A C-terminal domain in FosB, absent in FosB/SF and Fra-1, which is able to interact with the TATA binding protein, is required for altered cell growth

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Transcriptional regulation in eukaryotes is thought to occur through interactions between specific transcription factors and the general transcription machinery. We show that the regulatory protein FosB, but not FosB/SF or Fra-1, specifically and stably associates with the TATA box binding protein (TBP) and the multiprotein complex TFIID. The binding to TBP is specified by the last 55 C-terminal amino acids of FosB, requiring a small amino acid sequence, termed the 'TBP binding motif' (TBM). Deletion of the TBM affects transcriptional activity slightly, but it is adjacent to a proline-rich sequence which constitutes the major transactivation domain. However, both regions are required for the transformation of Rat-1A cells by FosB. Transfection experiments demonstrate that inhibition of transactivation due to excess levels of Gal4–FosB (squelching) can be partially relieved by the co-expression of TBP, which establishes that TFIID is a functional target of FosB. Since TBP binding is not exhibited by FosB/SF or Fra-1, we suggest that the activity mediated by the TBP interaction is one differentiating characteristic that distinguishes the FosB functions from those of FosB/SF and Fra-1.

Key words: FosB/TFIID/transcription regulation

Introduction

The transcription factor AP-1, which is composed of Fos and Jun family members, was originally discovered in mammalian cells as an activity that binds specifically to enhancers of the simian virus 40 and other vertebrate promoters that contain an AP-1 DNA binding site (Angel et al., 1987; Lee et al., 1987). A common structure of the Fos and Jun proteins is a highly conserved DNA binding and dimerization region, collectively termed a 'bzip' domain (Kouzarides and Ziff, 1988; Landschultz et al., 1988). The Jun proteins form dimers with each other and bind with varying affinities to AP-1 sites (Ryseck and Bravo, 1991). However, the Fos proteins (c-Fos, FosB, Fra-1 and Fra-2) are thought to modulate AP-1 activity, since they cannot dimerize among themselves but can with Jun proteins, significantly enhancing their DNA binding and transcriptional activity (reviewed in Curran

and Franza, 1988; Abate and Curran, 1990; Vogt and Bos, 1990; Angel and Karin, 1991). Interestingly, all of the *fos* gene family members are rapidly induced in quiescent fibroblasts following serum and growth factor stimulation; c-Fos and FosB are the earliest to be expressed, peaking at 1 h after stimulation, while Fra-1 and Fra-2 appear after 2 h and are maintained throughout the cell cycle (Kovary and Bravo, 1992). These data suggest that individual Fos proteins provide unique functions in the cell depending upon when they are expressed in the cell cycle (Bravo, 1990). Convincing evidence that Fos proteins play an important role in cell growth is the observation that inhibiting the activity of Fos family members, either by antisense expression or the microinjection of specific antibodies, blocks the induction of cell proliferation and cell cycle progression (Holt et al., 1986; Riabowol et al., 1988; Kovary and Bravo, 1991b, 1992).

It is unclear how the Fos molecules differ in their function. The Fos family members share intermittent regions of similarity, suggesting that they may show certain related properties. Conversely, there are non-overlapping activities associated with regions of the molecules which are not conserved between family members. For instance, AP-1 complexes containing c-Fos or FosB are potent activators of transcription which are able to transform certain established cell lines, while those containing Fra-2 or Fra-1 act, in certain cases, as repressors and fail to transform (Schutte et al., 1989; Angel and Karin, 1991; Suzuki et al., 1992). The *fosB* gene (Zerial et al., 1989; Lazo et al., 1992) encodes two functionally distinct molecules generated by alternative splicing, a 338 amino acid molecule, FosB, and a variant lacking 101 C-terminal amino acids, FosB/SF (Dobrzanski et al., 1991; Mumberg et al., 1991; Nakabeppu and Nathans, 1991; Yen et al., 1991).

Despite the compelling nature of these data, little is known about the mechanisms by which the Fos molecules exert their varying effects. It is generally believed that transcription is modulated by the dynamic interactions of regulatory factors, like AP-1, and the general transcription factors (GTFs) (Ptashne, 1988; Mitchell and Tjian, 1989). In TATA-containing genes, transcription initiation by RNA polymerase II (PolII) occurs following an ordered assembly of GTFs to form a multiprotein pre-initiation complex (PIC) at the transcriptional start site (Buratowski et al., 1989; Maldonado et al., 1990; Weinmann, 1992; Zawel and Reinberg, 1992; Hernandez, 1993). Activators, at least in part, work by increasing the rate of PIC assembly (Choy and Green, 1993). The formation of the PIC first requires the binding of the general transcription factor TFIID to the TATA box element (Davison et al., 1983; Buratowski et al., 1989). TFIID, which is a multiprotein complex composed of the TATA binding protein (TBP) and at least eight TBP-associated factors (TAFs), appears

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to be a target for both positive and negative effector molecules, some of which interact directly with the TBP subunit, like the viral transactivators VP16 (Stringler *et al.*, 1990; Lin *et al.*, 1991), E1a (Horikoshi *et al.*, 1991; Lee *et al.*, 1991), Zta (Liebermann and Berk, 1991; Liu *et al.*, 1993) and Tax1 (Caron *et al.*, 1993) and, more recently, the cellular transcription factors c-Rel (Kerr *et al.*, 1993; Xu *et al.*, 1993), p53 (Ragimov *et al.*, 1993), c-Myc (Hateboer *et al.*, 1993) and E2F (Hagemeir *et al.*, 1993a). Other activators function by contacting TAFs or other GTFs. For instance, the transcription factor SP1 functions by contacting TAF110 of the *Drosophila* TFIID complex (Hoey *et al.*, 1993).

We have been interested in understanding which are the mechanistic differences that contribute to certain discriminating activities observed with different Fos molecules. The objective of this study was to determine whether there is a causal relationship between the properties of FosB, FosB/SF and Fra-1 and functional interactions with the general transcription factor TFIID. In this report we present *in vitro* and *in vivo* data demonstrating that FosB contains two independent domains, located in the C-terminal 95 amino acids, which are required for full transcriptional activity and the efficient transformation of Rat-1A cells. One region (the TBP binding motif, TBM) located in the last 55 C-terminal amino acids interacts with the direct repeat of TBP, is not conserved in Fra-1 and shares sequence similarities with the TBP interacting region of E1a. The second region, rich in prolines, is the major activation domain and does not interact with TBP, but may function through a TBP-independent interaction with the TFIID complex. The functional significance of FosB−TBP contacts was demonstrated by transfection experiments, which show that inhibition (squelching) of transactivation by an excess of FosB can be partially relieved by the co-expression of TBP. Taken together, these data suggest that the activity mediated by TBP interactions may be one discriminating characteristic that distinguishes the transforming FosB molecule from FosB/SF and Fra-1 molecules.

