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Products of the *fos* and *jun* Proto-Oncogenes Bind Cooperatively to the AP1 DNA Recognition Sequence

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The products of the proto-oncogenes c-*fos* and c-*jun* form a tight protein complex that is a major component of the transcription factor AP1. To analyze the role of fos in the binding of this complex to the AP1 DNA recognition sequence and the mechanism of interaction in further detail, we have expressed a fos protein in *E. coli* using an expression vector containing the temperature-inducible λP_L promoter and a synthetic translational start codon. The fos protein encoded by this construct (termed Baf) was enriched by biochemical purification techniques and was found to form a specific complex with c-*jun* obtained by *in vitro* transcription/translation. As shown in gel retardation assays, the baf/jun complex binds to the AP1 DNA recognition sequence with high affinity, while no significant binding was observed with either of the individual protein components, indicating cooperative DNA binding of the two proteins. The fact that the bacterial baf protein does not undergo glycosylation indicates that the post-translational modification of eukaryotic c-*fos* with *N*-acetylglucosamine is not required for the formation of a stable fos/jun/DNA complex.

Introduction

The nuclear product (c-Fos) of the proto-oncogene c-*fos* has been implicated in a variety of biological processes including growth control, differentiation, and signal transduction in neurons (*1–3*). These multiple roles of c-Fos seem to be cell-type specific and may thus be due to the transregulation of defined sets of genes in specific cell types. A great number of results obtained within the last 2 years has provided overwhelming evidence that Fos indeed plays a pivotal role in transcriptional regulation (*4*). Based on the observation that c-Fos is present in a transcription complex bound to the promoter of the gene encoding the adipocyte protein -2 (aP2) (*5*), it could be demonstrated that the Fos protein complex binds to the DNA recognition sequence (TRE) of the transcription factor AP1 (*6–11*). AP1 had previously been identified by virtue of its sequence-specific interaction with the SV40 early promoter region and its inducibility by the tumor promoter TPA (*12–14*). Interestingly, a major component of AP1 is highly related to and probably identical to the product of the proto-oncogene c-*jun* (*15,16*), pointing to a direct link between the two nuclear proto-oncogene products. In addition, it is now clear that the Fos-associated protein p39, previously detected by virtue of its co-precipitation by Fos antibodies (*17–20*) is identical to AP1 (*8,10,21*). It could also be shown that Fos can transactivate AP1-dependent transcription in transient assays where Fos expression vectors were co-transferred with a chimeric TRE-HSVtK promoter-CAT construct into 3T3, HeLa, or F9 cells (*10,11,22,23*). In addition, Fos can transrepress certain promoters such as those of the c-*fos* and HSP70 genes, but the mechanism of transrepression is unclear (*9,22,24*).

In the present study we have investigated the reconstitution of a Fos/Jun complex *in vitro* to obtain direct evidence for the association of these proteins, to analyze the mechanism of their interaction, and to investigate the role of the complex formation in their binding to the TRE. For this purpose, we expressed Fos in *E. coli* and used a fraction enriched in Fos protein in *in vitro* reconstitution assays. Our results clearly show that the bacterially expressed Fos forms a complex with Jun and that this complex—in contrast to the individual proteins—possesses a high affinity for the TRE. We also show that glycosylation, which is found with cellular Fos but not with Baf protein, is not required for the formation of the Fos/Jun/TRE complex.

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Materials and Methods

Expression of Baf in *E. coli*

E. coli RR1 containing the pbaf plasmid (Fig. 1) were grown in a New Brunswick gyrotory shaker at 30°C in LB-medium supplemented with ampicillin (50 μg/mL). At early log-phase (OD_{600} 0.1–0.3), cultures were transferred for 30 min to a water bath at 42°C followed by 1 hr incubation at 39°C on the shaker. Cells were collected by centrifugation. The resulting pellet was resuspended in 50 mM Tris, pH 8.0, 2 mM EDTA, 0.1 mM DTT, 5% glycerol, 0.1 mM PMSF (buffer A, 3 mL/g cell pellet) and treated with lysozyme (1 mg/mL) for 15 min at 25°C. The cells were disrupted on ice with a sonicator (Branson Sonifier B-12) by three 5-sec bursts using a microprobe. The lysate was centrifuged at 1000*g* for 5 min at 4°C, and the resulting pellet was extracted twice in buffer A (5-mL/g starting material) containing 1 M urea. For each extraction step, cells were homogenized in extraction buffer using a Dounce homogenizer and were left for 45 min at 4°C on a rotating wheel. Insoluble material was sedimented by centrifugation at 4°C in a Beckman JA 20 rotor (14,000 rpm for 10 min). The resulting final pellet was extracted overnight with 5 M urea in buffer A (5 mL/g starting material). After centrifugation, as before, the supernatant was used for further purification of Baf. The presence of Fos in the various extraction steps was determined by immunoblotting.

