***In vitro* association between the Jun protein family and the general transcription factors, TBP and TFIIB**

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Transcriptional activator proteins interact with the general transcription factors TATA-binding protein (TBP), TFIIB and/or other TBP-associated factors (TAFs). Using affinity chromatography we demonstrate that members of the Jun family of transcriptional activators interact with both TBP and TFIIB *in vitro*. TBP binds to both the N-terminal activation domain and C-terminal bZIP regions of c-Jun, whereas TFIIB binds to only the c-Jun bZIP domain. This interaction requires the dimerization of the Jun protein. The ability of the N-terminal activation domains of c-Jun, JunB, JunD and v-Jun to interact with TBP *in vitro* correlates with their transcriptional activity *in vivo*. Domain

mapping experiments indicate that c-Jun interacts with the conserved C-terminus of TBP. Studies using a set of TFIIB in-frame deletion mutants demonstrate that C-terminal amino acids 178–201 and 238–316 play an important role in modulating the interaction between TFIIB and c-Jun. Although phosphorylation of the c-Jun N-terminal activation domain stimulates c-Jun transcriptional activity *in vivo*, it has no effect on the ability of c-Jun to interact with either TBP or TFIIB *in vitro*. These data suggest that the Jun family of activator proteins may activate transcription by interacting with the general transcription factors TBP and TFIIB.

INTRODUCTION

The Jun (c-Jun, JunB, JunD) and Fos (c-Fos, FosB, Fra-1, Fra-2) proteins are components of the AP-1 transcription factor (reviewed in ref. [1]). These proteins contain a basic region–leucine zipper (bZIP) domain that allows for DNA binding and dimerization respectively [2–4]. Structure–function analysis of the c-Jun protein has identified a potent transactivation domain located within the c-Jun N-terminus (A1 domain) with a less pronounced region adjacent to the basic domain (A2 domain) [5]. The A1 activation domain of c-Jun consists of three independent activation domains; I (amino acids 6–12), II (amino acids 68–81) and III (amino acids 105–120) [6]. Domains II and III contain sequences that are also found in the c-Fos protein and act co-operatively and heterologously to activate transcription [7]. c-Jun transcriptional activity is stimulated *in vivo* by the phosphorylation of two serine residues at positions 63 and 73 within the c-Jun N-terminal transcriptional activation domain [8–11]. This phosphorylation is induced by treatment with phorbol esters, growth factors, or expression of several transforming oncogenes (H-ras or v-src) [8–11]. The N-terminus of JunD, but not JunB or v-Jun, the viral transforming homologue of c-Jun, can also be phosphorylated in a similar manner both *in vivo* and *in vitro* [10,12–16]. c-Jun contains a 27-amino-acid sequence within its N-terminus termed the δ -domain which is lacking in v-Jun [5]. The c-Jun δ domain appears to act as a binding site for the c-Jun N-terminal protein kinase [13–15]. c-Jun transcriptional activity can be negatively regulated by a cell type-specific inhibitor which functionally interacts with this δ domain region [17]. The presence of this transcriptional repressor domain in c-Jun may provide the molecular basis for the difference in transcriptional activity between c-Jun and v-Jun [5]. Although several distinct transcriptional activation domains have been mapped within the c-Jun protein, the proteins within the general transcriptional machinery that mediate these transcriptional effects have not been identified.

Activator proteins enhance transcription by directly interacting with one or more components of the general transcriptional machinery [18,19]. The general initiating factors for RNA polymerase II transcription (TFIIA, -B, -D, -E, -F, -G/J, -H and -I) assemble over the TATA box in a highly ordered manner. The TATA-binding protein (TBP) is the DNA-binding subunit of TFIID and interacts with promoter DNA. TBP DNA binding is enhanced by interaction with TFIIA [20]. This DNA complex then interacts with TFIIB forming a minimal transcriptional initiation complex [21]. Activator proteins are thought to facilitate the formation of a stable preinitiation complex by enhancing one of these rate-limiting steps. The acidic activator VP16 interacts with TFIIB and enhances its recruitment into the preinitiation complex [22,23]. TFIIB has also been shown to mediate transcription by the *Drosophila* activator protein *fushi tarazu* [24]. The ZTA viral activator protein, however, mimics the effects of TFIIA by stabilizing the binding of TBP to the TATA box sequence [25]. TBP has been shown to interact with both viral activator proteins, VP16, E1A, IE2 and HTLV-I [25–30], and cellular DNA binding factors, c-Rel, p53, RB and c-Myc [31–35]. Coactivators or TBP-associated factors (TAFs) also play a key role in activated transcription [18,36]. The Sp1 activator protein interacts directly with *Drosophila* TAF_{II}110, which contacts TAF_{II}250, and partially restores Sp1-mediated transcription [37,38]. *Drosophila* TAF_{II}40 interacts with both TFIIB and the viral transactivator protein VP16 and mediates in part transcription by this enhancer binding protein [39].

