Volume 3 · Number 1 · 1993
GENE EXPRESSION

The bZIP domains of Fos and Jun mediate a physical association with the TATA box-binding protein

Lynn J. Ransone, Lawrence D. Kerr, Mark J. Schmitt, Penny Wamsley, and Inder M. Verma

Molecular Biology and Virology Laboratory, The Salk Institute, San Diego, California

Fos and Jun oncoproteins form a complex that regulates transcription from promoters containing AP-1 binding sites. These two proteins, like other transcriptional activators, are likely to stimulate transcription through direct and/or indirect interactions with members of the basal transcriptional machinery. The ability of c-Fos and c-Jun proteins to interact directly with the TATA box-binding protein (TBP), the general transcription factor required for initiating the assembly of transcription complexes, was investigated. Using co-immunoprecipitation and protein–protein association assays, we show that both c-Fos and c-Jun bind specifically and stably to TBP. Mutational analysis demonstrates that both the basic region and leucine zipper domains of c-Fos and c-Jun are necessary and sufficient for stable association with TBP. A 51-residue region from the conserved C-terminal region of TBP, previously shown to be the binding site for the viral activator protein E1A, interacts with c-Fos and c-Jun proteins. We propose that c-Fos and c-Jun proteins function as transcriptional activators, in part by recruiting TBP to form complexes to initiate RNA synthesis.

Transcriptional regulation of genes is controlled both by general transcription factors and by sequence-specific binding proteins (Maniatis et al., 1987; Mitchell and Tjian, 1989). The products of two nuclear proto-oncogenes, c-*fos* and c-*jun*, form a non-covalent association in some transcriptional complexes (Chiu et al., 1988; Rauscher et al., 1988b; Sassone-Corsi et al., 1988a). Activator protein 1 (AP-1) was first characterized as a nuclear factor that recognized the enhancer elements of SV40, the human metallothionein IIA gene, and the control regions of genes containing promoter elements responsive to TPA (12-O-tetra-decanoylphorbol-13-acetate; Angel et al., 1987; Lee et al., 1987b). AP-1, biochemically purified via its specific DNA-binding activity, was shown to contain several polypeptides ranging in size from 35 to 50 kDa (Lee et al., 1987a; Rauscher et al., 1988a). Several groups have demonstrated that these polypeptides include those encoded by the c-*fos* and c-*jun* proto-oncogenes and other members of the *jun* and *fos* gene families (reviewed in Vogt and Bos, 1990; Angel and Karin, 1991; Ransone and Verma, 1991). Jun and Fos proteins belong to the basic region/leucine zipper (bZIP) family of transcription factors (Johnson and McKnight, 1989). These bZIP proteins are characterized by the presence of a region of highly basic amino acids required for DNA binding (the basic region), and a heptad of leucine repeats that provides the dimerization interface (leucine zipper; Landschultz et al., 1988). The three Jun proteins, c-Jun, JunB, and JunD, form homodimers capable of binding to an AP-1 site (Nakabeppu et al., 1988; Ryseck and Bravo, 1991). Each

Received December 21, 1992; revision accepted January 22, 1993.

Correspondence: Inder M. Verma, Molecular Biology and Virology Laboratory, The Salk Institute, P.O. Box 85800, San Diego, CA 85800 Tel (619) 453-4100, ext. 462 Fax (619) 558-7454

 1052-2166/93/0301/037-12$2.00

of the three Jun proteins is also capable of forming a heterodimer with each of the four Fos family members—c-Fos, FosB, Fra-1, and Fra-2 (Kouzarides and Ziff, 1988; Halazonetis et al., 1988; Nakabeppu et al., 1988; Sassone-Corsi et al., 1988a,b; Rauscher et al., 1988b; Cohen et al., 1989; Nishina et al., 1990; Yen et al., 1991). This heterodimer binds DNA more tightly than the Jun homodimer and is more potent in activating transcription (Sassone-Corsi et al., 1988a,b; Zerial et al., 1989; Suzuki et al., 1992). The Fos proteins, unlike Jun, are unable to form homodimers and therefore have no specific intrinsic DNA-binding activity (Halazonetis et al., 1988; Nakabeppu et al., 1988; Cohen et al., 1989; Zerial et al., 1989; Ransone et al., 1990a; Suzuki et al., 1992).

c-Fos and c-Jun transactivation domains have been identified by both in vitro transcription assays and in vivo transfection experiments (Angel et al., 1989; Bohmann and Tjian, 1989; Hirai et al., 1989; Kelleher et al., 1990; Abate et al., 1990, 1991; Baichwal and Tjian, 1990). Analysis of c-Jun chimeras in different cell lines suggests that it contains an activator domain (A1) that is negatively regulated by a cell type-specific inhibitor (Baichwal and Tjian, 1990). A regulatory domain (δ), previously identified by in vitro experiments, also regulates transcriptional activation by c-Jun in vivo (Baichwal and Tjian, 1990). Stimulation of transcription by c-Fos requires two regions, one of which has the characteristics of an acidic domain postulated to be an activator, whereas the other has a high content of proline and acidic residues (Abate et al., 1991). The proline-rich region has also been shown to be important by co-transfection assays (Nakabeppu and Nathans, 1991; Wisdom et al., 1992).

Transcriptional activators such as c-Fos and c-Jun are thought to contact targets in the transcriptional machinery directly or indirectly (Ptashne, 1989; Ptashne and Gann, 1990). The current model contends that an activator protein once bound to a specific DNA sequence interacts with some component of the general transcription machinery, recruiting it to the DNA and/or changing its conformation on the DNA, and thereby initiating a cascade of events that leads to the initiation of transcription. The general initiation factors (TFIIA, B, D, E, F, G/J, H, and I), which are essential for RNA polymerase II to initiate transcription at promoter sites, can be assembled in a defined order (reviewed in Sawadogo and Sentenac, 1990; Roeder, 1991) that is initiated by the binding of TFIID to the TATA box element found in most promoters (Nakajima et al., 1988; Van Dyke et al., 1988, 1989; Buratowski et al., 1989). Addition of TFIIA then forms a complex that is competent for interaction with TFIIB, followed by RNA polymerase II and TFIIE/F (Buratowski et al., 1989; Van Dyke et al., 1989). Because of the central role TATA box-binding protein (TBP) plays in the initiation of transcription, it has been hypothesized to be one of the targets of upstream activator proteins (Ptashne, 1989; Ptashne and Gann, 1990). TFIID purified from HeLa cells contains additional factors, i.e., co-activators, that are required to mediate activation by upstream factors (reviewed in Ptashne and Gann, 1990; Dynlacht et al., 1991). The presence of these TBP-associated factors (TAFs) in the partially purified preparations raises the question whether activator proteins can contact TBP directly, or whether the additional proteins act as intermediaries or bridging factors (Ptashne and Gann, 1990). To address this issue, several viral transactivators have been examined for their ability to bind to TBP. Various groups have demonstrated physical interactions between TBP and the viral transactivators herpes simplex virus VP-16 (Stringer et al., 1990), adenovirus E1A (Lee et al., 1991), and Epstein-Barr virus Zta (Lieberman and Berk, 1991) proteins.