Results

FosB, but not FosB/SF or Fra-1, can interact with TBP

Activated transcription requires the holo-TFIID complex, of which TBP is the major functional component, that interacts with the TATA element (reviewed in Pugh and Tjian, 1992; Hernandez, 1993). To investigate whether FosB, FosB/SF or Fra-1 could differentially interact with TBP, the respective proteins were ^{35}S-labelled by *in vitro* translation and incubated with the non-conserved N-terminal (GST−nTBP, amino acids 1−163) or conserved C-terminal (GST−cTBP, amino acids 168−339) regions of TBP, which were produced in bacteria as GST fusion proteins. Association of labelled proteins with TBP was assayed by their retention on glutathione−Sepharose beads containing the GST−TBP protein. Interaction of FosB with the C- but not N-terminal portion of TBP was easily detectable (Figure 1A). Since retention was observed in the presence of a large amount of BSA and was maintained through a buffer wash containing a high salt concentration (500 mM KCl), we concluded that the binding was specific

and stable. In contrast, FosB/SF and Fra-1 did not associate with any portion of TBP (Figure 1A). The binding of FosB was resistant to the presence of 100 µg/ml ethidium bromide, which was used as an indicator of DNA-independent protein association. Pull-down assays using the complete TBP molecule fused to GST gave the same results (data not shown).

To determine the region of TBP required for the interaction with FosB, various fragments of TBP, produced as GST fusion proteins, were tested in a similar assay. Figure 1B demonstrates that FosB interacts strongly with C-terminal amino acids 168−339 of TBP (lane 2). No interaction is seen with GST fusion proteins containing amino acids 168−202 and 272−339 (lanes 4 and 5 respectively), while retention of FosB can be detected with TBP residues 202−272 and 202−339 (lanes 3 and 7 respectively). Taken together, these data demonstrate that FosB interacts with the fragment of TBP containing amino acids 202−272 in a weaker manner than the larger 168−272 fragment. This may be due to the GST polypeptide interfering with the residues of TBP interacting with FosB. Amino acids 202−272 of TBP contain the basic repeat structure positioned on the surface of the molecule, as predicted from the crystal structure (Nikolov *et al.*, 1992; Kim *et al.*, 1993a,b), the same area of TBP which contacts TFIIA (Buratowski and Zhou, 1992) and the adenovirus transcriptional activator E1a (Lee *et al.*, 1991).

The differential interactions of FosB and FosB/SF with TBP can be attributed to the C-terminal region of FosB, which is absent in FosB/SF. To further delineate which region(s) is important for the TBP binding activity, we performed a pull-down assay on two deletion mutants of FosB. Deletion of amino acids 243−282 (FosBΔ243−282) had little effect on TBP binding, whereas TBP interaction with FosBΔ283−310 was completely abolished (Figure 1C, lanes 2 and 3). Examination of this sequence revealed a short amino acid sequence, FVLTCPE, which is similar to a region of the adenovirus E1a protein (Figure 1C). This area of E1a was previously demonstrated as necessary for E1a−TBP interactions (Lee *et al.*, 1991). The deletion of these amino acids in FosB (Δ304−310) abolished any detectable interaction with TBP (Figure 1C, lane 4), suggesting that these residues may be functionally related to those in E1a. The importance of this region in TBP binding was demonstrated further by point mutants FV-RR and EV-RR, which demonstrate significantly decreased TBP binding activity (Figure 1C, lanes 5 and 8). Mutants containing changes in amino acids flanking these seven residues show varying abilities to interact with TBP. The binding of mutant TSS-RRR was significantly decreased, while TSS-EDD and AG-RR have little or no effect on TBP binding when compared with the wild-type molecule (Figure 1C, lanes 6, 7 and 9 respectively). To demonstrate that the C-terminal part of FosB is sufficient to confer TBP binding activity, Fra-1/FosB chimeric molecules were constructed where the C-terminal 67 amino acids of Fra-1 were replaced with the 55 C-terminal residues of FosB (Fra/B). The Fra/B chimera was able to bind TBP, while the same chimera carrying a seven amino acid deletion (Fra/BΔ304−310) failed to associate with TBP (Figure 1C, lanes 11 and 12 respectively). These data define a 55 amino acid region of FosB that is sufficient to confer TBP binding to Fra-1. Moreover, they confirm that the region
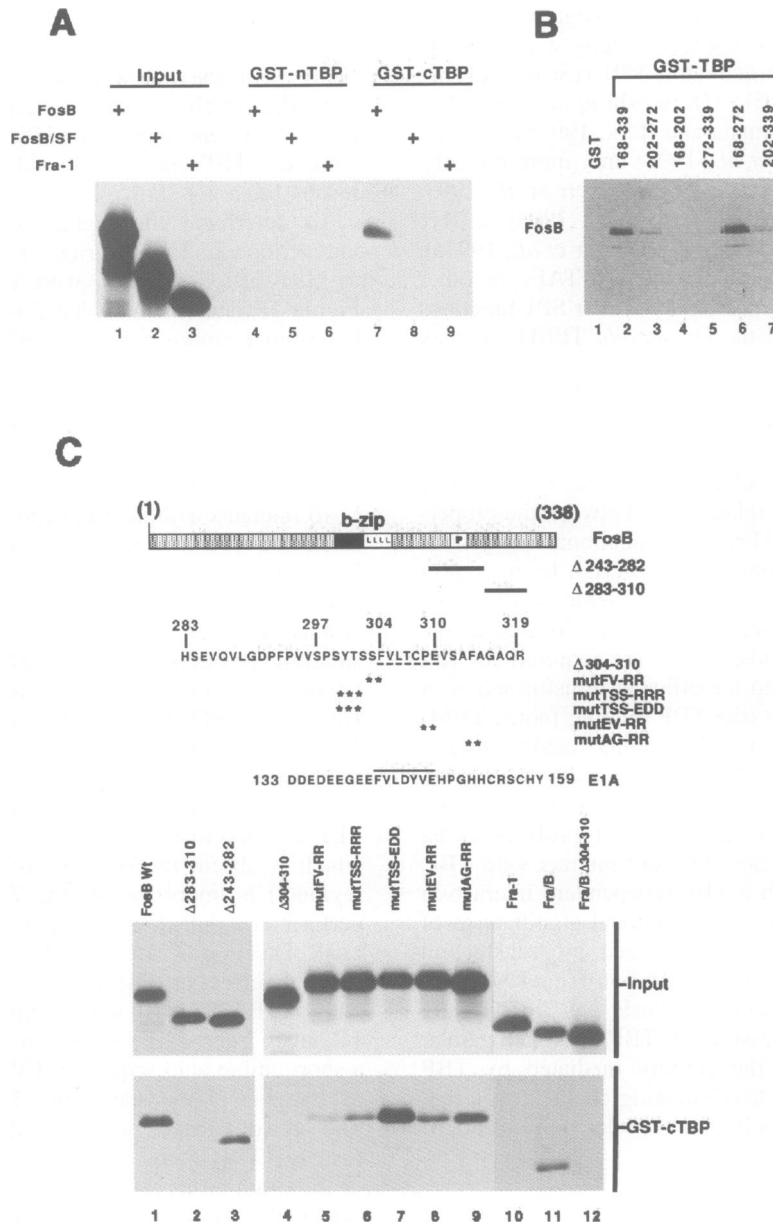
Fig. 1. FosB, but not FosB/SF or Fra-1, interacts *in vitro* with TBP. (**A**) *In vitro*-translated and ^{35}S-labelled FosB, FosB/SF and Fra-1 (Input, lanes 1–3) were incubated with GST−nTBP containing TBP residues 1–163 (lanes 4–6) or GST−cTBP containing residues 168–339 (lanes 7–9), and subjected to a GST pull-down assay with glutathione−Sepharose beads. (**B**) The indicated regions of TBP, expressed as GST fusions, were assayed for binding to *in vitro*-translated, ^{35}S-labelled FosB made in rabbit reticulocyte lysate. (**C**) The wild-type and mutant FosB molecules are diagrammatically represented. The sequence similarity between the C-terminus of FosB and E1a is indicated. The top panel (Input) indicates the *in vitro*-translated proteins added to the pull-down assay. The lower panel shows material retained by GST−cTBP. FosB, deletion mutants FosBΔ283–310 and FosBΔ243–282 (lanes 1–3), various double point mutants (lanes 4–9) and Fra-1 and Fra-1/FosB chimeras (lanes 10–12) are indicated.

