Transcriptional repression by the human bZIP factor E4BP4: definition of a minimal repression domain

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ABSTRACT

The bZIP factor E4BP4 overlaps in DNA binding site specificity with the transcriptional activator CREB and members of the ATF family of transcription factors, but is an active transcriptional repressor. In this study we have mapped the repressing activity of E4BP4 to a small 'domain' of 65 amino acids that retains its ability to repress transcription when transferred to the heterologous DNA binding domain of the yeast transcriptional activator GAL4. This segment of the E4BP4 polypeptide contains a high proportion of charged amino acids and does not resemble the repression domains that have been characterized so far from other active transcriptional repressors such as the *Drosophila* Krüppel, Engrailed or Even-skipped proteins. A mutation which changes the charge configuration of this repression module resulted in a complete loss of repressor activity. The E4BP4-GAL4 fusion protein is able to repress the residual transcription from minimal promoters containing the adenovirus E4 or E1b TATA box. This is consistent with a mechanism of action whereby E4BP4 interacts with some component of the general transcription machinery to cause repression of basal and activated transcription. Although a number of nuclear proteins are able to interact with the E4BP4 repression domain *in vitro*, these proteins do not appear to include the general transcription factors TFIIB or TBP.

INTRODUCTION

Genes transcribed by RNA polymerase II may be subject to control through both activation and repression of transcription. Transcriptional repression operates at several levels. Firstly, transcription is inhibited by histones, positioned nucleosomes and higher order chromatin structural features (1–5). Transcriptional activators increase the *in vivo* rate of transcription initiation by alleviating chromatin-mediated inhibition of transcription (anti-repression) (6–8) and by encouraging productive initiation complex formation at the transcriptional start site (activation) (9,10). In a second mode of transcriptional repression, transcriptional activators can be inhibited by repressor molecules that either compete for DNA binding sites or which titrate

particular activators through protein-protein interactions resulting in the formation of complexes with reduced or absent DNA binding activity (11–15). A third type of transcriptional repression can result from the action of so-called active transcriptional repressors that directly down regulate transcription of genes whose promoters they bind (15). Since both activators and active repressors are targeted to DNA in a sequence-specific manner and both modulate the rate of transcription initiation, active repressors can be considered to be analogous but opposite in effect to transcriptional activators. Active transcriptional repressors that have been characterized so far include the product of the Wilms tumour gene (WT1) (16,17), the *Drosophila* Engrailed (En) (18), Even-skipped (Ev) (19) and Krüppel (Kr) (20) proteins, the human Krüppel-related factor YY1 (21,22), the v-ErbA oncogene and its cellular counterpart, the thyroid hormone receptor c-ErbA (23,24) and the human bZIP factor E4BP4 (25).

Much work has centred recently on the mechanism of transcriptional activation. Studies, largely *in vitro*, have resulted in the working model whereby productive preinitiation complex formation is stimulated by transcriptional activators, resulting in enhanced rates of transcription initiation (9,26,27). In support of this, several transcriptional activators have been shown to bind directly to components of the general transcription machinery, specifically TFIID (28,29) and TFIIB (30,31). Discrete transcriptional activation domains have been defined for a number of positively acting transcription factors and these domains either directly (28,30–32) or indirectly (33) contact initiation factors presumably to bring about increased complex stability and thereby enhanced transcription initiation rates.

Less attention has been focused on the mode of action of transcriptional repressors, although a number of active transcriptional repressors have now been described. Certain of these repressors have been shown to contain transferable effector domains like the activation domains of positively acting transcription factors. Recurrent features of the repression domains that have been identified so far are an abundance of alanine, glutamine and/or proline residues and a relative scarcity of charged amino acids. This has led to the suggestion that the salient features of active transcriptional repression domains are that they are unstructured and relatively uncharged (34). In this work we have defined an apparently novel repression 'domain'

from the human bZIP factor E4BP4 which is unrelated in primary and predicted secondary structure to the alanine/glutamine/proline rich sequences of the *Drosophila* repressors.