In this article, we report that both c-Jun and c-Fos—but not Fra-1 and FosB, two other members of the *fos* gene family—physically associate with TBP. Mutational analysis demonstrates that both the basic region and leucine zipper domains of c-Fos and c-Jun are necessary and sufficient for stable association with TBP. Furthermore, we show that c-Fos and c-Jun interact with a 51-residue region in the conserved C-terminal region of TBP, previously shown to be the binding site for the viral activator protein E1A (Lee et al., 1991). We conclude that both c-Fos and c-Jun stimulate transcription in part through this direct interaction with TBP.

Materials and methods

Recombinant plasmids

All of the T7 c-Fos and c-Jun constructs (Ransone et al., 1989, 1990b), the FosB (Yen et al.,

1991) and Fra-1 cDNAs (Cohen and Curran, 1988), GST-Jun BR-LZ and GST-Fos BR-LZ (Bengal et al., 1992), the E12 and MyoD cDNAs (Davis et al., 1990), and the TBP and E1A cDNAs (Lee et al., 1991) have been previously described. The human TBP cDNA GST-derivatives were amplified from plasmid pKB104 (Lee et al., 1991) by polymerase chain reaction (PCR) using gene-specific oligonucleotides, which introduced a BamH I site at the 5′ end and an EcoR I site at the 3′ end of the protein coding regions. The PCR fragments that encoded either amino acids (aa) 1–337 or aa 221–271 of TBP were introduced into pT7-GT expression vector (Bengal et al., 1992) to generate GST-TBP and GST-TBP 221–271, respectively.

Protein cross-linking and immunoprecipitation

^{35}S-methionine-labeled proteins were synthesized in vitro using a rabbit reticulocyte lysate translation system as directed by the supplier (Promega). Lysate samples containing ^{35}S-labeled TBP were incubated with cold in vitro translated c-Fos, FosB, or Fra-1 protein, or with untranslated reticulocyte lysate, at room temperature for 30 minutes in a buffer containing 20 mM Hepes-KOH (pH 7.9), 2.5 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, and 10% glycerol. The cross-linking agent dithiobis-succinimidyl propionate (DSP) was added for an additional 15 minutes to a final concentration of 2 mM in a final volume of 50 μl. Five μl of 1M ethanolamine were added to quench the reaction, and a standard immunoprecipitation using the Fos monoclonal antibody 18H6 (DeTogni et al., 1988), the FosB-specific antibody 5108-1B (Yen et al., 1991), or Fos M antibody (Curran et al., 1985) was then carried out as previously described (Bengal et al., 1992). Immunoprecipitates eluted from protein A-sepharose by boiling in SDS sample buffer were subjected to sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) and fluorography.

Glutathione S-transferase fusion protein assay

Affinity purification of GST fusion resins. BL12(DE3) bacteria harboring either the parental pT7-GT or the recombinant glutathione S-transferase (GST) fusion plasmids were grown overnight. The cultures were diluted 1:10, and after 1 hour of additional growth, IPTG was added to a final concentration of 250 μg/ml. Three hours following the addition of IPTG, cells were harvested by centrifugation, resuspended in NETN (0.5% NP-40, 1 mM EDTA, 20 mM Tris, pH 8, and 100 mM NaCl), and sonicated as previously described (Bengal et al., 1992). Debris was spun down at 10,000 × g for 5 minutes at 4°C. The supernatant was mixed with glutathione-sepharose 4B (Pharmacia) that had been previously equilibrated with NETN (1:1) and left on a rotary shaker for 1 hour at 4°C. The resin was collected by low speed centrifugation and washed four times in NETN. To determine the purity and yields of the bound GST proteins, 20 μl of 1:1 beads were boiled in SDS-PAGE sample buffer and run on a SDS-polyacrylamide gel. The gel was subsequently stained with Coomassie blue (data not shown).

Binding of in vitro synthesized ^{35}S-labeled proteins to the immobilized GST proteins. Programmed ^{35}S-labeled reticulocyte lysates (2 μl) were analyzed by SDS-PAGE. Equivalent amounts of labeled in vitro translated protein were then diluted to 200 μl in NETN. This sample was precleared with 20 μl of GST resin (1:1 in NETN) for 1 hour on a rotary shaker at room temperature, followed by a low speed centrifugation to remove the GST beads. The supernatant was then mixed with either GST or the appropriate GST fusion resin and incubated for an additional hour at room temperature. The resin was washed four times in NETN at room temperature, and the bound proteins were eluted by boiling in SDS sample buffer and subjected to SDS-PAGE and fluorography.

Results

TATA-binding protein can be co-immunoprecipitated with c-Fos protein, but not with FosB or Fra-1

We tested the possibility that TBP might associate with various members of the *fos* family by conducting a co-immunoprecipitation experiment. Radiolabeled TBP was synthesized using a cell-free in vitro transcription/translation system. The ^{35}S-labeled TBP was incubated with unlabeled in vitro synthesized Fos, Fra-1, or FosB protein, followed by cross-linking with the reversible cross-linker dithiobis-succinimidyl propionate (DSP). After immunoprecipitation with the appropriate Fos antibody, the immune complexes were treated with reducing agent dithiothreitol (DTT) to reverse the cross-linker

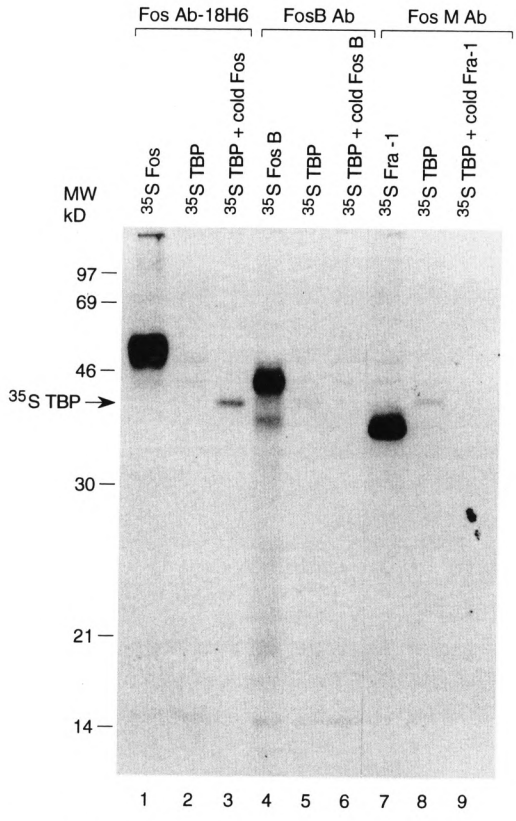

Figure 1. Cross-linking and co-immunoprecipitation of in vitro synthesized TBP and c-Fos protein. ^{35}S-labeled TBP and labeled and unlabeled c-Fos, FosB, and Fra-1 were prepared by programmed reticulocyte lysate, as previously described (Ransone et al., 1989). The radiolabeled TBP was mixed with unlabeled c-Fos, FosB, or Fra-1 programmed lysate or untranslated lysate as a control. Two mM DSP cross-linker were added, followed by a standard immunoprecipitation protocol (see Materials and Methods) using 2 μl of Fos monoclonal antibody 18H6 (lanes 1–3), FosB antibody (lanes 4–6), or Fos M antibody (lanes 5–9), as indicated. Immunoprecipitations of ^{35}S-labeled c-Fos, FosB, and Fra-1 were included as controls (lanes 1, 4, and 7). After reduction, the isolated immune complexes were resolved by SDS-PAGE and fluorography.