comprising amino acids 304–310 of FosB, which, for simplicity, we refer to as the 'TBP binding motif' or TBM, is required for TBP interactions.

FosB can interact directly with the TFIID complex

Cellular TBP is a component of the multiprotein complex TFIID. Therefore, an important question arising from our data is whether TBP can interact simultaneously with the TAFs and the TBM of FosB. This problem is compounded by the fact that all of the TAFs, GTFs and known interacting activators require only the C-terminal 180 amino acids of TBP (Zhou *et al.*, 1993). Thus, it was

important to determine whether FosB could still interact with TBP present in the holo-TFIID complex. To this end, the polymerase II (PolII)-specific TFIID, partially purified by phosphocellulose (PC0.7) fractionation of HeLa nuclear extracts (see Materials and methods), was incubated with a glutathione−Sepharose matrix containing bacterially expressed GST−FosB, −FosB/SF, −Fra-1 or GST alone. The retention of TFIID by the GST fusion proteins was determined by Western blot analysis using an affinity-purified antibody directed against the N-terminal 58 amino acids of human TBP. In Figure 2A, a 43 kDa polypeptide that co-migrates with *in vitro*-translated human
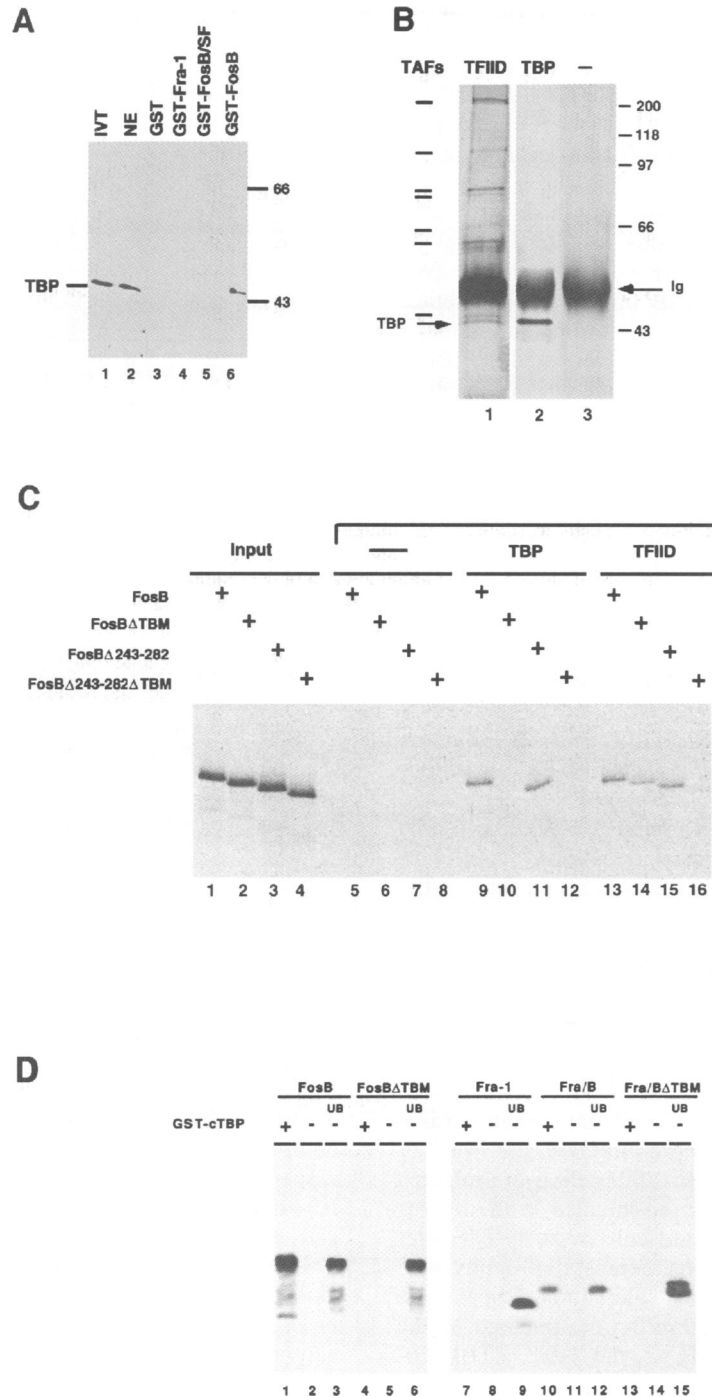
Fig. 2. FosB interacts with cellular TBP and TFIID. (**A**) HeLa nuclear extracts were mixed with glutathione−Sepharose beads containing GST, GST−FosB, GST−FosB/SF or GST−Fra-1. Material bound by the beads, following several washes, was analysed by SDS−PAGE and assayed for the presence of TBP by Western blot. Affinity-purified anti-TBP antibody recognizes the 43 kDa TBP polypeptide in rabbit reticulocyte lysates containing *in vitro*-translated (IVT) human TBP (lane 1), crude HeLa nuclear extracts (NE, lane 2) and material retained by GST−FosB (lane 6), but not in material retained by GST alone (lane 3), GST−Fra-1 (lane 4) or GST−FosB/SF (lane 5). (**B**) Silver-stained SDS−polyacrylamide gel (12.5%) of immune-purified TFIID (TBP plus TAFs, lane 1), TBP (lane 2) and anti-TBP antibody alone (lane 3). The arrows indicate the position of TBP and the immunoglobin heavy chain. (**C**) Protein A−Sepharose containing anti-TBP antibody (500 ng) alone (lanes 5−8) or complexed with cellular TBP (lanes 9−12) or TFIID (lanes 13−16) (prepared from HeLa nuclear extracts as described in Materials and methods) was incubated with radiolabelled FosB (lanes 1, 5, 9 and 13), FosBΔTBM (lanes 2, 6, 10 and 14), FosBΔ243-282 (lanes 3, 7, 11 and 15) or FosBΔ243-282ΔTBM (lanes 4, 8, 12 and 16) made in reticulocyte lysate. Input (lanes 1−4) shows the amounts of FosB molecules that were added to each pull-down assay. (**D**) Recombinant baculoviruses expressing FosB, FosBΔTBM, Fra-1, Fra/B (Fra-1 amino acids 1−204 and FosB243−338) or Fra/BΔTBM (Fra-1 amino acids 1−204 and FosB243−338DTBM) were used to infect Sf9 cells alone (labelled −, lanes 2, 3, 5, 6, 8, 9, 11, 12, 14 and 15) or as a co-infection with recombinant baculovirus expressing GST−cTBP (amino acids 168−339) (labelled +, lanes 1, 4, 7, 10 and 13). Extracts from infected cells were mixed with glutathione−Sepharose beads. Unbound (UB) material from infections without the GST−cTBP-expressing virus and material retained by the glutathione−Sepharose beads were separated by SDS−PAGE, Western blotted and probed with an anti-Fos family antibody (lane 15).

