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DNA-Binding Activity of Jun Is Increased Through Its Interaction With Fos

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Transcription factor AP-1 mediates induction of a set of genes in response to the phorbol ester tumor promoter TPA. Recently, AP-1 preparations from HeLa cells were shown to contain a product of the c-JUN protooncogene (Jun/AP-1) which forms a tight complex with the Fos protein. In this paper, we examine the role of the Fos protein in the DNA-binding activity of the AP-1 complex. We show that the DNA-binding activity of bacterially expressed trpE-Jun fusion proteins is increased many -fold upon their interaction with Fos (or a Fos-related antigen) expressed from a baculovirus vector. The site of Fos interaction is within the DNA-binding domain of Jun/AP-1, and anti-Fos antibodies interfere with the binding of affinity purified AP-1 to DNA. These results suggest that, by associating with Jun/AP-1, Fos is responsible for the formation of a multimeric protein complex that has greater affinity for the target sequence than does Jun/AP-1 alone.

Key words: transcription factor, activator protein-1, protooncogene, TPA, AP-1

Transcriptional activator AP-1 is responsible for induction of a number of genes in response to phorbol ester tumor promoters such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) [1,2]. TPA treatment causes a rapid increase in the DNA-binding activity of AP-1 [1,3]. A major constituent of purified AP-1 is the product of the c-JUN protooncogene [4–6]. This constituent is referred to as Jun/AP-1; "AP-1" describes the affinity-purified protein complex (see below). The viral counterpart of c-JUN, oncogene v-JUN [7], encodes a similar protein whose DNA-binding and transcriptional stimulatory activities are essentially identical to those of Jun/AP-1 [5,6,8,9]. A relationship between Jun/AP-1 and another protooncoprotein, Fos, was first indicated by identification of a DNA sequence to which Fos-containing complexes bound [10] and the observation that Fos-binding sequences conformed to the consensus binding site of AP-1, also known as TPA regulatory element (TRE) [11]. Direct physical interaction between these proteins was demonstrated by finding a protein complex containing Jun/AP-1 and

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Fos [6,12]. Whereas Jun/AP-1 appears to be primarily responsible for binding of this complex to DNA in a sequence-specific manner and is capable of activating transcription in the absence of Fos, the interaction with Fos potentiates its activity [6]. The interaction between Fos and Jun/AP-1 seems to be important for induction of AP-1 responsive target genes by TPA and transforming oncogene products such as Ras and Src [13]. To investigate further the mechanism of gene induction by TPA and transforming oncoproteins, we determined the region of the Jun/AP-1 molecule that interacts with Fos and examined the effect of Fos and anti-Fos antibodies on the activity of Jun/AP-1 in vitro. The results of these experiments indicate that the Fos protein is an important constituent of the protein complex originally described as AP-1. It is involved in the formation of a multimeric complex having greater affinity for the target sequence than Jun/AP-1 alone. While this manuscript was in preparation, several reports were published [14–18] that also indicate that Fos enhances the DNA-binding ability of Jun/AP-1. However, these papers did not directly address the role of Fos in the generation of the native AP-1 complex. Using antibody inhibition experiments, we show that Fos is an important component of the AP-1 complex purified from HeLa cells required for optimal DNA-binding activity.

MATERIALS AND METHODS

Cells and Transfections

F9 cells were grown and transfected as previously described [6]. Each 10 cm culture dish was incubated for 8 h with a precipitate containing 8 μg pSV-cFos and, when used, 8 μg of c-Jun, v-Jun, cJun/MUT, or vJun/DBD expression vectors. All procedures related to the baculovirus expression system were as described by Summers and Smith [19].

Cell Labeling and Immunoprecipitations

F9 cells were preincubated in methionine- and cysteine-free DME containing 1% dialyzed fetal calf serum (FCS) for 30–60 min and then labeled in fresh methionine- and cysteine-free DME (1% dialyzed FCS) with ^{35}S-Translabel (150 μCi/ml, ICN) for 60 min. Cells were then harvested, after washing with cold phosphate-buffered saline (PBS), in 1 ml of ice-cold RIPA buffer [6]. Cell lysates were incubated with preimmune rabbit serum and cleared with formalin fixed *Staphylococcus aureus* cells (Boehringer) and protein A-Sepharose by centrifugation at 14,000*g* for 10 min at 4°C and then diluted in RIPA buffer. The Jun proteins and the Jun/Fos complexes were immunoprecipitated using either anti-vJun antibodies [6] or anti-Fos antibodies provided by Dennis Slamon and Larry Souza (unpublished data). Immunocomplexes were washed with 1 ml ice-cold RIPA buffer followed by 1 ml ice-cold 0.5 M LiCl, 0.1 M Tris, pH 8.0, and then 1 ml of RIPA buffer. The immunocomplexes were resolved on 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Labeled proteins were visualized by fluorography of gels impregnated with DMSO/PPO. Gels were exposed to preflashed X-ray film at −70°C.

Preparation and Immunoblotting of Insect Cell Extracts

Spodoptera frugiperda (Sf9) cells (3×10^6) were seeded into each of three 60 mm tissue culture plates. One was mock infected; one was infected with wild-type baculovirus, *Autographa californica* nuclear polyhedrosis virus (AcNPV); and the third was

infected with recombinant c-Fos expressing baculovirus [19] (S. Agarwal, T. Curran, and T. Roberts, manuscript in preparation). All infections were at a multiplicity of infection of 10. At 40 h after infection, the cells were washed once in PBS and then lysed in 50 mM Hepes, pH 7.5, 100 mM sodium chloride, 1% nonidet P-40 (NP-40) containing 2 mM phenylmethylsulfonyl fluoride, 0.2 trypsin inhibitor units/ml of aprotinin, 0.02 mM leupeptin, 0.5 μg/ml pepstatin A, and 1 mM sodium vanadate. Protein concentrations were measured using the BioRad Laboratories protein assay kit. One hundred micrograms of total protein of each sample was resolved by SDS-PAGE and immunoblotted essentially as described previously [20]. Affinity-purified antibodies against the M-peptide of mouse c-Fos [21] were used at a dilution of 1:1,000. Protocols and reagents of the protoblot alkaline phosphatase immunoblotting system (Promega Biotech.) were used.

