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The adenovirus E1A binding protein BS69 is a corepressor of transcription through recruitment of N-CoR

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BS69 was first identified as a protein that interacts directly with the transactivation domain (conserved region 3) of the 289R adenovirus type 5 E1A protein. We show here that BS69 is a potent repressor of transcription. BS69 mediates repression, at least in part, through interaction with the co-repressor N-CoR. BS69 interacts with N-CoR through a MYND domain in its carboxyl terminus. A recently cloned splice variant of BS69, designated BRAM1, is also capable of interacting with N-CoR and E1A, but unlike BS69, is not able to repress transcription, indicating that N-CoR interaction is necessary but not sufficient for BS69 repression. Expression of E1A inhibits repression mediated by BS69. Our data suggest that BS69 participates in transcriptional repressor complexes and that E1A can modulate these complexes through interaction with BS69. *Oncogene* (2000) **19,** 1538–1546.

Keywords: BS69; BRAM1; N-CoR; E1A; MYND; repression

Introduction

Adenovirus E1A encoded proteins stimulate the cell cycle through their interaction with critical cell cycle regulatory proteins. The E1A region of human adenovirus type 5 encodes two differentially spliced mRNAs in transformed cells that encode a 243R (12S) and a 289R (13S) polypeptide. The E1A proteins of various serotypes share three conserved regions. Conserved region 1 and 2 are largely responsible for the cell cycle stimulatory activity (Lillie *et al*., 1987; Whyte *et al*., 1989). Conserved region 3 is unique to the larger 13S form of E1A and encodes a domain that exhibits strong transactivation potential (Green *et al*., 1988) and interacts with the mammalian Srb/Mediator complex (Boyer *et al*., 1999). The transforming activity of E1A, residing mainly in the conserved regions 1 and 2 (Whyte *et al*., 1998b), is brought about by interaction with a large number of cellular proteins, many of which are cell cycle regulatory proteins involved in transcription and/or chromatin modification such as Rb (Whyte *et al*., 1998a), p107 and p130 (Whyte *et al*., 1989; Hannon *et al*., 1993), cyclins A and E (Pines and Hunter, 1990; Giordano *et al*., 1991), the cdk inhibitor $p27^{kip1}$ (Mal *et al*., 1996) and the transcriptional adaptors p300 and CBP (Arany *et al*., 1994; Eckner *et al*., 1994).

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Acetylation and deacetylation of histones is known to be an important mechanism for regulation of transcription. Acetylation of N-terminal tails of histones weakens the interaction of histones with DNA and facilitates remodelling of the nucleosome structure into a more open conformation to allow access of transcription factors and the basal transcription machinery (Grunstein, 1997). Deacetylation of histone N-terminal tails can reverse this process and hypoacetylated regions of chromatin are transcriptionally less accessible. E1A interacts with various chromatin modifying proteins, (reviewed by Mymryk and Smith, 1997), some of which display histone acetyltransferase (HAT) activity, such as the transcriptional coactivators CBP/p300 (Ogryzko *et al*., 1996) and TAF_{II}-250 (Geisberg *et al*., 1995; Mizzen *et al*., 1996). Other E1A binding proteins are directly associated with histone deacetylase proteins (HDAc's), like the Rb family of transcriptional repressors (Luo *et al*., 1998; Brehm *et al*., 1998; Magnaghi *et al*., 1998; Ferreira *et al*., 1998) and the transcriptional regulators YY-1 (Lee *et al*., 1995; Lewis *et al*., 1995; Yang *et al*., 1997) and CtBP (Schaeper *et al*., 1995; Sundqvist *et al*., 1998).

We have previously cloned a novel E1A interacting protein, named BS69, a nuclear protein that interacts directly with conserved region 3 or adenovirus type 5 E1A. BS69 is able to efficiently inhibit E1A CR 3 mediated transactivation (Hateboer *et al*., 1995). Recently a splice variant of BS69 was cloned in a yeast two hybrid screen using BMP receptor type 1A as a bait (Kurozumi *et al*., 1998). BRAM1 (BMP Receptor Associated Molecule 1) is a cytoplasmic protein and contains the BS69 C terminus, amino acids 377–562, preceded by a stretch of twelve amino acids unique to BRAM1. Unlike the nuclear BS69 protein, BRAM1 has been shown to mediate binding of BMP-1A receptor to TAB1, an activator of the MAPKKK protein TAK1. BRAM1 may thus be involved in a cytosolic signalling cascade from the BMP receptor to TAK1.

At present, little is known about the function of BS69 in normal cell physiology. However, BS69 contains several domains that suggest a role for BS69 in regulation of transcription. First, BS69 contains a PHD finger, (amino acids 63–110 in BS69, Figure 1a) a plant homeodomain, which is a motif present in many genes involved in chromatin mediated transcriptional regulation (Aasland *et al*., 1995) such as helicases and transcription coactivators. A sequence closely homologous to the BS69 PHD finger is found in human Mi-2, the dermatomyositis-specific autoantigen. Mi-2 is a component of a large multisubunit complex (NuRD) that exhibits both histone deacetylase and nucleosome remodelling activities (Zhang *et al*.,

