholantrene-induced fibrosarcoma, known as MCO-1 (Figure 5, lane 6). These cells also had a reduced level of mbrm protein.

Discussion

The brm gene is down-regulated by the ras signal transduction pathway

Recent studies have shown that at least two versions of the SWI-SNF complex coexist in mammalian cells, containing either brm or BRG-1 and several common subunits (Wang *et al.*, 1996a,b). Initial studies suggested that brm and BRG-1 proteins share very similar properties. Both proteins were shown to stimulate the activity of nuclear hormone receptors in transient transfection studies. Furthermore, yeast two-hybrid and GST pull-down experiments revealed that both proteins interact with pRb family members via an LXCXE motif, also present in several viral oncogenes. This interaction was essential for the formation of flat G_0 -arrested cells, upon transient transfection of expression vectors for both proteins (Dunaief *et al.*, 1994; Singh *et al.*, 1995; Strober *et al.*, 1996). The interaction of the mammalian SWI2/SNF2 homologues with pRb, and their negative effect on cell growth, was somewhat unexpected for components of a nuclear complex that is involved in facilitating transcription. Recent observations bring a possible explanation to this paradox by demonstrating that hbrm co-operates with p105Rb in repressing E2F-1-dependent transcriptional activation (Trouche *et al.*, 1997). The inhibition of E2F-

Fig. 4. hbrm can counteract the effect of *ras* in mouse fibroblasts. **(A)** Growth in 0.25% serum. 10^5 cells from the DT cell line, three neomycin-resistant clones not expressing hbrm (neo1, neo2 and neo3), or the derived clones expressing the hbrm construct, were seeded in medium containing 0.25% FCS. After 7 days, cells were stained with trypan blue and counted. Results shown here are averaged from two independent experiments. **(B)** Growth in soft agar. The DT cells, a neomycin-resistant clone (neo3) and the clones expressing either hbrm WT or ATPmut, were plated in 60 mm dishes (10^3 or 10^4 cells per dish). The total number of visible colonies were scored after 15 days in culture and compared with the total number of cells initially seeded (plating efficiency in %). Results shown here are averaged from three independent experiments. **(C)** Several colonies from one of the soft agar experiments were re-isolated and propagated. Extracts from these cells were analyzed by Western blotting using either anti-hbrm (top panel) or anti-BRG-1 (bottom panel) antibodies. DT.1 and DT.2 are derived from DT cells, A2.1 and A2.2 are derived from A2, and DT21.1 and DT21.2 are derived from DT21 cells. **(D)** Tumor formation in nude mice. Mice were injected with 10^6 cells from the DT cell line or one of the derived neo3, DT19 and DT21 clones. For each clone, five mice were injected. Tumors were dissected 11 days after injection and weighed.

1-activated transcription may explain the flat cell forming activity of hbrm and BRG-1.

Previous studies did not clearly distinguish between brm and BRG-1, and raised the possibility that the two proteins fulfil identical functions. At present, we clearly demonstrate that this is not the case. We show that whereas the level of BRG-1 does not vary with the growth rate of the cells, mbrm levels are highly variable. The mbrm protein accumulates in quiescent mouse fibroblasts and strongly decreases after mitogenic stimulation, which activates the *ras/raf/ERK* pathway. In agreement with these observations, transformation of fibroblasts by *ras* or *raf* almost completely inhibits the accumulation of mbrm mRNA and protein, with no effect on BRG-1 protein or mRNA. Re-expression of hbrm from a constitutive promoter reverses the transformed phenotype of the cells,

without directly blocking the *ras/raf* pathway. This last conclusion is based on the finding that the typical pattern of expression and phosphorylation of Jun and Fos family members induced by *ras* transformation is not altered after re-introduction of hbrm (Mechta *et al.*, 1997; C.Muchardt and B.Bourachot, unpublished observations). The stable re-introduction of a constitutively expressed hbrm gene into DT cells is the first reported experiment allowing an assay of the effect of this protein on cells in which it is not aberrantly overproduced. In our system, expression of hbrm leads the *ras* transformed DT cells to recover a morphology and growth rate closer to those of normal NIH3T3 cells but, unlike hbrm transfected SW13 cells, growth arrest was only observed when cells were serum-starved. It is possible that the almost physiological levels of hbrm, which we obtain in the DT cells, are not