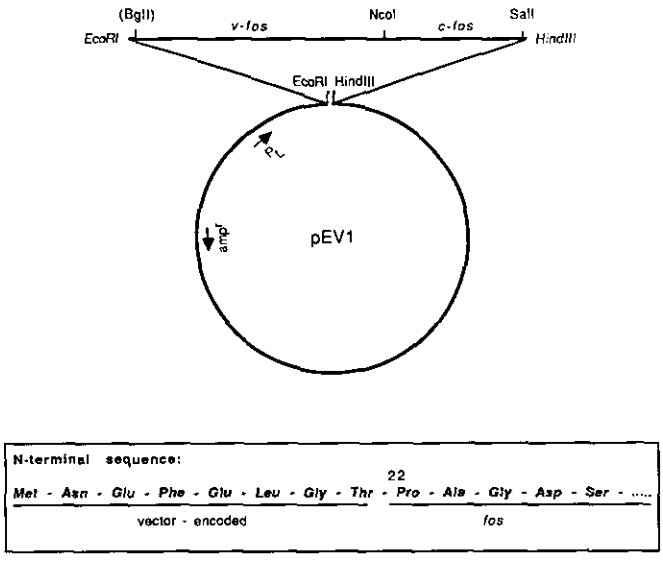


FIGURE 1. Structure of pbaf (top panel) and the encoded product (Baf; bottom panel). The indicated Bgl II-Nco I and NcoI-Sal I fragments from FBJ-MuSV and mouse *c-fos*, respectively, were ligated together via the Nco I sites, subcloned into the Sma I and Sal sites of pUC 19 after converting the overhang created by Bgl II to a blunt end in a fill-in reaction using the Klenow fragment of DNA polymerase I, exerting the insert with Eco RI and Hind III and cloning into the corresponding site of pEV I (*29*).

Ion-Exchange Chromatography

A Q-Sepharose column (1 × 5 cm, Pharmacia, Uppsala, Sweden) was equilibrated in 20 mM Tris-HCl pH 8.0, 2.5 M urea (buffer B). Five molar urea extract containing Fos protein was diluted 1:1 with buffer A and applied at a flow rate of 0.8 mL/min. After washing with buffer B, Fos protein was eluted with a linear 0 to 1 M NaCl gradient. Fractions of 1 mL were collected and assayed for Fos protein by immunoblotting.

Immunoblotting

Samples were resolved on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose paper by electroblotting in 25 mM Tris-HCl, pH 8.3, 193 mM glycine, and 20% methanol (2 hr, 100 V, 4°C). After saturation in 5% nonfat dry milk in 50 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Tween 20 (MTTBS) for 1 hr at 25°C, the membrane was incubated overnight at 4°C in a 1:400 dilution of anti-Fos antibody in MTTBS, washed five times for 5 min each with TTBS, incubated 1 hr at 25°C in MTTBS containing a 1:800 dilution of anti-rabbit IgG peroxidase conjugate (Dakopatts), and washed as before. The color reaction was carried out in TBS plus 4.4 μM H_2O_2, 16.6% methanol, and 4-chloro-1-naphthol (Serva, 0.5 mg/mL).

