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Protein–protein interaction between the transcriptional repressor E4BP4 and the TBP-binding protein Dr1

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ABSTRACT

We have previously mapped a repression domain from the active transcriptional repressor E4BP4 to a 65 amino acid segment near the C-terminus of the polypeptide. Here we show that the E4BP4 repression domain interacts specifically with the TBP binding repressor protein Dr1. Mutants that affect the ability of E4BP4 to bring about transcriptional repression are also deficient in their binding of Dr_1. The results are discussed in the light of evidence for squelching of a 'global' repressor by a DNA binding defective E4BP4 mutant.

INTRODUCTION

Transcriptional repression is an important component of gene regulation in eukaryotes and can be mediated through nucleosomal and higher-order chromatin structures, or more selectively through the action of specific transcriptional repressors. Such repressors fall into two functional classes that either down regulate the activity of one or more positively acting transcription factors by, for example, competing for their DNA binding sites or that possess intrinsic repressing activity. The latter group can be considered as active transcriptional repressors and include The *Drosophila melanogaster* proteins Krüppel (1), Even-skipped (2), Engrailed (3) and Snail (4), the mammalian protein WT1 (5) derived from the Wilm's tumour gene, the virally transduced oncogene vErbA, the thyroid hormone receptor in its unliganded state (6) and the bZIP factors ATF3 (7) and E4BP4 (8,9). In addition, the Retinoblastoma gene product Rb has recently been shown to repress transcription when targeted to promoter DNA (10).

Distinct mechanisms of transcriptional repression are suggested by variation in the ability of different repressors to reduce activated versus basal transcription. While evidence suggests that repressors such as snail and Rb interfere with or quench the communication between upstream activators and the basal transcription machinery (4,10), other repressors inhibit basal as well as activated transcription as evidenced by their ability to repress basal promoters lacking activator binding sites (2). These repressors presumably suppress transcription initiation (or some later stage) directly. The mammalian bZIP factor E4BP4 falls into the latter category of factors (11). Discrete transferable repression domains have been mapped for some repressors including E4BP4 (9,11) and as is the case for classical transcriptional activators there is little consensus in their amino acid sequence, composition or (predicted) secondary structure (9). By analogy with what is known about activators, active repressors such as E4BP4 might be predicted to destabilise or discourage some stage of initiation complex formation resulting in a decreased probability of initiation at a given promoter and hence a reduced initiation rate. This idea is supported by the work of Fondell *et al.* (12,13) who have shown *in vitro* that the thyroid hormone receptor (T_3R), which is an active repressor in the absence of its ligand, interacts directly with TBP and inhibits transcription at an early step during preinitiation complex formation. Um *et al.* (14) have shown a similar interaction between the *Drosophila* Even-skipped protein and TBP. Similarly, transcriptional repression by Krüppel appears to involve interaction with the β subunit of TFIIE (15).

Other active repressors could be expected to act through the same or similar targets which might include the family of TBP-associated factors (TAFs) that make up *holo*-TFIID or one or more of a number of recently purified and/or cloned TFIID/TBP-interacting factors, some of which, including NC1 (16), NC2 (17), Dr_1 (18), Dr_2 (topoisomerase I) (19,20), have inhibitory effects on transcription *in vitro*. Dr_1 is a general repressor and represses both basal and activated transcription in a reconstituted *in vitro* transcription system (18) and represses a range of promoters *in vivo* (21). Factors such as Dr_1 presumably add a level of transcriptional 'fine-tuning' and it is also possible that the activity of factors like this may be increased in the presence of appropriate DNA binding active repressors. This mode of repression has been suggested for the bZIP factor ATF3 (7) and for the Wilm's tumour gene product WT1 (22). In both cases, expression of non-DNA binding forms of the repressor resulted in transcriptional activation, potentially due to sequestration of a transcriptional inhibitory component. This is analogous

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to repression or squelching by the sequestration of co-activators through protein–protein interactions with over-abundant activators (23).

The transcriptional repressor E4BP4 is widely expressed in cell lines of human origin, is a member of the bZIP family of transcription factors and recognises an overlapping but distinct pattern of DNA binding sites to the CREB/ATF family of factors (8). The optimum binding sequences for E4BP4 (TTATGTAA or TTACGTAA) are also highly related to binding sites for the hlf/PAR family of bZIP factors (24,25). We have shown previously that E4BP4 is an active transcriptional repressor when transiently expressed in human cell lines and that the repressing activity resides fully in a 65 amino acid segment near the C-terminal of the protein (11). Here we present evidence that the E4BP4 repression domain interacts with the TBP-binding repressor protein Dr_1 and discuss the significance of this in relation to evidence for titration of a negatively acting factor by E4BP4 *in vivo*.