To understand further how the Jun family of enhancer proteins activate transcription, we have examined whether c-Jun interacts with either TFIIB or TBP. Here we demonstrate that all the Jun family members are capable of binding both TBP and TFIIB *in vitro*. TBP can interact with both the N-terminal transcriptional activation domain and the C-terminal dimerization/DNA-binding domain of c-Jun, whereas TFIIB can interact only with the C-terminal domain of c-Jun. Mutational analysis suggests that dimerization of the c-Jun protein is necessary for the interaction

Abbreviations used: TBP, TATA-binding protein; TFI, general initiating factor for RNA polymerase II transcription; TAF, TBP-associated factor; bZIP, basic region–leucine zipper; GST, glutathione transferase.

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of c-Jun with both TBP and TFIIB. Mapping experiments demonstrate that the conserved C-terminal domain of TBP plays an important role in mediating the interaction with c-Jun. c-Jun interacts with both the basic domain and C-terminus of TFIIB; however, multiple regions within TFIIB appear to be important. Unlike the prominent regulatory role of c-Jun N-terminal phosphorylation in regulating c-Jun transcriptional activity *in vivo*, phosphorylation had no effect on the ability of c-Jun to interact with either TBP or TFIIB *in vitro*. Together these results suggest that the interaction of the Jun family of proteins with the TBP and TFIIB plays a role in regulating transcriptional by these enhancer binding proteins.

MATERIALS AND METHODS

Construction of glutathione S-transferase (GST) fusion proteins

All GST fusion proteins were prepared by cloning into the *Bam*HI–*Eco*RI site of either the pGEX-2T or pGEX-3X vector (Pharmacia, Piscataway, NJ, U.S.A.). The N-terminal GST–JunB and GST–v-Jun fusions, the full-length GST–c-Jun fusion and all c-Jun N-terminal constructs have previously been described [10,13,14]. GST–JunD(1–115) was constructed by cloning an N-terminal murine JunD (gift from D. Nathans) PCR fragment into pGEX-2T. The GST–c-Jun(190–334) and GST–v-Jun(148–301) plasmids were constructed by ligating C-terminal PCR fragments from human c-Jun and chicken v-Jun (gift from T. Bos) into pGEX-2T respectively. The GST–JunB(196–344) and GST–JunD(206–341) fusion proteins were gifts from L. H. K. Defize and J. Woodgett [16]. The wild-type and mutant GST–TFIIB fusion proteins were gifts from S. Roberts and M. Green [40].

Expression and purification of GST fusion proteins

Overnight cultures of *Escherichia coli* DH5 α transformed with the recombinant fusion constructs were diluted 1:10, grown for 1 h and stimulated with 0.4 mM isopropyl β -D-thiogalactoside for an additional 3 h. Cells were harvested, resuspended in TBSTE (15 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA) containing 0.5 mM phenylmethanesulphonyl fluoride, 0.5 mM benzamidine and 5 mM dithiothreitol and sonicated three times for 30 s. The lysate was centrifuged and the supernatant was incubated with glutathione–Sephadex beads (Sigma, St. Louis, MO, U.S.A.) for 30 min at 4 °C with mixing. The beads were washed twice with TBSTE, twice with TBSTE containing 2 M NaCl, twice with TBSTE and once with TNEN (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P40). The beads were stored at 4 °C in TNEN containing 10% glycerol. The integrity, quantity, and purity of fusion proteins bound to beads were determined by SDS/PAGE (see Figure 3b) and Bradford analysis.

In vitro transcription and translation of protein probes

[³⁵S]Methionine-labelled protein probes were generated by standard *in vitro* transcription–translation protocols (Promega, Madison, WI, U.S.A.). Probe cDNAs were linearized with the appropriate restriction enzymes and transcribed with either SP6 or T7 RNA polymerase. pSP64–c-Jun was prepared by cloning the full-length human c-Jun cDNA into the *Eco*RI site of pSP64. pRC-CMV–c-Jun(Δ 234–334) and c-Jun(Δ 1–217) (pGEMjunP; gift from P. Leder) were prepared as previously described [3,10]. c-Jun(L297P) was prepared by cloning the full-length c-Jun

