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Cross-coupling of the NF-κB p65 and Fos/Jun transcription factors produces potentiated biological function

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NF-κB and AP-1 represent distinct mammalian transcription factors that target unique DNA enhancer elements. The heterodimeric NF-κB complex is typically composed of two DNA binding subunits, NF-κB p50 and NF-κB p65, which share structural homology with the c-*rel* proto-oncogene product. Similarly, the AP-1 transcription factor complex is comprised of dimers of the c-*fos* and c-*jun* proto-oncogene products or of closely related proteins. We now demonstrate that the bZIP regions of c-Fos and c-Jun are capable of physically interacting with NF-κB p65 through the Rel homology domain. This complex of NF-κB p65 and Jun or Fos exhibits enhanced DNA binding and biological function via both the κB and AP-1 response elements including synergistic activation of the 5′ long terminal repeat of the human immunodeficiency virus type 1. These findings support a combinatorial mechanism of gene regulation involving the unexpected cross-coupling of two different classes of transcription factors to form novel protein complexes exhibiting potentiated biological activity.

Key words: AP-1/leucine zipper/NF-κB/Rel/transcription factor heterocomplexes

Introduction

The NF-κB and AP-1 families of transcription factors each exert pleiotropic regulatory effects on the expression of an array of cellular genes that are induced in response to various growth factors, mitogens, tumor promoters, DNA damaging agents or oxygen radicals (Angel *et al.*, 1987; Lee *et al.*, 1987; Curran and Franza, 1988; Greene *et al.*, 1989; Lenardo and Baltimore, 1989; Stein *et al.*, 1989a, 1992; Vogt and Bos, 1990; Angel and Karin, 1991; Baeuerle, 1991; Baeuerle and Baltimore, 1991; Grilli *et al.*, 1993; and references therein). Prior studies have demonstrated that these two families of transcription factors bind to distinct enhancer motifs and that each is structurally related to distinct oncogenes (reviewed in Angel and Karin, 1991; Baeuerle, 1991). Specifically, the 50 kDa (Bours *et al.*, 1990; Ghosh *et al.*, 1990; Kieran *et al.*, 1990) and 65 kDa (Nolan *et al.*, 1991; Ruben *et al.*, 1991) subunits of NF-κB share N-terminal homology with the v-*rel* oncogene product from the avian reticuloendotheliosis virus (Gilmore, 1990). This 300 residue N-terminal Rel homology domain mediates the DNA binding, dimerization and nuclear targeting functions, as well as interaction with IκB (Gilmore and Temin, 1988; Ballard *et al.*, 1990; Bours *et al.*, 1990; Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Nolan *et al.*, 1991; Ruben *et al.*, 1991; Beg *et al.*, 1992; Ganchi *et al.*, 1992). AP-1 DNA binding activity is composed of various dimers of the c-*fos* and c-*jun* proto-oncogene products or closely related polypeptides (Angel *et al.*, 1987; Lee *et al.*, 1987; Curran and Franza, 1988; Franza *et al.*, 1988; Rauscher *et al.*, 1988a,b; Schönthal *et al.*, 1988a; Cohen *et al.*, 1989; Distel and Spiegelman, 1990; Ransone and Verma, 1990). The members of this transcription factor family together with ATF/CREB and C/EBP belong to the class of bZIP proteins. These proteins are characterized by a bZIP region containing a basic region domain involved in DNA binding and a leucine zipper motif involved in dimerization (Kouzarides and Ziff, 1988; Landschulz *et al.*, 1988; Gentz *et al.*, 1989; Hai *et al.*, 1989; Maekawa *et al.*, 1989; Turner and Tjian, 1989; Vinson *et al.*, 1989; Busch and Sassone-Corsi, 1990; and references therein).