In Vitro Reconstitution and Immunoprecipitation

Linearized T7 expression plasmid, 1 μg containing the c-*jun* cDNA (*25*) was incubated with 0.5 mM nucleoside triphosphates (ATP, CTP, GTP, UTP), 1 unit of RNAsin (Genofit), 0.4 OD_{260} units CAP nucleotide (Boehringer) and 5 units of T7 RNA polymerase for 30 min at 37°C in a total volume of 50 μL transcription buffer (40 mM Tris, pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 10 μM dithiothreitol, 5 μg bovine serum albumin). Another 5 units of enzyme were added, and the reaction mixture was incubated for 30 min. The RNA was checked for integrity on a 1% agarose gel. *In vitro* transcribed RNA was mixed with 60 μL of reticulocyte lysate (Amersham) plus, if the protein was to be labeled, 30 μL (450 μCi) of ^{35}S-methionine. The reaction mixture was adjusted to 150 μL with H_2O and incubated at 30°C for 1 hr.

Complex formation was carried out by incubating *in vitro* translated ^{35}S-labeled c-Jun and unlabeled extracts (13 μL and 10 μL, respectively), in 30 μL H_2O for 1 hr at 30°C. After the addition of 5 μL of 10 × RIPA buffer, the mixture was incubated with 4 μL of antiserum 455 (*26,27*) for 1 hr on ice followed by incubation with 40 μL Pansorbin (Calbiochem) for 1 hr. Immune complexes were sedimented and washed twice with RIPA buffer. SDS sample buffer was added to the pellet, the samples heated to 95°C for 3 min, and separated on a 11.5% SDS-polyacrylamide gel. After electrophoresis, gels were processed in Amplify (Amersham), dried, and exposed to Fuji RX film.

Gel Retardation Analysis

Binding reactions were performed by preincubating 10 μL Baf and 5 μL *in vitro* translated c-Jun, either alone or combined, with 1 μg of poly(dI-dC) in a buffer containing 10 mM Hepes, pH 7.9, 60 mM KCl, 4% Ficoll, 1 mM EDTA, and 1 mM DTT for 60 min at 25°C. Double-stranded oligonucleotide, ^{32}P-labeled with polynucleotide kinase, 3.5 fmole, was added and incubation was continued for 30 min at room temperature. The reaction mixtures were separated on 4% polyacrylamide gels at room temperature at 10 V/cm (*28*). Gels were dried and exposed to Kodak RP film.

Lectin Binding

Wheat germ agglutinin (WGA) immobilized on Agarose (Pharmacia) was washed three times with TTBS (50 mM Tris, pH 7.4, 150 mM NaCl, 0.05% TWEEN 20), resuspended in 1 volume TTBS, and incubated for 16 hr at 4°C with an equal volume of HeLa cell or bacterial cell extract, respectively (300 μg protein). Prior to the lectin binding reaction, the bacterial cell extract was incubated with reticulocyte lysate for 2 hr at 25°C. After extensive washing in TTBS, the beads were extracted at 95°C for 5 min with SDS sample buffer. The extracts were resolved by SDS-PAGE and analyzed by Western blotting using anti-Fos antibodies (*27*). For competition experiments the WGA beads were suspended in 2 volumes of TTBS containing 0.5 M *N*-acetylglucosamine for 2 hr at 4°C on a rotating wheel. The binding reaction was carried out in the presence of 0.25 M *N*-acetylglucosamine.

Results

Expression of Fos Protein in *E. coli* and Enrichment for Baf by Ion-Exchange Chromatography

As the expression vector for production of Fos in *E. coli* we chose pEV1 containing the heat-inducible P_L promoter, a 5′ located start codon and multiple cloning site (*29*). To generate the Fos insert, we first subcloned an intronless hybrid v-*fos*/c-*fos* gene into pUC19, released the insert with EcoRI and Hind III, and introduced this fragment into pEV1 via the corresponding restriction sites generating the pbaf construct (Fig. 1). The product encoded by pbaf consists of 8 N-terminal vector-derived amino acids fused to 359 amino acids of Fos (residues 22–380 of c-Fos) (Fig. 1). Otherwise, this protein which will be subsequently referred to as Baf, differs from c-Fos only in the 5 FBJ-MSV-specific single amino acid changes (*30*). The pbaf construct was introduced into *E. coli*. RR1 cells and individual colonies were analyzed for Baf expression. Two different clones were analyzed in Figure 2 and used for subsequent analyses, one clone expressing Baf only after heat induction and one clone expression Baf constitutively (Fig. 2). The