Protein Extracts and Antisera

AP-1 was purified from whole cell extracts of HeLa S3 cells as described previously [1]. TrpE-vJun and trpE-cJun fusion proteins were expressed in *Escherichia coli,* semipurified, and renatured as described [5]. TrpE-vJun was further purified after renaturation by heparin-agarose chromatography [1]. This preparation was approximately 80–90% pure. Fos was produced in cultured insect cells by infection with a baculovirus vector containing the c-FOS gene (S. Agarwahl, T. Curran, and T.M. Roberts, manuscript in preparation) as described above. It is estimated that 1 μl of extract of these cells contains approximately 1–5 ng of Fos protein as determined by immunoblotting with anti-Fos antisera (Roman Herrera and S. Agarwahl, unpublished results). Anti-Fos antibodies obtained from Dennis Slamon and Larry Souza were directed against the C-terminal two-thirds of the Fos protein produced as a fusion protein (unpublished results). Anti-PEP1 and anti-PEP2 were obtained from T. Bos and P. Vogt and were raised as described [4]. Anti-Jun antisera was raised against the trpE-cJun fusion protein [6].

DNA-Binding Assays

The gel retardation assay was performed generally as described [22,23]. End-labeled TRE collagenase probe, a 173 bp EcoRI-PvuI fragment of pTRE-TK CAT [1], was used at 3–6 ng (2,500–5,000 cpm) per reaction, in the presence of 500 ng to 1 μg poly-(dI-dC), and was incubated with protein or protein-antibody mixtures on ice for 15 min and at room temperature for 2 min and then electrophoresed on a prechilled 4% nondenaturing polyacrylamide gel at 4°C.

Footprinting reactions and probes were as previously described [1]. TrpE-vJun and BV-Fos or BV-WT were mixed in approximately equimolar amounts and incubated in 6 M urea at 37°C for 10 min and then allowed to renature by dialysis at 4°C for 2 h. Proteins were incubated with an end-labeled 360 bp hMT-II_A promoter fragment at 4°C. DNaseI was added for 2 min at room temperature.

RESULTS

We have previously shown that Jun/AP-1 and Fos proteins produced by transiently transfected expression vectors in F9 embryonal carcinoma cells associate to form a stable complex [6]. Due to the presence of very low levels of Jun/AP-1 and Fos in

undifferentiated F9 cells, the formation of detectable amounts of the Jun/AP-1:Fos complex in these cells is strictly dependent on cotransfection of Jun/AP-1 and Fos expression vectors [6]. This assay was used to examine whether the v-Jun protein is also capable of this interaction and to determine the region of the protein important for binding to Fos. Viral and cellular Jun proteins, a truncated version of the viral protein containing only its DNA-binding domain (DBD), and a mutant not expressing the DNA-binding domain (cJM) were expressed in F9 cells by transient transfection of the appropriate expression vectors (Fig. 1A). In the absence of a cotransfected Fos expression vector, none of these proteins were immunoprecipitated by anti-Fos antibodies [6] (T. Smeal, unpublished results). However, expression of exogenous Fos protein from a cotransfected pSVcFos vector led to formation of a stable complex between it and each of the Jun proteins except cJM as evidenced by their coprecipitation using anti-Fos antibodies (Fig. 1B). The mutant Jun protein, cJM, truncated by a frame-shift mutation and thereby consisting of only the aminoterminal half of Jun/AP-1 (Fig. 1A), does not interact with Fos, as determined by its ability to be immunoprecipitated with anti-PEP2 [4] but not with anti-Fos antibodies (Fig. 1B). Anti-PEP2 antibodies are directed against an aminoterminal sequence in the Jun protein and are used instead of the anti-Jun antiserum, which does not recognize this portion of the protein. These results with DBD and cJM indicate that the target site for Fos interaction is within the DNA-binding domain of v-Jun, which, except for two amino acid substitutions, is identical to the equivalent domain of Jun/AP-1 [5].

The finding that Fos interacts with the DNA-binding domain of Jun/AP-1 and the previous observation that TPA treatment leads to increased DNA-binding activity of AP-1 [1,3] prompted us to examine whether Fos might potentiate the action of Jun/AP-1 by increasing its DNA-binding activity. Since affinity-purified preparations of AP-1 from HeLa cells contain Fos protein [12] (E.A. Allegretto and J. Meek, unpublished results), we used a bacterially expressed trpE-vJun fusion protein as an alternative source of Jun protein devoid of Fos. This fusion protein is an active DNA-binding protein exhibiting sequence specificity identical to AP-1 [5]. As a source of Fos protein, we used extracts of insect tissue culture cells infected with a recombinant baculovirus vector harboring a c-FOS gene (S. Agarwahl, T. Curran, and T. Roberts, manuscript in preparation). These extracts contain high levels of Fos protein and may also contain Fos-induced Fos-related antigens (Fig. 2, lane 3). As suggested by the DNA-binding assay (Fig. 3A), these extracts, referred to as BV-Fos, do not contain functional "AP-1-like" activity. Extracts of insect cells infected with wild-type baculovirus do not contain detectable amounts of Fos protein (Fig. 2, lane 2) and are referred to as WT extract.