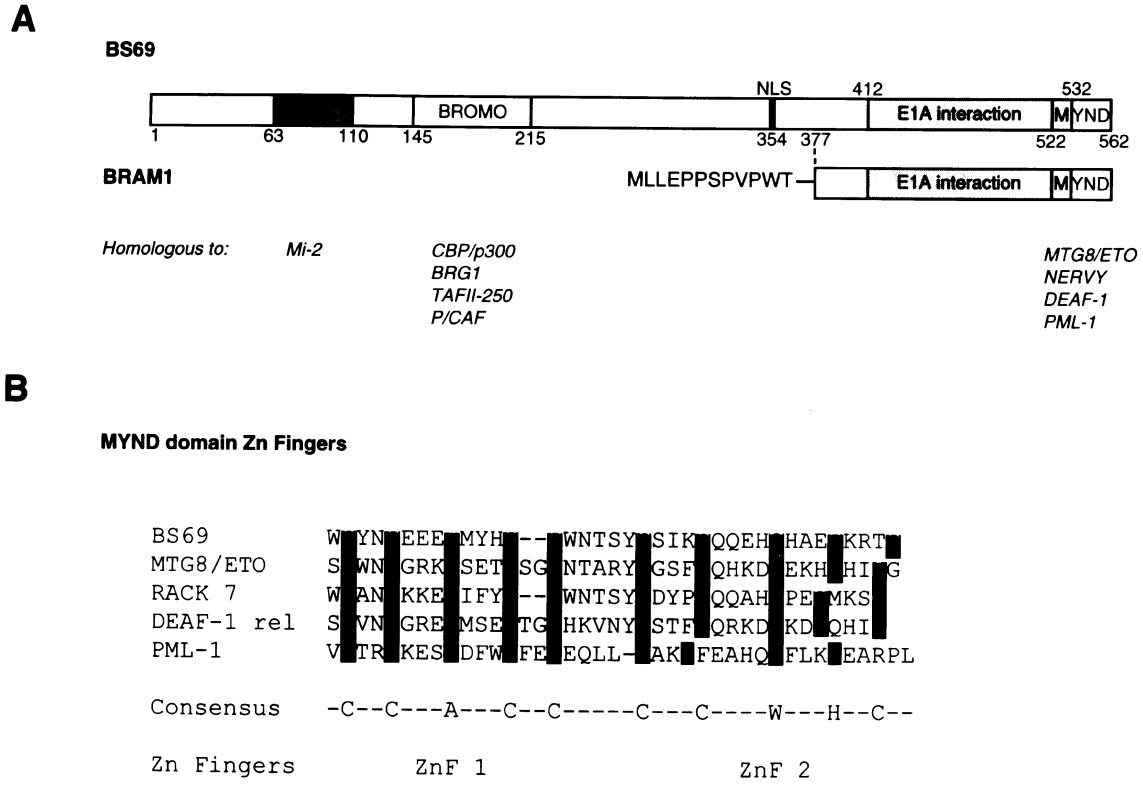

Figure 1 (**a**) Schematic representation of the BS69 protein and the BRAM1 splice variant, showing their functional domains and indicating human proteins containing homologous domains. (**b**) Alignment of the MYND motif containing human proteins and the derived consensus sequence of the two putative zinc fingers

1998). HPV E7, a viral oncoprotein with overlapping functions to E1A (Vousden and Jat, 1989) was found to bind Mi-2 and histone deacetylase activity to promote cell growth (Brehm *et al*., 1999).

Secondly, BS69 has a bromodomain (amino acids 145–215). Bromodomains are present in many proteins involved in transcriptional regulation, such as the histone acetyl transferases P/CAF, CBP/p300 and chromatin remodelling proteins such as hSNF2alpha/hBrm and hBRG/hSNF2beta (Jeanmougin *et al*., 1997). The bromodomain of the P/CAF histone acetyltransferase was shown to bind *in vitro* to acetylated lysines in a peptide derived from histone H4 (Dhalluin *et al*., 1999) and bromodomain containing proteins may thus be targeted to chromatin (Winston and Allis, 1999).

The third motif involved in transcription regulation is found in the extreme C-terminus of BS69, a region that is also present in the splice variant BRAM1. The C-terminus harbours a region with homology to the MYND domain, a two zinc finger motif first identified in DEAF-1 (Gross and McGinnis, 1996) and in MTG8/ETO (Lutterbach *et al*., 1998a). This domain was shown to be required for both repression of basal transcription by the AML/ETO fusion protein and binding of the transcriptional corepressors N-CoR and SMRT (Lutterbach *et al*., 1998b; Gelmetti *et al*., 1998; Wang *et al*., 1998). N-CoR and its close homologue SMRT were cloned as co-repressors of the thyroid-hormone and retinoic acid receptors (Chen and Evans, 1995; Horlein *et al*., 1995). Both N-CoR and SMRT have been shown to be components of large protein complexes that include histone deacetylase (Taunton *et al*., 1996; Nagy *et al*., 1997) and Sin3 (Ayer *et al*., 1995; Schreiber *et al*., 1995). Moreover, N-CoR is involved in Mad repression, reviewed by (Schreiber and DePinho, 1998). Mad interacts with Sin3A or B, which recruits HDAc 1/2 and N-CoR/SMRT containing complexes to repress transcription from E-box sequences, bound by Max/Mad(Mxi-1) heterodimers.

N-CoR and SMRT can also interact directly with components of the basal transcription machinery (Muscat *et al*., 1998; Wong and Privalsky, 1998). N-CoR inhibits a functional interaction between $TF_{II}B$ and $TAF_{II}32$, which is critical in initiation of transcription (Muscat *et al*., 1998). $TF_{II}B$ was also identified as a target for both SMRT and Sin3A mediated repression (Wong and Privalsky, 1998). N-CoR/SMRT and Sin3 may thus exert their repression by interference with general transcription factors. This implies a dual role for N-CoR/SMRT and Sin3A/B repression, one involving histone deacetylation and chromatin remodelling and another involving inhibition of the basal transcription machinery.

In this report we investigate the involvement of BS69 in transcriptional repression and identify a functional interaction of BS69 with co-repressor complexes.

Results

Interaction of BS69 with N-CoR through the MYND domain

Figure 1a shows a schematic representation of the functional domains of BS69 and its splice variant BRAM1. BS69 contains a PHD finger (amino acids 63–100) and a bromodomain (amino acids 145–215).