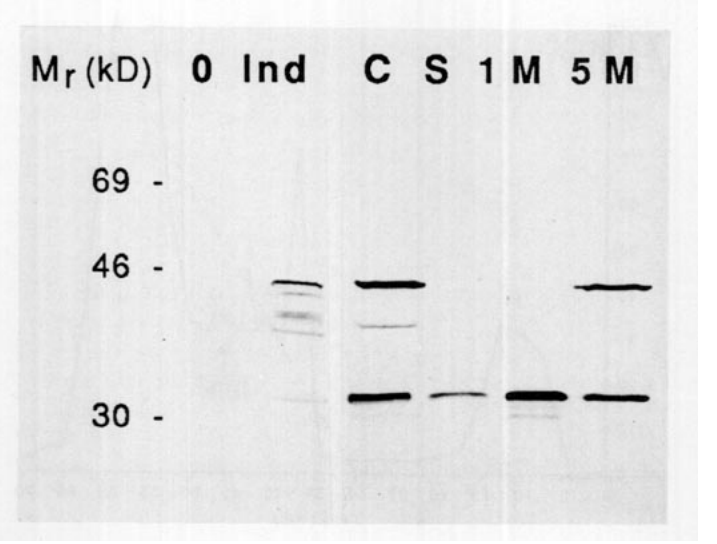


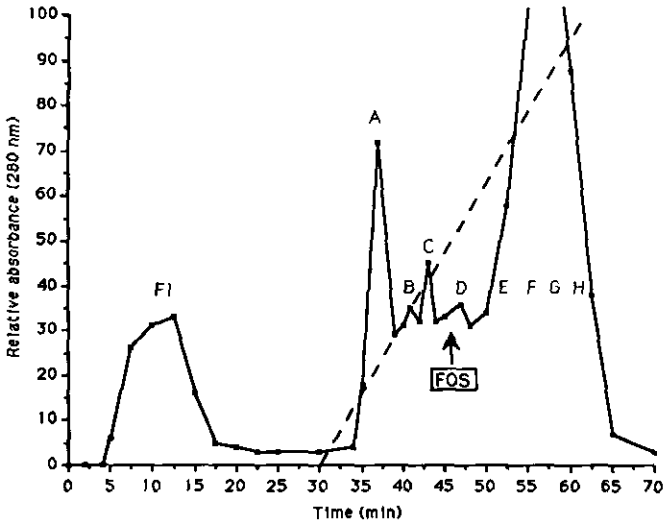
FIGURE 2. Expression and extraction of Baf. Proteins were separated by SDS-PAGE and analyzed by Western blotting using a Fos-specific antiserum (*38*). Left panel: inducible clone (0, before induction; Ind, after heat induction for 2 hr). Right panel: constitutively expressing clone (C, whole cells; S, supernatant after cell lysis and centrifugation; 1 M and 5 M, urea extracts of pellets. M, molecular mass of marker proteins).

M_r of full-length Baf is approximately 45 kd, the other bands visible on the Western blot in Figure 2 presumably represent degradation products of Baf. The absolute amount of Baf produced in these cells is relatively low, since we were not able to detect Baf reproducibly on Coomassie blue-stained polyacrylamide gels without further purification (not shown). In the bacterial extract, the majority of the Baf protein was found to be insoluble. After centrifugation of the homogenate the supernatant did not contain any detectable amount of the full-length product and only a small fraction of the shorter M_r form (Fig. 2). However, Baf protein could be readily solubilized from the pellet by dissolving in 5M urea (Fig. 2).