MATERIALS AND METHODS

Expression plasmids

Expression plasmids pSVKP4, pSVKP4 δ Apa and pSVKP4 δ Pst have been described previously (25). Plasmid pSVKP4 δ BST was made by digesting pSVKP4 with BstB1 and XhoI, filling in the recessed ends with Klenow fragment of DNA polymerase I and religating. The GAL4₁₋₁₄₇ expression plasmid pSG424 was described by Sadowski and Ptashne (1989) (35). GAL-CT2 and GAL-CT3 plasmids contain PCR-amplified segments of E4BP4 cDNA encoding amino acids 299 to 462 or 299 to 429 respectively, cloned into the BamHI site of pSG424. In both cases the 5' PCR primer was CGGATCCATTCTCCAGTT and the 3' primers were ATTCTAGCATAATACAAA and CAGGCTCG-AGAACTTTGTAGCCACTGTCTTT respectively. In each case the fidelity of the cloned PCR products was checked by DNA sequencing. GAL δ BstB was constructed by digesting GAL-CT3 with BstB1 and XbaI, filling in the recessed ends and recircularising by ligation. The plasmid pKB104 has been described (36) and pIIIB (37) was a gift from D.Reinberg. pGEX-CT4 was constructed as follows: a fragment encoding the C-terminal region of E4BP4 was generated by PCR amplification from pSVKP4 using the PCR primers AAGGGATCCATCC-ATTCTCCAGTT and ATTCTAGCATAATACAAA. This fragment was ligated into the EcoRV site of pBluescript and checked for PCR-errors by DNA sequencing. The fragment was excised by digestion with BamHI and cloned into BamHI digested pGEX2TK (Pharmacia).

Reporter constructs

The reporter plasmid p π S12 (34) CAT has been described previously (25), p π GAL-CAT was similarly derived by ligation of two copies of the double stranded oligonucleotide AGCTTAGTCGGAGGACAGTACTCCGACA into the HindIII site of pSS0.2CAT (38) (corresponding to position -94 in the human GST- π promoter). Plasmids pG5E1BCAT and pG5E4CAT have been described by Lillie and Green (1989) (39) and Flint and Jones (1991) (40) respectively.

Cell culture and transfection assays

HepG2, HeLa and COS-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. Transfection was carried out by the calcium phosphate procedure (41) using 3 μ g of reporter plasmid and unless otherwise stated, 1 μ g of expression plasmid. For transfection of COS-1 cells prior to preparation of nuclear extracts for EMSA 20 μ g of expression plasmid was used per plate. Chloramphenicol acetyl transferase (CAT) assays were performed as described previously (25,41) using ¹⁴C-labelled acetyl-CoA. Reactions are extracted with ethyl acetate and the organic phase was added to a scintillation cocktail. Transfer of ¹⁴C-acetyl groups to chloramphenicol was assessed by liquid scintillation counting. Transfections were performed in duplicate. Each experiment was repeated at least three times. Mean relative CAT activities and the standard deviation of the data were calculated.

Electrophoretic mobility shift analysis (EMSA)

Binding reactions were carried out in GRB (25mM HEPES-KOH (pH7.9), 1mM EDTA, 5mM DTT, 150mM NaCl, 10% glycerol)

and contained 10 μ l of crude nuclear extract from transfected COS-1 cells (approximately 30 μ g protein), 0.2 ng labelled double-stranded probe and a cocktail of non-specific competitor DNA consisting of 2 μ g poly(dI/dC), 2 μ g poly(dA/dT), 2 μ g sonicated salmon sperm DNA and 2 μ g of the single stranded oligonucleotide TCCGGATCAAAGGCAAACCCATGCAGATCA. Binding mixes were incubated for 20 minutes on ice prior to loading directly onto an 8% polyacrylamide (Acrylamide:bis 44:0.8) gel in 0.5% TBE. The double stranded GAL4 oligonucleotide probe AGCTTAGTCGGAGGACAGTACTCCGTCA was labelled by filling-in with the Klenow fragment of DNA polymerase in the presence of ³²P-dCTP.