Figure 1 Interaction of c-Jun with both TBP and TFIIB *in vitro*

Detection of c-Jun interaction by binding to immobilized fusion proteins. *In vitro* translated [³⁵S]-labelled c-Jun (lanes 1–5), TBP (lanes 6–8) or TFIIB (lanes 9–11) was incubated with immobilized GST, GST–TBP, GST–TFIIB or GST–c-Jun fusion protein for 1 h at 25 °C. After extensive washing, bound protein was eluted by boiling in SDS sample buffer and analysed by SDS/PAGE followed by fluorography. The input lane contains one-tenth that used for the binding reaction.

cDNA into M13 followed by site-directed mutagenesis to change Leu-297 to a proline residue (CTG to CCG) using the Amersham oligonucleotide-directed system (Arlington Heights, IL, U.S.A.). The c-Jun insert was then cloned into the *Hind*III–*Xba*I site of pRC-CMV (Invitrogen, San Diego, CA, U.S.A.). The *Bam*HI–*Eco*RI fragment of the wild-type and double-mutant yeast TBPs were excised from pUC-IIDORF (gift from S. Buratowski) and cloned into the *Bam*HI–*Eco*RI site of pSP72. pSP72–TFIIB was prepared by cloning a full-length TFIIB (gift from I. Ha and D. Reinberg) PCR product into the *Hind*III–*Cl*aI site of pSP72. Human TBP and the C-terminal deletion mutants were transcribed directly from a hTBP BS(SK[–]) plasmid (gift from A. Hoffman and R. Roeder) [41] after digestion with the appropriate restriction enzymes.

Complementary RNA isolated from the transcription of these constructs was used to programme either rabbit reticulocyte lysates or wheat germ extracts for *in vitro* translation in the presence of [³⁵S]methionine according to the manufacturer's instructions. Incorporation of [³⁵S]methionine was determined by fluorography of SDS/polyacrylamide gels and by trichloroacetic acid precipitation of radiolabelled proteins.

Assay of binding to immobilized GST fusion proteins

GST fusion proteins (10 μ g) immobilized on glutathione–Sephadex beads were preincubated for 1 h at 25 °C in 0.3 ml of TNEN containing 3% BSA, 1 mM dithiothreitol, 5 mM MgCl₂, 0.5 mM phenylmethanesulphonyl fluoride, 0.5 mM benzamidine and 0.4 mg/ml ethidium bromide. The beads were then incubated for 1 h at 25 °C in fresh buffer containing approx. 300 000 c.p.m. of trichloroacetic acid-precipitable [³⁵S]methionine-labelled *in vitro* translated protein. The beads were washed five times with TNEN at 25 °C and the bound proteins eluted by boiling in 1 $\times$ Laemmli sample buffer. Bound proteins were resolved by SDS/PAGE and visualized by fluorography. In all Figures the lanes marked input represent one-tenth the amount utilized in the binding reactions. The relative amounts of bound protein were quantified by densitometric scanning of the relevant autoradiographs using a Bio-Rad GS-670 Imaging densitometer.

RESULTS

c-Jun interacts with both TBP and TFIIB *in vitro*

To understand the mechanism by which members of the Jun family of proteins regulate transcription, the potential physical association of c-Jun with the general transcriptional machinery was investigated. TBP and/or TFIIB interact with several transcriptional activators and were therefore likely candidates to interact with the Jun family of transactivator proteins. An affinity-chromatography technique was utilized to examine whether c-Jun could bind to either of these proteins *in vitro*. c-Jun, TBP and TFIIB were expressed in *E. coli* as GST fusion proteins and coupled to glutathione-Sepharose beads [42]. [³⁵S]Methionine-labelled c-Jun protein synthesized by *in vitro* transcription/translation of human c-Jun cDNA was incubated with the immobilized fusion proteins. After extensive washing, bound ³⁵S-labelled c-Jun was eluted by boiling the beads in SDS sample buffer, followed by electrophoresis on SDS/polyacrylamide gels and fluorography (Figure 1, lanes 1–5). c-Jun bound to both GST–TBP (lane 3) and GST–TFIIB (lane 4), but not to control beads coupled to GST alone (lane 2). As a positive control, c-Jun was also found to dimerize with an immobilized GST–c-Jun fusion protein (lane 5). Inversely, radiolabelled *in vitro* translated TBP (lanes 6–8) and TFIIB (lanes 9–11) were also found to bind to the GST–c-Jun fusion. Identical results were obtained utilizing ³⁵S-labelled protein probes synthesized in either rabbit reticulocyte lysates or wheat germ extracts (results not shown). Ethidium bromide was included in the incubation buffer to ensure that these interactions were not mediated by contaminating DNA [43].