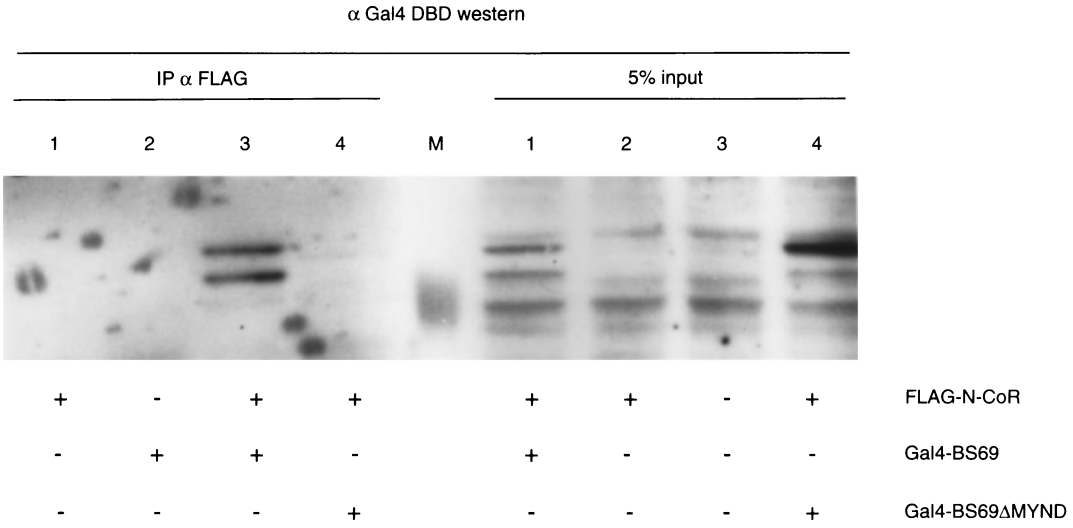

Figure 2 BS69 co-precipitates in a FLAG-N-CoR immunoprecipitation. U2OS cells were transiently transfected with 10 μg FLAG-N-CoR and 10 μg GAL4-BS69 or GAL4-BS69ΔMYND. Thirty six hours post transfection cells were lysed and immune precipitations were carried out using the M2 anti FLAG monoclonal antibody. Immunocomplexes were separated on 10% SDS–PAGE and subjected to Western blot analysis using the 5C1 anti GAL4 DBD monoclonal antibody. FLAG tagged N-CoR can co-precipitate BS69 (lane 3), which is dependent on the presence of a MYND motif (lane 4). The right panel serves as a control for expression of the proteins. GAL4-BS69 appears as a doublet due to limited proteolytic degradation

Both these domains are present in many proteins involved in transcriptional regulation (Aasland *et al*., 1995; Jeanmougin *et al*., 1997). The extreme C-terminus of BS69 and its splice variant BRAM1 contain a motif consisting of two zinc fingers, first identified in *Drosophila* DEAF-1 (Gross and McGinnis, 1996) and the mammalian transcription factor MTG8/ETO (Lutterbach *et al*., 1998a). Figure 1b shows the homology of the human BS69 MYND domain to the domains of human MTG8/ETO and the human DEAF-1 related protein. Additionally promyelocytic leukaemia genes human PML-1, 2 and 3 were found to have substantial homology to the MYND motif, as does RACK7, a potential PKC binding protein. A search of GenBank reveals that many more proteins featuring this domain can be found in various organisms, indicative of an evolutionary conserved function.

Since the MYND domain is a putative interaction molecule for the nuclear co-repressors N-CoR and SMRT (Lutterbach *et al*., 1998a; Gelmetti *et al*., 1998; Wang *et al*., 1998) we tested whether the MYND domain of BS69 also mediates N-CoR binding in a co-immunoprecipitation experiment. We transfected human U2OS osteosarcoma cells with expression vectors for FLAG tagged N-CoR and full-length human BS69 fused to the DNA binding domain of the yeast transcription factor GAL4 at the N-terminus (Figure 2). As a control we used GAL4-BS69ΔMYND, a deletion mutant of BS69 harbouring an in frame stop codon at position 522, creating a deletion of the MYND domain starting at the first cysteine of the zinc fingers. Figure 2 shows that only full-length BS69 co-precipitated with N-CoR. Significantly, BS69 was not immunoprecipitated when FLAG-N-CoR was omitted from the transfection, indicating that the M2 FLAG antibody did not recognize BS69 directly. Similar results were obtained using a C-terminal HA-tag on full-length BS69 (data not shown). GAL4 tagged BS69 appears as a doublet of approximately 77 and 80 kD, due to limited proteolytic degradation of the protein (see also Figure 5b).

Repression of BS69 is mediated by the MYND domain

To ask whether BS69 can mediate repression of transcription we used GAL4-BS69 (amino acids 1–147 of the yeast GAL4 transcription factor DNA binding domain) and subsequently tested the ability of GAL4-BS69 fusion protein to repress a luciferase reporter gene driven by a thymidine kinase promoter flanked by five upstream GAL4 binding sites. Figure 3 shows that GAL4-BS69 was able to repress transcription from this luciferase reporter in transiently transfected U2OS cells in a dose-dependent fashion. Significantly, the BS69 mutant lacking the N-CoR interaction domain had a severely reduced ability to repress transcription. We conclude that BS69 can act as a repressor of transcription when recruited to a promoter and that the MYND domain is required for full repression.

BRAM1 binds E1A and N-CoR but is unable to repress transcription

The recently cloned splice variant of BS69, BMP-Receptor type 1A Associated Molecule (BRAM1), contains both the E1A-interaction domain and the N-CoR binding MYND motif. In contrast to BS69, it is reported to be cytoplasmic (Kurozumi *et al*., 1998). We therefore tested whether BRAM1 could interact with the nuclear E1A and N-CoR proteins after transient transfection in U2OS cells. Figure 4a shows that HA-BRAM1 can readily be co-precipitated in an E1A immunoprecipitation. Consistent with this we observe that BRAM1 translocates to the nucleus when co-expressed with E1A (data not shown). Thus, BRAM1 can be recruited to the nucleus through interaction with a protein that harbours a nuclear localization signal.

To ask whether BRAM1 can interact with N-CoR, we co-expressed GAL4-BRAM1 and FLAG-N-CoR in U2OS cells. Figure 4b shows that like BS69, Gal4-