Protein secondary structure prediction

Potential secondary structure features of residues 299–363 of E4BP4 were determined using the Garnier and GBSN methods (42,43) as implemented by IntelliGenetics 'PC-GENE' software (v6.70)

In vitro transcription and translation

Capped TFIIB and TBP RNAs were synthesised *in vitro* by transcription of pIIIB or pKB104 respectively using T7 or T3 RNA polymerase as described previously (25). RNA was subsequently translated *in vitro* using a rabbit reticulocyte lysate (Promega)

Bacterial synthesis and purification of GST fusion protein

E.coli BL21(DE3) were transformed with pGEX2T or pGEX-CT4. In a standard preparation, 500 ml of medium (LB) at 30°C was inoculated with 10 ml of an overnight culture of the appropriate plasmid in BL21(DE3). The culture was incubated at 30°C until an O.D.₆₀₀ of 0.5 was reached, at which time IPTG was added to 0.3 mM. Incubation at 30°C was carried on for a further two to four hours. Cells were collected by centrifugation and resuspended in 20 ml of ice cold phosphate buffered saline (PBS) containing 1 mM DTT, 0.5 mM PMSF, 1 mg/ml lysozyme. After holding on ice for 30 min, the suspensions were rapidly frozen in a dry-ice/methanol bath. The frozen suspensions were then thawed in a 30°C water bath. Sodium chloride was added to a final concentration of 0.4 M and the lysate was cleared by centrifugation. The supernatant was diluted with an equal volume of cold PBS and 0.5–1.0 ml of a slurry of glutathione–Sephacrose (GSH–Sephacrose) was added. Binding was allowed to occur for 30 min at 4°C after which time the GSH–Sephacrose was pelleted in a bench-top centrifuge and washed with three changes of 50 ml of PBS containing 0.5 % NP40. The loaded GSH–Sephacrose was finally suspended in PBS and stored at 4°C.

In vivo labelling

HeLa cells were grown to approximately 70 % confluence in complete medium containing 10 % FCS. The medium was replaced with DMEM minus methionine and the cells were incubated for 10 min after which time the medium was replaced with 3 ml per 90 mm plate of fresh minus-methionine medium containing 300 μ Ci of [³⁵S]-methionine (>1000 Ci/mmol). Cells were grown for a further 8 hr before preparing nuclear extracts as described by Hurst *et al.* (1990) (44).

Pull-down assays

Binding reactions contained 10 μ l of labelled nuclear extract or 1–5 μ l of *in vitro* translated material and 25 μ l protein-loaded GSH–Sephacrose beads equilibrated in 25 mM HEPES, pH 7.9,

1 mM EDTA, 5 mM dithiothreitol, 150 mM NaCl. Each reaction contained 0.5–1.0 μ g of recombinant protein as judged by SDS PAGE and protein staining with coomassie blue. Fresh, non-loaded beads were added where necessary so that all each reaction contained similar quantities of GSH–Sepharose. The final volume of the binding reaction was made up to 50 μ l with the same buffer. Binding was carried out at 10–15°C for 20 minutes. The bound complexes were briefly sedimented and washed twice with 1 ml each time of 25 mM Tris (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol, 0.05% NP40 and twice with the same buffer lacking NP40. The pelleted GSH–Sepharose beads were finally boiled in 50 μ l of SDS-PAGE sample buffer and bound proteins were resolved by SDS PAGE.

RESULTS

Previous work has shown that E4BP4 represses transcription in a DNA binding site-dependent manner when expressed in a transient transfection assay with a suitable reporter plasmid. The transcriptional repression activity of E4BP4 was shown to require sequences in the C-terminal half of the protein (25). Figure 1 shows the effect of a series of C-terminal deletions on repressing activity of E4BP4. In these experiments plasmids expressing various C-terminal deleted E4BP4 proteins were cotransfected with the reporter plasmid p π S12 (34) CAT which contains three E4BP4-specific DNA binding sequences introduced upstream of the human glutathione transferase- π basal promoter driving the chloramphenicol acetyl transferase gene (see ref. (25)). The results show that sequences essential for transcriptional repression lie between the deletion points of pSVKP4 δ Bst and pSVKP4 δ Apa, that is between residues 297 and 363. C-terminal truncation as far as residue 208 did not affect DNA binding or nuclear localization of E4BP4 as determined by EMSA of E4BP4 DNA binding activity in nuclear extracts from transfected cells (data not shown). The truncated protein encoded by pSVKP4 δ Bst

Figure 1. C-terminal deletion of E4BP4 localizes residues essential to repression between amino acids 298 to 363. Plasmids expressing full-length or truncated E4BP4 (1 μ g) were co-transfected into HepG2 cells with 3 μ g of the CAT reporter plasmid p π S12 (34) CAT. CAT activities were determined as described in Materials and Methods. CAT activities were normalized against the activity obtained with cotransfection of the 'empty' expression vector (pSVK3) and p π S12 (34) CAT. The data shown represent the mean result of at least three experiments. Crossed bars represent the value of one standard deviation from the mean value. The organisation of the reporter plasmid is shown at the bottom of the figure. The large open arrow represents the GST- π promoter sequence and the solid arrows represent specific E4BP4 binding sites.

appears to be slightly impaired in its repressing activity compared to full-length E4BP4 in Figure 1. However the difference is within the standard error of the data and is probably not significant. In several individual experiments, pSVKP4 δ Bst and pSVKP4 were equally active.