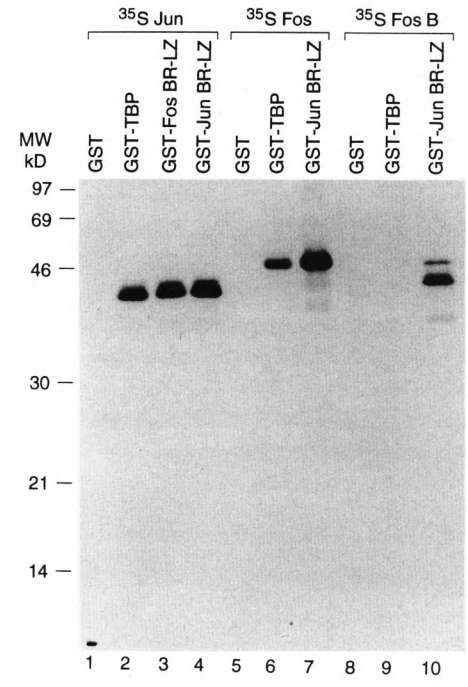

Figure 2. c-Jun and c-Fos—but not FosB—associate with immobilized TATA-binding protein. Equivalent amounts of in vitro translated c-Jun (lanes 1–4), c-Fos (lanes 5–7); and FosB (lanes 8–10) were precleared as described in Materials and Methods. Supernatants containing the diluted radiolabeled proteins were mixed with GST (lanes 1, 5, and 8), GST-TBP (lanes 2, 6, and 9), GST-Fos BR-LZ (lane 3), or GST-Jun BR-LZ (lanes 4, 7, and 10) resin for 1 hour at room temperature. The labeled proteins which specifically bound to the resin were analyzed by SDS-PAGE, followed by fluorography.

and analyzed by SDS-PAGE, followed by fluorography. Immunoprecipitation of a mixture of ^{35}S-labeled TBP and cold c-Fos protein by the Fos monoclonal antibody 18H6 specifically precipitated labeled TBP (Fig. 1, lane 3). This was not due to cross-reaction of the Fos 18H6 antibody with TBP, since labeled TBP was not precipitated when the incubation was carried out with untranslated reticulocyte lysate rather than c-Fos protein (lane 2), thus demonstrating the specificity of the reaction. Interestingly, no ^{35}S-labeled TBP was detected in co-immunoprecipitations with either FosB (compare control lane 5 to lane 6) or Fra-1 (compare control lane 8 with lane 9). Unlabeled c-Fos, FosB, and Fra-1 proteins were used in all of the co-immunoprecipitations because of the similarity in molecular mass of the *fos* gene family members and TBP. Specificity of each antibody was verified by immunoprecipitation of individually radiolabeled Fos proteins (lanes 1, 4, and 7). We estimate that approximately 15–20% of the input labeled TBP associates with c-Fos. These data, however, rely on the ability of the c-Fos antibody to immunoprecipitate all of the free and TBP-complexed, unlabeled Fos protein present in the reaction mixture. Our results demonstrate that while c-Fos, FosB, and Fra-1 are all members of the same bZIP family of transcription factors and capable of forming heterodimers with Jun proteins, only c-Fos protein is capable of physical interactions with TBP.

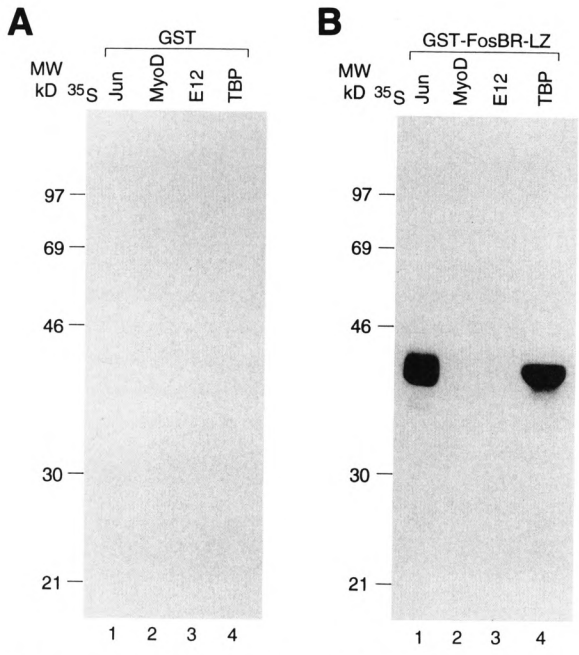

Figure 3. The bZIP domain of c-Fos protein mediates interaction with TBP. Equivalent amounts of in vitro translated c-Jun, MyoD, E12, and TBP that had been pre-cleared with GST resin were incubated with either immobilized GST (**A**) or GST-FosBR-LZ resin (**B**). Following one hour incubation at room temperature, the resins were washed extensively and eluted, and ^{35}S-labeled proteins were analyzed by SDS-PAGE and fluorography.

Direct interaction between c-Jun and TBP

Previous studies have demonstrated that the c-Fos protein does not form homodimers and is usually found as a heterodimeric complex with c-Jun protein (Halazonetis et al., 1988; Kouzarides and Ziff, 1988; Nakabeppu et al., 1988; Sassone-Corsi et al., 1988b). We therefore asked whether c-Jun protein, like c-Fos, was capable of forming a complex with TBP. We took an alternative approach to monitor the interactions between c-Jun and TBP, as c-Jun antibodies work very poorly in co-immunoprecipitation experiments (Sassone-Corsi et al., 1988b). In this experiment, we used affinity chromatography in which the entire TBP cDNA was fused in frame to the glutathione S-transferase gene (GST-TBP). GST-TBP fusion protein was expressed in bacteria and immobilized on glutathione-sepharose beads. Additionally, the bZIP (also referred to as BR-LZ or basic region/leucine zipper) domains of Jun (Jun BR-LZ) and Fos (Fos BR-LZ) fused to GST were prepared as binding controls. Radiolabeled c-Jun, c-Fos, and FosB were synthesized by in vitro transcription/translation in a rabbit reticulocyte lysate. The in vitro labeled translation products were incubated individually with either GST-TBP, GST-Jun BR-LZ, GST-Fos BR-LZ, or GST-control resin and washed as described in Materials and Methods. The bound material was eluted and analyzed by SDS-PAGE. None of the ^{35}S-labeled proteins bound to the control GST resin (Fig. 2, lanes 1, 5, and 8). As expected, greater than 80% of the input labeled c-Jun protein bound very strongly to resin containing either the Fos bZIP domain (lane 3) or Jun bZIP domain (lane 4). Interestingly, ^{35}S-labeled c-Jun protein also bound to the GST-TBP resin (lane 2) with approximately the same affinity as it did to the bZIP resins. When ^{35}S-c-Fos and ^{35}S-FosB were analyzed by the same assay, once again the ability to physically interact with TBP could be demonstrated only with c-Fos (lane 6), while both c-Fos and FosB were able to bind to GST-Jun BR-LZ (lanes 7 and 10, respectively). In these experiments, well over 80% of the input labeled c-Fos protein was retained on the GST-TBP resin. Thus, the results obtained by two different approaches clearly indicate that TBP can physically associate with c-Fos but not FosB. Additionally, we have demonstrated the c-Jun, like its partner c-Fos, is capable of binding to TBP.