MATERIALS AND METHODS

Plasmid constructs

Eukaryotic expression plasmids. pGALδBstB has been described previously (11). pCMVP4 was constructed by ligating the *Bam*HI–*Xho*I fragment, containing the full-length E4BP4 coding sequence, from pSVKP4 (8) into plasmid pCDNA3 (Invitrogen). Plasmid pP4δZIP, which encodes the E4BP4 leucine zipper deletion mutation was generated by digesting pSVKP4δBstB (11) with *Nde*I and *Sal*I, filling in the recessed ends with Klenow fragment of *Escherichia coli* DNA polymerase I and dNTPs and then recircularizing. Plasmids pGAL-pm1 and pGAL-pm4 were constructed as follows. A fragment was amplified by PCR from pP4RS2 (8) using the primers: 5′-TCCGGATCGAAGCCG-AAGCCATGCAGATC-3′ or 5′-TCCGGATCGCAGCCGCAG-CCATGCAGATC-3′ respectively and 5′-TCTAGAAATTGTC-TTTTAGATGTC. The resulting fragments were ligated into the *Eco*RV site of pBluescript (Stratagene) to generate pBS-pm1 and pBS-pm4. pBS-pm1 and pBS-pm4 were digested with *Bsp*EI and *Xba*I and the fragments were ligated into pGAL-CT3 (11) cleaved with the same enzymes. Mutations were verified (as was the fidelity of the PCR reaction) by DNA sequencing. Plasmids dz-pm1 and dz-pm4 were created as follows: BS-pm1 and BS-pm4 were digested with *Bsp*EI and *Xho*I and the resulting fragments were ligated into pPSVKP4 digested with the same enzymes.

Reporter plasmids. Plasmids pπS12(34)CAT, pπGALCAT (11) and G5E1BCAT (26) have been described previously.

Bacterial expression plasmids. Plasmid pGSTδBstB was generated by digesting pGST-CT4 (11) with *Bst*BI and *Sma*I and recircularizing the plasmid after filling the recessed ends.

Plasmids for in vitro expression. Plasmids pET11aDr_1 (18) and pBShTOP1 (27) [containing the human topoisomerase I (Dr_2) cDNA in pBluescript] have been described elsewhere. pET11aDr_1 was a gift from D. Reinberg and pBSTopI was a gift from R. Hania and J. C. Wang. The E1a expression plasmids pSPNC, pSPCS, pSP5/3x pSP13S and pSP12S containing various E1a deletion mutations and cDNA encoding the 13S and 12S forms of E1a are all based on the vector pSP65a and have been described previously (28).

In vitro translation

cDNAs to be transcribed and translated were added as plasmid DNAs to a coupled transcription translation rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions. Transcription required variously T3, T7 or SP6 RNA polymerase and was carried out in the presence of [^{35}S]L-methionine. Transcription/translation reactions were generally performed for 60 min at 30°C after which time an equal volume of 50 mM HEPES–KOH, pH 7.9, 150 mM NaCl, 20% (v/v) glycerol was added.

Cell culture and transfection assays

HeLa cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS. Transfections and CAT assays were performed as described in reference (11) with details given in figure legends.

Bacterial synthesis and purification of GST fusion proteins and *in vitro* protein interaction assays

Escherichia coli BL21(DE3) were transformed with plasmids pGEX2TK (Pharmacia) or pGEXδBstB. Glutathione transferase (GST) or GST fusion proteins were prepared from 500 ml cultures of each strain by standard procedures (11). Purified proteins were stored bound to GSH agarose beads in PBS containing 0.5 mM DTT and 0.2 mM PMSF at 4°C.

For *in vitro* protein interaction assays (GST pull down assays) a standard binding reaction contained up to 10 µl of diluted rabbit reticulocyte lysate and 10 µl protein-loaded GSH–Sepharose beads equilibrated in 25 mM Tris–HCl, pH 7.9, 1 mM dithiothreitol, 150 mM NaCl, 0.01% NP-40. Each reaction contained an equivalent quantity of recombinant fusion protein as judged by SDS–PAGE and protein staining with Coomassie blue. The final volume of the binding reaction was made up to 50 or 100 µl with the same buffer. Unless stated otherwise binding was carried out at 20–22°C for 20 min. The bound complexes were briefly sedimented and washed twice with 1 ml each time of 25 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol, 0.05% NP-40. Beads were transferred to fresh tubes and were washed twice with the above buffer lacking NP-40. The pelleted GSH–Sepharose beads were finally boiled in 20–50 µl SDS–PAGE sample buffer and bound proteins were resolved by SDS–PAGE. After electrophoresis, gels were fixed in 25% isopropanol, 10% acetic acid and treated with Amplify (Amersham) according to the manufacturer's instructions. Gels thus treated were dried and exposed to film at –70°C.