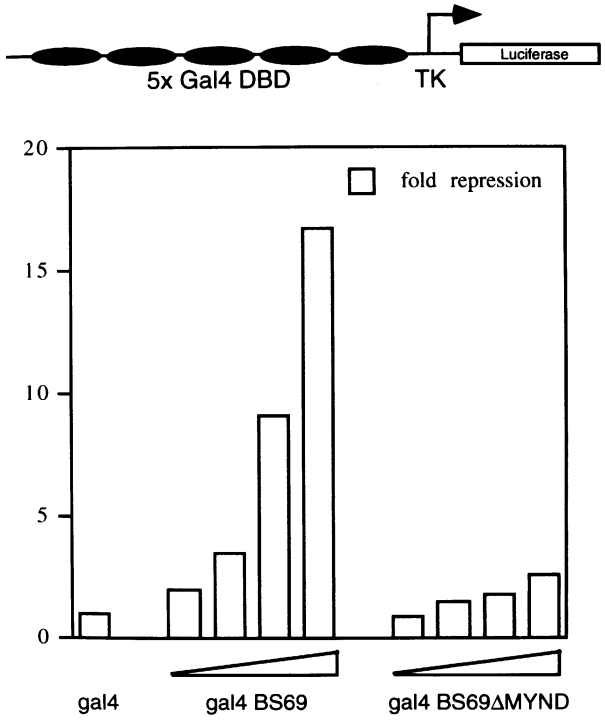

Figure 3 GAL4-BS69 represses transcription from a GAL4-luciferase reporter. U2OS cells were transiently transfected in duplicate with 3 μg luciferase reporter, 0.5 μg CMV β-galactosidase, and 0, 0.1, 0.5, 2.5 and 5 μg GAL4-BS69 or GAL4-BS69ΔMYND per transfection. Two days post transfection lysates were prepared and luciferase and β-galactosidase activities were determined. Relative luciferase activities were calculated by normalization for β-galactosidase activity after subtraction of background activities. The fold repression was determined compared to expression of GAL4-DNA binding domain alone. GAL4-BS69 represses the expression from the luciferase reporter in a dose dependent fashion, the GAL4-BS69ΔMYND mutant is severely compromised in mediating repression

BRAM1 can be co-precipitated in an immune precipitation for FLAG tagged N-CoR. A mutant BRAM1 lacking the MYND domain, analogous to the BS69ΔMYND mutant, was not co-precipitated.

Since BRAM1 can also to interact with the co-repressor N-CoR, we next tested whether GAL4 fusions of BRAM1 and BRAM1ΔMYND were able to repress transcription from an active luciferase reporter, as shown for BS69 in Figure 3. Figure 4c shows that unlike GAL4-BS69, Gal4-BRAM1 is not able to repress transcription from this reporter, although both proteins are expressed to similar levels (see Figure 5b). Similarly the BRAM1ΔMYND deletion mutant was inactive, as its BS69ΔMYND counterpart, in mediating repression. Although tested in a very wide range of concentrations (0.001 to 10 μg), GAL4-BRAM1 had no significant effect on the luciferase activity.

Involvement of BS69 N-terminus in repression

BS69 can function as a transcriptional repressor via N-CoR interaction, but its splice variant BRAM1 is unable to do so, even though it does bind N-CoR. This suggests that sequences in the BS69 N-terminal domain contribute to the MYND domain dependent repression. To examine a possible role for the N-terminus of BS69 in mediating repression, we created a series of BS69 deletion mutants (Figure 5a) each fused to GAL4-DBD and tested their ability to repress transcription. Western blot analysis of these GAL4-BS69 fusion proteins transiently expressed in U2OS cells is shown in Figure 5b. Although the fragments of the BS69 N-terminus (1–263) alone are unable to repress transcription when expressed, the N-terminus appears to be required for repression mediated by the MYND domain in native BS69. The absence of repression cannot be explained by a difference in subcellular localization, since the N-terminal deletion mutants 56–562, 138–562 and 210–562, all containing the repression mediating MYND domain, are still nuclear, as determined by immunohistochemistry using a GAL4 antibody (Figure 5a) but unable to repress transcription. The nuclear localization of the mutants correlates with the presence of a putative nuclear localization domain around amino acid 354 in BS69 (Hateboer *et al*., 1995). The cytoplasmic BS69 mutants 1–263, 56–263 and 138–263 appear to be less stable, although degradation products can also be seen for full-length BS69, the nuclear BS69 mutants and BRAM1 (Figure 5b, the aterisk indicates the presence of the stretch of twelve amino acids unique to BRAM1).

E1A inhibits repression by BS69

To investigate a role for the binding of adenovirus 5 E1A proteins to BS69, we tested whether co-expression of E1A would affect the repression mediated by GAL4DBD-BS69. To circumvent induction of apoptosis encountered when expressing higher levels of E1A in U2OS cells, we used the p5Xhocc4 construct (Bernards *et al*., 1982) which expresses moderate levels of 12S and 13S E1A as well as the 19kd E1B protein which inhibits induction of apoptosis by E1A. Figure 6 shows that expression of E1A inhibits the repression of BS69 in a dose dependent manner. In contrast, repression of GAL4 Mad-SID (Sin3 interaction domain) which also represses via recruitment of N-CoR containing complexes, is not relieved by expression of E1A but rather is slightly enhanced, as is repression by the GAL4-BS69ΔMYND mutant. Similar results were obtained with a HA tagged 13S E1A expression vector, but the higher expression levels of this construct resulted in significant apoptotic cell death which complicated the interpretation of this particular experiment (data not shown).

Genomic localization BS69/BRAM1 gene

The genomic localization of the gene encoding BS69 and BRAM1 was determined by FISH analysis using DAPI banding as shown in Figure 7a,b. The BS69 gene was mapped to the tip of the short arm of chromosome 10, without additional signals, to approximately 10p14 (Figure 7c). Interestingly, this locus is found deleted in human malignancies (Mitelman *et al*., 1997).

Discussion

We have previously cloned a novel E1A interacting protein named BS69, a nuclear protein that binds directly to conserved region 3 of E1A and strongly inhibits E1A CR 3 mediated transactivation (Hateboer *et al*., 1995). We show here that BS69 can act as a