TBP interaction with c-Fos and c-Jun is mediated through their bZIP domains

Having established that TBP physically associates with both c-Fos and c-Jun proteins, we next asked which domains of the two proteins are involved in the association. Heterodimer formation is known to be mediated through the leucine zipper domain of both c-Fos and c-Jun. To determine whether this domain is involved in the association with TBP, we performed GST association assays using radiolabeled TBP and GST-Fos BR-LZ resin (Fig. 3). Control ^{35}S-proteins c-Jun, MyoD, and E12, as well as TBP, were incubated with GST-Fos BR-LZ resin, washed extensively, and subjected to SDS-PAGE analysis. Again, none of the ^{35}S-proteins bound non-specifically to the GST resin (Fig. 3A). Both c-Jun and TBP bound specifically to the GST-Fos BR-LZ resin (Fig. 3B, lanes 1 and 4), while binding of MyoD and E12 was not detected (Fig. 3B, lanes 2 and 3). In these experiments, well over 80% of the labeled TBP bound to the bZIP

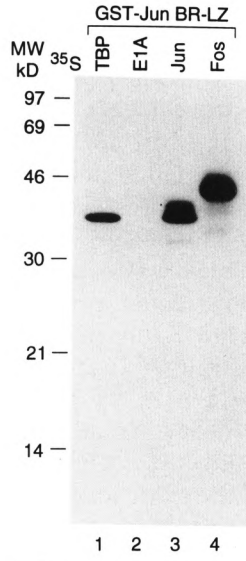

Figure 4. The bZIP domain of c-Jun protein mediates interaction with TBP. Equivalent amounts of in vitro translated c-Jun, c-Fos, E1A, and TBP that had been pre-cleared with GST resin were incubated with either immobilized GST (data not shown) or GST-Jun BR-LZ resin. Following one hour incubation at room temperature, the resins were washed extensively and eluted, and ^{35}S-labeled proteins were analyzed by SDS-PAGE and fluorography.

resin. These data demonstrate that a minimal region of c-Fos protein, namely the bZIP domain, is sufficient to mediate specific interaction with TBP.

To determine whether the bZIP domain of c-Jun is also involved in TBP-c-Jun complex formation, a GST-Jun BR-LZ association assay was carried out with ^{35}S-labeled TBP. As with c-Fos protein, the c-Jun bZIP domain was sufficient to direct specific radiolabeled TBP binding (Fig. 4, lane 1). As before, none of the labeled proteins bound non-specifically to GST resin (data not shown). In this experiment, ^{35}S-labeled E1A, which has been demonstrated to bind to TBP (Lee et al., 1991), was included as a negative control (lane 2), while ^{35}S-labeled full-length c-Jun and c-Fos were included as positive controls (lanes 3 and 4). These data demonstrate that c-Jun, like c-Fos, interacts with TBP through its bZIP domain.

The basic region/DNA-binding domain of both c-Jun and c-Fos is required for efficient binding of TBP

Having established that the bZIP domain of both c-Fos and c-Jun is necessary for association with TBP, we next investigated whether we could further delineate the binding domain by using c-Fos and c-Jun mutants containing precise deletions in either the basic region (ΔRK) or the leucine zipper (ΔLZ). These mutant proteins were translated in vitro and mixed with GST-TBP resin in a standard GST association assay. For all radiolabeled c-Jun proteins, GST-Fos BR-LZ resin was included as a control. As shown in Figure 5A, ^{35}S-labeled c-Jun wild-type protein (lane 2) and not Jun ΔRK protein (lane 5)

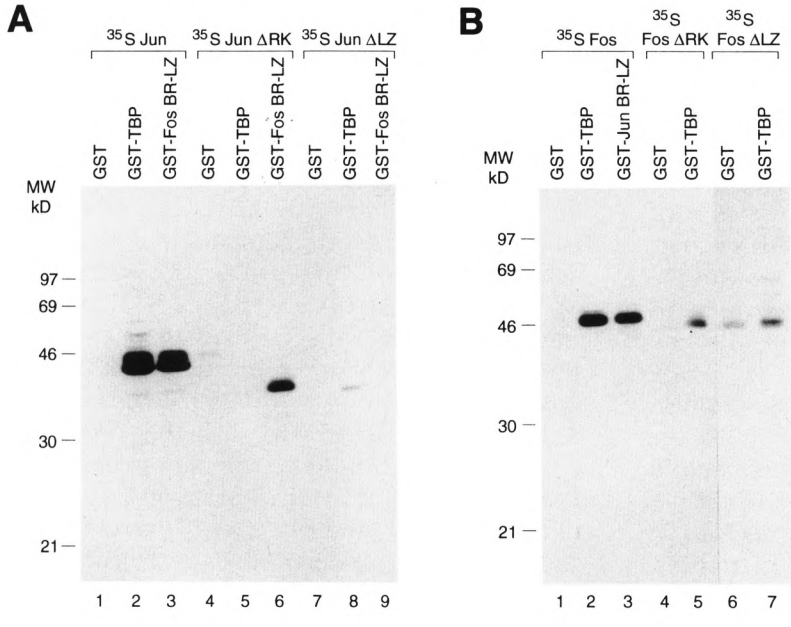

Figure 5. Deletion of the leucine zipper in c-Fos or c-Jun does not completely abolish binding to TBP. ^{35}S-labeled c-Jun (**A**) and c-Fos (**B**) basic region (ΔRK) and leucine zipper (ΔLZ) deletion mutant proteins were synthesized in vitro, diluted, pre-cleared, and mixed with either GST, GST-TBP, GST-Fos BR-LZ, or GST-Jun BR-LZ resin, as indicated. Specifically bound proteins were analyzed by SDS-PAGE and fluorography.

bound to GST-TBP resin. This result was somewhat surprising, in that the basic region of c-Jun protein is functionally defined as the DNA-binding domain (Nakabeppu et al., 1988; Gentz et al., 1989; Kouzarides et al., 1989; Neuberg et al., 1989; Ransone et al., 1990a,b) and can be removed without disrupting heterodimer formation with c-Fos protein (lane 6; Ransone et al., 1990b). Deletion of the leucine zipper domain of c-Jun protein (JunΔLZ, lanes 7–9) greatly reduced binding to the GST-TBP resin and, as expected, completely abolished binding to GST-Fos BR-LZ (Ransone et al., 1989).

The results obtained with the ^{35}S-labeled FosΔRK and FosΔLZ mutants (Fig. 5B) were slightly different from those found with the c-Jun deletion mutants (Fig. 5A). As shown in Figure 5B, each c-Fos deletion mutant bound to the GST-TBP resin, albeit with decreasing affinity compared to wild-type levels (compare lanes 5 and 7 with wild-type lane 2). These data demonstrate that the dimerization interface between TBP and c-Fos – or TBP and c-Jun – is not simply bounded by the leucine zipper domain, but also includes a region previously thought to be required only for DNA binding (Nakabeppu et al., 1988; Gentz et al., 1989; Kouzarides et al., 1989; Neuberg et al., 1989; Ransone et al., 1990a,b).