RESULTS

The repression domain of E4BP4 interacts with the TBP-binding factor Dr_1

Most models for the mechanism of action of a eukaryotic repressor involve direct or indirect interaction with components of the general transcription machinery. We therefore set out to identify nuclear proteins capable of interacting with E4BP4. The minimal repression domain from E4BP4 (residues 299–363) fused to *S.japonicum* glutathione transferase (GST) and thus bound to glutathione (GSH) agarose beads was used as a substrate to test for the binding of candidate proteins. We have previously used this assay to test for interaction of E4BP4 with human TBP,

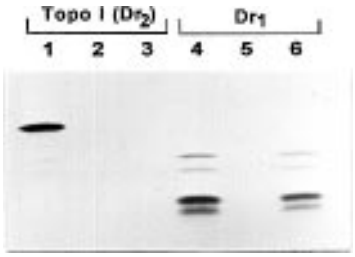

Figure 1. The E4BP4 repression domain interacts with Dr_1 *in vitro*. Dr_1 and Dr_2 were translated *in vitro* in a rabbit reticulocyte lysate system. Eight microlitres of each were incubated with 10 µl GST (lanes 2 and 5) or GSTδBstB (consisting of E4BP4 residues 299–363 fused to GST) (lanes 3 and 6) loaded beads containing equivalent quantities of GST or fusion protein, in a total volume of 100 µl. After binding for 20 min and washing, bound proteins were eluted into 20 µl SDS–PAGE sample buffer. Four microlitres of the input lysates were run on the same gel (lanes 1 and 4).

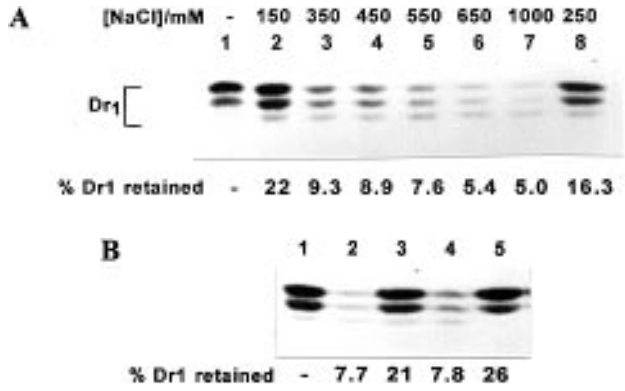

Figure 2. Effect of sodium chloride concentration on binding of Dr_1 to immobilized E4BP4 repression domain. (**A**) *In vitro* translated Dr_1 was incubated with GSH–agarose beads loaded with GSTδBstB fusion protein. Binding reactions contained 8 µl Dr_1 lysate in 100 µl binding buffer containing 25 mM Tris–HCl pH 7.8, 0.5 mM DTT, 0.2 mM PMSF, 0.05% NP-40 and 150 mM NaCl (lane 2), 250 mM (8), 300 mM NaCl (3), 450 mM NaCl (4), 600 mM NaCl (5), 750 mM NaCl (6) or 1 M NaCl (7). Beads were washed in the same buffers and then in buffer without NP-40 and containing 150 mM NaCl prior to elution into SDS–PAGE sample buffer. Eluted proteins were visualized by fluorography after SDS–PAGE using a 10 cm 12.5% gel run overnight. The three bands in each lane only resolve on large gels and are all due to Dr_1 as they do not appear in lysates unprogrammed with pET11Dr_1 (not shown) and presumably represent internal starts or termination within the Dr_1 sequence during translation. The relative abundance of the lower forms varied with different batches of reticulocyte lysate. Bands were cut from the dried gel and counted by liquid scintillation counting to estimate the relative amounts of Dr_1 retained on the beads. 1/4 of the amount of lysate used in the binding reaction was applied directly to lane 1. (**B**) Dr_1 was bound to GSTδBstB beads as above in buffer containing either 1 M (lanes 2 and 4) or 150 mM (lanes 3 and 5) NaCl. Beads were washed in fresh binding buffer (lanes 2 and 3) or were washed briefly and then incubated at room temperature for 1 h in buffer containing 1 M NaCl (lanes 4 and 5). One quarter of the amount of lysate used in the binding reaction was applied directly to lane 1.

TFIIB (11) and TFIIEβ (unpublished results) with negative results. We therefore extended the analysis of proteins that might interact with the repression domain of E4BP4 to the TBP-associated regulatory factors Dr_1 and Dr_2 (DNA topoisomerase I).