TBP is present in HeLa nuclear extracts (lanes 1 and 2 respectively) and can be detected in material retained by GST—FosB (lane 6). In contrast, TBP was not detected in material retained by GST alone, GST—Fra-1 or GST—FosB/SF (lanes 3–5). In addition, the TBP retained by GST—FosB was active for PolII transcription in an *in vitro* assay (data not shown).

Despite these data, we could not rule out the TFIID—FosB interaction being due to intermediary or 'bridging' factors present in the nuclear extracts. We therefore asked whether the FosB protein could interact with holo-TFIID which was immune-purified and immobilized on protein A—Sepharose beads. We used the phosphocellulose (PC0.7) fraction as a source of PolII-specific TFIID. For a positive control, immobilized TFIID was stripped of associated TAF molecules so that only cellular TBP remained (Figure 2B). TBP was represented in each sample at approximately the same amount. *In vitro*-translated ^{35}S-labelled FosB, FosBΔTBM, FosBΔ243–282 and FosBΔ243–282ΔTBM were added to the immune-purified TFIID or cellular TBP, washed extensively and analysed for retention by SDS—PAGE (Figure 2C). This interaction was not due to the anti-TBP antibody recognizing FosB, since the antibody bound to protein A—Sepharose alone did not retain any of the FosB molecules (lanes 5–8). Both the TFIID and TBP immune complexes retained FosB and FosBΔ243–282 to similar extents. Surprisingly, the TFIID complex also retained significant amounts of FosBΔTBM, but little, if any, FosBΔ243–282ΔTBM. In contrast, the interaction of FosB with cellular TBP was dependent on the presence of the TBM. These data demonstrate that TBP, when complexed with TAFs, is accessible to FosB via the TBM. Furthermore, they suggest that there is an additional TFIID interacting region (FosB amino acids 243–282) that may require the tightly associated factors (TAFs) present in the TFIID complex.

To investigate whether FosB can also associate with TBP *in vivo*, we constructed recombinant baculoviruses expressing GST—cTBP, FosB, FosBΔTBM and Fra-1 or chimeras Fra/B and Fra/BΔTBM. Sf9 cells were infected with each virus separately or co-infected with a virus expressing GST—cTBP. Infected cells were lysed under mild detergent conditions and the expressed proteins were assayed for their retention on glutathione—Sepharose beads (Figure 2D). FosB and Fra/B were retained by the matrix (lanes 1 and 10) only in the GST—cTBP co-infected cells. None of the FosB proteins were retained in the absence of GST—cTBP (lanes 2, 5, 8, 11 and 14). FosBΔTBM, Fra-1 and Fra/FosBΔTBM, which fail to bind TBP *in vitro*, were also not retained by the glutathione—Sepharose beads following co-infection with GST—cTBP (lanes 4, 7 and 13). All of the proteins were detected in the unbound material (UB, lanes 3, 6, 9, 12 and 15). These data demonstrate that FosB is able to interact with the C-terminal region of TBP in the cell.

The TBP binding motif plays a role in transactivation

The TBP binding regions of E1a and VP16 function to promote transcription (Ingles *et al.*, 1991; Lee *et al.*, 1991). In order to determine whether the TBP binding motif of FosB serves as an activation domain, we per-
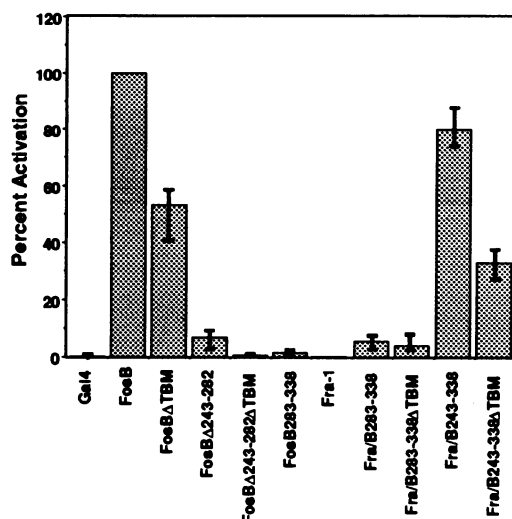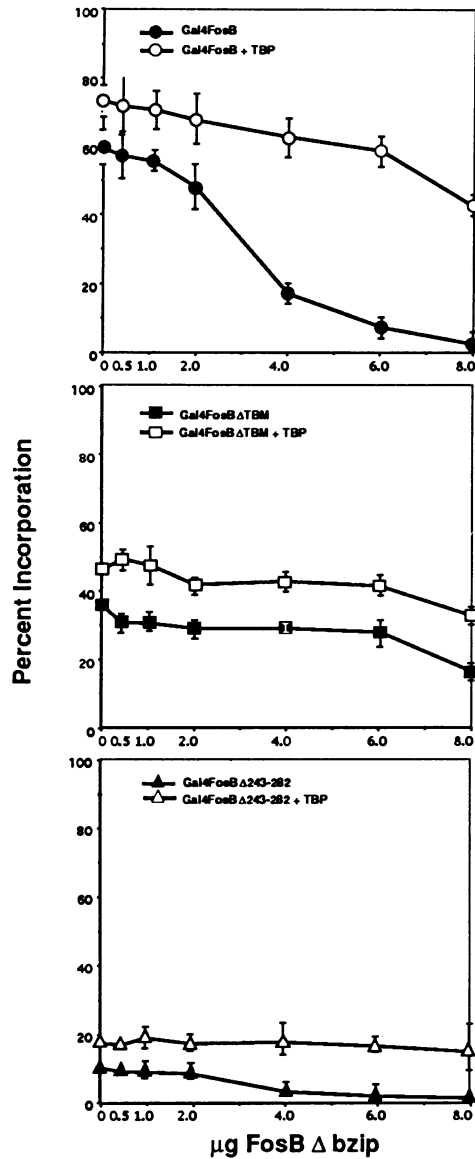