Point mutations in the basic region of c-Fos and c-Jun can abolish binding to TBP

The results presented so far seem to indicate that both domains in the bZIP region of c-Jun and c-Fos play a role in protein–protein interaction with TBP. To investigate further the contribution of specific amino acids within the basic region motif of both proteins in association with TBP, point mutants of c-Jun and c-Fos were analyzed. We chose specific valine substitution mutants, which display wild-type heterodimer formation to their respective c-Fos or c-Jun counterpart but exhibit reduced DNA-binding activity (Ransone et al., 1989, 1990b), to test in a GST-TBP resin association assay. The mutations were introduced at amino acid positions 262, 273, 276 (all in the basic domain), and 283 (leucine 1 of the zipper, or L1) of c-Jun protein, and amino acid positions 144 and 159 (basic domain) of c-Fos protein, as previously described (Fig. 6C; Ransone et al., 1989, 1990b). ^{35}S-labeled in vitro translated c-Jun and c-Fos point mutants were mixed with either GST-Fos BR-LZ resin, GST-Jun BR-LZ, or GST-TBP resin, washed extensively, and analyzed by SDS-PAGE followed by fluorography. As expected, mutations in the c-Jun basic region had no effect on the binding of these ^{35}S-labeled proteins to GST-Fos BR-LZ (Fig. 6A, lanes 3, 6, 9, and 12). Likewise, the ^{35}S-labeled c-Fos basic region mutants were retained on the GST-Jun BR-LZ resin (Fig. 6B, lanes 3 and 6). Jun V262, V273, and JunL1 all maintained the ability to bind to GST-TBP resin (Fig. 6A, lanes 2, 5, and 11 respectively). Surprisingly, the substitution of valine for lysine at amino acid residue 276 (Jun V276) completely abolished the ability of c-Jun to bind to the GST-TBP resin (Fig. 6A, lane 8). In a similar manner, c-Fos mutants V144 and V159 also lost the capacity to bind to the GST-TBP resin (Fig. 6B, lanes 2 and 5). These results further illustrate that the basic region of both c-Jun and c-Fos represents an important contact point for physical association with TBP. Furthermore, these data demonstrate that the protein dimerization motif of bZIP proteins is not restricted exclusively to the leucine zipper domain.

Amino acids 221–271 of TBP are sufficient to interact with c-Jun and c-Fos

Having established that the basic region motif is necessary for interaction with TBP, we next investigated the domains of TBP required for association with c-Fos and c-Jun. Previous studies have demonstrated that adenovirus large E1A protein binds to a 51-residue region (amino acid residues 221–271) from the conserved carboxyl-terminal domain of TBP (Lee et al., 1991). We tested the possibility that c-Jun and c-Fos might also bind to this region of TBP, which includes a repeat of basic residues between the homologous direct repeats, by expressing this region as a GST fusion protein for use in an affinity binding assay. ^{35}S-labeled E1A, c-Jun, and c-Fos were incubated with glutathione-sepharose resin coupled to either full-length TBP or amino acid residues 221–271 of TBP. As expected, ^{35}S-labeled E1A was retained by the full-length GST-TBP resin, and to a lesser extent by GST-TBP 221–271 (lanes 2 and 3). Similarly, ^{35}S-labeled c-Jun and c-Fos proteins bound to both full-length (Fig. 5B, lanes 2 and 5) and truncated GST-TBP 221–271 (lanes 3 and 6) resins in approximately the same ratios as E1A (Fig. 7A, lanes 2 and 3). The reduction in binding observed

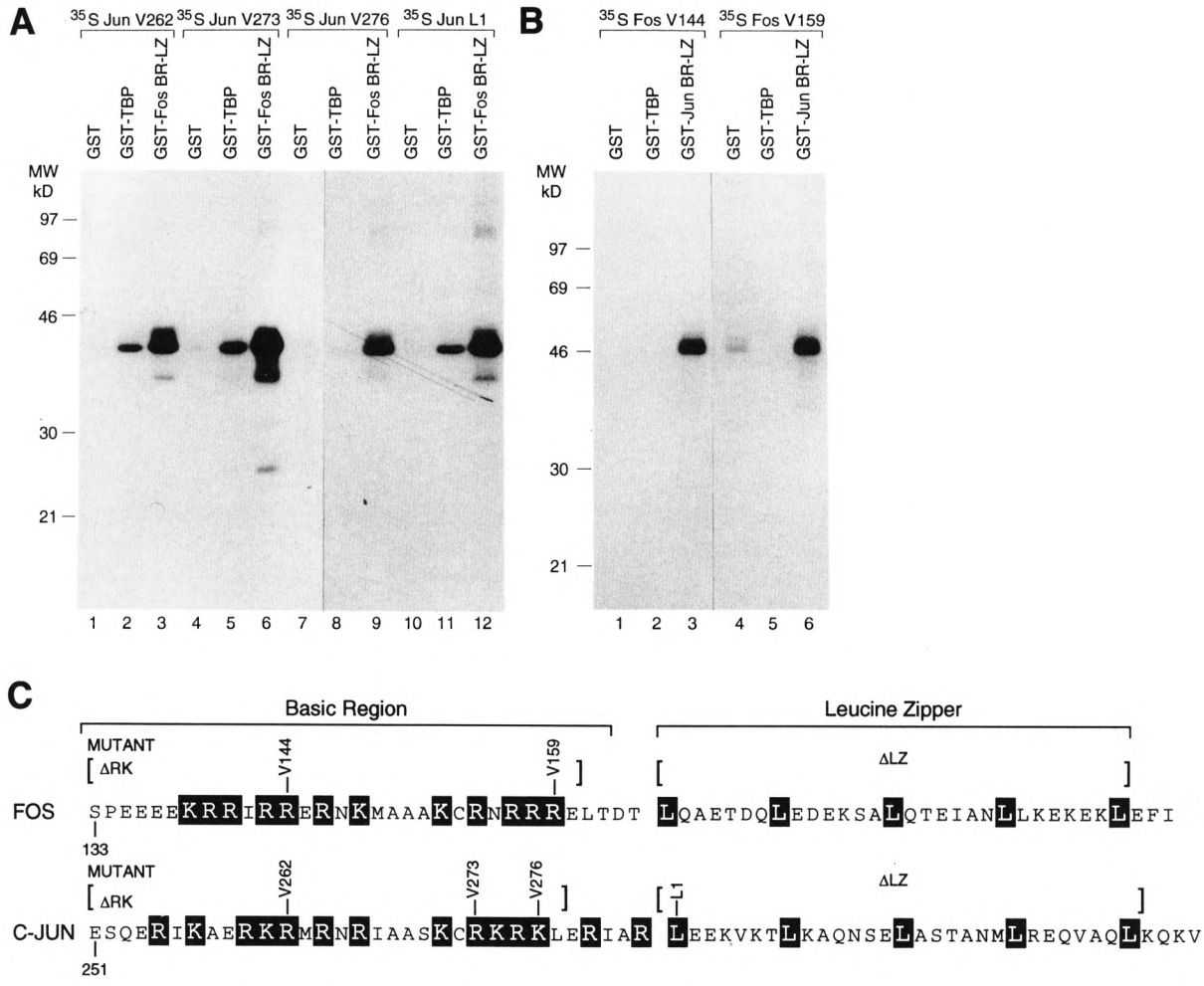

Figure 6. Point mutations in the basic region interfere with TBP binding. ^{35}S-labeled c-Jun (**A**) and c-Fos (**B**) proteins containing single amino acid substitutions at the indicated residues were tested for their ability to bind to immobilized GST-TBP in a standard association assay. Equivalent amounts of radiolabeled protein were added in each reaction. The binding control for the mutant c-Jun proteins was GST-Fos BR-LZ (**A**, lanes 3, 6, 9, and 12), and for the mutant c-Fos proteins was GST-Jun BR-LZ (**B**, lanes 3 and 6). **C.** DNA-binding mutants. The amino acid sequence encompassing the basic region and leucine zipper domains of Fos and Jun is shown. The positions of point mutations are indicated above the amino acid sequences (in single letter code) of Fos and Jun. The deleted regions (ΔRK and ΔLZ) are designated by brackets.

between the full-length GST-TBP and GST-TBP 221–271 for E1A and c-Fos and c-Jun is most likely due to differences in the concentration of fusion protein bound to the glutathione resin (data not shown). Based on these results, we conclude that amino acid residues 221–271 of TBP are necessary and perhaps sufficient to mediate interaction of c-Jun and c-Fos with TBP.