Dr_1 and Dr_2 polypeptides were translated *in vitro* in the presence of [^{35}S]methionine (Fig. 1, lanes 1 and 4). GSH–agarose beads loaded with either GST (Fig. 1, lanes 2 and 5) or a GST E4BP4 repression domain fusion protein (GSTδBstB beads, Fig. 1, lanes 3 and 6) were incubated with rabbit reticulocyte lysates containing either *in vitro* translated Dr_1 or Dr_2. The beads were washed and any bound proteins eluted into SDS–PAGE sample buffer. Eluted proteins were visualized by fluorography after SDS–PAGE. Dr_2 (topoisomerase I) was not retained on the GSTδBstB beads (Fig. 1, lane 3), but Dr_1 was specifically retained on the GSTδBstB, but not control GST beads (Fig. 1, lanes 6 and 5). Binding was not affected by inclusion of ethidium bromide in the binding and washing buffers, nor was it affected appreciably by the inclusion or omission of the non-ionic detergent NP-40 up to 0.05% (data not shown).

Since the E4BP4 repression domain contains a number of charged residues (11), it was important to test the effect of salt concentration on the binding reaction between E4BP4 and Dr_1. Increasing the sodium chloride concentration in the binding reaction in steps from 150 mM to 1 M resulted in a gradual loss of binding, but significant binding still occurred even in buffer containing 1 M sodium chloride (23% of that retained in 150 mM NaCl; Fig. 2A). The length of time allowed for binding between E4BP4 and Dr_1 (20 min) was sufficient to allow maximum binding. Prolonged washing of the GST–E4BP4 beads in wash buffer containing 1 M NaCl failed to significantly elute pre-bound Dr_1 (Fig. 2B). Thus it is unlikely that the retention of Dr_1 by GSTδBstB is due to non-specific electrostatic interactions between the two proteins.

The ability to bind Dr_1 correlates with E4BP4 repression activity

Two mutations in the E4BP4 repression domain were created that either partially or fully abolish transcriptional repression activity in transient expression assays (see below, Fig. 3). Mutant pm1 in which two lysine residues (Lys-330 and Lys-332) were changed to glutamate residues has been described previously (11) and renders the repression domain inactive when transiently expressed in HeLa cells as a chimera with the GAL4 DNA binding domain (GAL-pm1, Fig. 3a). In the same assay the wild-type repression domain fused to the GAL4 DNA binding region resulted in an ~10-fold repression of transcription (GALδBstB, Fig. 3A). Computer analysis of the primary structure of the E4BP4 repression domain predicts an α-helical structure for the central part of the domain (11). We therefore constructed the mutant pm4, in which Lys-330 and Lys-332 where exchanged for alanine residues. This represents a less drastic charge change than pm1 and would not be expected to disrupt potential α-helical structure. In transient transfection assays, a $GAL4_{1–147}$–pm4 fusion protein (GAL–pm4) was approximately four times less effective as a repressor than the wild-type repression domain (Fig. 3A). Minimal 65 amino acid repression domains containing these mutations were synthesised as GST fusion proteins and compared with the wild type for the ability to bind Dr_1. As shown in Figure 3B, mutant pm1, which was inactive in the *in vivo* repression assay, was negative for Dr_1 binding (lane 4) while mutant pm4 that was compromised for repression, but which retained some activity also retained the ability to bind Dr_1, albeit with reduced efficiency compared to the wild-type repression domain (lanes 5 and 7). Thus, on the basis of these mutants at least, the ability of E4BP4 to bind Dr_1 correlates well with its transcriptional repression activity.