Fig. 5. The mbrm protein is down-regulated in several transformed cell lines. Extracts from either NIH3T3 (lanes 1 and 5), EHneo (lane 2), F111 (lane 3), PYF3.2 (lane 4) and MCO-1 (lane 6) cells were analyzed by SDS-PAGE and Western blotting with either anti-hbrm (top panel) or anti-BRG-1 (bottom panel) antibodies.

sufficient to counteract the mitogenic signals induced both by serum and activated *ras*. Suppression of the *ras* transformed phenotype requires functional hbrm, since a mutant in the ATP-binding domain is quasi inactive. Deletion of the E7 homology, which abolishes pRb binding in the yeast two-hybrid system (Strober *et al.*, 1996) reduced, but did not abolish growth in agar, raising the possibility that agar growth suppression is not exclusively dependent on pRb binding (C.Muchardt and B.Bourachot, unpublished results).

Possible functional differences between brm and BRG-1

The studies cited above clearly demonstrate that brm and BRG-1 may have both common and distinct roles. Both affect the activity of nuclear hormone receptors, interact with pRb and may be important for controlling G₁ progression. However, only brm is down regulated by *ras*. Another difference between brm and BRG-1 was observed during the study of embryonic carcinoma (F9) and embryonic stem (ES) cells. While non-differentiated cells contain predominantly BRG-1, brm accumulated upon *in vitro* differentiation (C.Muchardt and J.-C.Reyes, unpublished observations). One has to recall that undifferentiated ES or EC cells have a very short G₁ phase, and that F9 embryonic carcinoma cells are tumorigenic before, but not after, differentiation (Dony *et al.*, 1986; Savatier *et al.*, 1994). In this respect, a recent study by Sumi-Ichinose *et al.* (1997) has shown that inactivation of one of the two *BRG-1* genes by homologous recombination in F9 cells, affected their capacity to proliferate, but not to differentiate. No viable cells lacking both copies of *BRG-1* were obtained after drug selection. On the contrary, we have isolated viable ES cells that lack both copies of *mbrm* (J.-C.Reyes, unpublished results). These studies strongly suggest that BRG-1, but not brm, is essential for viability of early embryonic cells. The persistence of BRG-1 in faster growing cells, and the accumulation of brm in cells that become slow growing or arrested, raise the possibility that a partial switch from BRG-1 to brm-containing complexes occurs when cells slow their growth. Part of the chromatin sites occupied by BRG-1-containing

complexes may be replaced by brm-containing complexes. In addition, new sites may be occupied by the brm complex. This differential targeting could be mediated by the structure of promoters or by DNA-binding proteins with affinity for either brm or BRG-1. This may facilitate the expression of genes that are important for the maintenance of the quiescent or terminally differentiated state of cells. SWI-SNF complexes containing brm may be important for the activation of growth arrest related genes, as well as genes that promote the formation of cell-cell or cell-matrix contacts and the stabilization of the cytoskeletal network. Studies of mutant mice lacking brm or BRG-1, and eventually of cells isolated from such mice, will permit us to test our hypothesis. A switch of protein isoforms between rapid early embryonic cell divisions and later slower somatic divisions has frequently been reported. Besides, it is well documented that several species, including *Xenopus leavis* or sea urchin, use different repertoires of histones H2A and H2B and linker histone genes during development, leading to changes in the degree of DNA compaction (Wolffe, 1995).

Chromatin associated factors and cellular transformation

In *Drosophila*, the polycomb group of proteins, involved in homeotic gene regulation, has been shown to create local heterochromatin-like structures, leading to transcriptional repression. This repression is antagonized by members of the trithorax family of proteins, by a mechanism that is not yet understood (for review, see Orlando and Paro, 1995). The bmi-1 protein was the first mammalian homologue of a member of the polycomb group to be isolated. This protein is functionally related to its *Drosophila* homologue, Psc, and was initially identified as a proto-oncogene, able to accelerate lymphomagenesis in Eμ-myc transgenic mice (Haupt *et al.*, 1991; Alkema *et al.*, 1995). In parallel, mutations of the *Drosophila* brm gene can antagonize Polycomb mutations, and brm is considered to be a member of the trithorax group of genes (Brizuela *et al.*, 1994; Tamkun, 1995). In the present study, we provide evidence that the mammalian homologue of *Drosophila* brm is involved in growth control, and that deregulation of this protein may be one of the events leading to cellular transformation. bmi-1 and mammalian brm may not directly antagonize the effect of each other, but they provide evidence that proteins involved in transcriptional regulation at the level of chromatin may be important regulators of cell growth.