The association characteristics of the c-Jun–TBP and c-Jun–TFIIB complexes were examined under conditions of increasing ionic strength using the immobilized-fusion-protein-binding assay described above. GST–TBP and GST–TFIIB beads were incubated with ³⁵S-labelled c-Jun in the presence of increasing concentrations of NaCl (Figure 2). Optimal binding of c-Jun to both TBP and TFIIB occurred at NaCl concentrations below 100 mM (lane 2 and results not shown). The c-Jun–TBP complex forms more efficiently in higher ionic strength buffers than the c-Jun–TFIIB complex as judged by its resistance to

Figure 2 Effect of ionic strength on c-Jun–TBP and c-Jun–TFIIB complex-formation

Immobilized GST–TBP, GST–TFIIB and GST–c-Jun were incubated with *in vitro* translated ³⁵S-labelled c-Jun in buffer containing the indicated concentrations of NaCl for 1 h at 25 °C. The beads were then washed extensively with incubation buffer containing the same NaCl concentration. c-Jun remaining associated with the beads was analysed by SDS/PAGE followed by fluorography.

Figure 3 Interaction of TBP with the N-terminal activation domain of all the Jun family members

GST–Jun fusion proteins encoding the N-terminal activation domains of c-Jun(5–89), JunB(5–100), JunD(1–115), v-Jun(5–62) and a c-Jun deletion mutant lacking amino acids 30–56 [c-Jun(Δ30–56)] were constructed by PCR cloning the indicated fragments into pGEX-2T and the expressed fusion protein was bound to glutathione-Sepharose beads. (a) ³⁵S-labelled *in vitro* translated TBP was incubated with 10 µg of the indicated N-terminal GST–Jun fusion protein, washed extensively, and bound protein analysed by SDS/PAGE followed by fluorography. (b) Analysis of the purity and integrity of the N-terminal GST–Jun fusion proteins. Equivalent amounts of the indicated GST fusion proteins (2 µg) were resolved by SDS/PAGE and the gel stained with Coomassie Blue. The positions of prestained molecular-mass markers are indicated on the left.

increasing salt concentrations (lanes 3–7). The relative sensitivities of these complexes to increasing salt concentrations was much greater than that of the c-Jun–c-Jun homodimeric complex-formation which occurred at NaCl concentrations of up to 1 M.

Mapping the interaction domain(s) of the Jun family members

The c-Jun protein is a bipartite molecule composed of an N-terminal transcriptional activation domain and a C-terminal bZIP domain which mediates DNA binding and dimerization. The N-terminal 89 amino acids of c-Jun have been shown to be sufficient to activate transcription *in vivo* [10]. To evaluate whether this region was capable of interacting with TBP and/or TFIIB, N-terminal GST fusions of the Jun family of proteins corresponding to amino acids 5–89 of c-Jun were prepared and coupled to glutathione-Sepharose beads. *In vitro* translated ³⁵S-labelled TBP and TFIIB were incubated with the N-terminal fusion protein beads and binding was analysed. Whereas no significant interaction between TFIIB and these beads could be detected (results not shown), TBP bound to the N-terminus of all the Jun family members (Figure 3a). Densitometric scanning analysis indicates that the v-Jun N-terminal fusion protein consistently bound threefold more TBP than the c-Jun fusion protein, which bound much more than either the JunB or JunD fusion proteins (14 and 33 % that of c-Jun respectively). These differences in binding were not due to variability in the amount of fusion protein present as the quantity and integrity of all

Figure 4 Interaction of both TBP and TFIIB with the C-terminal bZIP region of the Jun protein family

GST–Jun fusion proteins corresponding to the C-terminal domains of c-Jun(190–334), JunB(196–344), JunD(184–341) and v-Jun(148–301) were constructed, expressed in bacteria and coupled to glutathione–Sepharose beads. 35 S-labelled *in vitro* translated TBP, TFIIB or c-Jun was incubated with these immobilized fusion proteins, washed extensively, and bound protein analysed by SDS/PAGE.

fusion proteins utilized were found to be similar as judged by Coomassie staining of an SDS/polyacrylamide gel (Figure 3b).

The major difference between the N-terminus of c-Jun and v-Jun is the presence of an additional 27 amino acids in c-Jun termed the δ domain, which is necessary for c-Jun N-terminal phosphorylation [12,13,15] and negatively regulates c-Jun transcriptional activity [5]. To determine whether the c-Jun δ domain regulates TBP binding, it was deleted from the N-terminus of c-Jun and this mutant expressed as a GST fusion protein. This c-Jun mutant, c-Jun(Δ 30–56), bound twofold more TBP than the wild-type GST–c-Jun fusion protein (Figure 3a, compare lanes 6 and 2), suggesting that the δ domain is not essential for the TBP interaction and may have an inhibitory effect on TBP binding. This result indicates that the TBP interaction domain within the N-terminus of c-Jun spans either amino acids 5–30 or 56–89 which are present in this mutant construct. These regions contain the transcriptional activation domains I and II respectively [6,7].