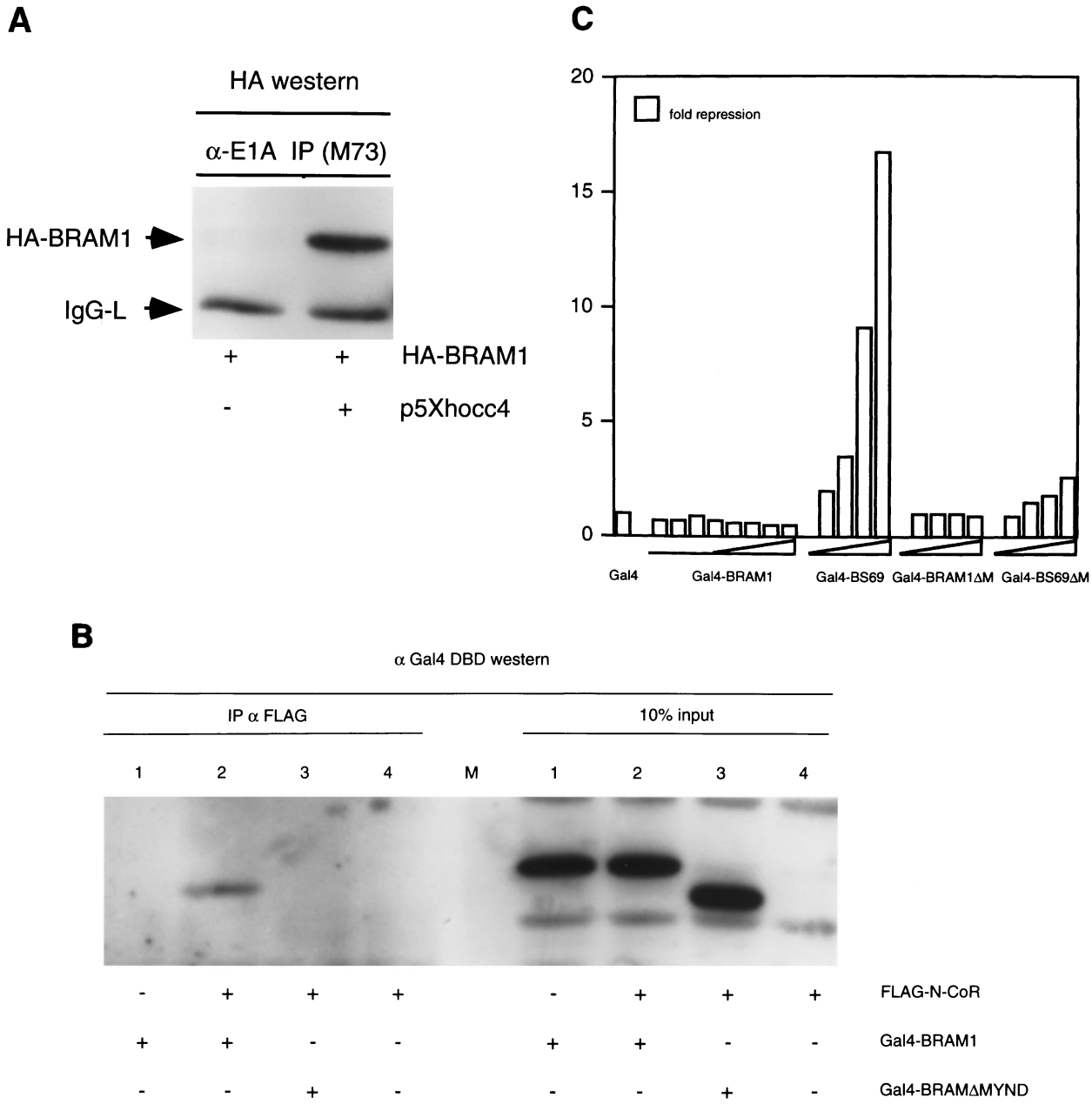

Figure 4 The BS69 splice variant BRAM1 also interacts with E1A and N-CoR but does not repress transcription. (**a**) U2OS cells were transiently transfected with 10 μg HA-tagged BRAM1 in the presence and absence of E1A expressing p5Xhocc4 (5 μg). Thirty-six hours after transfection cell lysates were prepared and subjected to immuno-precipitation using the M73 anti E1A monoclonal antibody. HA-BRAM1 was detected in the immunocomplex only when E1A was present. (**b**) U2OS cells were transiently transfected with 10 μg FLAG-N-CoR and 10 μg GAL4-BRAM1 or GAL4-BRAM1ΔMYND. Thirty-six hours post transfection cell lysates were prepared and subjected to immuno-precipitation using the M2 anti FLAG monoclonal antibody. Immuno-complexes were separated on 10% SDS–PAGE and Western blotted. GAL4-BRAM1 is coprecipitated by the anti FLAG antibody only when FLAG-N-CoR is present (lane 2), whereas the BRAM1ΔMYND deletion mutant is not (lane 3). The right panel serves as a control for proper expression of the transfected constructs. (**c**) GAL4-BRAM1 is inactive in repressing transcription from a GAL4-luciferase reporter. U2OS cells were transiently transfected in duplo with 3 μg luciferase reporter, 0.5 μg CMV β-galactosidase, and 0.001, 0.01, 0.05, 0.1, 0.5, 1.0, 5.0 and 10 μg for GAL4-BRAM1 per transfection and 0, 0.1, 0.5, 2.5 and 5 μg GAL4-BS69, GAL4-BS69ΔMYND and GAL4-BRAM1ΔMYND, per transfection. Two days post transfection lysates were prepared and luciferase and β-galactosidase activities were determined. Relative luciferase activities were calculated by normalization for β-galactosidase activity after subtraction of background activities. The fold repression was determined compared to expression of GAL4-DNA binding domain alone. GAL4-BRAM1 does not repress the transcription from the luciferase reporter at any concentration tested, in contrast to full-length BS69. The GAL4-BRAM1ΔMYND mutant is also inactive, whereas its BS69 counterpart GAL4-BS69ΔMYND has some residual activity

repressor of transcription through interaction with the co-repressor N-CoR via a carboxyl terminal MYND domain in BS69 (Figures 1b and 2). A similar motif was previously shown to be responsible for binding of N-CoR to the ETO/MTG8 protein (Lutterbach *et al.*, 1998a,b). Our present data indicate that the N-CoR binding MYND domain of BS69 is necessary (Figure 3), but not sufficient for BS69 repression (Figure 5a). The BS69 splice variant BRAM1 also interacts with E1A and N-CoR (Figure 4a,b), but unlike BS69, is not able to repress transcription when tethered to a promoter (Figure 4c), indicating that the BS69 N-