Discussion

TATA box-binding protein (TBP) is one of several general factors required for initiation of eukaryotic gene transcription by RNA polymerase II (reviewed in Sawadogo and Sentenac, 1990; Roeder, 1991). TBP binds in a sequence-specific manner to promoter DNA and interacts with the general initiation factors TFIIB and TFIIA (Buratowski et al., 1989; Maldonado et al., 1990). Basal transcription may be regulated by TBP via direct and/or indirect interactions with activator proteins (Ptashne, 1988; Ptashne and Gann, 1990). It becomes important then to determine whether known activator proteins can interact directly with this general transcription factor. In this paper, we have examined the abil-

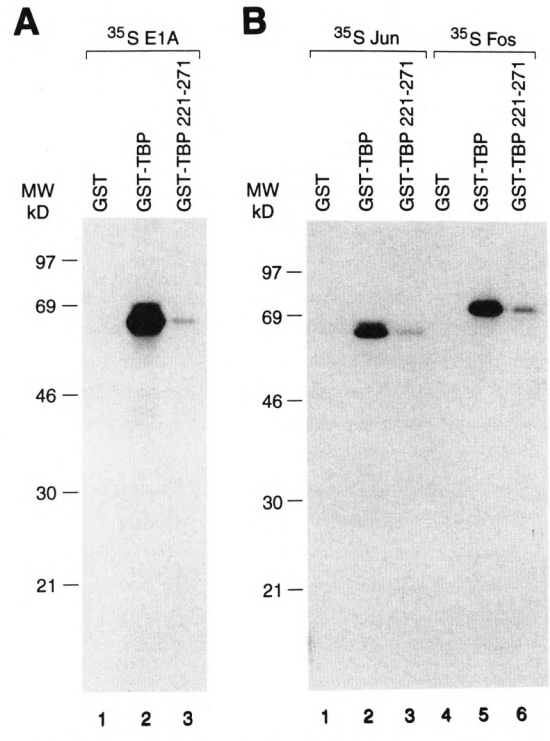

Figure 7. Amino acids 221–271 of TBP, which compose the basic repeat, mediate the physical association with c-Jun and c-Fos. ^{35}S-labeled E1A (**A**) and c-Jun and c-Fos (**B**) were tested for their ability to bind to immobilized GST-TBP (**A**, lane 2; **B**, lanes 2 and 5) or GST-TBP 221–271 (**A**, lane 3; **B**, lanes 3 and 6) in a standard association assay. Equivalent amounts of radiolabeled protein were added in each reaction. Specifically bound proteins were analyzed by SDS-PAGE and fluorography.

ity of *fos* and *jun* gene family members to associate directly with TBP.

Using a co-immunoprecipitation analysis (Fig. 1) and GST association assays (Figs. 2–4), we have shown that oncoproteins c-Fos and c-Jun, both members of the bZIP family of transcription factors, can associate directly with TBP. The bZIP region of these proteins is both necessary and sufficient to mediate binding to TBP (Figs. 3 and 4). Interestingly, we were unable to detect any physical association of TBP with FosB or with Fra-1 (Figs. 1 and 2). This result was rather surprising, in that the bZIP domains contain the highest degree of homology between *fos* and *jun* gene family members (reviewed in Vogt and Bos, 1990; Angel and Karin, 1991; Ransone and Verma, 1991). Recently, Kovary and Bravo (1992) demonstrated that different Fos/Jun complexes exist during the G_0-to-G_1 transition and during exponential growth in mouse fibroblasts. They found that while c-Fos is the major Fos protein associated with the Jun proteins (c-Jun, JunB, and JunD) soon after serum stimulation, at later times Fra-1 and Fra-2 are the predominant Fos proteins associated with Jun. This differential requirement for the various Fos proteins suggests that each has specific functions in the cell, although the missing activity of any given Fos family member can be partially compensated for by the function of any other Fos family member (Kovary and Bravo, 1992). The repertoire and complexity of regulation by the AP-1 complex arising from all the possible combinations of Jun and Fos proteins is further expanded by the ability or lack thereof to bind to specific general transcription factors. Recently, it has been demonstrated that both Fra-1 and Fra-2 can have a stimulatory or inhibitory effect on Jun activity, depending on the specific partner in the heterodimer (Suzuki et al., 1992). c-Jun activity is inhibited by Fra-1 and Fra-2, while JunD activity is stimulated. Proteins such as FosB and Fra-1 may exert their regulatory effects by binding to bridging factors, which in turn bind to TBP and/or bind directly to other members of the basal transcriptional machinery.

Several viral transactivators, such as herpes simplex virus, VP-16, adenovirus E1A, and Epstein-Barr virus Zta, have been shown to bind directly to TBP (Stringer et al., 1990; Horikoshi et al., 1991; Lee et al., 1991; Lieberman and Berk, 1991). The site on TBP that is bound by the viral transactivator E1A was mapped to a 51-residue region within the conserved C-terminal domain (Lee et al., 1991). Preliminary experiments using ^{35}S-labeled C-terminal truncation mutants of TBP demonstrated that amino acids 201–337 are required for binding to both GST-Fos BR-LZ and GST-Jun BR-LZ (data not shown). Since this region encompasses the TBP-binding domain for E1A, we tested the ability of c-Fos and c-Jun to bind to the same 51 aa residue region (Fig. 7). The results indicate that amino acids 221–271 of TBP, which bind to E1A, are also required for binding to c-Fos and c-Jun. However, additional residues outside of this region may also contribute, since the binding of labeled E1A, c-Fos, and c-Jun to GST-TBP 221–271 was not as efficient as binding to the full-length TBP resin (Fig. 7).

Recently, the crystal structure of TBP has been determined by X-ray crystallography (Nikolov et al., 1992). It is a highly symmetric α/β struc-

ture that contains a new DNA-binding fold resembling a "molecular saddle" that sits on the DNA. The convex outer surface of the protein contains the regions previously found to interact with a large number of proteins (Stringer et al., 1990; Meisterernst and Roeder, 1991; Horikoshi et al., 1991; Lee et al., 1991; Lieberman and Berk, 1991; Inostroza et al., 1992). One of these proteins is the general transcription factor TFIIA (Buratowski et al., 1989; Maldonado et al., 1990; Cortes et al., 1992). TFIIA recognizes a region containing a positively charged α-helix on the convex upper surface of TBP's first conserved domain (Nickolov et al., 1992; Ranish et al., 1992). This region is adjacent to the conserved basic repeat recognized by E1A (Lee et al., 1991) and by c-Jun and c-Fos, as shown in this work. In addition to the viral activators and general transcription factors, TBP is also associated with several TAFs to form a multiprotein complex (Sharp, 1992; Pugh and Tjian, 1992). Basal transcription can be carried out in vitro in the absence of TAFs; however, activated transcription requires the presence of these proteins to achieve full activation (Buratowski et al., 1989; Hoffman et al., 1990; Peterson et al., 1990).