E4BP4 contains a transferable repressing domain

To further analyse the requirements for transcriptional repression by E4BP4, plasmid constructs were made which encoded fusion proteins containing the DNA binding domain of the yeast transcriptional activator GAL4 and various fragments of the C-terminal portion of E4BP4 (see Fig.2). The reporter plasmid p π GAL-CAT was used for these experiments. This is similar to p π S12 (34) CAT, but contains a pair of GAL4 binding sequences 5' to the human GST- π promoter (see Materials and Methods and Fig.2). Sequences between residues 297 and 363 were shown to be required for repression in Fig.1. As shown by the transcriptional repressing activity of the GAL-CT2 fusion construct (see Fig. 2), a C-terminal segment of E4BP4 containing these residues is sufficient when expressed as a fusion protein to convert GAL4_{1–147} into a transcriptional repressor. The extreme C-terminal portion of E4BP4 (amino acids 364–462) was neither necessary nor sufficient for repression. Full repressing activity was found to lie in the sequence contained between amino acids 299 and 363 as evidenced by the repressing activity of GAL δ Bst (Fig.2). The apparent small difference in repression efficiency data for GAL-CT2 and GAL-CT3 in Figure 2 is probably not significant, since it was not reproducible between experiments. This data is consistent with the results shown in Figure 1, where the C-terminally truncated protein encoded by pSVKP4- δ BstB (lacking residues 364–462) functioned as a repressor, but that encoded by pSVKP4- δ Apa (lacking residues 297–462) did not. Thus, the repressing activity of E4BP4 lies in the 65 amino acids between positions 299 and 363. Furthermore, this region presumably forms an independent protein folded domain since it retains its repressing activity when

Figure 2. E4BP4 contains a transferable repression domain. Cells were cotransfected with plasmids expressing GAL4_{1–147} or one of the GAL4-E4BP4 fusion proteins indicated on the left of the figure (1 μ g) together with 3 μ g of the reporter plasmid p π GAL-CAT. GAL_{1–147} represents the DNA binding domain of GAL4, contained in residues 1–147 of the protein. GAL-CT2 represents a fusion construct containing amino acids 1–147 of GAL4 joined to residues 299–462 of E4BP4. GAL-CT3, GALCT7 and GAL δ Bst are similarly constructed but contain smaller subregions of the E4BP4 sequence fused to GAL4_{1–147}. Relative CAT activities and the standard deviations of the data are shown as in Fig.1.

Figure 3. E4BP4 contains a novel repression domain. (A) The repression domain of E4BP4 is compared to regions containing the repressing activities of Kr (20,45) En (34) and Ev (19). Charged residues are indicated. A predicted α -helical subdomain of E4BP4 is indicated by lettering above (a-g) representing 28 residues of potential α -helix. (B) 'Helical Wheel' representation of residues 326–353 of E4BP4. Residues 326 to 353 of E4BP4 are displayed down the axis of a schematic α -helix. The most amino-terminal amino acid, His-326 is placed at the 'a' position. Each successive amino acid is depicted as being displaced from the last by approximately 100°. A 28 residue segment is depicted, occupying four turns of the idealized alpha helix ending with Ser-353. The placement of charged amino acids is shown for clarity.

removed from its native environment and fused to a heterologous DNA binding protein such as GAL4_{1–147}, although the presence of the GAL4 DNA-binding region may be important to stabilize the domain.

E4BP4 contains a novel type of repression domain

Transferable repression or silencing domains have been reported for several active transcriptional repressors including the *Drosophila* En, Ev and Kr proteins (19,20,34,45) the Wilms tumour gene product, WT1 (17) and the thyroid hormone receptor (23). The effector domains of these repressors are not homologous at the primary amino acid sequence level, but the *Drosophila* repressors and the WT1 protein all contain an abundance of alanine, proline and to a lesser extent glutamine residues and in addition have a deficit of charged amino acids.