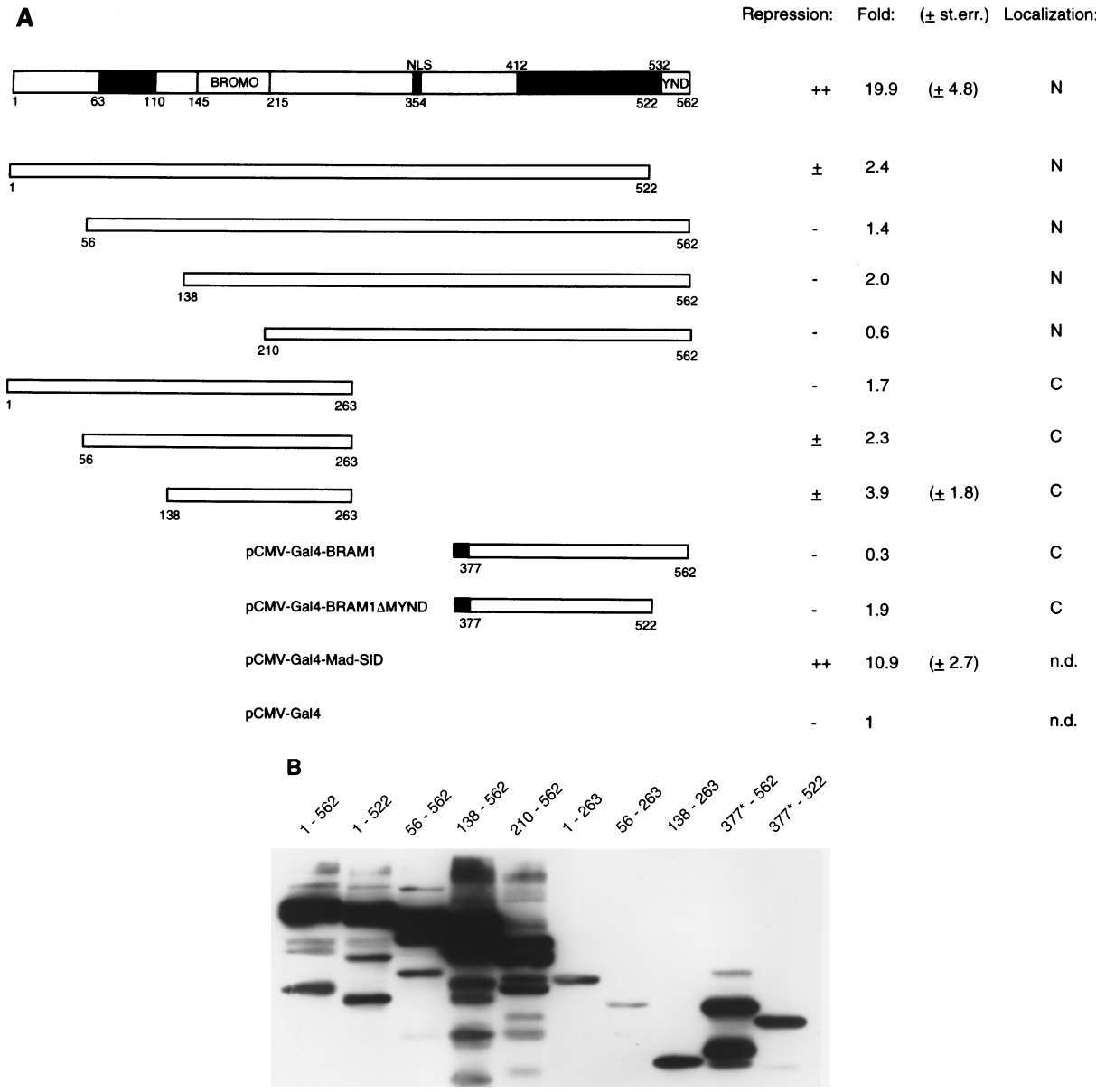

Figure 5 The N-terminal domains of BS69 are required for mediating repression. (**a**) Schematic representation of the Gal4-BS69 and Gal4-BRAM1 deletion mutants and their relative ability to repress transcription from the Gal4 luciferase reporter in transiently transfected U2OS cells (triplicates), using 5 μg of each mutant, 3 μg luciferase reporter and 0.5 μg CMV β-galactosidase for normalization as in Figures 3 and 4c. GAL4-Mad and GAL4-DNA binding domain proteins are included as positive and negative control respectively. The subcellular localization was determined on paraformaldehyde fixed U2OS cells, 36 h after transient transfection of the various mutants, detection using the 5C1 monoclonal antibody. Although the mutants 56–562, 138–562 and 210–562 all contain the MYND domain, are nuclear and expressed to similar levels (panel B), they are unable to significantly repress transcription, demonstrating the importance of the BS69 amino terminus for mediating repression. (**b**) Western blot analysis of GAL4-BS69 and GAL4-BRAM1 deletion constructs. U2OS cells were transiently transfected with 5 μg of each construct and lysed 36 h post transfection. Lysates were separated on 10% SDS–PAGE and Western blotted on immobilon-P membrane, detection using the 5C1 monoclonal antibody directed against the GAL4 DNA binding domain

terminus plays a crucial role in repression. This apparent requirement for the N-terminal domain of BS69 to repress transcription may be explained by recruitment of other co-factors by the N-terminus. The BS69 PHD finger is a candidate motif for binding such proteins. Plant Homeo Domains are frequently found in proteins involved in chromatin-mediated transcriptional regulation (Aasland *et al*., 1995). The PHD finger is highly homologous to residues 245–337 of human Mi-2, a component of the NuRD histone deacetylase and chromatin remodelling complex (Zhang *et al*., 1998). The PHD domain in BS69 may contact components of this complex. Bromo domains have recently been reported to bind to acetylated histones *in vitro* (Dhalluin *et al*., 1999), suggesting that this domain in BS69 may help in targeting or maintaining bromo domain containing protein complexes to relevant domains in the chromatin (Winston and Allis, 1999) and thus contribute to repression.