It was recently reported that TBP can also interact with the C-terminal bZIP domain of c-Jun [44]. As TFIIB binds full-length c-Jun but not the N-terminal activation domain, it was of interest to determine whether the bZIP region also mediates this interaction. The C-terminal bZIP domains of all the Jun family members were expressed as GST fusion proteins, coupled to glutathione–Sepharose beads, and TBP and TFIIB binding was assessed. TFIIB and TBP were found to bind the C-terminal GST fusion proteins of all the Jun family members (Figure 4). Both TBP and TFIIB were found to bind the c-Jun fusion protein most efficiently. The relative binding of TBP for the other Jun fusion proteins was JunD > v-Jun > JunB (60, 50 and 19% that of c-Jun respectively). The relative binding of TFIIB to the other Jun fusions was of the order v-Jun > JunD > JunB (49, 29 and 15% that of c-Jun respectively). These C-terminal Jun fusions were tested for their ability to dimerize with c-Jun and were found to be equally capable of binding 35 S-labelled c-Jun, indicating that equivalent amounts of functional fusion protein were utilized for these studies.

Because the C-terminus of Jun contains the leucine zipper–dimerization domain, we examined whether these interactions

Figure 5 The bZIP region of c-Jun is required for binding to both TBP and TFIIB

Various c-Jun mutants were analysed for their ability to interact with either TBP or TFIIB using the immobilized-fusion-protein-binding assay. A C-terminal deletion [c-Jun(Δ 234–334)] was prepared by inserting a termination codon at residue 234 of the full-length c-Jun cDNA. An N-terminal c-Jun deletion mutant containing only the bZIP region of c-Jun [c-Jun(Δ 1–217)] was a gift from P. Leder [3]. c-Jun(L297P) was prepared by site-directed mutagenesis, altering the codon for the third leucine of the c-Jun leucine zipper to encode a proline residue [4]. These proteins were radiolabelled by *in vitro* transcription/translation in the presence of [35 S]methionine and incubated with immobilized GST, GST–TBP, GST–TFIIB or GST–c-Jun fusion protein. The ability of each mutant to associate with these fusion proteins was determined after extensive washing.

were dependent on Jun protein dimerization. Three mutant c-Jun proteins were used to evaluate the role of the leucine zipper in the formation of the c-Jun–TBP and c-Jun–TFIIB complex. A c-Jun C-terminal deletion mutant [c-Jun(Δ 234–334)], and a full-length c-Jun protein containing a leucine to proline point mutation within the leucine zipper [c-Jun(L297P)] [4] were prepared which lack the ability to dimerize. An N-terminal deletion mutant containing only the bZIP domain of c-Jun was also utilized [c-Jun(Δ 1–217)] [3]. These proteins were translated *in vitro* and binding to immobilized GST–TBP, GST–TFIIB or GST–c-Jun was analysed (Figure 5). Whereas none of these proteins interacted with the control GST fusion protein, wild-type c-Jun and c-Jun(Δ 1–217), both of which contain an intact leucine zipper domain, bound to all of the immobilized fusion proteins. Interestingly, c-Jun(Δ 234–334) was not capable of binding any of the fusion proteins (< 5% that of c-Jun wild-type), and the leucine to proline point mutation dramatically reduced the ability of the full-length c-Jun protein to interact with c-Jun, TBP and TFIIB (< 15% that of c-Jun wild-type). These results demonstrate that the C-terminal dimerization domain of c-Jun is not only necessary for Jun/Jun homodimer formation, but also for the association of the c-Jun protein with both TBP and TFIIB.

Mapping the interaction domain of TBP

The C-terminus of TBP is highly conserved amongst species whereas the N-terminus varies greatly in both size and amino acid content [19]. The conserved C-terminus of TBP is composed

Figure 6 c-Jun interacts with the conserved C-terminal domain of human TBP

(a) Diagram of human TBP and the deletion mutants used to map the interaction domain of TBP. The white and black areas represent the variable N-terminus and conserved C-terminus of TBP respectively. The arrows indicate the location of the imperfect direct repeats, and the plus marks represent the lysine-rich basic core domain. (b) ³⁵S-labelled full-length TBP (TBP/Xba) and C-terminal deletion mutants were prepared by *in vitro* translation from complementary RNA synthesized from TBP cDNA digested at unique restriction sites with *StuI*, *ApaLI* or *SspI*, in the presence of [³⁵S]methionine. Labelled protein probes were incubated with immobilized GST or GST-c-Jun fusion protein, washed extensively and bound proteins analysed.