TrpE-vJun fusion protein and AP-1 were incubated with a TRE-containing DNA probe, and their binding was analyzed by gel retardation [22,23]. Two specific complexes of different mobility, C1 and C2, were detected upon incubation with AP-1 and trpE-vJun, respectively (Fig. 3A, lanes 4 and 6). This differential mobility is likely due to the molecular weight difference between the two proteins (AP-1, 40 K; trpE-vJun, 75 K) and/or the charge dissimilarities of the proteins owing to the trpE portion of the vJun fusion protein. Upon addition of BV-Fos to trpE-vJun, the amount of TRE DNA migrating as a protein-DNA complex (C2) was increased by five- to tenfold, as determined by densitometry (Fig. 3A, compare lanes 6 and 8). This effect was not seen when the WT extract is added to trpE-vJun (lane 7). In contrast, when either WT

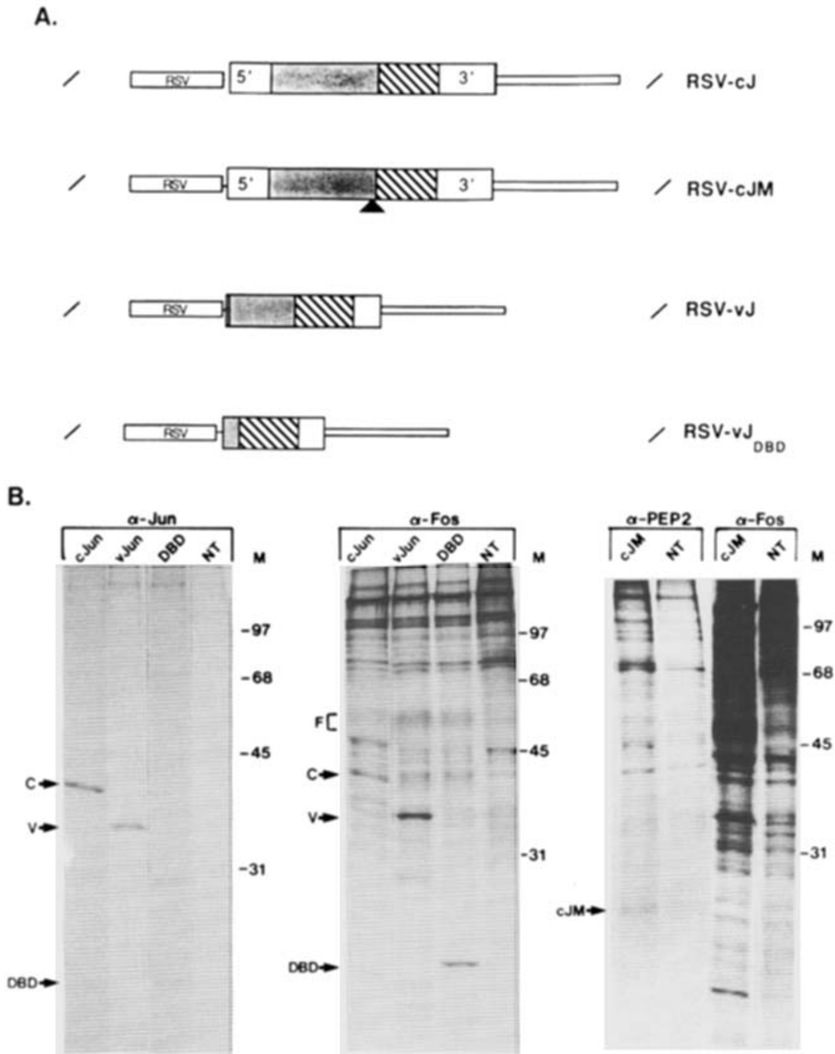

Fig. 1. Complex formation between transiently expressed Jun proteins and Fos in F9 cells. **A:** Maps of Jun expression vectors whose construction is described previously [9]. Hatched bars denote the highly conserved DNA-binding domain of c-Jun and v-Jun proteins and the shaded bars, the remainder of the coding sequence. Open boxes represent 5′ or 3′ untranslated sequences (c-Jun) or viral sequences (v-Jun). The arrowhead indicates the location of a frame shift mutation that destroys the DNA-binding domain. In RSV-vJun/DBD, only the domain sufficient for DNA-binding in vitro [5] is expressed. The sequences containing RNA splicing and polyadenylation signals are located in a 0.85 kb fragment derived from SV40 that is inserted downstream from the Jun sequences (thin bars). **B:** Examination of complex formation between Jun and Fos proteins in F9 cells. F9 cells were cotransfected with the various Jun expression vectors, as indicated at the top of B and pSV-cFos. NT, nontransfected cells. Twelve hours after transfection, cellular proteins were labeled in vivo with ^{35}S-methionine and ^{35}S-cysteine for 1 h, and cellular extracts were prepared. Jun proteins were identified by immunoprecipitation with anti-Jun antibodies, and formation of the Fos:Jun complex was detected by immunoprecipitation with anti-Fos antibodies, as indicated at the top of B. The migration positions of Jun/AP-1 (C), v-Jun (V), v-Jun/DBD (DBD), c-Jun/MUT (cJM), and Fos (F) proteins are indicated. The size of ^{14}C-labeled protein molecular weight markers (M) are as indicated in kilodaltons.