The MYND domain is essential for BS69 repression (Figure 3), presumably via recruitment of N-CoR and possibly SMRT containing complexes. Although we have not yet been able to test SMRT binding to BS69, the MYND domain of AML/ETO fusion protein does interact with SMRT (Gelmetti *et al*., 1998) suggesting that BS69 will also be able to associate with SMRT. A

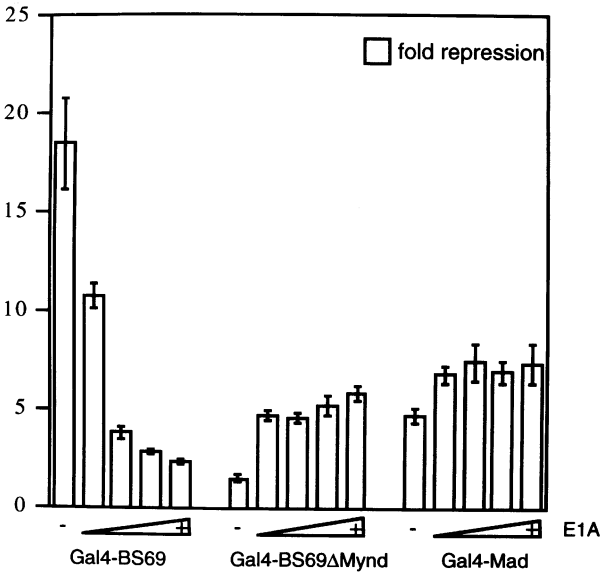

Figure 6 Repression of GAL4-BS69 is inhibited by expression of adenovirus E1A protein. U2OS cells were transiently transfected in triplicate with 3 μg luciferase reporter, 0.5 μg CMV β-galactosidase, and 5 μg GAL4-BS69, GAL4-BS69ΔMYND or GAL4-Mad in the absence and presence of increasing amounts of the E1A expressing p5Xhocc4 construct, concentrations increasing from 0, 0.1, 0.25, 1.0 to 2.5 μg per transfection. Two days post transfection lysates were prepared and luciferase and β-galactosidase activities were determined. Relative luciferase activities were calculated by normalization for β-galactosidase activity after subtraction of background activities. The fold repression was determined compared to expression of GAL4-DNA binding domain in the presence of the same concentration of E1A. GAL4-BS69 mediated repression is inhibited in a dose dependent manner by E1A expression, whereas GAL4-Mad repression is not compromised. The residual activity of GAL4-BS69ΔMYND is slightly enhanced in the presence of E1A, but does not increase with the dose

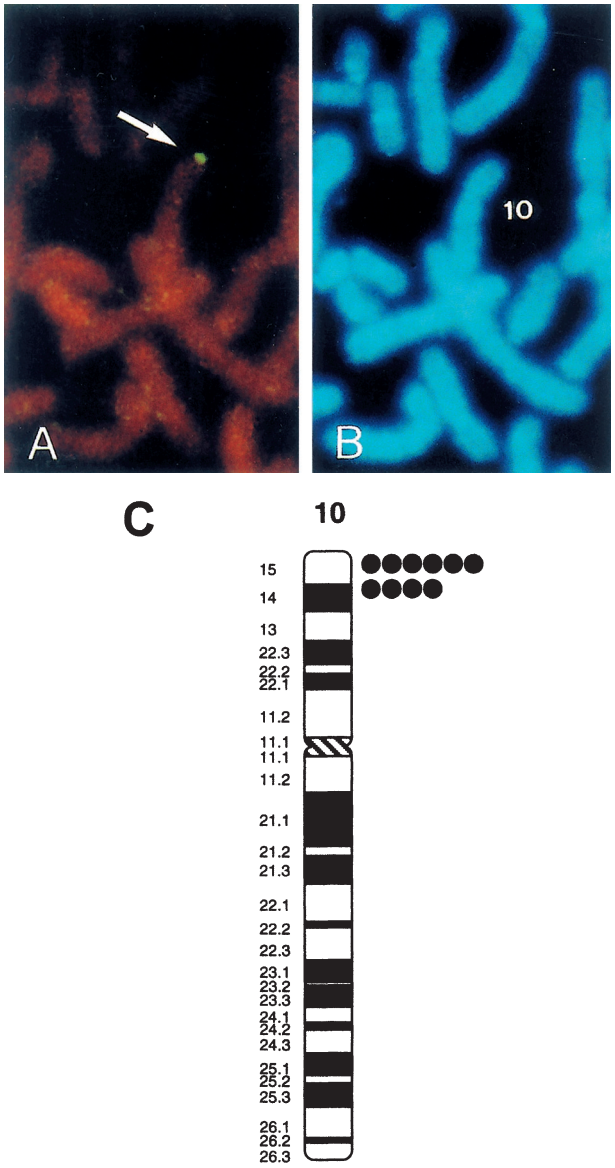

Figure 7 FISH mapping of BS69 cDNA on human peripheral blood lymphocytes to chromosome 10p. (**a**) FITC signal of the BS69 cDNA probe on a representative mitotic spread. (**b**) DAPI staining of the same frame. (**c**) Diagram of FISH mapping results, each dot representing the double FISH signals as detected on the tip of human chromosome 10p

larger SMRT cDNA that is highly homologous to N-CoR, has only recently been isolated (Ordentlich *et al*., 1999; Park *et al*., 1999). N-CoR and SMRT have been shown to be a component of large protein complexes that include histone deacetylase 1 and 2 (Heinzel *et al*., 1997; Nagy *et al*., 1997) and Sin3A/B (Alland *et al*., 1997; Hassig *et al*., 1997; Soderstrom *et al*., 1997). The N-CoR corepressor is involved in repression by nuclear hormone receptors (Chen and Evans, 1995; Horlein *et al*., 1995) and Mad/Mxi-1 mediated repression, reviewed by (Schreiber and DePinho, 1998). Apart from interacting with Sin3 and histone deacetylase protein containing complexes, N-CoR also interacts directly with components of the basal transcription machinery such as $TAF_{II}32$, $TF_{II}B$ and $TAF_{II}70$ and inhibits the binding of $TF_{II}B$ to the $TAF_{II}32$ subunit of $TF_{II}D$ (Muscat *et al*., 1998). Additionally, both SMRT and Sin3A repressor proteins were found to physically interact with $TF_{II}B$, and overexpression of $TF_{II}B$ could partially overcome SMRT mediated repression (Wong and Privalsky, 1998). Repression mediating complexes containing SMRT and N-CoR may thus exert their repression via multiple mechanisms, by direct interference with transcription pre-initiation complex and/or by histone deacetylation and chromatin remodelling.