In the present study, we report the discovery and features of a functional and physical interplay of the NF-κB and AP-1 families of transcription factors. Our experiments originated from the observation that antisense c-*fos* and c-*jun* reduce the phorbol ester response of an NF-κB-dependent promoter, which suggested synergism between Fos and NF-κB at a promoter that carries no AP-1 binding site. Precedent for such unexpected interactions of distinct transcription factors, a phenomenon termed cross-coupling (Schüle *et al.*, 1991), is provided by the well-documented interactions of Fos and Jun with the nuclear receptors for glucocorticoids and retinoic acid (Diamond *et al.*, 1990; Jonat *et al.*, 1990; Lucibello *et al.*, 1990; Schüle *et al.*, 1990, 1991; Yang-Yen *et al.*, 1990) and of Jun with MyoD (Bengal *et al.*, 1992). Further, we and others have recently shown the cross-family interaction of NF-κB and C/EBP family members (LeClair *et al.*, 1992; Stein *et al.*, 1993). We now demonstrate that both c-Fos and c-Jun functionally synergize with the 65 kDa subunit of NF-κB leading to potentiated biological function *in vivo* through either the κB or AP-1 enhancer elements. We further show that the cross-coupling of these transcription factors is limited to the nucleus and results in increased DNA binding activity of NF-κB. Both c-Fos and c-Jun can physically associate through their bZIP regions with the Rel homology domain of NF-κB p65, suggesting that the physical interaction represents the molecular basis of the functional synergism. Together, these findings illustrate an interplay of diverse transcription factor families to form novel complexes displaying enhanced biological activity.

Results

Fos and Jun activate the HIV-1 LTR in a κB enhancer-dependent manner

When HeLa cells were cotransfected with Jun or Fos expression vectors and a chloramphenicol acetyltransferase (CAT) reporter plasmid, either HIV-1 LTR-CAT, containing the human immunodeficiency virus type 1 long terminal repeat (HIV-1 LTR), or Δ-121 HIV-1 LTR-CAT, containing a 5′ truncated form of this LTR retaining both κB enhancers, a 3- to 10-fold activation over the basal level of CAT expression was observed (Figure 1A, lines 1 and 3).