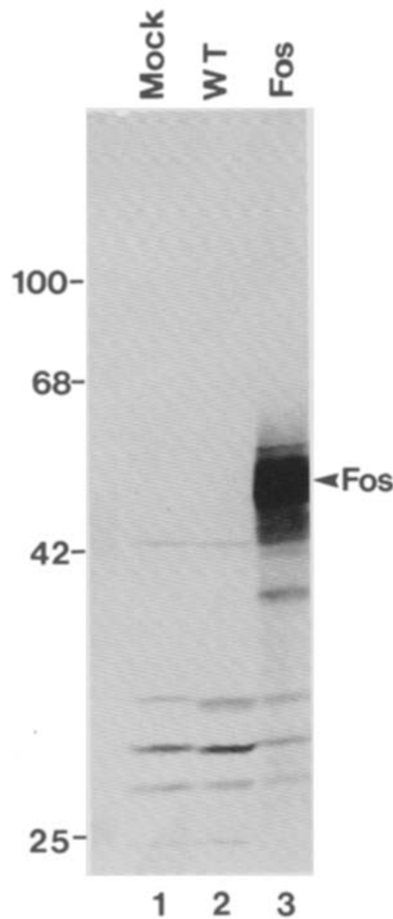

Fig. 2. Overproduction of Fos protein using a baculovirus expression system. Cell lysates were prepared from Sf9 cells, which had been mock infected (lane 1), infected with wild-type baculovirus (lane 2), or infected with c-Fos encoding recombinant baculovirus (lane 3). One hundred micrograms of each lysate were resolved by electrophoresis on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with anti-Fos M-peptide antibodies. The sizes of prestained protein molecular weight markers are indicated on the left in kilodaltons.

extract or BV-Fos is incubated with HeLa cell AP-1, the resultant complexes formed with the DNA are of similar intensity (lanes 4 and 5). Neither of the baculovirus-infected cell extracts exhibited formation of a complex with the TRE with similar mobility to complexes C1 and C2 in the absence of trpE-vJun or AP-1, suggesting that both extracts lack an "AP-1-like" binding activity (lanes 2 and 3). On the other hand, both extracts form a small amount of a complex of faster mobility with the TRE probe (complex NS). However, formation of this complex does not correlate with the presence or absence of Fos, suggesting that the Fos protein does not bind to the TRE probe on its own to any detectable extent.

Formation of the C2 protein-DNA complex is specific because it is inhibited by addition of excess unlabelled TRE-containing DNA fragment (Fig. 3B, lanes 4 and 5), but not by incubation with an excess of a DNA fragment containing a T to G

transversion (Δ-72) in the TRE sequence (lanes 6 and 7). This mutation was previously shown to reduce greatly the binding of AP-1 to this sequence [1,5].

The lack of effect of BV-Fos on the DNA-binding activity of AP-1 is most probably due to the fact that this protein preparation is already saturated with Fos [12] (E.A. Allegretto and J. Meek, unpublished results). The presence of nearly equal amounts of Jun/AP-1 and Fos polypeptides in affinity-purified AP-1 suggested that the active DNA-binding species recognizing the TRE sequence in HeLa cells is the Jun/AP-1:Fos complex. To test this possibility, we incubated affinity-purified AP-1 with anti-Fos or anti-Jun antibodies prior to incubation with the TRE probe. The presence of anti-Fos antibodies led to almost complete inhibition of protein-DNA complex formation, and addition of the anti-Jun antibodies led to substantial but incomplete inhibition (Fig. 3A, lanes 10 and 11). Nonimmune rabbit serum had no effect (lane 9). The incomplete inhibitory activity of the anti-Jun antibodies is consistent with their inability to react with Jun/AP-1 present within the Jun/AP-1:Fos complex [6] and suggests that these antibodies may inhibit binding activity by reacting with the Jun/AP-1 molecules that slowly dissociate from the complex [12]. In addition, the residual binding activity could be due to other members of the JUN family such as Jun-B [14], which are likely to be minor components of the AP-1 complex.

In Figure 4A it can be seen that preincubation of BV-Fos with anti-Fos antibodies greatly reduces the enhancement of trpE-vJun binding to DNA (see lanes 7 and 10). These anti-Fos antibodies may act either by blocking interaction of the trpE-vJun:Fos complex with the DNA or by disallowing Fos or a Fos-related antigen to bind to trpE-vJun. Correspondingly, trpE-vJun is also participating in formation of the retarded protein-DNA complex, because anti-vJun peptide antibodies [4] severely interfere with formation of that complex (lanes 8 and 9). No inhibition was seen upon incubation with nonimmune rabbit serum (data not shown; but see Fig. 3A, lane 9). Therefore, it is apparent from these data that Fos protein (or a Fos-related antigen) has an important stimulatory role in the interaction of the trpE-vJun fusion protein with the TRE sequence.

Titration experiments show that addition of increasing amounts of BV-Fos to a constant amount of trpE-vJun results in a linear increase in formation of the protein-DNA complex, C2 (Fig. 4A, lanes 1–4). Densitometric quantitation of the complex indicates five- to sixfold enhancement of trpE-vJun binding to DNA upon addition of nearly saturating amounts of BV-Fos (data not shown). We estimate that, under these conditions, formation of the complex requires similar amounts of trpE-vJun and Fos (approximately 8 and 5 ng, respectively). Order-of-addition experiments indicate that less protein-DNA complex is formed if trpE-vJun is preincubated with the TRE probe prior to addition of BV-Fos. Preincubation of the TRE probe with BV-Fos followed by addition of trpE-vJun was as efficient in forming the complex as preincubation of the two proteins before addition of the DNA probe (lanes 5–7).

We also tested the ability of BV-Fos to enhance the DNA-binding activity of a trpE-cJun fusion protein, which contains only the DNA-binding domain of Jun/AP-1 [5]. Figure 4B illustrates that trpE-cJun binds to the TRE (lane 4) and forms a complex (C3) that migrates faster than the trpE-vJun:DNA complex (C2; lane 2). BV-Fos is shown to enhance binding of both trpE-vJun (lane 3) and trpE-cJun (lane 5) to the TRE. These data lend further credence to the previous observation that Fos interacts with the DNA-binding domain of Jun/AP-1 (see Fig. 1).