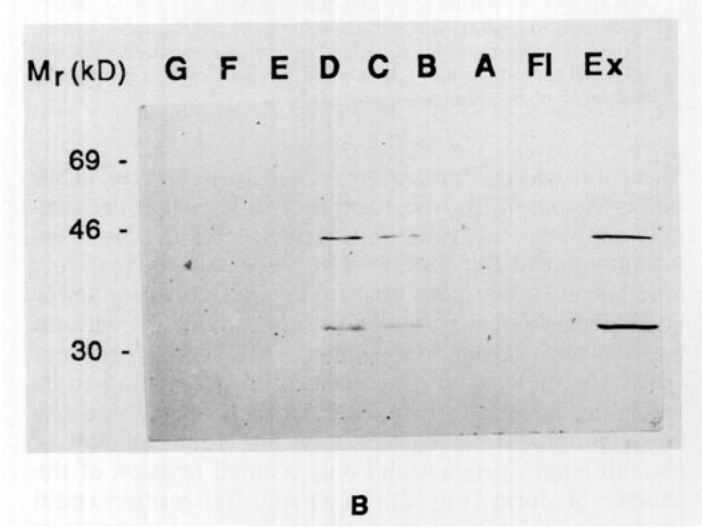
The extracted Baf protein was further enriched by chromatography on a Q-Sepharose ion exchange column run in the presence of 2.5 M urea and eluted with a linear gradient of 0 to 1 M NaCl (Fig. 3*A*). The protein eluted from this column was collected in approximately 60 fractions, which were analyzed for the presence of Baf by Western blotting. Figure 3*B* shows that Baf eluted within a narrow range, approximately in pools C and D, corresponding to fractions 43 to 48 in Figure 3*A*. These fractions were pooled and used for the subsequent binding studies.

Complex Formation between Baf and *In Vitro* Translated c-Jun

Baf protein was then used in an *in vitro* assay to reconstitute a Fos/Jun complex. For this purpose, radioactively labeled c-Jun was synthesized by *in vitro* transcription/translation of a c-Jun cDNA in a T7 poly-



A



B

FIGURE 3. Fractionation of bacterial extract (urea-subsidized pellets; see Fig. 2) on Q-Sepharose. (*A*) OD_{280}; NaCl gradient (0–1 M). Letters indicate pooled fractions analyzed in (*B*). (*B*) Western blot analysis of pooled fractions A–G. Ex, extract applied to column; Fl, flow through.

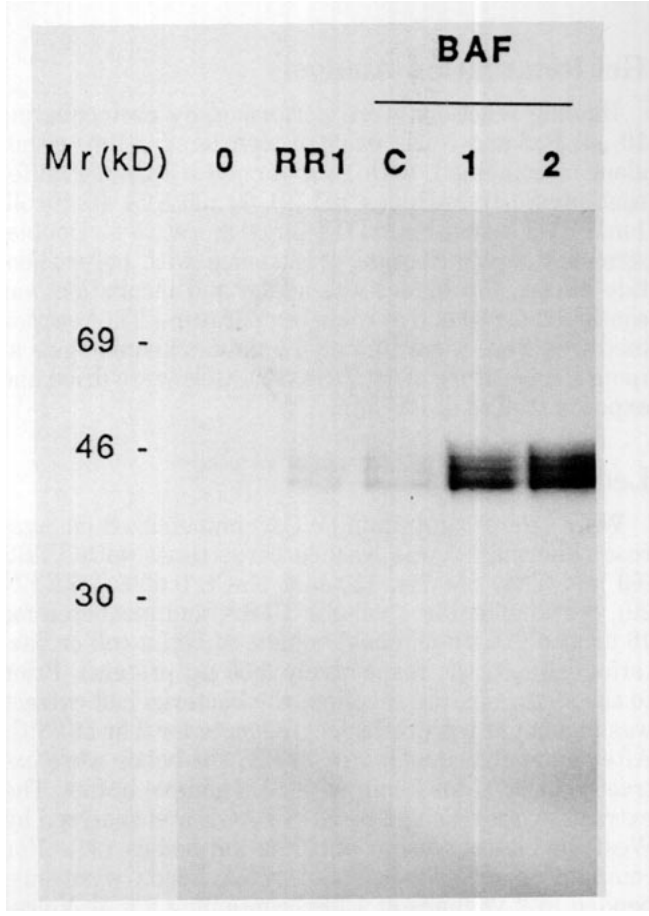


FIGURE 4. *In vitro reconstitution of a Baf/c-Jun complex.* ^{35}S-labeled, *in vitro* translated c-Jun was incubated with buffer only (lane 0), with extract from RR1 bacteria not expressing Baf (lane RR1) or with two different preparations of Baf (lanes 1 and 2) and immunoprecipitated with Fos-specific antibodies. C, control for lane 1 where Fos antibodies were omitted.