Fig. 3. FosB contains two activation regions, a proline-rich region and the TBM. A target reporter vector (pBLG$_1$TCAT) containing a single Gal4 binding site and TATA box sequence from the MLP of adenovirus was transfected into NIH3T3 cells along with 1 μg plasmid expressing Gal4, Gal4—FosB, Gal4—FosBΔTBM, FosBΔ243–282, FosBΔ243–282ΔTBM, Fra-1, Fra/B243–338, Fra/B283–338, Fra/B243–338ΔTBM or Fra/B283–338ΔTBM. The CAT activity was calculated as per cent conversion. Typically, FosB gave 60–70% conversion, which was arbitrarily assigned as 100% activation and represented a 70-fold increase in CAT activity over activity obtained from Gal4 alone, under the conditions used. Activities from the other transfections were recorded as the percentage of activity with respect to FosB.
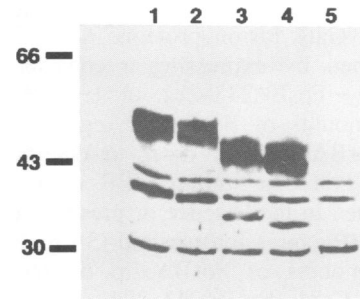
formed transient transfection experiments. The transactivation properties of the various proteins were assayed directly as fusions with the DNA binding domain (amino acids 1–147) of Gal4 and monitored by a reporter plasmid containing a single Gal4 DNA recognition site upstream from the adenovirus major late promoter TATA element and the CAT gene. In addition, to eliminate the possible aberrant effects resulting from Gal4 fusions forming complexes with endogenous Jun proteins, the leucine zippers of FosB and Fra-1 were mutated by changing leucines 197 of FosB and 147 of Fra-1 to prolines. The activity was adjusted as a percentage of the activation observed with the wild-type Gal4—FosB, arbitrarily assigned as 100%. Similar to previous reports (Dobrzanski *et al.*, 1991; Wisdom *et al.*, 1992), FosB is a potent activator of transcription, which was typically 50- to 100-fold higher than the activity shown by the Gal4 DNA binding domain alone (Gal4, Figure 3). Deletion of the TBM results in an average 60% reduction in transcriptional activation, suggesting that the TBM provides a modest activation function for the FosB molecule. However, a greater reduction in activity is observed with the deletion FosBΔ243–282, which provides only 5% of the wild-type activity. FosBΔ243–282 possesses some activity, 3- to 4-fold above that observed with Gal4 alone, which is further reduced by the TBM deletion FosBΔ243–282ΔTBM. The Gal4 fusion containing only the C-terminal 55 amino acids (including the TBM) of FosB activates weakly, which is nevertheless 3- to 4-fold greater than the negative control. Taken together, these data provide evidence that FosB contains two independent domains which contribute to activation—the TBM and a proline-rich region defined by

Fig. 5. FosB, FosBΔTBM, FosBΔ243–282 and FosBΔ243–282ΔTBM are all expressed at similar levels in Neo-resistant stable cells lines. Protein (20 μg) from whole cells lysates from stable neo-resistant Rat-1A cells transfected with pMexneo-FosB, -FosBΔTBM, -FosBΔ243–282, -FosBΔ243–282ΔTBM or pMexneo alone (lanes 1–5 respectively) were analysed by Western blot analysis using an affinity purified anti-FosB antibody.

Fig. 4. The TBP protein relieves the inability of FosB to activate at high concentrations. An increasing amount of pMexneoFosBΔbzip was transfected into NIH3T3 cells along with a constant amount of a target promoter containing a single Gal4 binding site and the TATA box sequence from the MLP of adenovirus and 1 μg plasmid expressing either Gal4−FosB, −FosBΔTBM or −FosBΔTBM. Transfections were carried out in the absence or presence (3 μg) of a TBP expression vector. The CAT activity represents percentage incorporation of ^{14}C into chloramphenicol.

chimera containing the C-terminal 55 amino acids of FosB (including the TBM), Fra/B283–338, provides only a marginal 5-fold increase in activation over that of Gal4−Fra-1. However, the Gal4−Fra-1/FosB chimera containing both activation domains in the C-terminal 95 amino acids of FosB, Fra/B243–338, possesses the highest activity, which is nearly equivalent to levels obtained with Gal4−FosB. The deletion of the TBM again causes a >50% reduction in activity. As in the FosB molecule, the sum of the activities provided by the two activation domains separately is less than the activity seen with the combined domains. This leads us to conclude that the TBM cooperates with the proline-rich activation domain and that this effect is independent of the N-terminal residues (amino acids 1–242) of FosB.

The observed differences in activity between FosB, FosBΔ243–282 and FosBΔTBM are not due to variations in their expression levels, since these proteins are expressed to similar extents as Gal4 fusions (data not shown) or when expressed in stable cells lines in the absence of Gal4 (Figure 5).

residues 243–282. The activity of the complete FosB molecule is greater than the sum of the activities observed from FosBΔ243–282 and FosBΔTBM, suggesting that both domains cooperate in activating transcription.

In order to determine whether the proline-rich and TBM regions of FosB can function when bound to a heterologous protein such as Fra-1, we constructed expression plasmids that express a chimeric Gal4−Fra-1/FosB polypeptide by replacing the C-terminal 67 amino acids of Fra-1 with different regions of wild-type FosB or mutant FosBΔTBM (Figure 3). Gal4−Fra-1 has low transcriptional activation properties on its own, consistent with previous data (Wisdom and Verma, 1993). The Gal4−Fra-1/FosB

Functional correlation between TBP binding and levels of TBP in the cell

It has been demonstrated previously for several transcriptional activators that over-expression in tissue culture cells often leads to an inhibition of transcription. This phenomenon, termed squelching, is believed to be a consequence of the titration of cellular target factors by an excess of exogenously added activator (Ptashne, 1988). Since TBP is thought to be limiting for PolII transcription (Colgan and Manly, 1992), the functional significance of the TBP interaction in vitro could be assessed in vivo by performing squelching experiments where increasing levels of TBP relieve the inhibiting effects caused by over-expressing the TBP-interacting molecule. In fact, these types of experiment have been recently used to show that TBP is the functional target for E1a (Boyer and Berk, 1993), Tax1 (Caron et al., 1993) and c-Rel (Kerr et al., 1993; Xu et al., 1993). Therefore, similar experiments were carried out. Increasing the levels of Gal4− FosB resulted in diminished activity (squelching) that could be relieved by the co-expression of TBP (data not shown). In order to confirm that the squelching was due to a region

on FosB and not due to an artifact of expressing high levels of the Gal4 fusion proteins, a squelching curve was determined by expressing a constant amount of Gal4−FosB, −FosBΔ243−282 or −FosBΔTBM with increasing amounts of FosB carrying a leucine zipper mutation (FosBΔbzip). In order to determine whether inhibition was due to limiting TBP, these transfections were performed in the absence or presence of a constant amount of TBP expression plasmid (3 μg). The effect of increasing amounts of FosBΔbzip on the activity of Gal4−FosB, Gal4−FosBΔ243−282 and Gal4−FosBΔ-TBM on transactivation was evaluated by co-transfection studies. The stimulatory effect of Gal4−FosB and Gal4−FosBΔ243−282 begins to be inhibited by FosBΔbzip at 2–4 μg (Figure 4). This inhibition can be overcome by the co-expression of TBP. Conversely, FosBΔTBM is inhibited by FosBΔbzip at a much higher DNA concentration, which is not affected by TBP expression. Even though the overall levels of activity are higher in the presence of TBP, the inhibition curve for FosBΔbzip DNA is unchanged for FosBΔTBM. These data suggest that the squelching observed in the absence of exogenous TBP is due to the limiting amount of TBP or TFIID and that TBP is a functional target for FosB. Inhibition at higher amounts of FosBΔbzip DNA (6–8 μg) is observed with Gal4−FosB and Gal4−FosBΔ243−282 constructs, but this inhibition is not affected by TBP expression, suggesting that an additional component(s) may become limiting, which does not depend on the expression of TBP.