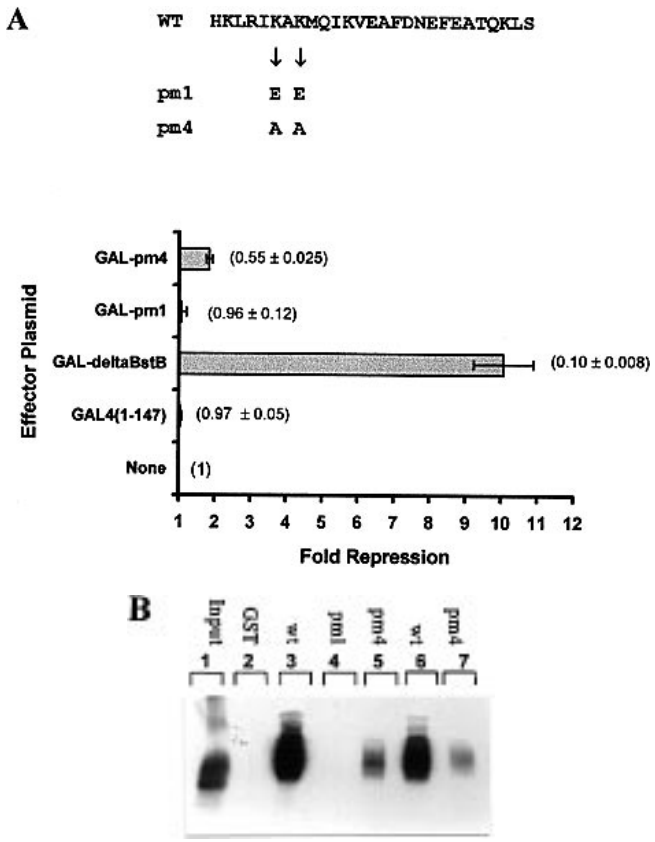

Figure 3. The ability of wild-type and mutant forms of E4BP4 to repress transcription *in vivo* mirrors its ability to bind Dr_1 *in vitro*. (**A**) Top: diagram of the point mutants pm1 and pm4. Bottom: HeLa cells were cotransfected with the reporter plasmid pπGalCAT (3 µg) and 1 µg of plasmids expressing the GAL4 DNA binding domain [$GAL_{1–147}$, pSG424 (38)], GALδBstB (Fig. 1) or analogous proteins containing a mutated E4BP4 repression domain as indicated. The histogram represents fold repression over the average CAT value obtained with the reporter alone. Numbers in brackets are CAT activities obtained relative to transfection with the reporter alone. The results are the averages of at least two independent transfections each performed in duplicate. Error bars represent the standard errors of the mean values obtained. (**B**) *In vitro* translated Dr_1 (8 µl) was incubated with GSH–agarose beads loaded with GST (lane 2), GSTδBstB (WT, lanes 3 and 6), GSTδBstB–pm1 (pm1, lane 4) or GSTδBstB–pm4 (pm4, lanes 5 and 7). GSTδBstB–pm1 and GSTδBstB–pm4 fusion proteins contain the respective mutated forms of E4BP4 299–363 fused to GST. Two independent preparations of GSTδBstB and GSTδBstB–pm4 were used in lanes 3 and 6 and lanes 5 and 7 respectively. Binding reactions and washing conditions were as described for Figure 2. Lane 1 contains 2 µl of the input protein. Each binding reaction contained the same quantity of GST fusion protein as judged by SDS–PAGE and Coomassie blue staining (not shown).

Figure 4. Alignment of the E4BP4 repression domain and TBP. TBP-H, human TBP; E4BP4, repression domain (residues 299–363) of E4BP4; TBP-Y, *Saccharomyces cerevisiae* TBP. The bars represent β strands and helix H2 of the crystal structure of TBP (39) as designated. Amino acid identities are shown as | and conservative substitutions are designated *.

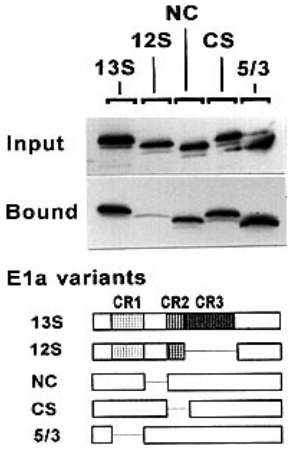

Figure 5. The E4BP4 repression domain binds the 289 R form of the adenovirus E1a protein. 12S and 13S E1a cDNAs were transcribed and translated *in vitro* as were the E1a deletion mutants shown. Two microlitres of each lysate are shown in the top panel. Eight microlitres of the same lysates were incubated with GSTδBstB loaded GSH agarose beads. Binding and washing conditions were as described in Figure 2. Bound proteins were eluted into SDS–PAGE sample buffer and visualized by fluorography. In a parallel experiment (not shown) no binding to beads loaded with GST was observed.

The E4BP4 repression domain interacts with the adenovirus E1a gene product

Dr_1 is a TBP binding protein and in the light of the above findings it is interesting to note that careful comparison of the amino acid sequences of the E4BP4 repression domain with yeast and human TBP revealed a region of similarity (Fig. 4) overlapping the basic repeat of TBP, suggesting that E4BP4 and TBP might contain a similar Dr_1 binding motif (see below). A further similarity between the TBP basic repeat and the E4BP4 repression domain concerns their affinity for the adenovirus E1a product. In Figure 5 we present data showing that GSTδBstB will bind to E1a proteins containing the constant region 3 (CR3) peptide; a binding preference also shown by TBP (29). It should be pointed out that E1a also has also been shown to bind DR_1 *in vitro* (30), but this binding depends on the N-terminal region of E1a whereas interaction between E1a and both TBP and E4BP4 involves constant region 3. Together this evidence suggests that the basic repeat region of TBP and the repression domain of E4BP4 present a similar binding surface, both capable of binding Dr_1 and E1a.