merase/reticulocyte system and incubated with Baf. The mixture was then subjected to immunoprecipitation using a Fos-specific antibody that does not cross-react with Jun (Fig. 4). The precipitation of ^{35}S-labeled c-Jun in the presence of Baf indicates the formation of a protein complex between Baf and Jun. No significant amount of Jun was precipitated when control preparation from the parental RR1 cells (which do not harbor the pbaf plasmid) were incubated with the labeled c-Jun or when the fos-specific antibody was omitted from the immunoprecipitation. These findings strongly suggest that the Baf protein is capable of forming a complex with c-Jun protein.

Baf and c-Jun Act Synergistically to Form a Complex of High Affinity for the AP1 Binding Site

To investigate the role of complex formation between Fos and Jun, we analyzed the binding properties of Baf and Jun individually or as a complex for the AP1 DNA recognition sequence. This experiment was performed by using these proteins and a ^{32}P-labeled synthetic oligonucleotide (designated ap1) containing a palindromic AP1 binding site in a gel retardation assay. Figure 5 shows that neither c-Jun nor Fos produced any detectable band shift, while the Baf/c-Jun complex gave a very strong shift. The Baf/Jun/DNA complex formation was also performed in the presence of excess unlabeled competitor oligonucleotides. Figure 6 shows that only the specific ap1 oligonucleotide but not the random oligonucleotide (rd) was able to abolish the band shift. This demonstrates that the retarded band indeed represents a specific complex between Baf, c-Jun, and the AP1 binding site. In addition, a complex of very similar elec-

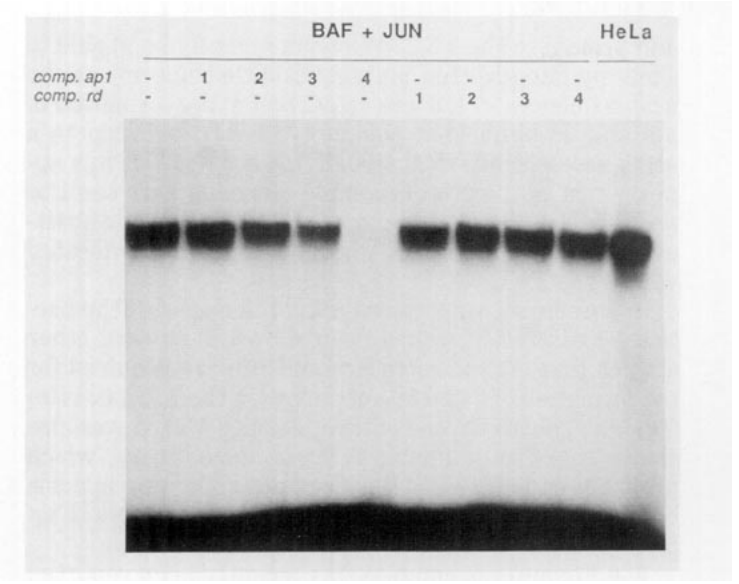


FIGURE 5. Formation of a stable, specific complex between Baf, Jun and the TRE. Synthetic double-stranded oligonucleotide (upper strand: 5′ AAGCA TGAGTCAGACAC; TRE underlined) was end-labeled with ^{32}P, incubated with either the *in vitro* translated Jun, Baf, or the Jun/Baf complex, and separated by nondenaturing PAGE. Unbound oligonucleotides are visible at the bottom of the gel; the weak shifted band in the Baf lane is nonspecific as shown in competition experiments (not shown).

trophoretic mobility was found when nuclear extract from serum-stimulated HeLa cells was used in the gel retardation experiment (Fig. 6).