Both the proline-rich and TBM regions of FosB are required for transformation

It has been previously reported that in Rat-1A cells the constitutive expression of FosB efficiently transformed the cells, as determined by their capacity to form foci and grow well under low serum conditions, whereas FosB/SF did not (Kovary et al., 1991; Mumberg et al., 1991; Wisdom et al., 1992). To determine whether transformation by FosB is dependent on the proline-rich and/or TBM regions, the immortalized rat fibroblast cell line Rat-1A was transfected with FosB, FosBΔTBM, FosBΔ243−282 or FosBΔ243−282ΔTBM. Each protein contained a wild-type bzip domain which allows for the dimerization with endogenous Jun partners. Since extreme levels of expression may affect transactivation, as predicted from our squelching experiments, we performed several transfections using different amounts of various expression vectors. The number of foci were scored 14 days after transfection. Consistently, FosB yielded far greater numbers of foci at every DNA concentration than the corresponding FosBΔTBM, FosBΔ243−282 and FosBΔ243−282ΔTBM DNAs (see Table I). Interestingly, FosBΔ243−282 and FosBΔTBM resulted in similar numbers of foci, even though they differ somewhat in their ability to transactivate (Figure 3). The difference in transformation is not due to differences in steady-state levels of the proteins, since stable neo-resistant cell lines express the various proteins at the same levels (Figure 5). The lack of correlation between transcriptional activity and transformation for various AP-1 components has been noted before (Lucibello et al., 1991, reviewed in Angel and Karin, 1991). Nevertheless, our data demonstrate that efficient transformation by FosB requires both the TBM

Table I. Both the proline-rich and the TBM regions of FosB are required for transformation

DNA (μg)	Number of foci 1	3	5	15
FosB	153	260	382	>500
FosBΔTBM	0	15	34	84
FosBΔ243−282	0	35	60	91
FosBΔ243−282ΔTBM	0	22	52	72
pMexneo	0	0	0	0

Transfections were performed in duplicate on 2×10^5 Rat-1A cells with the indicated expression plasmids (the DNA was adjusted to 15 μg with pMexneo). pMexneo alone and various amounts of pMexneo plasmid containing the coding regions of FosB, FosBΔTBM, FosBΔ243−282 or FosBΔ243−282ΔTBM were assayed. The number of foci observed were scored 14 days following transfection. In separate experiments, clones selected with neomycin were pooled and examined for protein expression by Western assay (data not shown).

and proline-rich regions, each of which may function through different mechanisms.

Discussion

The Fos family includes c-Fos, FosB, FosB/SF, Fra-1 and Fra-2. Each displays varying activities with regard to their spatial and temporal patterns of expression, AP-1-dependent gene regulation and cellular transformation. The discernible differences in the transactivation and transformation properties among Fos proteins are generally believed to be due to different functional domains which predicate a particular activity. FosB and FosB/SF are products derived from the same gene through alternatively spliced mRNAs. Each is functionally distinct from the other concerning their transactivating and transformation properties (Dobrzanski et al., 1991; Wisdom et al., 1992; Wisdom and Verma, 1993). Fra-1 possesses little ability to transactivate or transform established cell lines. Since FosB, FosB/SF and Fra-1 are able to form dimers with Jun molecules and bind TRE sequences with similar or identical affinities, it is reasonable to assume that the cellular protein targets, through which they function, differ. A common target for several transcriptional activators is the multiprotein complex holo-TFIID (Boyer and Berk, 1993). Therefore, we explored whether the interaction with holo-TFIID may be one characteristic that differentiates the functional properties of FosB, FosB/SF and Fra-1. In this report we present evidence that the C-terminal region of FosB contains two separable domains. One region (TBM) interacts with the TBP directly and provides an auxiliary role in transcriptional activation. The other is rich in proline residues and constitutes the major activating region of FosB and may function by interacting with holo-TFIID. Finally, the transformation of Rat-1A cells requires both regions of FosB.

A variety of biochemical experiments, similar to those previously used to demonstrate protein−protein interactions in vitro (Lee et al., 1991; Boyer and Berk, 1993; Hagemeir et al., 1993a,b; Kerr et al., 1993; Xu et al., 1993), were employed to explore whether FosB, FosB/SF and/or Fra-1 interact with the major subunit of TFIID, TBP. Using a GST pull-down assay, we show that FosB can associate with TBP. This interaction was specific for

the C-terminal region of FosB, since FosB/SF did not associate with the TBP molecule. Furthermore, the FosB–TBP complex was stable, since it was resistant under stringent washing conditions (0.5 M KCl and 0.5% NP40), and was independent of DNA intermediates, since it was also observed at high concentrations of the DNA intercalating agent, ethidium bromide (100 μg/ml). Fra-1 failed to interact with TBP in this assay, suggesting further that the interaction we observed with FosB was due to a unique structure present in the molecule. The examination of a number of FosB mutations revealed the requirement of a seven amino acid region, which we called the 'TBP binding motif' (TBM), since it showed some sequence homology with a region in E1a required for TBP association. The requirement for the TBM was supported by in vivo experiments where FosB–TBP interactions were observed in Sf9 cells co-infected with FosB and GST–cTBP-expressing baculoviruses. These complexes displayed the same specificity and stability as those observed in vitro. The C-terminal 55 amino acids of FosB, containing the TBM, are sufficient for in vitro and in vivo interactions with TBP, since this region was able to confer TBP binding activity to a Fra-1/FosB chimera in a manner that was dependent on the TBM.

One of the conceptual problems arising from our current thinking on transcriptional activation is understanding how the C-terminal 180 amino acids of TBP can interact simultaneously with so many polypeptides, including TAFs, other GTF molecules, such as TFIIA and TFIIB, and FosB. The three-dimensional structure of the TBP conserved core (Nikolov et al., 1992; Kim et al., 1993a,b) predicts that the DNA-bound TBP molecule has a considerabe surface area, which could accommodate interactions with several proteins. Two of the eight known TAF molecules (TAF250 and TAF125) contact TBP directly (Takada et al., 1992; Zhou et al., 1993). We provide evidence that FosB interacts with holo-TFIID based on the following two criteria. First, in affinity chromatography experiments holo-TFIID, present in the phosphocellulose (PC0.7) fraction of HeLa nuclear extracts, was specifically retained by the matrix containing FosB, but not by the FosB/SF support. Moreover, the holo-TFIID retained by the FosB matrix was able to support PolII transcription in vitro using a TFIID-depleted HeLa nuclear extract (data not shown). Second, holo-TFIID, immune-purified from the PC0.7 fraction and immobilized on protein A–Sepharose, was able to specifically and stably bind in vitro-translated FosB. Several viral and cellular proteins have been demonstrated to interact with TBP in vitro (Stringler et al., 1990; Horikoshi et al., 1991; Lee et al., 1991; Liebermann and Berk, 1991; Lin et al., 1991; Inostroza et al., 1992; Caron et al., 1993; Kerr et al., 1993; Liu et al., 1993; Xu et al., 1993). The direct association of FosB with the holo-TFIID complex implies that the interaction may provide an important function in regulating transcription.