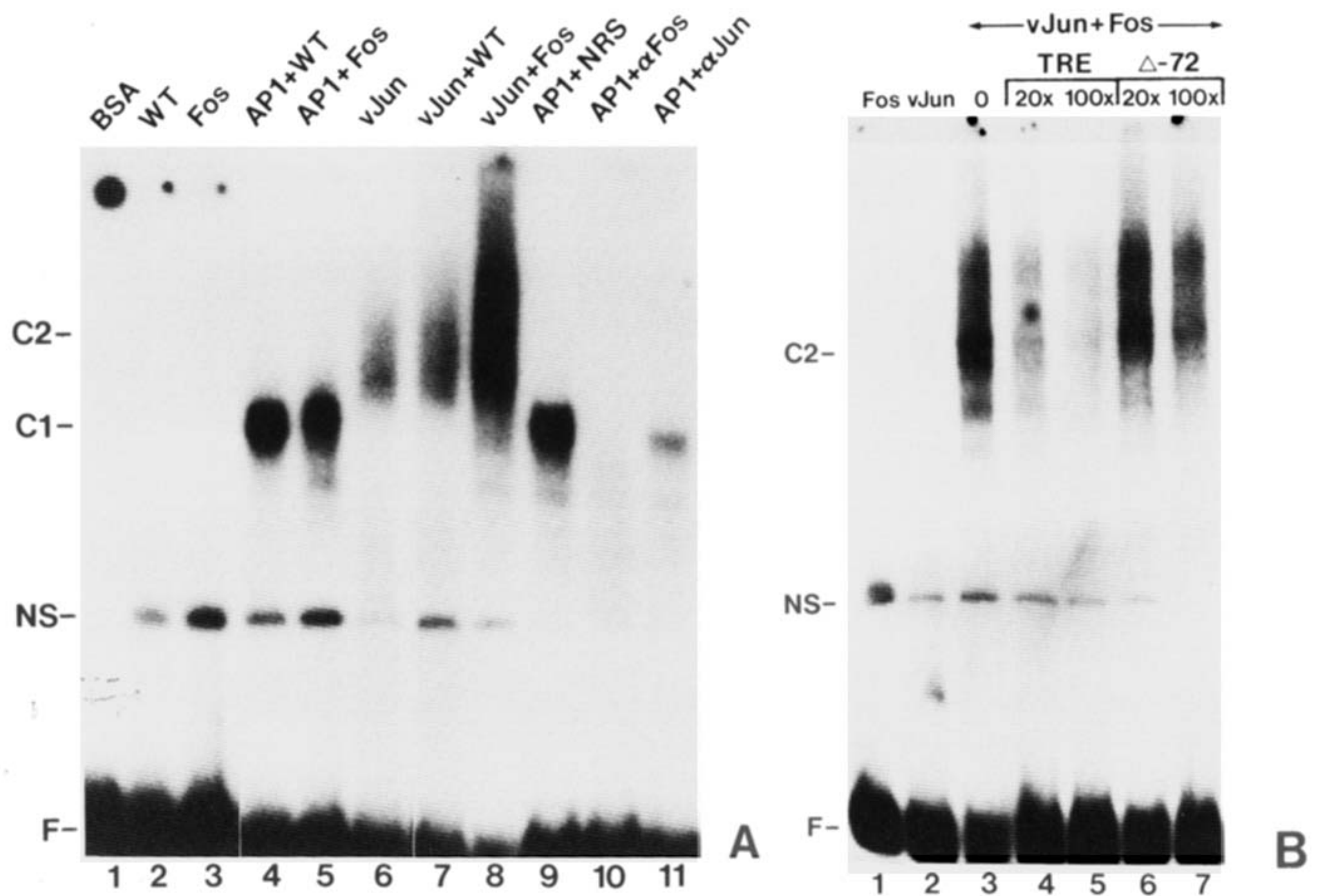

Figure 3

The enhancement of trpE-vJun binding to the TRE can also be seen by DNaseI footprinting experiments. Figure 5A shows complete protection of the hMT-II_A TRE by AP-1 purified from HeLa cells (lane 2). Addition of BV-Fos does not affect the binding of AP-1 (not shown), but, upon denaturation and renaturation of BV-Fos mixed with trpE-vJun, an enhancement of protection of the same DNA site is exhibited (lane 4) compared to that observed using the same amount of denatured and renatured trpE-vJun mixed with wild-type extact (lane 5). BV-Fos in the absence of trpE-vJun does not protect the TRE (lane 3). This denaturation/renaturation procedure was necessary for footprinting but not for gel retardation assays. This finding may indicate that a tighter complex is formed upon denaturation/renaturation of the two proteins, which is necessary to withstand treatment with DNaseI.

The association of Fos with affinity-purified AP-1 is also demonstrated in Figure 5B by the fact that preincubation with anti-Fos antibodies greatly reduces protection of the hMT-II_A TRE by AP-1 (compare lanes 2 and 3). This is likely to be caused by steric hindrance produced by binding of the antibodies to the Jun/AP-1:Fos complex and probably not to the dissociation of the complex because these antibodies do not affect the stability of the complex [6]. No inhibition was observed upon incubation with nonimmune rabbit serum (data not shown).