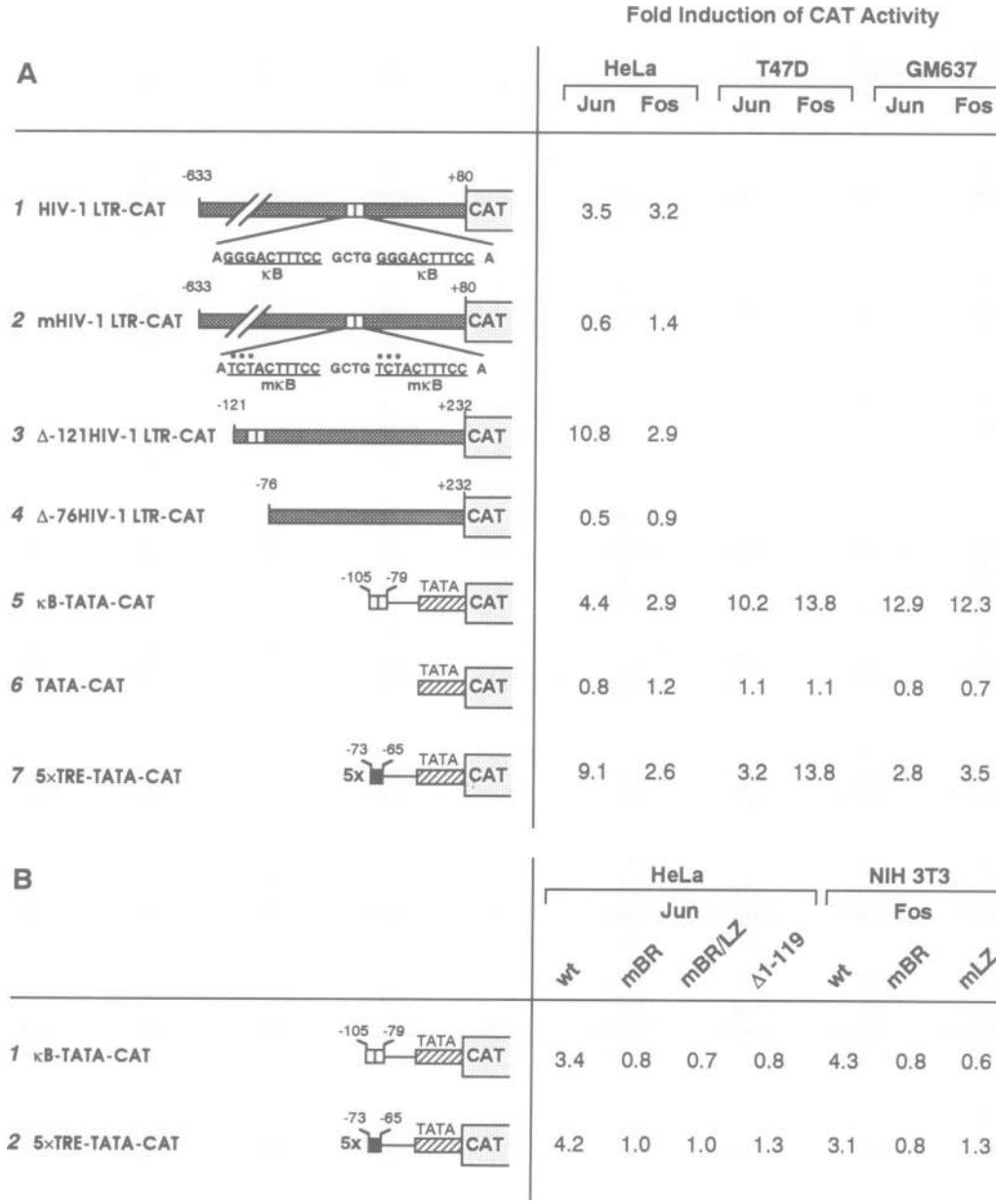

Fig. 1. Fos and Jun potentiate κB enhancer-dependent expression of the HIV-1 LTR. (**A**) Thymidine kinase-deficient HeLa epithelial cells (HeLa tk⁻), human GM637 and mouse NIH 3T3 fibroblasts, and human T47D mammary carcinoma cells were transiently cotransfected with 5 μg of the indicated CAT reporter plasmids [wild-type HIV-1 LTR-CAT (−633 to +80), mHIV-1 LTR-CAT containing point mutations in each of the two κB enhancers (GGG → TCT), Δ-121 HIV-1 LTR-CAT retaining both κB enhancers, Δ-76 HIV-1 LTR-CAT lacking both κB enhancers, κB-TATA-CAT containing one copy of the reiterated κB enhancers from the HIV-1 LTR (−105 to −79) linked to the albumin TATA box of *Xenopus laevis*, 5×TRE-TATA-CAT containing a pentad of the AP-1 site from the human collagenase gene (−73 to −65) and the enhancerless parental TATA-CAT plasmid] and 10 μg of expression vector DNA encoding Jun or Fos (RSVc-Jun and RSVc-Fos). Eight hours after transfection, the cells were washed and the medium was changed to 0.5% FCS. CAT activity was measured 42 h later. Data are presented as fold induction of CAT activity obtained with Jun or Fos relative to CAT activity produced by transfection of identical amounts of expression vector DNA lacking a *jun* or *fos* cDNA insert (RSVneo). Values shown represent the mean inductive response from at least three independent experiments. The standard errors were consistently less than 20% of the means. The *jun* and *fos* expression vectors were shown to direct the synthesis of similar levels of protein and both proto-oncogene products were almost exclusively localized in the nucleus as shown by immunofluorescence analysis (data not shown). (**B**) HeLa tk⁻ and NIH 3T3 cells were transfected with the indicated mutated versions of Jun and Fos in the presence of either κB-TATA-CAT (line 1) or 5×TRE-TATA-CAT (line 2). Each mutant is described in Materials and methods. The basal level of CAT activity for the promoter−CAT constructs shown was 2.0, 0.16, 5.1, 0.87, 20.4, 0.52 and 0.42% acetylation of [^{14}C]chloramphenicol in lines A1−7 respectively, and 21.6 and 0.31% in lines B1 and B2.

Unexpectedly, these inductive effects of Jun and Fos on HIV-1 LTR promoter constructs proved dependent on the κB enhancer as both Fos and Jun failed to activate mutant forms of the HIV-1 LTR either altered at three bases within each κB enhancer element (mHIV-1 LTR-CAT, GGG → TCT; Figure 1A, line 2) or deleted to base −76, which removes both of these enhancers (Δ-76 HIV-1 LTR-CAT; Figure 1A, line 4). These functional effects of Fos and Jun were also confirmed using a synthetic CAT transcription unit comprised of only the duplicated κB enhancer motifs located between nucleotides −105 and −79 in the HIV-1 LTR linked to the TATA box of the *Xenopus laevis* albumin gene (κB-TATA-CAT). A pentad of AP-1 binding sites from the human collagenase gene linked to the same TATA box (5×TRE-TATA-CAT) served as a reference plasmid. Fos and Jun significantly activated both of these minimal enhancer CAT plasmids in each of three different cell lines, including HeLa, T47D human mammary carcinoma cells and GM637 human fibroblasts (Figure 1A, lines 5 and 7). Fos and Jun were stronger activators of the κB enhancer than of the AP-1 enhancer in T47D and GM637 cells. The fold stimulation of transactivation varied between these different cell lines, probably reflecting differences in the endogenous levels of Jun and Fos expression found in each. In contrast, neither Jun nor Fos stimulated the parental TATA-CAT plasmid lacking these enhancer elements (Figure 1A, line 6). These data permit several interpretations: (i) Fos and Jun could enhance the synthesis or activation of NF-κB, (ii) AP-1 could cause the synthesis of a factor that cooperates somehow with NF-κB, or (iii) a transcription factor containing Jun, Fos or both could act directly on the κB enhancer element. These points are addressed in the following experiments.