Figure 4. Charged residues in the central part of the E4BP4 repression domain are essential for repressing activity. (A) Sequence of the wild-type E4BP4 repression domain and the position of the two substitutions in the pm1 mutant. (B) Cells were transfected with p_{tr}GAL-CAT and either 1 or 2 μ g of plasmids expressing GAL1_{1–147}, GAL δ Bst or GALpm1 which contains the mutant E4BP4 repression domain fused to residues 1–147 of GAL4. Relative CAT activities are shown as in Figure 1. (C) EMSA of nuclear extract prepared from mock transfected COS-1 cells (lane 1) or COS-1 cells transfected with GAL δ Bst (lane 2), GALpm1 (lane 3) or GAL1_{1–147} (lane 4) plasmids (20 μ g). The probe consisted of an oligonucleotide containing a GAL4 binding site (see Materials and Methods for conditions)

The amino acid sequence of the repression domain of E4BP4 (amino acids 299–363) is shown in Figure 3a, where it is compared with the analogous effector regions of Kr, En and Ev. Notably, the E4BP4 repression domain lacks abundant alanine, proline or glutamine residues and 17 out of 65 (26%) of the amino acids in this segment are charged. E4BP4 is thus clearly distinct from the alanine/glutamine/proline rich *Drosophila* proteins and potentially represents a new type of transcriptional repressor. Amino acid sequence comparison with entries in the SwissProt protein sequence database (release 25) revealed no significant

Figure 5. E4BP4 represses transcription from a minimal promoter. (A) HepG2 cells were cotransfected with increasing amounts of plasmids expressing either GAL4₁₋₁₄₇ (open triangles) or GAL-CT2 (see Fig.2) (open circles) together with the reporter plasmid pG5E4CAT (3µg). Relative CAT activities, normalized against the activity obtained with the reporter alone are shown. (B) GAL-CT2, GAL4₁₋₁₄₇ or full-length GAL4 (GAL4WT) expression plasmids were transfected together with either pG5E4CAT or pG5E1BCAT as indicated.

similarities between residues 299–363 and other known protein sequences.

The distribution of charged residues is not random in the E4BP4 repression domain. At each end of the region there is a segment of approximately zero net charge, while the central 22 amino acid segment consists of an amino terminal part with a net charge of +5 and a carboxyl part with a net charge of –4 (Fig.3). Secondary structure algorithms using the Garnier (43) and GGBSN (42) methods predict an α -helical structure for the bipolar central segment (data not shown). Displaying this probable α -helical region as a helical wheel projection reveals a strikingly non-random arrangement of charged or polar and hydrophobic residues (Fig.3b). For the first three turns of the predicted helical segment positions c and g are occupied exclusively by hydrophobic residues. Similarly, positions b and e are predominantly hydrophobic. Conversely, all amino acids at positions a, d and the first three amino acids at position f are either charged or polar residues. This segment is thus predicted to assume a helical conformation with a strongly hydrophobic face flanked on both sides by charged/polar surfaces. Furthermore, since the first two helical turns in this segment contain positively but not negatively charged residues while the third and fourth turns are predominantly negatively charged, the a, d and f faces of the predicted helical region present a positively charged amino terminal end and negatively charged carboxyl end.

To test the importance of the pattern of charged residues in the E4BP4 repression domain a mutant was constructed in which Lys-330 and Lys-332 were each changed to glutamate residues. As shown in Fig.4 this mutation completely abolished the repression activity of the amino acid 299–363 segment. This mutation did not appear to affect the expression or stability of the fusion protein as judged by EMSA of nuclear extracts prepared from transfected cells (Fig. 4c).

Mechanism of repression by E4BP4

In trying to uncover the mechanism of repression by E4BP4 we have tested its ability to repress minimal promoter constructs.

Transcriptional repressors such as E4BP4 might interact with nearby transcriptional activators and prevent them from stimulating transcription (anti enhancement). Alternatively, a repressor might interact with the general transcription machinery itself to cause transcriptional repression (direct repression). In the latter but not the former case, both basal and activated transcription could be repressed. Thus, addressing whether E4BP4 can repress promoters that lack binding sites for known transcriptional activators provides an important clue as to its mechanism of action.