Overexpression of E4BP4 titrates a factor involved in Pol II transcriptional regulation

Since E4BP4 apparently represses both activated and basal transcription *in vivo* (11) we surmised that the mechanism of repression was likely to involve interference with the basic machinery of transcription initiation at the promoter. This could involve interaction with a component of the general transcription machinery such as one of the general RNA polymerase II transcription factors or accessory factors such as one of the TAFs. In order to test this we constructed a non-DNA binding E4BP4 mutant, reasoning that overexpression of such a protein *in vivo*

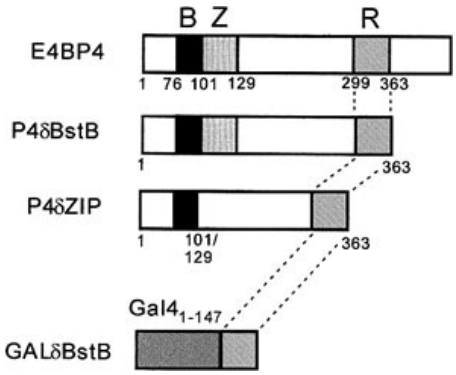

Figure 6. Schematic diagram comparing the primary structure of wild-type E4BP4, the C-terminally truncated P4δBstB and P4δZIP. B, basic region; Z, leucine zipper; R, repression domain.

would titrate any interacting proteins, potentially resulting in a detectable change in the level of transcription of a reporter gene. This would be analogous to the squelching effect first described by Gill and Ptashne (23). To this end a variant of E4BP4 was constructed (P4δZIP; Fig. 6) that lacks the leucine zipper dimerization domain (residues 102–128) but retains a basic DNA binding region that overlaps a predicted nuclear localization signal sequence (31,32). P4δZIP was not expected to exhibit specific DNA binding activity as dimerization is necessary for DNA binding of bZIP factors. When P4δZIP was synthesised by *in vitro* translation no DNA binding activity was detected towards an adenovirus E4 promoter ATF site to which full-length E4BP4 binds avidly (data not shown).

HeLa cells were transfected with the reporter plasmid pπS12(34)CAT in which the CAT reporter gene is driven by the human GST-π gene promoter (33,34) containing three artificial upstream E4BP4 binding sites (Fig. 7A insert). Cotransfection with 1 µg of the E4BP4 expression plasmid pCMVP4 resulted in a 5-fold repression of the promoter (Fig. 7A). However, simultaneous transfection with increasing amounts of the P4δZIP expression construct reduced and ultimately abolished E4BP4-mediated repression. Interestingly, as shown in Figure 7B, when P4δZIP was introduced into HeLa cells in the absence of exogenous wt E4BP4 a significant increase in transcription of the reporter gene was observed.

Essentially identical results were obtained when the E4BP4 binding sites in pπS12(34)CAT were replaced by GAL4 binding sites (pπGALCAT). Repression by the GAL4–E4BP4 fusion protein GalδBstB was suppressed by P4δZIP and the reporter alone was activated by P4δZIP (data not shown).

To determine whether this was a general effect, the experiment was repeated using the reporter plasmid G5E1BCAT, which consists simply of five GAL4 binding sites upstream of a TATA box derived from the adenovirus E1B gene driving the CAT gene (Fig. 7C insert). Increasing amounts of P4δZIP caused a significant increase in CAT activity from this basal promoter, confirming that the apparent activation or derepression properties of P4δZIP are not promoter specific. As previously shown (11), a DNA-binding competent GAL4–E4BP4 repression domain fusion construct (GALδBstB) was an efficient repressor of this promoter.

The data described above are consistent with the titration or squelching of a negatively acting transcriptional component by high level expression of P4δZIP. To determine the effect of the