of two direct repeats separated by a basic-core domain rich in lysine residues (see Figure 6a). To determine which region(s) of TBP interacted with c-Jun, C-terminal deletion mutants of human TBP were prepared. Human TBP cDNA was cleaved at several unique restriction sites and the resulting deletion mutants were radiolabelled by *in vitro* transcription/translation in the presence of [³⁵S]methionine. The radiolabelled proteins were incubated with immobilized GST-c-Jun and binding was analysed (Figure 6b). Deletion of the second direct repeat (lanes 4–6) and basic-core domain of TBP (lanes 7–9) resulted in a gradual reduction in the ability of TBP to bind c-Jun (72 and 29% that of wild-type TBP respectively). Further deletion of the first direct repeat of TBP (lanes 10–12), leaving a protein spanning only the variable N-terminus of TBP, completely abolished c-Jun binding (lane 12). These findings indicate that several regions of the conserved C-terminus of TBP influence the formation of c-Jun–TBP complex.

Yeast TBP exhibits a high degree of similarity to human TBP within the conserved C-terminal domain including the two direct repeats and lysine-rich basic domain, but is very dissimilar within the variable N-terminus of the protein [41]. The basic-core domain of yeast TBP is necessary for the interaction between it and the general transcriptional factor TFIIA [20]. To evaluate

Figure 7 Interaction of c-Jun with yeast TBP at a site(s) unique from that of TFIIA

Wild-type yeast TBP and two double mutants lacking the ability to interact with TFIIA [20] were transcribed and translated *in vitro* in the presence of [³⁵S]methionine. The ability of these proteins to interact with immobilized GST-c-Jun fusion protein was analysed as previously described.

the ability of c-Jun to bind to this protein, wild-type yeast TBP and two yeast TBP double point mutants which are incapable of binding TFIIA [20] were radiolabelled by *in vitro* transcription/translation in the presence of [³⁵S]methionine. These proteins were incubated with immobilized GST-c-Jun fusion protein and the associated proteins analysed as previously described. Wild-type yeast TBP bound to GST-c-Jun, and the basic-core domain mutations, which abolished the yeast TBP–TFIIA interaction, did not affect this binding (Figure 7). Together these results using human and yeast TBP suggest that c-Jun interacts with the conserved C-terminal domain of TBP.

Mapping the interaction domain of TFIIB

Like TBP, the TFIIB protein contains two C-terminal imperfect direct repeats, a basic domain and an N-terminal tail [45]. A set of TFIIB in-frame deletion mutations expressed as GST fusion proteins have been used to map the interaction domain between TFIIB and the viral activator protein VP16 [40]. To determine the region(s) of TFIIB responsible for the binding of c-Jun, these same GST–TFIIB deletion mutants were incubated with ³⁵S-labelled *in vitro* translated c-Jun protein (Figure 8). TFIIB mutations previously shown to decrease or block VP16 binding also reduced the binding of radiolabelled c-Jun. TFIIB deletion mutants lacking the C-terminal amino acids 178–201 and 238–316 (lanes 9 and 12 respectively) resulted in the largest reduction in c-Jun binding (47 and 29% of wild-type TFIIB respectively). However, unlike VP16, none of the mutations investigated completely abolished the binding of c-Jun to the TFIIB fusion protein. Furthermore, point mutations within the basic domain of TFIIB (178–201) that abolished both VP16 binding and the ability of TFIIB to mediate VP16-activated transcription *in vitro* [40] had little effect on the ability of c-Jun to interact with TFIIB (results not shown). Interestingly, deletion of amino acids 2–123 of TFIIB resulted in a twofold enhancement of c-Jun binding (lane 6). These results suggest that there may be multiple sites within TFIIB that regulate its ability to interact with c-Jun.

Figure 8 Mapping of the c-Jun site of interaction on TFIIB

Various TFIIB deletion mutants expressed as GST fusion proteins were immobilized on glutathione-Sepharose beads [40]. ^{35}S -labelled c-Jun protein was prepared by *in vitro* transcription/translation, and binding to the immobilized GST-TFIIB fusion proteins was analysed as previously described.

Figure 9 The phosphorylation state of the c-Jun N-terminal activation domain has no effect on the ability of c-Jun to interact with either TBP or TFIIB

^{35}S -labelled wild-type c-Jun or c-Jun containing a double serine to leucine point mutation at residues 63 and 73 [c-Jun(S63,73L)] were prepared by *in vitro* translation using rabbit reticulocyte lysate. Radiolabelled protein probes were analysed for their ability to bind immobilized GST, GST-TBP or GST-TFIIB as previously described.