Although we extensively tried, we have failed to detect association of BS69 with endogenous Sin3A and HDAc-1 proteins and overexpressed HDAc-2 in U2OS and COS cells. Sin3/HDAc complexes were found to associate with AML/ETO, however ETO can interact with Sin3A directly and independently of N-CoR, which may strengthen the interaction significantly (Lutterbach *et al*., 1998b). BS69 repression appeared to be highly refractory to inhibition by the histone deacetylase inhibitor Trichostatin A at concentrations ranging from 40 nM to 1.2 μM (data not shown). Interference with the transcription initiation complex by N-CoR may at least be partially responsible for the ability of BS69/N-CoR to repress transcription.

Further study is required to identify the cellular targets of BS69 mediated repression. Recent evidence indicates that BS69 interacts with the product of the proto-oncogene c-*myb* and B-*myb*. Binding of BS69 to the negative regulatory domain of c-Myb downregulates c-Myb transactivation, which is reversed by E1A. (Collins and Lipsick, submitted). Interestingly, we have mapped the genomic locus encoding BS69 and

BRAM1 (Figure 7) on the short arm of chromosome 10, to approximately 10p14. This matches closely to more detailed mapping data provided by a recent human gene map (Deloukas *et al*., 1998) that pinpoints the exact location of the BS69 gene to chromosome 10p12.48, a region frequently mutated in human cancer (Mitelman *et al*., 1997). The high number of deletions in the 10p12, 10p13 and 10p14 region in human leukaemias and solid tumours may cause derepression and deregulation of proto-oncogenes such as c-*myb*. BS69 may therefore have tumour suppressor-like properties, by down regulating transcription factors that have oncogenic potential. In transformed cells BS69 function may be impaired either by expression of transforming oncogenes such as adenovirus E1A or by deletion of the locus encoding BS69 and BRAM1. Further study is ongoing to ask whether BS69 has tumour suppressor-like properties.

Materials and methods

Plasmids

HA and GAL4 DBD tagged BS69 constructs as described in (Hateboer *et al*., 1995). BRAM1 cDNA was obtained by PCR from GAL 4-BS69 template using primers (forward 5′-CCA TCGATAATG CTGCTGGAACC ACCTTCACCTGT-GC CCTGGACAGCAGT AAGTTC TAGCCA GGAAATA-CCC-3′ and reverse 5′-TCGGAATTCTCATCTTTTCCGG-CGGCAGGAGCG-3′), confirmed by sequencing and cloned into pMV-HA-tag and pRcCMV-GAL4 DBD(1–147) vectors. The pRcCMV-GAL4 DBD BS69 deletion constructs were obtained by PCR. p5Xhocc4 as described in (Bernards *et al*., 1982). The 2×FLAG-N-CoR cDNA was *Not*I digested from pBSK-N-Cor, kindly provided by H Stunnenberg, and ligated into pRcCMV (Invitrogen). The 5×GAL4-TK-luciferase reporter was kindly provided by M Timmers.

Cell culture and transfections

U2OS cells were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum. Transfections were carried out overnight using standard calcium precipitation method, pRcCMV empty vector was added to adjust DNA concentrations to 20 μg per ml HBS buffer.

Immunoprecipitation and Western blotting

Transiently transfected U2OS cells in 10 cm petri dishes were washed in PBS, lysed in 1 ml NP40 lysis buffer (50 mM Tris pH 7.5, 150 mMNaCl, 1% Nonidet P 40) containing a protease inhibitor cocktail (Complete™, Boehringer) on ice. The cells were collected, sonicated (five bursts of 0.5 s) and spun down for 10 min at 4°C. Immunoprecipitations were carried out for 1 h at 4°C, using preformed complexes of protein A and G sepharose (Pharmacia) and immunoglobulins. Immunocomplexes were washed four times with NP40 lysis buffer and separated on SDS/10% polyacrylamide gel and transferred to a nylon membrane (Immobilon™- P Millipore), detection and visualization using ECL (Amersham).

Antibodies

Anti-Flag monoclonal antibody (M2) was purchased from Sigma. Anti HA (12CA5), anti Gal4 DNA Binding Domain (5C1) and anti E1A (M73) monoclonal antibodies were obtained from hybridoma tissue culture supernatants. Dilutions used were 1:500 for Western blotting experiments and 2 μg M2 antibody per FLAG immunoprecipitation.

Immunohistochemistry

U2OS cells were fixed in 4% paraformaldehyde in PBS for 15 min, and permeabilized using 0.2% Triton X-100. Blocking, antibody incubation and washes were done in PBS containing 1% BSA, 0.5% non fat milk (Protifar, Nutricia) and 0.2% Tween 20. Antibodies were used in 1:50 dilutions. Goat α Mouse-FITC conjugate was obtained from Pierce.

Luciferase and β-galactosidase assays

U2OS cells were transiently transfected in duplicates or triplicates in six-well tissue culture plates, using 3 μg 5×GAL4-TK-luciferase (pBSK containing 5×GAL4 binding sites followed by a TK(−109/+18) minimal promoter and a luciferase gene) and 0.5 μg pCMV β-galactosidase (Clontech) and the indicated amount of GAL4 construct per well. Cells were lysed in a 0.25 ml lysis buffer per well (lysis buffer containing 25 mM glycylglycine pH 7.8, 1% Triton X-100, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT) 48 h after removal of the calcium precipitate. After 1 h incubation at 4°C, the crude lysate was used to determine β-galactosidase and luciferase activities. Luciferase was measured using the Luclite™ system (Packard) in microtiter plates in a Packard Topcount microtiter scintillation counter. After subtraction of the background levels, luciferase values were normalized to β-galactosidase activity and standard errors calculated from three independent observations. All data shown are a representative of at least three independent experiments.

FISH/genomic mapping

The 1.7 kB BS69 cDNA probe was biotinylated with dATP using the BRL BioNick labelling kit. The procedure for FISH detection on human lymphocytes was performed according to (Heng *et al*., 1992). FISH signals and the DAPI banding pattern was recorded separately by taking photographs, and the assignment of the FISH mapping data with chromosomal bands was achieved by superimposing FISH signals with DAPI banded chromosomes (Heng and Tsui, 1993).

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