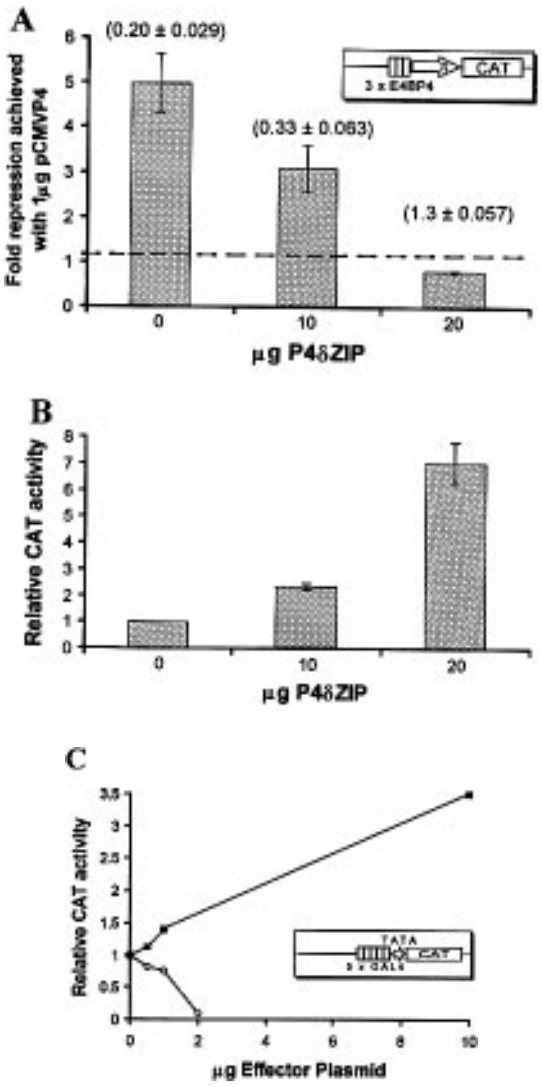

Figure 7. E4BP4 interacts with a titratable corepressor. (**A**) HeLa cells were transfected with 3 µg pπS12(34)CAT, 1 µg of the wt E4BP4 expression plasmid pCMVP4 (or CMV vector) and 0–20 µg pP4δZIP. The amount of P4δZIP plus empty expression vector (pSVK3) was maintained at 20 µg throughout. The CAT activity obtained with the reporter plus 20 µg pSVK3 and 1 µg CMV vector was arbitrarily set at 1.0. The chart shows fold repression obtained when CMV vector was replaced by CMVP4. Numbers in brackets are the relative CAT activities obtained. The values represent the mean of three experiments ±SEM. (**B**) Cells were treated as in (A) but the E4BP4 expression plasmid was omitted. The CAT activity obtained with the reporter plus 20 µg pSVK3 was arbitrarily set at 1.0. (**C**) HeLa cells were transfected with the reporter plasmid G5E1BCAT and increasing amounts of P4δZIP (■) or GALδBstB (○). As before, the amount of pSVK3-derived plasmid was the same in each transfection.

repression domain mutations pm1 and pm4 (see above and Fig. 3) on this phenomenon, experiments analogous to those shown in Figure 7 were performed in which HeLa cells were transfected either with P4δZIP, dz-pm1 or dz-pm4 which contain the pm1 or pm4 mutation respectively in the context of P4δZIP. As shown in Figure 8 while transfection of HeLa cells with 10 µg P4δZIP resulted in an ~3-fold increase in CAT activity from the pπS12(34)CAT reporter as before, dz-pm1 had no effect or resulted in marginal repression of the reporter. A small (1.2-fold)

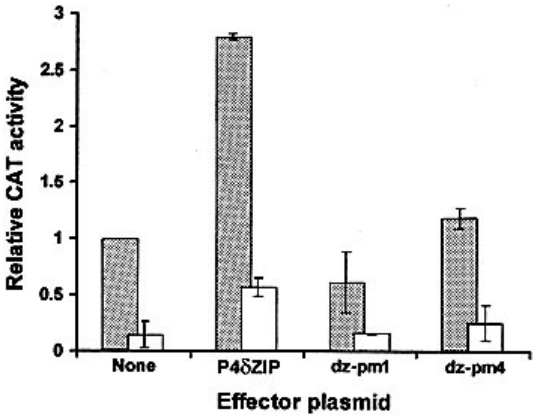

Figure 8. Mutations affecting repression activity and Dr_1 binding also affect corepressor titration. HeLa cells were co-transfected with 3 µg reporter plasmid pπS12(34)CAT, 1 µg wt E4BP4 expression plasmid pCMVP4 (open bars) or 1 µg CMV vector pCDNA3 (shaded bars) and 10 µg of one of the following: SV40 expression plasmid pSVK3 (none), pP4δZIP (P4δZIP), plasmid pdzpm1 expressing the P4δZIP protein containing the pm1 repression domain mutation (dz-pm1), plasmid pdzpm4 expressing the P4δZIP protein containing the pm4 repression domain mutation (dz-pm4). CAT activities are reported relative to the activity obtained with 3 µg reporter plasmid, 1 µg pCDNA3 vector and 10 µg pSVK3 vector. The values represent the average of three experiments each performed in duplicate ± SEM.

increase in CAT activity was noted for dz-pm4 (shaded bars in histogram). A similar pattern emerged when cells were simultaneously transfected with P4δZIP-derivatives and the wild-type E4BP4 expression plasmid pCMVP4 (Fig. 8, open bars) in that P4δZIP partially relieved repression by WT E4BP4 but little if any relief of repression was observed with dz-pm1 or dz-pm4. Thus, the ability of wild-type versus mutant E4BP4 to repress transcription correlates with their apparent squelching ability.