DISCUSSION

The experiments described above demonstrate that Fos or an immunologically cross-reactive protein thereof is an important component of the DNA-binding activity known as AP-1. Although AP-1 was originally thought to represent a single protein species [24], purification of AP-1 from HeLa cell extracts indicated that even highly purified preparations of this factor contained several polypeptide species [1,2]. Now it is known that two of these polypeptides correspond to the primary translation product of the c-JUN gene, Jun/AP-1, and a proteolytic cleavage product thereof [4–6]. Renaturation experiments indicated that these polypeptides exhibit sequence-specific DNA-binding activity. In these experiments, only a fraction of the DNA-binding activity of the AP-1 excised from the gel was recovered, which was attributed to its incomplete

Fig. 3. In vitro interactions between Jun proteins and Fos. **A:** An AP-1 specific collagenase-TRE probe (1, approximately 6 ng) was incubated with:Bovine serum albumin (BSA) (lane 1, 1 μg), extract of WT-BV infected cells (lane 2, WT, 1 μl), extract of BV-Fos infected cells (lane 3, FOS, 1 μl containing approximately 5 ng Fos), affinity-purified AP-1 and WT-BV extract (1 ng and 1 μl, respectively, lane 4), AP-1 and BV-Fos (1 ng and 1 μl, respectively, lane 5), trpE-vJun (8 ng, lane 6), trpE-vJun and WT-BV extract (8 ng and 1 μl, respectively, lane 7), trpE-vJun and BV-Fos (8 ng and 1 μl, respectively, lane 8), AP-1 preincubated with nonimmune rabbit serum (lane 9), AP-1 preincubated with anti-Fos antibodies (lane 10) and AP-1 preincubated with anti-Jun antibodies (lane 11). Coincubation of trpE-vJun AP-1 with WT extract and BV-Fos was for 10 min on ice and preincubation with antisera was for 2 h on ice prior to addition of the TRE probe. Protein-DNA complexes (C1, C2, and NS) were resolved from free DNA (F) by electrophoresis at 4°C on a 4% polyacrylamide gel. C1 and C2 denote the AP-1 and trpE-vJun specific protein-DNA complexes, respectively, and NS indicates a protein-DNA complex formed by an endogenous insect cell protein. **B:** An AP-1-specific collagenase-TRE probe (approximately 4 ng) was incubated with BV-Fos (1 μl containing 5 ng Fos, lane 1), trpE-vJun (8 ng, lane 2), and trpE-vJun and BV-FOS extract (8 ng and 1 μl, respectively) without competitor DNA (lane 3), in the presence of a 20-fold excess (lane 4) or a 100-fold excess (lane 5) of unlabeled TRE fragment or with a 20-fold excess (lane 6) or a 100-fold excess (lane 7) of unlabeled Δ-72 mutant TRE fragment. Incubation conditions and electrophoresis was as in A. TrpE-vJun (lane 2) exhibits binding upon longer exposure (see A, lane 6).