Cells were cotransfected with a plasmid expressing the GAL-CT2 fusion protein (see Fig.2) together with either pG5E4CAT (40) or pG5E1bCAT (39) reporter plasmids (Fig.5). In these plasmids, the reporter genes are driven by minimal promoters consisting of the adenovirus E4 or E1b TATA box respectively and a series of upstream GAL4 sites. Although the reporter genes are transcribed at a low level in the absence of activator, CAT expression was detectable and quantifiable in the assays described here. As shown in Figure 5, CAT activity from both G5E4CAT and G5E1bCAT was repressed by GAL-CT2. It is unlikely that repression by GAL-CT2 in these experiments is due to steric hindrance of initiation complex formation by promoter-bound GAL-CT2, since wild type GAL4 (see Fig.5b) and a number of other GAL-fusion proteins activate the same reporter constructs and GAL4₁₋₁₄₇ alone is neutral or weakly activating in the same cotransfection experiments (Fig.5). Thus, the repression of G5E1bCAT and G5E4CAT transcription by GAL-CT2 leads to the probable conclusion that E4BP4 represses basal transcription. However the possibility cannot be excluded that the transcription we have observed from the pG5E4CAT and pG5E1bCAT is not really basal transcription but activated transcription resulting from cryptic binding sites for cellular transcription factors in the vector DNA of the reporter plasmid. Nevertheless, this data and evidence that E4BP4 represses different types of promoter (Figure 1, Figure 5) are more consistent with the idea that E4BP4 acts through interactions with the general transcription machinery rather than by inhibiting the action of specific activators. By

Figure 6. E4BP4 does not interact *in vitro* with TFIIB or TBP. (A) Glutathione Sepharose beads loaded with a C-terminal fragment of E4BP4 fused to GST (lane 1) or with GST (lane 2) were incubated with TFIIB translated *in vitro* in the presence of ^{35}S -methionine. After washing, beads were boiled in SDS-PAGE loading buffer to elute any bound proteins. Input protein is shown in lane 3. (B) 'Pull-down' assay as described above but using *in vitro* translated TBP. Lane 1, input protein; lane 2, eluant from GSH Sepharose beads loaded with E4BP4 fusion protein; lane 3, eluant from GSH Sepharose beads loaded with GST. (C) E4BP4 fusion protein loaded beads were incubated with a nuclear extract prepared from HeLa cells grown in the presence of ^{35}S -methionine. Lane 1, Input (1/100th of the amount of extract used in lanes 2 and 3); lane 2, material retained on E4BP4 fusion protein loaded beads; lane 3, material retained on GST-loaded beads. Molecular weights are indicated on the left hand side of the figure. In A, B and C samples were resolved by SDS PAGE and detected by fluorography.

analogy with transcriptional activators (30,46) (47–49) it is possible to imagine a mechanism for active transcriptional repression that involves a physical interaction between the

repressor molecule and general transcription factors such as TFIIB or TFIID. Such an interaction might inhibit one or more steps involved in transcription initiation. To test for any such interaction we used an immobilised E4BP4 C-terminal domain to try and identify nuclear proteins that bind to the E4BP4 *in vitro*. For the purpose of this 'pull-down assay' (49) a glutathione transferase (GST) fusion protein was constructed containing the C-terminal segment of E4BP4 (residues 299–462), fused to the C-terminal of *S.japonicum* GST. The fusion protein, GEX-C-T4, was synthesised in *E.coli* and bound to glutathione Sepharose beads. We investigated the possible interaction between GEX-C-T4 and *in vitro* translated TBP (the DNA-binding subunit of TFIID) and TFIIB (see Materials and Methods). No binding could be detected between GEX-CT4 and TFIIB (Fig.6a) and only a low level of non-specific binding could be detected between GEX-CT4 (or GST alone) and TBP (Fig.6b). Thus, using conditions similar to those used by other workers who have observed specific interactions between TFIIB or TBP and particular transcription factors (30,31,49,50) no specific interaction could be detected between E4BP4 and either TFIIB or TBP. Fusion-protein loaded GSH–Sepharose was also incubated with nuclear extracts prepared from HeLa cells metabolically labelled with ^{35}S -methionine (see Materials and Methods) in an attempt to identify other nuclear proteins with which E4BP4 might interact. A small number of proteins were selectively retained on the GSH–Sepharose loaded with GST-E4BP4 fusion protein (GEX-CT4 beads), while no proteins were detected on GSH–Sepharose loaded with GST alone (GEX2T beads) (Fig.6c). Particularly notable amongst the retained proteins was a doublet of bands at approximately 70 KDa. Other retained bands included a species at approximately 100 KDa and one with an apparent molecular weight of approximately 30 KDa. A series of bands in the range 18 to 30 KDa were also observed in some experiments (data not shown). Some of the retained bands, including the prominent 70 KDa doublet correspond in mobility to abundantly labelled species in the nuclear extract. Determining which, if any, of the retained bands represent proteins with which E4BP4 interacts *in vivo* and are important for its action as a repressor awaits further analysis.