In addition to these data, we detected a FosB–TFIID interaction which is independent of the TBM but requires the amino acid region 243–282. This finding is not unreasonable, since it is possible that this region contacts one of the TAF molecules independently of the TBM–TBP association. This notion is supported by the fact that when both the amino acid 243–282 and the TBM regions are

deleted, little or no residual binding to the holo-TFIID complex is observed (Figure 2C). It has been demonstrated that certain activation domains transduce their signal by making contact with TAFs (Brou et al., 1993). For instance, SP1 functions by contacting TAF110 (Hoey et al., 1993). VP16 is able to make contacts with TBP, TFIIB and TAF40 (Goodrich et al., 1993; Choy and Green, 1993 and references therein). Moreover, Tanese et al. (1991) have shown that the proline-rich activation region of CTF requires TAFs in order to function. Protein blotting experiments using holo-TFIID and labelled FosB were performed, however technical difficulties precluded any definitive conclusion as to whether this region interacts with a particular TAF molecule. On the basis of the data presented in this paper demonstrating that the amino acid 243–282 region is a potent activation domain, it is reasonable that FosB may make two independent contacts with the TFIID complex.

The interaction between activator molecules and TBP has been associated with enhanced transcription (Boyer and Berk, 1993). We have shown that the TBM is required for the full expression of the transactivation property of FosB. However, the proline-rich region, amino acids 243–282, constitutes the major C-terminal activation domain. Alone, the C-terminal 55 amino acids containing the TBM of FosB activate weakly, 3- to 4-fold above baseline activity (Gal4 alone). Taken together, these data suggest that the interaction of the TBM with TBP provides a support function that cooperates with the proline-rich activation domain. In an effort to establish whether TBP is a functional target we determined a squelching curve similar to those determined previously to establish the functional significance of E1a (Boyer and Berk,1993), Tax1 (Caron et al., 1993) and c-Rel (Kerr et al., 1993; Xu et al., 1993) interactions with TBP. These data demonstrate that co-expression of TBP can relieve the inhibition caused by high levels of FosB expression and establish that TBP is a functional target of FosB. Due to the nature of these experiments, one cannot prove conclusively that TBP is a direct target of TBM in vivo, however, the observed relief of squelching by TBP is dependent on TBM. These results, taken together with the accompanying in vitro and in vivo data demonstrating a tight association between FosB and TBP, are strong evidence that this interaction is specific and occurs in vivo. Nevertheless, it is clear from these results that the mechanism by which FosB activates transcription is more complex than the FosB–TBP interaction, since high FosBΔTBM levels also inhibited transcription, which was not dependent on TBP levels. Therefore, additional cellular target proteins are likely to be limiting at high concentrations of the FosB activator.

The disparate transforming properties of FosB, FosB/SF and Fra-1 have been reported by several groups (Kovary et al., 1991; Mumberg et al., 1991; Wisdom et al., 1992). The fact that FosB is the only polypeptide of the three which activates transcription supports the notion that transactivation is required for efficient transformation. However, our data indicate that the major activation region, amino acids 243–283, and the TBP binding module, which has only a modest effect on activation, are both required for transformation. These suggest that transcriptional activity may not necessarily

be linked to the efficacy of transformation. Indeed, there are examples for c-Fos where mutations affecting transformations do not have a corresponding effect on transcriptional activation (Mumberg *et al.*, 1991). Therefore, one cannot exclude the possibility that the TBP binding activity present in FosB provides a specific function in regulating those genes involved in transformation, which may not necessarily be linked to the direct activation of transcription *per se*. One of the difficulties in making such a correlation is that we studied activation using a simple reporter construct. In the transformation studies, the target genes and the complex make-up of their regulatory machinery are not known. Therefore, it is reasonable to assume that the TBM may play a more or less important role in transcriptional regulation depending on the architecture of the given promoter region. This leads one to the obvious question, 'what role does the interaction of the TBM with TBP play in transcriptional regulation?'. Given our current understanding of transcriptional regulation there are several possibilities which can be suggested. These include the following: (i) to aid in the assembly of the general transcription machinery, similar to activator proteins ATF and VP16; (ii) to relieve transcriptional repression by the removal of transcriptional inhibitors that function by interacting with TBP, like DR1 and NCI (Meisterernst and Roeder, 1991; Inostroza *et al.*, 1992); (iii) to assist in the juxtaposition of an activator domain(s) with the cellular target molecules (TAFs) (Hernandez, 1993). Recent studies by Choy and Green (1993) suggest that activator proteins stimulate transcription in a stepwise process which leads to increased PIC formation. On the basis of the data presented here, the TBM and proline-rich regions of FosB may provide independent mechanisms which aid in the assembly of the PIC by operating at different rate-limiting steps.

It should be noted that c-Fos lacks a proline-rich activator region but contains activation domains termed HOB1 and HOB2 (Sutherland *et al.*, 1992). c-Fos also contains a C-terminal TBP binding motif, which is interchangeable with that of FosB (Metz *et al.*, 1994). However, the TBM region of c-Fos is absent in v-Fos and therefore may be dispensable for transformation. These data are not in conflict with the data presented here, since the deletion of the c-Fos TBM does not completely abolish TBP binding activity. Indeed, there appears to be an additional region on the c-Fos protein which can support TBP interaction. Nevertheless, the data presented here provide evidence that the major activation domain of FosB, 243–282, and the TBM provide independent activities which are required for the transformation of Rat-1A cells.

Materials and methods

Recombinant DNA

The FosB, FosB/SF and Fra-1 cDNAs have been described previously (Zerial *et al.*, 1989; Dobrzanski *et al.*, 1991; Ryseck and Bravo, 1991). Mutants FosBΔTBM and point mutants were introduced by site-directed mutagenesis using the Altered Sites *In Vitro* Mutagenesis System (Promega). Constructs encoding FosBΔ243–282, FosBΔ243–282ΔTBM, FosBΔ283–310, Fra/B283–338, Fra/B282–338ΔTBM, Fra/B243–338 and Fra/B243–338ΔTBM were cloned by ligating the appropriate polymerase chain reaction (PCR)-generated fragments containing unique *Bam*HI sites introduced at codon junctions 242-3, 282-3 and 310-1 of *fos*B and *fos*BΔ*TBM* and 206-7 for *fra-1*. To generate GST−FosB,

−FosB/SF and −Fra-1 fusion proteins, the respective cDNAs were cloned in-frame with the open reading frame (ORF) of GST in pGex-3X (Smith and Johnson, 1988). The Gal4 fusion expression vectors were generated by cloning the various reading frames in-frame with the ORF of Gal4 in the pHKG expression vector (Sutherland *et al.*, 1992). The *fos*B and *fra-1* coding regions used to make the pHKG expression vectors all contained a mutated leucine zipper, where leucine 197 of FosB and leucine 147 of Fra-1 were changed to proline by site-directed mutagenesis. pMexneoFosBΔbzip contains the coding region of FosB (leucine 197 to proline) cloned into the pMexneo vector. For the production of recombinant baculoviruses the coding regions for FosB, FosBΔTBM, Fra-1, Fra/B283–338, Fra/B283–338ΔTBM and GST−cTBP were cloned into plasmid pVL1393 (Summers and Smith, 1987). For the focus forming assay the FosB, FosBΔTBM, FosBΔ243–282 and FosBΔ243–282ΔTBM coding regions containing a wild-type bzip domain were cloned into the pMexneo vector (Martin-Zanca *et al.*, 1989). All cloning junctions were verified by dideoxy sequencing following their cloning into Bluescript KS+, which was also used to generate *in vitro* synthesized mRNA for *in vitro* translation reactions.