Interaction between TBP and c-Fos or c-Jun requires a functional basic region/leucine zipper domain (Figs. 3 and 4). Using basic region and leucine zipper deletion mutants, we demonstrate that the basic region, which is dispensable for Jun/Fos heterodimer formation (Ransone et al., 1990b), is required for TBP-Jun and TBP-Fos complex formation (Fig. 5). Furthermore, we have identified specific point mutations in the basic region of both c-Fos and c-Jun that completely abolish the ability of these proteins to bind to TBP. Again, these mutations in no way alter the ability to form a Fos/Jun heterodimer, although their ability to bind to DNA is disrupted (Ransone et al., 1990b). It is thought that bZIP proteins bind to DNA via a "scissors-grip" structure (Vinson et al., 1989). In this model, the positively charged side chains of conserved basic residues are naturally disposed toward the edges of the major groove, where they might interact with the negatively charged phosphodiester backbone of the DNA (Vinson et al., 1989). This would leave the opposite face of the domain free to interact with other regulatory proteins or transcription factors, such as TBP, as demonstrated in this work. We hypothesize that the exposed face of the Fos-Jun heterodimer basic region/leucine zipper is free to interact with the protein-binding surface of TBP. The residues in TBP shown to bind to Jun, Fos, and E1A reside along a region encompassing helix H2′, the basic linker connecting the domains of TBP, strand S1, and the majority of helix H1′, as demonstrated by crystallography (Nikolov et al., 1992). Presumably the DNA between the two factors (AP-1 and TBP) can loop out, thereby allowing direct interaction between the two protein binding sites. Clearly, our results demonstrate that the basic region not only facilitates DNA-binding to an AP-1 site but can also function as a dimerization interface for protein–protein interactions, specifically with TBP. Interestingly, this region has not been targeted by previous analyses to be a transactivation domain (Angel et al., 1989; Baichwal and Tjian, 1990; Abate et al., 1991).

We have not addressed the exact composition of the interacting components in the Fos-Jun-TBP complex. Jun homodimers or Fos-Jun heterodimers may interact with TBP, which of course is also associated with its own set of TAFs. In the cell, the presence of other AP-1 family members must be considered. Thus the question arises whether the Jun-Fos heterodimer can form a complex of higher order with TBP. The experiments conducted so far cannot distinguish between TBP binding individually to c-Fos or to Jun and TBP binding to the heterodimer (this work and L. Ransone, unpublished results). The complexity increases when one considers the other components involved in the initiation complex. To accommodate all of these interactions, the assortment of proteins that exist in complex with TBP must be constantly changing. Consistent with this is the observation that TBP exists in HeLa cell extracts in at least two multi-protein complexes with distinct biochemical properties (Timmers and Sharp, 1991). The in vitro binding studies presented here are just beginning to address the complex interactions between Fos and Jun and a member of the general transcription machinery.

Results from several groups have demonstrated that there are multiple regions in the c-Jun protein that participate in transcriptional activation in an interdependent manner (Angel et al., 1989; Baichwal and Tjian, 1990; Abate et al., 1991). Although transactivation by c-Fos is dependent on Jun protein, it has also been

shown to contain transactivation domains (Angel et al., 1989; R. Ofir and V. J. Dwarki, unpublished data). In vitro transcription assays were used by Abate et al. (1991) to determine the transcriptional regulatory domains of a series of truncated c-Fos and c-Jun proteins. They demonstrated that transcriptional stimulation by c-Jun requires an N-terminal domain corresponding to amino acids 90–186. The region rich in proline and glutamine (aa 215–255) does not function in transcriptional stimulation, although it acts as an ancillary DNA-binding domain (Abate et al., 1991). This contrasts with previous work identifying the region (aa 215–255) as an activation domain in vitro (Bohmann and Tjian, 1989). In vivo co-transfection experiments demonstrated that the proline/glutamine-rich region (aa 215–255 in c-Jun) is unable to stimulate transcription (Angel et al., 1989; Hirai et al., 1989; Baichwal et al., 1990; Kelleher et al., 1990), while the N-terminal region defined by Abate et al. (1991) functions in vitro and in vivo. Fos was found to contain two regions which stimulated transcription: (1) an acidic region adjacent to the basic region (aa 116–139), and (2) a region rich in acidic and proline residues C-terminal to the leucine zipper (Abate et al., 1991). Additionally, both c-Fos and c-Jun contain inhibitory regions that have potent effects on transcriptional repression (Baichwal and Tjian, 1990; Abate et al., 1991). Recently, Oehler and Angel (1992) suggested that a common intermediary factor recognizing an "acidic blob" type of domain is required for transcriptional activation by the Jun proteins. It appears then that c-Jun and c-Fos must perform other functions in addition to binding to TBP in order to activate transcription. We propose that multiple factors, multiple domains, and possibly multiple pathways are all involved in the AP-1-activated initiation of transcription.

Acknowledgments

We thank Dr. Arnie Berk for the TBP constructs and Pat McClintock for help in the preparation of the manuscript.

This work was supported by funds from the National Institutes of Health, American Cancer Society, the H. N. and Frances C. Berger Foundation, and the Council for Tobacco Research USA, Inc. to I. M. Verma.

L. J. Ransone was supported by a Leukemia Society of America Special Fellowship, and L. D. Kerr by the Cancer Research Institute/Janet Maureen Grace Fellowship. I. M. Verma is an American Cancer Society Professor of Molecular Biology.

The costs of publishing this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC Section 1734 solely to indicate this fact.

References

C. Abate, D. Luk, E. Gagne, R. G. Roeder, and T. Curran (1990), Mol Cell Biol 10, 5532–5535.

C. Abate, D. Luk, and T. Curran (1991), Mol Cell Biol 11, 3624–3632.

P. Angel, M. Imagawa, R. Chiu, B. Stein, R. J. Imbra, H. J. Rahmsdorf, C. Jonat, P. Herrlich, and M. Karin (1987), Cell 49, 729–739.

P. Angel, T. Smeal, J. Meek, and M. Karin (1989), New Biol 1, 35–43.

P. Angel and M. Karin (1991), Biochim Biophys Acta 1072, 129–157.

V. R. Baichwal and R. Tjian (1990), Cell 63, 815–825.

E. Bengal, L. Ransone, R., Scharfmann, V. J. Dwarki, S. J. Tapscott, H. Weintraub, and I. M. Verma (1992), Cell 68, 507–519.