DISCUSSION

In this paper we present evidence for a novel type of transcriptional repressing domain in the bZIP transcription factor E4BP4. As recently described for the *Drosophila* En and Ev proteins, the E4BP4 repressing domain is small (in this case 65 amino acids) and its activity can be transferred to a heterologous DNA binding domain such as GAL4_{1–147}. The repressing domains of Ev (19) and En (34) and the N-terminal repression domain of Kr (20) are rich in alanine, proline and glutamine residues and contain few charged amino acids which has prompted Han and Manley (1993) (19) to suggest that a minimal requirement for a repression domain might be a small, relatively hydrophobic, unstructured region containing few charged residues. This analysis is also largely true for the Wilms tumour gene product, WT1, which contains a transferable repressing domain that is rich in proline and glutamine residues (17). However, the E4BP4 repressing domain has a very different amino acid composition to the *Drosophila* repressors. Although it does contain a number of hydrophobic residues, it is also rich in charged amino acids and contains an unusual central bipolar arrangement of acidic and basic residues that is predicted to have

an α -helical structure. Thus, E4BP4 appears to be distinct from the En/Ev/Kr/WT1 group of transcriptional repressors. Although the repressing domain of E4BP4 appears unique amongst the natural repressors characterized so far, a high content of charged amino acids is also a feature of an artificial repressor encoded in a random segment of *E. coli* genomic DNA described by Saha *et al.* (51). This polypeptide repressed LexA-containing promoters in yeast when fused to the LexA DNA binding domain and is extraordinarily rich in lysine and arginine residues and thus has a large net positive charge. Saha *et al.* went on to show that a synthetic peptide (AK₄)₃KK will also repress transcription in yeast when targeted to a promoter, albeit with a reduced efficiency. Interestingly, the En and Ev repression domains are also rather basic in nature due to a high histidine content and the N-terminal segment of the silencing domain of the v-ErbA oncogene contains an abundance of charged amino acids with a net positive charge (23). The repressing domain of E4BP4 contains many charged residues, but has a net charge of only +3. However, taking just the N-terminal 39 amino acids, the net charge is +5 and this region also contains four histidine residues, further increasing the basic nature of this sub domain. Interestingly, a double point mutant that reduces the net charge of this 37 amino acid segment abolishes the activity of the promoter (Fig 4).

Determining which features of the E4BP4 repressor domain are essential for transcriptional repression awaits further experimental analysis, but it is conceivable that the central segment of this domain, which is predicted to form an amphipathic α -helix, provides an interface for interaction with other transcriptional components such as members of the general transcription factor family or their accessory proteins. We were unable to see any interaction between E4BP4 and TFIIB or TBP. However, E4BP4 may interact with other transcriptional components, which might include *holo*-TFIID or one of its accessory factors. Alternatively, the data presented here is not inconsistent with a chromatin mediated mechanism for transcriptional repression whereby E4BP4 could inhibit transcription through nucleosome positioning effects or by encouraging incorporation of histone H1 into the vicinity of the promoter. That the mutation Lys-300, Lys-332 → Glu abolishes repression suggests that the precise arrangement of the charged residues is important for activity.

We have reported here a novel type of transcriptional repression domain in the human transcription factor E4BP4. It appears that active repressors, like transcriptional activators, fall into more than one structural and possibly functional class. We are currently trying to identify proteins with which E4BP4 interacts in order to determine the mechanism of repression by this protein.

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