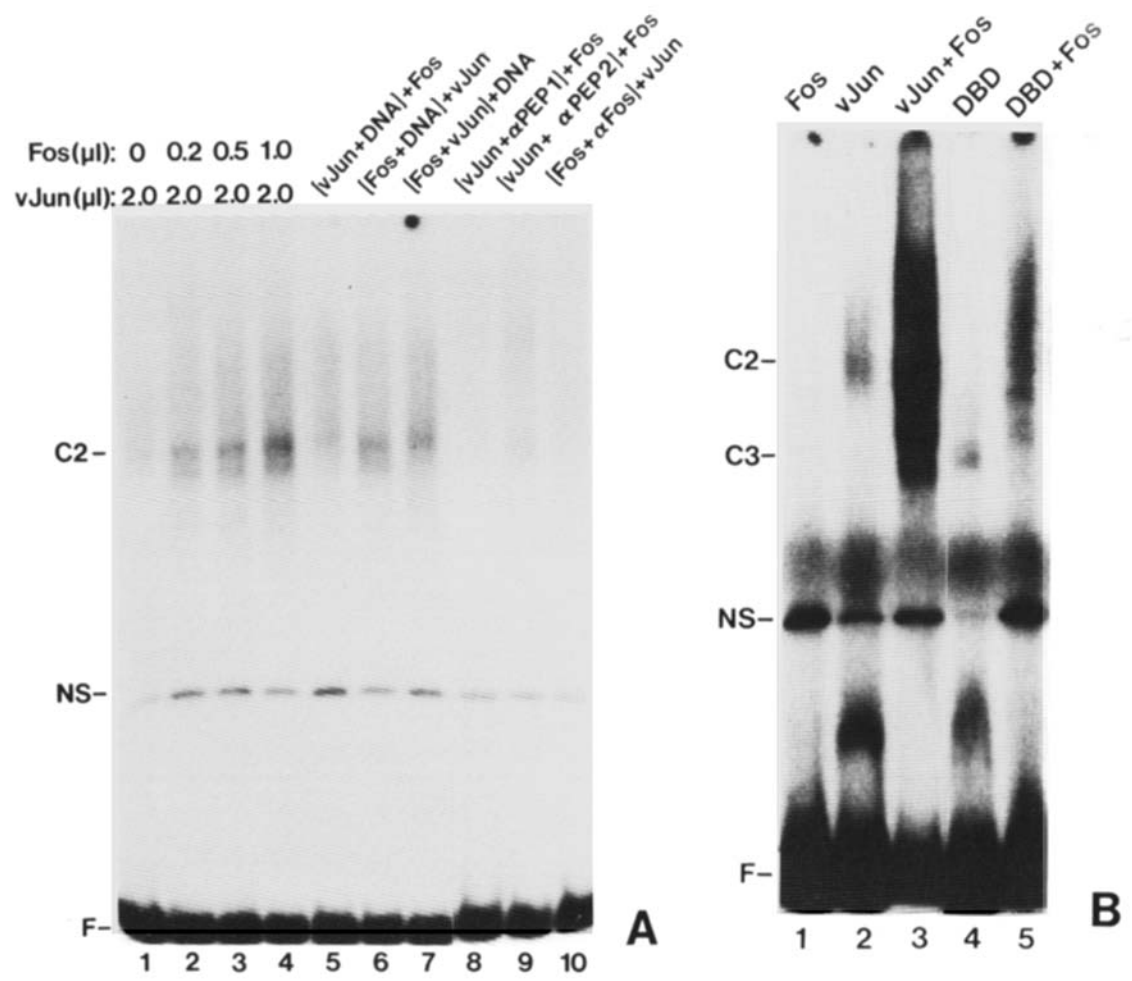

Figure 4

renaturation [2]. Another component of affinity-purified preparations of AP-1 is the Fos gene product, which forms a tight complex with Jun/AP-1 [6,12]. Although Fos copurifies with Jun via AP-1-specific affinity columns [12] (E. Allegretto and J. Meek, unpublished results), it does not exhibit sequence-specific DNA-binding activity of its own [12] (see Figs. 3–5). We show here that a dramatic decrease in the DNA-binding activity of HeLa AP-1 occurs upon incubation with anti-Fos antibodies and that addition of Fos to this AP-1 does not further increase its DNA-binding ability. These data argue that Fos is an important component of the active DNA-binding species known as AP-1, which, as is discussed below, is most likely a Jun-Fos complex. This finding is interesting, and further work will involve determination of the existence and distribution of Jun-Fos heterodimers and Jun or Fos monomers and/or homodimers in the cell and their significance in the signal transduction pathway.

Although the present and previous [10,11,14–18] results strongly suggest that Fos is part of the active AP-1 DNA-binding complex, it is still formally possible that a Fos-related antigen induced by transient expression of Fos in F9 cells and insect cells is the protein that interacts with v-Jun and Jun/AP-1. We consider this possibility unlikely since immunoprecipitation analysis failed to show the appearance of a Fos-inducible, Fos-related antigen in transiently transfected F9 cells [6]. Although there are some cross-reactive bands migrating faster than the intact Fos protein in recombinant c-FOS encoding baculovirus-infected cell extracts (Fig. 2), they are present in much lower levels than Fos and most likely represent Fos degradation products. Some of these degradation products can presumably participate in complex formation and may account for the heterogenous mobility of the Fos:Jun:DNA complexes. Another factor that is likely to contribute to the formation of heterogenous protein-DNA complexes is the heterogenous nature of the baculovirus expressed Fos protein (Fig. 2). This heterogeneity could be due to extensive posttranslational modification, as was shown previously for the mammalian expressed Fos protein [25].

The dyad symmetry of the AP-1 consensus sequence, which is essentially identical to the recognition sequence of the yeast transcriptional activator GCN4 [1,2,4], and the sequence similarity between the DNA-binding domains of GCN4, v-Jun, and Jun/AP-1 [5,7], suggest that, like GCN4 [26], the Jun proteins may bind to DNA as dimers. Indeed, recent experiments indicate that this is the case [14,15,27–30]. As with GCN4, dimerization of the Jun proteins could be brought about by an interaction between their DNA-binding domains. Interestingly, these domains contain the "leucine-zipper" motif,

Fig. 4. Fos increases the ability of Jun fusion proteins to bind to the TRE. **A:** Lanes 1–4: TrpE-vJun (approximately 8 ng) was incubated for 15 min on ice with increasing amounts of BV-Fos (Fos protein content was from 0 to approximately 5 ng), and then 6 ng of ^{32}P-end-labeled TRE probe was added. Incubation of the proteins with the DNA was for 15 min on ice and then 2 min at room temperature. Lanes 5–7: TrpE-vJun (lane 5) and BV-Fos (lane 6) were first incubated with the TRE probe for 15 min on ice prior to the addition of BV-Fos (lane 5) or trpE-vJun (lane 6). In lane 7, BV-Fos and trpE-vJun were incubated together for 15 min on ice prior to the addition of the probe, which was followed by 2 min incubation at room temperature. Lanes 8–10: TrpE-vJun was preincubated with anti-PEP1 (lane 8) or anti-PEP2 (lane 9) antibodies for 2 h on ice prior to the addition of BV-Fos and DNA. BV-Fos was preincubated on ice for 2 h with anti-Fos antibodies prior to addition of trpE-vJun and DNA (lane 10). **B:** BV-Fos (1 μl, lane 1), trpE-vJun (8 ng, lane 2), BV-Fos and trpE-vJun mixture (1 μl and 8 ng, respectively, lane 3), trpE-cJun DNA-binding domain fusion protein (DBD) (5 ng, lane 4), or DBD and BV-Fos mixture (5 ng and 1 μl, respectively, lane 5) was incubated with end-labeled TRE probe and electrophoresed as described for Figure 3. C2 and C3 denote the trpE-vJun and trpE-cJun/DBD protein-DNA complexes, respectively.