D. Bohmann and R. Tjian (1989), Cell 59, 709–717.

S. Buratowski, S. Hahn, P. A. Sharp, and L. Guarente (1989), Nature 334, 37–42.

R. Chiu, W. J. Boyle, J. Meek, T. Smeal, T. Hunter, and M. Karin (1988), Cell 54, 541–552.

D. R. Cohen and T. Curran (1988), Mol Cell Biol 8, 2063–2069.

D. R. Cohen, P. C. Ferreira, R. Gentz, B. R. Franza, Jr., and T. Curran (1989), Genes Dev 3, 173–184.

P. Cortes, O. Flores, and D. Reinberg (1992), Mol Cell Biol 12, 413–421.

T. Curran, C. Van Beveren, N. Ling, and I. M. Verma (1985), Mol Cell Biol 5, 167–172.

R. L. Davis, P.-F. Cheng, A. B. Lassar, and H. Weintraub (1990), Cell 60, 733–746.

P. DeTogni, H. Niman, V. Raymond, P. Sawchenko, and I. M. Verma (1988), Mol Cell Biol 8, 2251–2256.

B. D. Dynlacht, T. Hoey, and R. Tjian (1991), Cell 66, 563–576.

R. Gentz, F. J. Rauscher III, C. Abate, and T. Curran (1989), Science 243, 1695–1699.

T. D. Halazonetis, K. Georgopoulous, M. E. Greenberg, and P. Leder (1988), Cell 55, 917–924.

S. I. Hirai, R. P. Ryseck, F. Mechta, R. Bravo, and M. Yaniv (1989), EMBO J 8, 1433–1439.

A. Hoffman, E. Sinn, T. Yamamoto, J. Wang, A. L. Roy, M. Horikoshi, and R. G. Roeder (1990), Nature 346, 387–390.

M. Horikoshi, K. Maguire, A. Kralli, E. Maldonado, D. Reinberg, and R. Weinmann (1991), Proc Natl Acad Sci USA 88, 5124–5128.

J. A. Inostroza, F. H. Mermelstein, I. Ha, W. S. Lane, and D. Reinberg (1992), Cell 70, 477–489.
P. F. Johnson and S. L. McKnight (1989), Annu Rev Biochem 58, 799–829.
R. J. Kelleher III, P. M. Flanagan, and R. D. Kornberg (1990), Cell 61, 1209–1215.
T. Kouzarides and E. Ziff (1988), Nature 336, 646–651.
T. Kouzarides and E. Ziff (1989), Nature 340, 568–571.
K. Kovary and R. Bravo (1992), Mol Cell Biol 12, 5015–5023.
W. H. Landschultz, P. F. Johnson, and S. L. McKnight (1988), Science 240, 1759–1764.
W. Lee, P. Mitchell, and R. Tjian (1987a), Cell 49, 741–752.
W. Lee, A. Haslinger, M. Karin, and R. Tjian (1987b), Nature 325, 368–372.
W. S. Lee, C. C. Kao, G. O. Bryant, X. Liu, and A. J. Berk (1991), Cell 67, 365–376.
P. M. Lieberman and A. J. Berk (1991), Genes Dev 5, 2441–2454.
E. Maldonado, I. Ha, P. Cortes, L. Weiss, and D. Reinberg (1990), Mol Cell Biol 10, 6335–6347.
T. Maniatis, S. Goodbourn, and J. A. Fischer (1987), Science 236, 1237–1245.
M. Meisterernst and R. G. Roeder (1991), Cell 67, 557–567.
P. Mitchell and R. Tjian (1989), Science 245, 371–378.
Y. Nakabeppu and D. Nathans (1991), Cell 64, 751–759.
Y. Nakabeppu, K. Ryder, and D. Nathans (1988), Cell 55, 907–915.
N. Nakajima, M. Horikoshi, and R. G. Roeder (1988), Mol Cell Biol 8, 4028–4040.
M. Neuberg, M. Schuermann, J. B. Hunter, and R. Mueller (1989), Nature 334, 314–319.
D. B. Nikolov, S.-H. Hu, J. Lin, A. Gasch, A. Hoffman, M. Horikoshi, N.-H. Chua, R. G. Roeder, and S. K. Burley (1992), Nature 360, 40–46.
H. Nishina, H. Sato, T. Suzuki, M. Sata, and H. Iba (1990), Proc Natl Acad Sci USA 87, 3619–3623.
T. Oehler and P. Angel (1992), Mol Cell Biol 12, 5508–5515.
M. G. Peterson, N. Tanese, P. F. Pugh, and R. Tjian (1990), Science 248, 1625–1630.
M. Ptashne (1988), Nature 335, 683–689.
M. Ptashne and A. F. Gann (1990), Nature 346, 329–331.
B. F. Pugh and R. Tjian (1992), J Biol Chem 267, 679–682.
J. A. Ranish, W. S. Lane, and S. Hahn (1992), Science 255, 1127–1129.
L. J. Ransone, J. Visvader, P. Sassone-Corsi, and I. M. Verma (1989), Genes Dev 3, 770–781.
L. J. Ransone, P. Wamsley, K. L. Morley, and I. M. Verma (1990a), Mol Cell Biol 10, 4565–4573.
L. J. Ransone, J. Visvader, P. Wamsley, and I. M. Verma (1990b), Proc Natl Acad Sci USA 87, 3806–3810.
L. J. Ransone and I. M. Verma (1991), Annu Rev Cell Biol 6, 539–557.
F. J. Rauscher III, P. J. Voulalas, B. R. Franza, and T. Curran (1988a), Genes Dev 2, 1687–1699.
F. J. Rauscher III, D. R. Cohen, T. Curran, T. J. Bos, P. K. Vogt, D. Bohmann, R. Tjian, and B. R. Franza, Jr. (1988b), Science 240, 1010–1016.
R. G. Roeder (1991), Trends Biochem Sci 16, 402–408.
R.-P. Ryseck and R. Bravo (1991), Oncogene 6, 533–542.
P. Sassone-Corsi, W. W. Lamph, M. Kamps, and I. M. Verma (1988a), Cell 54, 533–560.
P. Sassone-Corsi, L. J. Ransone, W. W. Lamph, and I. M. Verma (1988b), Nature 336, 692–695.
M. Sawadago and A. Sentenac (1990), Annu Rev Biochem 59, 711–754.
P. A. Sharp (1992), Cell 68, 819–821.
K. F. Stringer, C. J. Ingles, and J. Greenblatt (1990), Nature 345, 783–786.
T. Suzuki, H. Okuno, T. Yoshida, T. Endo, H. Nishina, and H. Iba (1992), Nucleic Acids Res 19, 5537–5542.
H. Th. M. Timmers and P. A. Sharp (1991), Genes Dev 5, 1946–1956.
M. Van Dyke, R. G. Roeder, and M. Sawadogo (1988), Science 241, 1335–1338.
M. Van Dyke, M. Sawadogo, and R. G. Roeder (1989), Mol Cell Biol 9, 342–344.
C. R. Vinson, P. B. Sigler, and S. L. McKnight (1989), Science 246, 911–916.
P. K. Vogt and T. J. Bos (1990), Adv Cancer Res 55, 2–36.
R. Wisdom, J. Yen, D. Rashid, and I. M. Verma (1992), Genes Dev 6, 667–675.
J. Yen, R. Wisdom, I. Tratner, and I. M. Verma (1991), Proc Natl Acad Sci USA 88, 5077–5081.
M. Zerial, L. Toschi, R.-P. Ryseck, M. Schuermann, R. Muller, and R. Bravo (1989), EMBO J 8, 805–813.