Materials and methods

Cell extracts and immunoblot analysis

All cell lines were maintained at 37°C in 7% CO₂. Hela, NIH3T3, EHneo, F111, PYF3.2 and MCO-1 were grown in DMEM (Sigma) supplemented with 7% FCS, unless otherwise indicated. Cells were lysed in 200 μl of Buffer B (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris, pH 8). Protein concentrations were determined using the Bio-Rad Bradford reagent. For Western blot analysis, 20 μg of extract were fractionated by SDS-PAGE and transferred to nitro-cellulose membranes. The membranes were then blocked with PBS/0.2% Tween-20/10% horse serum, and incubated with the various antibodies. Enhanced chemiluminescence (ECL) reagents (Amersham) were used for detection. Antibodies used for detection of hbrm and BRG-1 have previously been described (Muchardt *et al.*, 1996). Polyclonal rabbit antibodies against Jun-B were kindly provided by D.Lallemand. The *ras* oncogene was

detected using a monoclonal mouse anti-*ras* antibody from Oncogene Science (cat# OP40). HA-tagged hbrm was detected using the 12CA5 monoclonal mouse antibody.

RNA preparation and RT-PCR

Total RNA was purified on CsCl cushion, as previously described (Sambrook *et al.*, 1989). 2 µg of this RNA were then used for cDNA synthesis, using 200 ng of random hexamer primer and 200U of M-MLV RT in 20 µl, under conditions recommended by 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 13 cycles in a two step PCR reaction (1 min at 95°C and 1 min at 60°C) using Taq polymerase and mbrm, mBRG-1 or GPDH specific primers (total volume: 100 µl). Preliminary experiments have shown that the PCR reactions were within the linear range between 10 and 15 cycles of amplification. 5 µl of the PCR product was resolved on a 10% polyacrylamide gel and analyzed by Southern blotting, using a *Bam*H1/*Sac*I restriction fragment from the hbrm cDNA and a Asp718/*Sac*I fragment from the BRG-1 cDNA (respectively, nucleotide 274–548 and 245–599 of the published sequences). Primer sequences: mbrm sense, CCCGGACCTCCAGCGTC; mbrm reverse, GCCAGGCAA-GGTCCTTTT; mBRG-1 sense, CACCCAGGGGCTGGAGG; mBRG-1 reverse, TCCTGTGGCGGACACTGA.

Preparation of stable cell lines

Wild type and mutant hbrm constructs were inserted as *Eco*RI fragments downstream of the MoMuLV LTR of pVLMNP1 (Marty *et al.*, 1990). hbrm WT and ATPmut have previously been described (Muchardt *et al.*, 1995). The Ki-*ras* transformed NIH3T3 cells (DT) were seeded at a density of 5×10^5 per 100 mm plate and transfected the next day with 10 µg of plasmid DNA using the calcium phosphate coprecipitation method. After 2 days, each plate was divided in two and 500 µg/ml G418 was added to the medium. 18 days after the transfection, individual colonies were picked, subcloned and characterized by PCR on the genomic DNA and by immunoblotting. DT cells were also transfected with pVLMNP1 vector without insert and several neomycin clones were also picked for control purposes. These clones were visible 9–10 days after the transfection. The Ha-*ras* transformed clones, R2 and R3, were derived from our NIH3T3 cells. They have been previously described (Pfarr *et al.*, 1994; Mechta *et al.*, 1997).

Transformation assays

Growth at low serum concentrations was tested by plating 10^5 cells in DMEM containing 0.25% FCS. Cells were then counted after 7 days. For assay of cell growth in soft agar, 10^3 and 10^4 cells were plated in 0.3% agar (Difco) prepared in DMEM plus 7% FCS, over a cushion of 0.6% agar in 60 mm dishes. Colonies were counted after growth for 15 days. For tumor growth assays, 10^6 cells in midlog-phase of growth were harvested, washed with PBS and resuspended in 250 µl of PBS, and injected subcutaneously into the scapular region of Swiss 3T3 nude mice. After 11 days of growth, the mice were sacrificed and the tumors were dissected out and weighted.

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