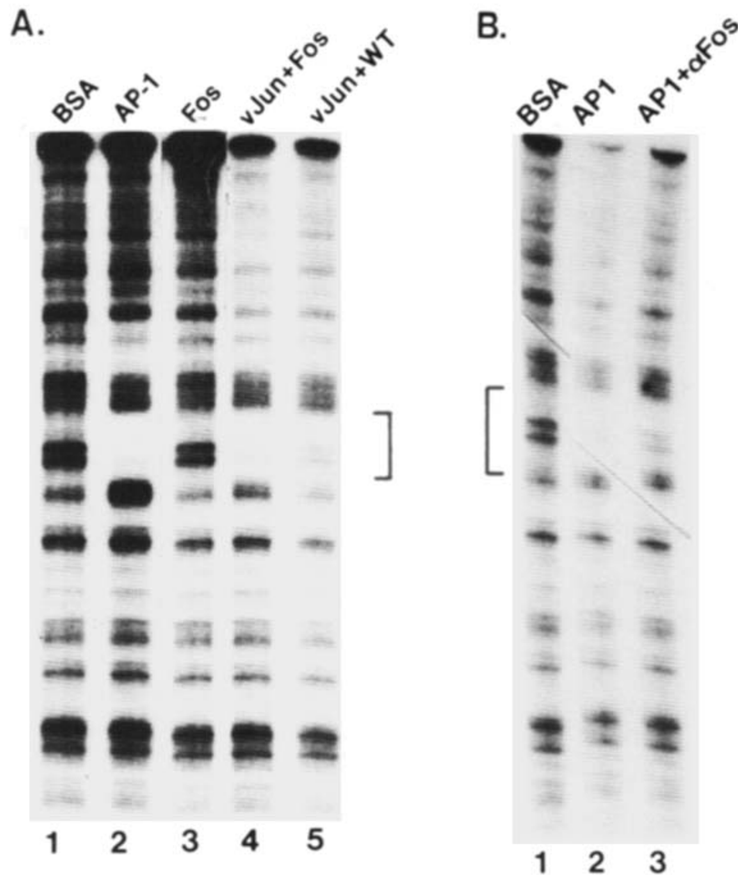

Fig. 5. DNaseI footprinting analysis. **A:** BSA (lane 1, 25 μg), AP-1 (lane 2, 4 ng), BV-Fos (lane 3, 4 μl extract containing approximately 20 ng Fos), trpE-vJun plus BV-Fos (lane 4, approximately 7 ng trpE-vJun and 2 μl of BV-Fos), or trpE-vJun plus WT extract (lane 5, approximately 7 ng trpE-vJun and 2 μl WT extract) was incubated on ice for 15 min in the presence of ^{32}P-labeled hMT-II$_A$ promoter probe and treated with DNaseI for 2 min. The resulting DNA fragments were electrophoresed on a 8% polyacrylamide sequencing gel. **B:** BSA (lane 1), AP-1 (lane 2), or AP-1 preincubated with anti-Fos antibodies (lane 3) as incubated with hMT-II$_A$ probe and treated with DNaseI as described for A.

which is thought to play a role in protein dimerization [31]. Fos itself was found to have a "leucine-zipper" motif [31], and mutations that destroy this motif were shown to interfere with its ability to interact with Jun [16] and transactivate a TRE-CAT reporter gene [32]. This activity is mediated by the interaction of Fos with Jun/AP-1 [6]. It has been established that Fos seems to interact with the Jun proteins to form heterodimers with a higher level of DNA-binding activity than the Jun homodimers [14,15,27–30]. Our data are consistent with this body of evidence. The addition of the Fos extract to the truncated trpE-cJun protein (DBD) in a 1:1 molar ratio results in formation of a slower migrating protein-DNA complex. The mobility of the protein-DNA complex formed by the larger trpE-vJun is not dramatically affected by addition of BV-Fos, which makes sense if the mobility of a trpE-vJun:Fos heterodimer is not significantly different from that of a trpE-vJun homodimer, whereas the trpE-cJunDBD homodimer migrates faster

than the trpE-cJunDBD:Fos heterodimer. Whether Fos is directly involved in the contact with DNA is not known conclusively; however, experiments cross linking Fos to the TRE sequence [10] and the inhibition of AP-1 DNA-binding by anti-Fos antibodies provide evidence in its favor. Gentz et al. [29] report that basic regions of both Jun and Fos are necessary for association with DNA. Regardless of the exact nature of the interaction between Jun, Fos, and DNA, the in vitro findings reported here are of biological significance and explain previous observations made in vivo. For instance, it has been shown that treatment of cells with TPA leads to a rapid increase in the DNA-binding activity of AP-1 [1,3]. This effect does not depend on de novo synthesis of Jun/AP-1 and instead could be mediated by increased formation of a Jun/AP-1:Fos complex [9]. Accordingly, it was shown that cotransfection with an antisense FOS construct that blocks Fos synthesis prevents the induction of a TRE-CAT reporter gene by TPA [13]. In addition, the interaction with Fos increases the transcriptional stimulatory activity of both Jun/AP-1 and v-Jun [6]. Interestingly, the magnitude of this effect (four- to sixfold) is very similar to the increase in the DNA-binding activity of Jun proteins seen here in vitro upon interaction with Fos (five- to tenfold) and the in vivo increase in AP-1 binding activity after TPA treatment (fourfold [1]). This suggests that the increased binding activity of the Fos:Jun complex to DNA may be responsible for these in vivo effects. Other workers [14–18] have used in vitro transcription and translation to produce Jun and Fos protein for use in gel retardation assays and reported Fos enhancement of Jun DNA binding to be as high as 1,000-fold [17]. This clearly does not agree with our data and may be a function of the difference in the systems used. Since the other workers were unable to detect a band shift with Jun protein alone, it may have been difficult to gauge the -fold enhancement upon addition of Fos. The actual difference using this in vitro transcription/translation system may be closer to 25–30-fold, as determined by titrations with known amounts of protein and DNA [15,18]. Additionally, we have used Jun fusion proteins produced in *E. coli,* which are not posttranslationally modified, and thus may be more efficient DNA binders than the Jun protein produced in rabbit reticulocytes.

Although interaction between two nuclear factors to form a more active DNA-binding species is not without precedence [33–35], the interaction between Jun/AP-1 and Fos is likely to be of great importance in the control of cellular proliferation and tumor promotion. Thus the availability of an in vitro system for studying this interaction should now allow the examination of the effect that various protein kinases and phosphatases involved in signal transduction [36] have on this interaction.

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