DISCUSSION

Expression in cells of either wt E4BP4 or non-DNA binding mutant P4δZIP produced apparently opposite transcriptional effects, with wt protein repressing promoters containing it's cognate binding site while the mutant stimulates both basal and activated transcription (Fig. 7). These observations are reminiscent of the 'squelching' phenomenon documented for non-binding versions of a number of transcriptional activators (23). Consequently, although it is formally possible that overexpression of P4δZIP may disrupt the normal mechanism of E4BP4 repression, the simplest explanation of these results is that P4δZIP sequesters an inhibitory factor or 'global' repressor that normally mediates the repression function of E4BP4.

In the light of the *in vitro* protein interaction data we present here, a possible candidate for this E4BP4 repression cofactor is Dr_1. Not only does Dr_1 bind specifically and tightly to the repression domain of E4BP4 (Figs 1 and 2), but mutants within this domain that are either negative or defective for Dr_1 binding *in vitro* are similarly compromised for repression activity *in vivo* (Fig. 3). In addition, we have also detected a weak but reproducible *in vivo* interaction between E4BP4 and Dr_1 using the yeast two hybrid system (35) (not shown). Furthermore, the same mutations that cripple the E4BP4 repression domain and disrupt its interaction with Dr_1 also interfere with the ability of the non-DNA binding form of E4BP4 to stimulate transcription (Fig. 8). The ability of Dr_1 to down regulate both basal and activated transcription at a variety of promoters has been well documented (18,21,30) and this would correlate with our findings in Figure 7 discussed above. Unfortunately, our attempts to determine the effects of expressing exogenous Dr_1 in cells co-transfected by the P4δZIP expression construct have yielded variable and unreproducible results probably due to modulation of the P4δZIP expression construct by exogenous Dr_1 (unpublished results). We therefore cannot unequivocally say that the sequestered factor is Dr_1.

Dr_1 also binds to the basic repeat region of the TATA binding factor TBP. Indeed, the E4BP4 repression domain and this domain of TBP share certain sequence similarities (Fig. 4) and the ability to bind the adenovirus E1a product constant region 3 (Fig. 5). This suggests that Dr_1 binds to either TBP or E4BP4 and we have certainly failed to find evidence for a ternary complex containing all three components *in vitro* (unpublished data). Therefore, if Dr_1 is the physiological target for E4BP4 the question arises as to the role of this interaction. Simply tethering Dr_1 to a promoter does not result in transcriptional repression (21) suggesting that it is not sufficient for E4BP4 to simply recruit Dr_1 to a promoter to cause repression. It also seems unlikely that E4BP4 'passes' Dr_1 to TBP as Dr_1 appears to dissociate from E4BP4 very slowly under *in vitro* conditions (Fig. 2B). However, this situation may be different *in vivo* where other factors may be involved. Notably, Merlmelstein *et al.* (36) have recently described a DR_1-associated protein DRAP1 that increases the stability of the DR1–TBP–TATA complex and it may be that this protein also modifies the interaction of Dr_1 with E4BP4.

It is also possible that E4BP4 represses transcription through both corepressor (Dr_1) dependent and independent mechanisms. *In vitro* transcription experiments using a transcription system composed largely of recombinant factors have shown that E4BP4 (in the form of GALδBstB, Fig. 6) is still capable of repressing a GAL–MLP chimeric promoter in reactions apparently lacking Dr_1 (K. Leung and D. Reinberg, personal communication). This would be analogous to the suggestion made by Fondell *et al.* (13) to explain the fact that the thyroid hormone receptor T_3R has been shown to interact directly with TBP *in vitro* and to interfere with preinitiation complex assembly (13) while persuasive evidence also exists that a corepressor, N-CoR, mediates ligand independent repression by T_3R (37) thus indicating that two pathways leading to transcriptional repression exist.

We have recently identified a novel protein by yeast two-hybrid screening that is distinct from Dr_1 but which also binds E4BP4. We are currently examining whether this protein is involved in a distinct mechanism of repression by E4BP4 or whether it is a further component of a Dr_1-mediated repression mechanism indicated by the protein interaction and transfection data presented here.

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