O-Glycosylation of Fos Is Not Required for the Formation of a Stable Fos/Jun/TRE Complex

It has recently been shown that the transcription factor SP-1 purified from HeLa cells is post-translationally modified by *O*-glycosylation and binds to wheat germ agglutinin (WGA) (*31*). The presence of the *O*-linked *N*-acetylglucosamine residues appears to be important for transcriptional activation by SP1 (*31*). In the same study it was shown that other transcription factors, among them the AP1/Fos protein complex, can be labeled *in vitro* by ^{3}H-galactose and galactosyl transferase (an enzyme transferring galactose onto terminal GlcNAc residues), but the nature and role of this modification was not investigated further. To elucidate some of these questions, we analyzed the cellular Fos protein complex and Baf protein for their affinity for WGA. Figure 7 shows that the Fos protein complex from nuclear HeLa cell extract binds to WGA and that this binding can be blocked by *N*-acetylglucosamine. In contrast, Baf protein does not bind to any significant extent, even after incubation with reticulocyte extract. (This control was important to show that no glycosylation occurs during complex formation with the *in vitro* translated c-Jun.) These results therefore indicate that

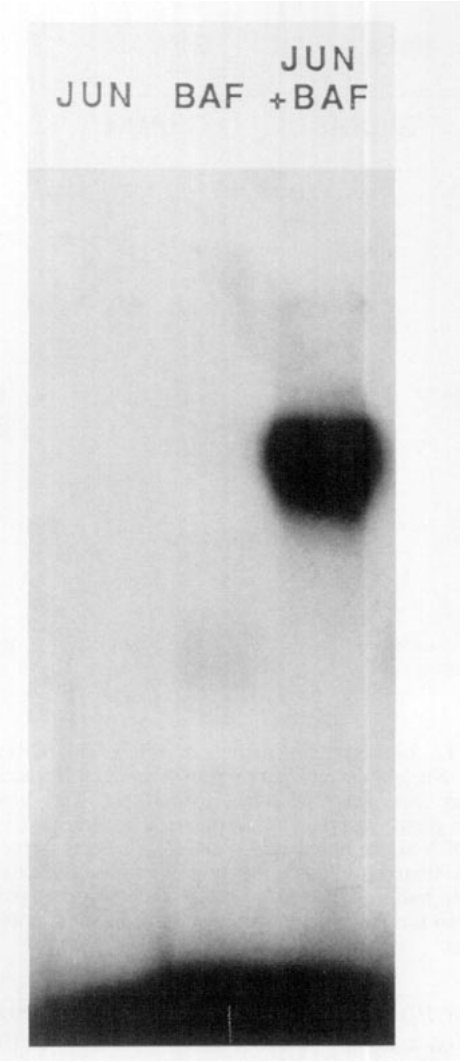


FIGURE 6. Competition of Baf/Jun/TRE complex formation by specific (ap1) and nonspecific (rd) oligonucleotides. Lanes 1, 2, 3, and 4 represent 10-, 100-, 1000- and 10,000-fold excesses of competitor DNA. The sequence of ap1 is identical to the labeled oligonucleotide; rd has the sequence 5′ GCGACTAACATCGATCG (upper strand). The right-most lane shows a band shift with nuclear extract from serum-stimulated HeLa cells.

the formation of a stable Fos/Jun/TRE complex does not require *O*-glycosylation of Fos.

Discussion

It is now well established that Fos protein plays a crucial role in the regulation of AP1-dependent transcription. This is mainly based on two lines of evidence. First, Fos is a constituent of transcription complexes forming over AP1 DNA recognition sequences (*5–8,10,32*), and second, Fos can transactivate promoters containing AP1 binding sites (TREs), such as the human collagenase promoter or the HSV-tk-promoter, after insertion of 5 TRE elements (*8,10,11,22*). In these transcription complexes Fos tightly associates with AP1/