The TBP regions 1–163 (nTBP), 202–272, 272–339 and 202–339 were cloned into pGEX 2T, whereas residues 168–339 (cTBP), 168–202 and 168–272 were cloned into pGEX-3X, and the human TBP was cloned into the expression vector pHK3, described elsewhere (Metz *et al.*, 1994). The reporter plasmid (pBLG$_1$TCAT) contains a single Gal4 DNA binding site (AGCTTCGGAAGACTCTCCTCCGA) and the adenovirus major late promoter TATA (GATCCGGGGGGCTATAAAA-GGGGTG) double-stranded oligonucleotides cloned into the *Hin*dIII and *Bam*HI sites of pBL3CAT (Luckow and Schutz, 1987).

In vitro and in vivo GST pull-down assays

Appropriate cDNAs were transcribed, translated and ^{35}S-labelled *in vitro* using the TnT7-coupled reticulocyte lysate system as described by the manufacturer (Promega).

The GST fusion proteins were expressed and purified as previously described (Hagemeier *et al.*, 1993b). Interaction studies involving GST fusions and *in vitro* translated products were carried out as described (Hagemeir *et al.*, 1993b).

Recombinant baculoviruses were generated using the pVL1393 transfer vector containing FosB, FosBΔTBM, Fra-1, Fra/BΔ283–338, Fra/BΔ283–338ΔTBM and GST−cTBP coding regions, as described (Summers and Smith, 1987). Sf9 cells were infected with individual recombinant baculoviruses or co-infected with GST−cTBP-expressing virus as described. After 24 h, the cells were harvested and lysed in NP40 lysis buffer (25 mM HEPES, pH 7.5, 12.5 mM MgCl$_2$, 250 mM KCl, 0.5% NP40, 1 mM DTT, 20 μM ZnSO$_4$, 0.5 mM PMSF, 10 μg/ml leupeptin and 1% aprotinin) by incubating on ice for 10 min. After adjusting to 8% glycerol, the cellular extracts were cleared by centrifugation (10 000 *g* for 15 min), incubated with glutathione−Sepharose beads for 15 min at 4°C, washed three times with 1 ml NETN, boiled in SDS sample buffer and resolved by SDS−PAGE. The retention of baculovirus-expressed proteins was detected by Western blot analysis.

FosB interaction with native TBP and TFIID immune complexes

HeLa cells were grown in suspension media (sMEM) containing 5% fetal bovine serum (FBS). Nuclear extracts were prepared as described (Dignam *et al.*, 1987) and fractionated over a phosphocellulose column (P11) as described by Timmers and Sharp (1991). Native TFIID was immune-purified from the PC0.7 fraction as described previously (Tanese *et al.*, 1991) by incubating 500 μg protein with 2 μg affinity-purified anti-TBP antibody (directed against amino acids 1–58 of human TBP) at 4°C for 4–5 h, followed by the addition of protein A−Sepharose and incubation overnight. The immune complexes were collected by centrifugation and washed in BC buffer (25 mM HEPES−KOH, pH 7.9, 12% glycerol, 3 mM MgCl$_2$ and 1 mM DTT containing 100–500 mM KCl). A sample of the TFIID immune complex was analysed quantitatively and qualitatively by SDS−PAGE. To generate cellular TBP (free of TAFs), the TFIID complex was washed with BC buffer containing 1 M guanidine−HCl as described previously (Tanese *et al.*, 1991).

Immune complexes containing ~100 ng native TBP or TBP in the holo-TFIID complex were incubated with *in vitro*-translated ^{35}S-labelled FosB, FosBΔ243–282, FosBΔTBM or FosBΔ242–282ΔTBM under the same conditions as described for the GST pull-down experiments (described above). Proteins retained by the immune complexes were resolved by SDS−PAGE and detected by autoradiography.

For GST−FosB interactions with holo-TFIID present in HeLa nuclear extracts, 2 μg GST fusion protein linked to glutathione−Sepharose [previously incubated with 0.25% BSA in BC buffer (BC100)] were added to 500 μg protein from a phosphocellulose PC0.7 fraction of HeLa nuclear extracts. The mixture was incubated for 4 h at 4°C. The beads were washed four times each with BC100 and BC250 containing 0.5% NP40, boiled in 1× SDS sample buffer and subjected to SDS−PAGE and Western blot analysis.

Antibodies

The anti-TBP antibody was generated against the entire human TBP protein, which was produced in bacteria as a fusion protein with MS2 polymerase using the pEx34 vector (Strebel *et al.*, 1986). Positively reacting serum was affinity purified on a GST−TBP (amino acids 1−58 of human TBP) fusion protein affinity column (Bio-Rad P10). The anti-FosB-specific antibody and the 'pan-Fos' antibody were generated as described (Kovary and Bravo, 1991a,b). Protein−antibody interactions on Western blots were detected by the ECL kit, using the manufacturer's instructions (Amersham).

Transfections

For transient activation studies, ~5×10⁵ NIH3T3 cells were transfected using the standard calcium phosphate co-precipitation method (Van der Eb and Graham, 1980). DNAs included 2 μg CAT reporter construct (pBLG₁TCAT) and different amounts (0.01−10 μg) of pHK3-derived plasmids expressing various Gal4 fusion proteins. In the experiments indicated, 3 μg pHKGTBP and/or increasing amounts of pMexneo-FosBΔbzip were transfected. The final DNA amount was adjusted to 15 μg with Bluescript KS+ plasmid DNA. After 12 h the cells were washed and re-fed. Thirty six−forty eight hours after transfection, extracts were prepared and assayed for CAT activity, as described (Gorman *et al.*, 1982). Equivalent amounts of protein were used for each reaction. Protein quantitation was determined by the Bio-Rad protein assay kit.

Stable transfections were carried out as previously described (Kovary *et al.* 1992), where 1, 3, 5 or 15 μg each pMexneo-based expression vector were transfected into 4 ×10⁵ Rat-1A cells (kindly provided by M.J.Bishop; Smale *et al.*, 1987). DNA concentrations were adjusted to 15 μg with pMexneo. Twelve hours after transfection the cells were washed and re-fed with fresh medium. After an additional 24 h, the cells were split 1:4. Cells were fed with fresh medium every 3–4 days. Foci were counted 14 days after transfection. In some cases (using 15 μg expression plasmid) the cells were split 1:4 and selected for neomycin resistance using medium supplemented with 800 μg neomycin. Neomycin-resistant colonies were analysed for protein expression by Western analysis.

Acknowledgements

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