Phosphorylation of the N-terminus of c-Jun does not regulate the interaction between c-Jun and TBP

The transcriptional activity of c-Jun *in vivo* is enhanced by the phosphorylation of serines 63 and 73 within the c-Jun N-terminal transcriptional activation domain [8–11]. As TBP interacted with this region of c-Jun *in vitro* (see Figure 3), we examined whether c-Jun N-terminal phosphorylation played a role in regulating the interaction between c-Jun and TBP. Both wild-type c-Jun and a c-Jun double mutant containing serine to leucine mutations at amino acids 63 and 73 [c-Jun(S63,73L)] were utilized for this purpose. Rabbit reticulocyte lysates contain an unknown protein kinase activity that results in the hyperphosphorylation of serines 63 and 73 of c-Jun upon translation *in vitro* [8,10,12]. This feature was exploited to synthesize highly phosphorylated c-Jun protein *in vitro*. Both wild-type and double-mutant c-Jun proteins were translated *in vitro* using complementary RNA-programmed rabbit reticulocyte lysates, and binding to immobilized GST-TBP and GST-TFIIB was analysed (Figure 9). Wild-type and mutant c-Jun bound the TBP and TFIIB fusion proteins equally well. Alternatively, wild-type c-Jun translated *in vitro* using pro-

grammed wheat germ extracts, which does not result in the hyperphosphorylation of the c-Jun N-terminus [8,12], also bound to the TBP and TFIIB beads as efficiently as the hyperphosphorylated c-Jun produced in rabbit reticulocyte lysates (results not shown). Furthermore, dephosphorylation of c-Jun produced in reticulocyte lysates with calf intestinal alkaline phosphatase had no effect on its ability to interact with either TBP or TFIIB (results not shown).

As TFIIB only interacted with the C-terminal bZIP region of c-Jun (Figure 4), it is not surprising that this interaction was not affected by the phosphorylation of the c-Jun N-terminus. However, TBP interacted with both the bZIP domain and the N-terminal transactivation domain of c-Jun (Figures 3 and 4). To evaluate further the effect of c-Jun phosphorylation on TBP binding to the N-terminus of c-Jun, an N-terminus GST-c-Jun construct was prepared that contained serine to leucine mutations at serines 63 and 73, GST-c-Jun(5–89)(S63,73L). This double mutation had no effect on the ability of TBP to bind to the c-Jun N-terminal (results not shown). Furthermore, phosphorylation of GST-c-Jun(5–89) *in vitro* with a protein kinase purified from U937 human leukaemic cells that specifically phosphorylates serines 63 and 73 of c-Jun [13,14] also had no effect on the ability of ^{35}S -labelled TBP to associate with the c-Jun N-terminus (results not shown). Taken together these findings indicate that the phosphorylation state of the c-Jun N-terminal transactivation domain does not affect the ability of c-Jun to interact with the general transcription factors TBP or TFIIB.

DISCUSSION

The general transcriptional factors TBP and TFIIB are necessary for both basal and activated transcription (reviewed in ref. [19]). The observation that these proteins are capable of directly interacting with both viral and cellular enhancer binding proteins suggests that TBP and TFIIB might play a role in regulating transcriptional by the Jun family of upstream activator proteins. We demonstrate that members of the Jun family of proteins are capable of interacting with both TBP and TFIIB *in vitro*. This finding is similar to that of the VP16 viral activator protein which is also capable of interacting with both TBP and TFIIB [23,26,40]. The N-terminal activation domains of the Jun family members exhibit different binding characteristics for TBP; that is, v-Jun > c-Jun > JunD ≈ JunB. Likewise, v-Jun is a more potent transactivator *in vivo* than c-Jun, with JunB being the weakest [17,46–48]. Therefore the ability of Jun family members to activate transcription factors *in vivo* correlates with their ability to interact with TBP *in vitro*.

The interaction between both TBP and TFIIB and the Jun family of proteins requires a functional leucine zipper and dimerization of the Jun protein, as demonstrated by the inability of TBP or TFIIB to interact with either a truncated c-Jun protein lacking a bZIP domain or a c-Jun leucine zipper mutant lacking the ability to dimerize efficiently. Mapping of the c-Jun domains that interact with TBP and TFIIB demonstrates that both the N-terminal transcriptional activating region (amino acids 5–89) and the C-terminal bZIP region (amino acids 190–334) are capable of binding TBP, whereas TFIIB only interacts with the bZIP domain of c-Jun. These regions of c-Jun contain the previously identified transcriptional activation domains A1 and A2 respectively [5], suggesting that these regions may be important for interactions with TBP and/or TFIIB.