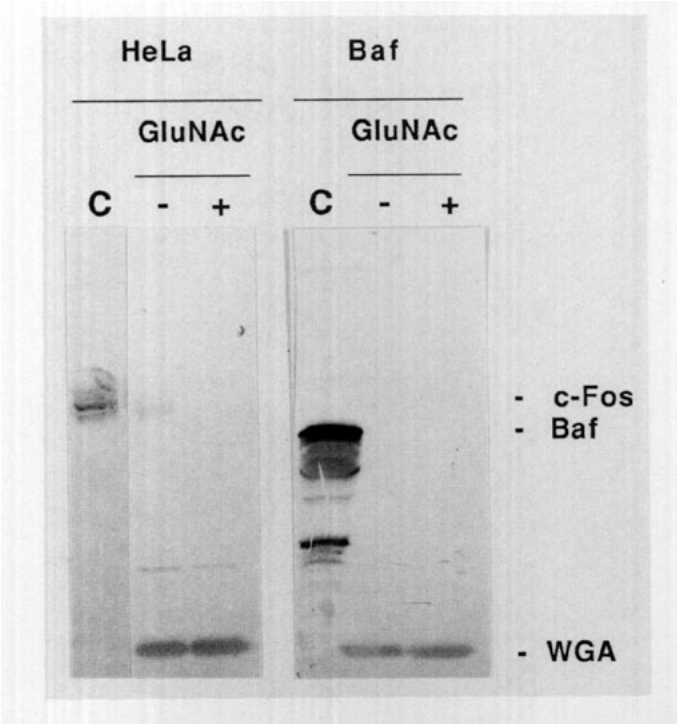


FIGURE 7. Lectin binding activity of cellular and bacterial Fos proteins. Nuclear extract from serum-stimulated HeLa cells and urea extract from bacterial cells, respectively, were incubated with wheat germ agglutinin (WGA) agarose in presence (+) or absence (−) of *N*-acetylglucosamine (GluNAc), a competitive inhibitor of WGA. Bound proteins were eluted by heating the matrix in SDS-sample buffer, resolved on a 10% SDS-polyacrylamide gel, and assayed for Fos by immunoblotting. C, material applied to WGA matrix.

Jun (*6,8,10*) which is identical with the previously identified Fos-associated protein p39 (*17–20*). In the present study we have attempted to reconstitute a protein complex between Fos and Jun proteins. For this purpose, we have expressed and partially purified Fos protein in bacteria. This Baf protein is largely identical with c-Fos, except for the substitution of 21 amino acids at the N-terminus with 6 vector-derived amino acids and 5 internal point mutations (Fig. 1). All these alterations, however, have no noticeable effect on the biological properties of the Fos oncogene product (*11,26*; unpublished data).

Our data clearly show that Baf forms a specific complex with c-Jun protein obtained by *in vitro* translation (Fig. 4) and that only this complex binds with high affinity to the AP1 DNA recognition sequence (Fig. 5). In contrast, the individual Baf and Fos proteins do not bind to any detectable extent in this assay. It has been described that bacterially expressed Jun can bind to the TRE (*15*). This may be due to the high concentration of Jun present in bacterial extract, which may compensate for the low affinity of Jun protein for the TRE. In a recent study, it was indeed shown that *in vitro* translated Jun protein binds to an oligonucleotide containing an AP1 site when the concentration of operator DNA is very high (*32*). Taken together these observations suggest that Fos protein stabilizes the weak binding of Jun protein to the TRE. We have recently been able to show by site-directed mutagenesis that specific amino acid sequences in Fos are required for the formation of a stable Fos/Jun/TRE complex (*33–35*), pointing to a sequence-specific DNA recognition by Fos. It thus appears that in the protein complex forming between Fos and Jun via the "leucine zipper" (*36–38*) both constituents bind to specific DNA sequences, probably located within the TRE.

In agreement with our results obtained with the bacterial Fos protein, it has been shown in several other studies that both *in vitro* Fos and Jun are required for the formation of a stable complex with the TRE *in vitro* (*32,33,37,39,40*). In one of these studies (*39*), it was also shown that Fos is unable to form homodimers, which probably explains why Baf does not show any specific binding to the TRE in our gel retardation assays (Fig. 5).

The Fos protein complex has recently been reported to be labeled *in vitro* by ^{3}H-galactose and galactosyl transferase (*31*). We therefore investigated whether *O*-glycosylation of Fos protein is required for the formation of a Fos/Jun/TRE complex. Our findings confirm the post-translational addition of sugar residues to cellular Fos protein complex that could be shown to bind to WGA, indicating the presence of *N*-acetylglucosamine (Fig. 7). In contrast, no such modification is present in Baf (Fig. 7). This observation shows that *O*-glycosylation of Fos is not required for the sequence-specific DNA binding of the Fos/Jun complex, an observation that is in agreement with the data obtained for the transcription factor SP1 (*31*). It may be that, as in the case of SP1, glycosylation of Fos is required for its transregulatory properties.

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