The ability of the N-terminal activation domain of the Jun family of proteins to bind TBP in the absence of protein dimerization (Figure 3) may be explained by the manner in which these experiments were performed. The N-termini of the Jun

proteins were expressed as GST fusion proteins and immobilized on glutathione beads. This coupling may allow the close packing of the N-terminal activation domains similar to what might be found in a dimeric protein complex. In contrast, the requirement for dimerization is demonstrated by producing c-Jun protein *in vitro* and binding this protein to an immobilized TBP fusion protein.

The cloning of the human TBP cDNA reveals that the C-terminus is highly conserved amongst species and is composed of two imperfect direct repeats separated by a basic-core domain [41,49–51]. In contrast, the N-terminus of human TBP which contains long stretches of glutamine and proline residues is not conserved amongst species. Deletion analysis indicates that c-Jun interacts with the conserved C-terminus of TBP *in vitro*. The mapping of the c-Jun binding site to the conserved C-terminus of human TBP and the ability of c-Jun to bind to yeast TBP, which contains a highly divergent truncated N-terminus relative to human TBP, suggests that the variable N-terminus does not play a significant role in regulating the interaction between c-Jun and TBP. Furthermore, the ability of c-Jun to bind two yeast TBP mutants that are incapable of interacting with the general transcription factor TFIIA [20] suggests that c-Jun binds at a site distinct from that of TFIIA. TFIIA and ZTA, a bZIP activator protein encoded by the Epstein–Barr virus, interact with the basic-core domain of TBP and function to stabilize or enhance TBP TATA-box binding [20,35]. As c-Jun does not appear to interact with the same region of TBP as these regulatory proteins, it is possible that c-Jun functions by a different mechanism, perhaps recruiting TFIIB to the preinitiation complex.

The TFIIB protein shares several structural similarities with TBP. Both proteins contain two C-terminal imperfect repeat regions separated by a basic-core domain which potentially forms an amphipathic α -helix [45]. Whereas TBP binds to the basic-core domain of TFIIB [21], the interaction between the VP16 activator protein and TFIIB requires both the basic-core domain and the C-terminus of the second direct repeat [40]. The interaction between TFIIB and c-Jun is apparently more complex than that for either TBP or VP16. Using GST–TFIIB fusion proteins encoding various TFIIB deletion mutants identical with those used in the VP16 studies, we found that deletions that were capable of abolishing VP16 binding resulted in only modest reductions in c-Jun binding. These results suggest that c-Jun interacts with multiple sites on TFIIB, but that similar regions in the TFIIB protein interact with c-Jun and VP16.

We and others have previously demonstrated that phosphorylation of the N-terminus of c-Jun stimulates c-Jun transcriptional activity *in vivo* [8–11]. We attempted to address whether this phosphorylation may activate c-Jun transcription by altering the ability of c-Jun to interact with the general transcriptional machinery. To examine the role of phosphorylation in regulating the ability of c-Jun to interact with TBP, the c-Jun N-terminal activation domain was phosphorylated *in vitro* by a variety of methods. While c-Jun N-terminal phosphorylation enhances c-Jun transcriptional activity *in vivo*, it has no effect on the ability of c-Jun to interact with either TBP or TFIIB. Recent studies examining the transactivation potential of c-Jun *in vitro* were not able to demonstrate a role for N-terminal phosphorylation in c-Jun transactivation [52]. It was proposed that the *in vitro* system utilized may lack a cofactor necessary for the stimulatory effect of c-Jun phosphorylation to be evident [52]. Several studies have also suggested that an inhibitory factor(s) is associated with the N-terminus of c-Jun [17,53] and the negative regulatory function of this factor is overcome by various stimuli that enhance c-Jun transcriptional activity [53]. It is possible that such cofactors may regulate the ability of c-Jun to interact with TBP in response to

c-Jun N-terminal phosphorylation, but are not present in the *in vitro* system used in these studies. Recent studies demonstrate that the VP16 activator protein not only binds to TBP and TFIIB, but also to the *Drosophila* TAF, dTAF_{II}40 [39]. TAFs have also been implicated in the regulation of activated transcription mediated by Sp1 [36,37]. Although present experiments demonstrate that c-Jun is capable of associating with both TFIIB and TBP *in vitro*, additional experiments are required to examine fully the role of TBP, TFIIB and TAFs in mediating the transcriptional effects of the Jun family of transcriptional enhancers.

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