To explore how Fos and Jun could act on an 'inappropriate' promoter, we examined the Jun and Fos domains needed for the κB (as well as 5×TRE) enhancer stimulation. Interestingly, the same domains were required for both enhancers. Mutation of recognized functional domains within these proto-oncogene products including the basic region (mBR), the C-terminal bZIP region (mBR/LZ) or the N-terminal activation domains of Jun (Δ1−119) was associated with a complete loss of transcriptional enhancement via the κB enhancer as well as the AP-1 binding site (Figure 1B; the Fos mutants were tested in NIH 3T3 fibroblasts because these cells have lower endogenous Fos protein levels than HeLa cells; see also the relatively low inductions by Fos in HeLa cells in Figure 1A, lines 5 and 7). These results indicate that the same transcription factor domains of Fos and Jun are needed for the stimulation of both κB- and AP-1-dependent enhancers.

Since several growth factors, phorbol esters or DNA damaging agents induce both the translocation of NF-κB to the nucleus and the post-translational activation of Fos and/or Jun (Baeuerle and Baltimore, 1988a,b; Herrlich *et al.*, 1989; Stein *et al.*, 1989a,b; Angel and Karin, 1991; Devary *et al.*, 1992; Radler-Pohl *et al.*, 1993; and references therein) conditions could be created where the described effect of Fos and Jun on κB enhancers occurred physiologically. To assess the putative contribution of Fos and/or Jun to the phorbol ester and UV-induced transcription of the HIV-1 LTR, cells were specifically deprived of c-Fos and c-Jun by the antisense technique (Schönthal *et al.*, 1988a,b). These data show a functional involvement of c-Fos and c-Jun in the κB-dependent HIV-1 LTR response to phorbol esters and UV (Figure 2, left panel). The inhibition of κB-dependent HIV-1 LTR expression in these antisense experiments was of similar magnitude to the inhibition of an AP-1 enhancer-dependent promoter (Figure 2, right panel). The antisense constructs did not inhibit expression of various control promoters lacking NF-κB and AP-1 binding sites (Δ-76HIV-1 LTR-CAT, TK-CAT; data not shown). Further, induced κB enhancer activity was also significantly down-regulated in HeLa and COS cells by the addition of dexamethasone (Collart *et al.*, 1990; B.Stein, unpublished data), an agent that has been shown to inhibit AP-1 activity at the collagenase AP-1 binding site, presumably by direct association of Fos and Jun with the glucocorticoid receptor (Diamond *et al.*, 1990; Jonat *et al.*, 1990; Lucibello *et al.*, 1990; Schüle *et al.*, 1990; Yang-Yen *et al.*, 1990; König *et al.*, 1992; Ponta *et al.*, 1992). It is possible that dexamethasone acts on the κB enhancer by eliminating the direct or indirect contribution of Fos and/or Jun. The antisense experiments document that part of the normal κB enhancer-dependent HIV-1 LTR control *in vivo* is exerted by c-Fos and/or c-Jun.

Fos and Jun act through NF-κB p65 to stimulate the κB enhancer synergistically

To explore whether Fos and Jun influence the endogenous NF-κB level, Western blot analyses were performed (not shown). NF-κB p65 was barely visible and there was no significant increase in transient or stable RSV-Fos or RSV-Jun transfectants. Because these data do not convincingly rule out effects on NF-κB synthesis, we decided to select NF-κB and AP-1 negative cells and to examine the putative cooperation between these factors by exogenous overexpression under the control of constitutive promoters. Expression vectors were chosen which transcribe the NF-κB and AP-1 subunits under the control of the cytomegalovirus (CMV) promoter. Using these individually

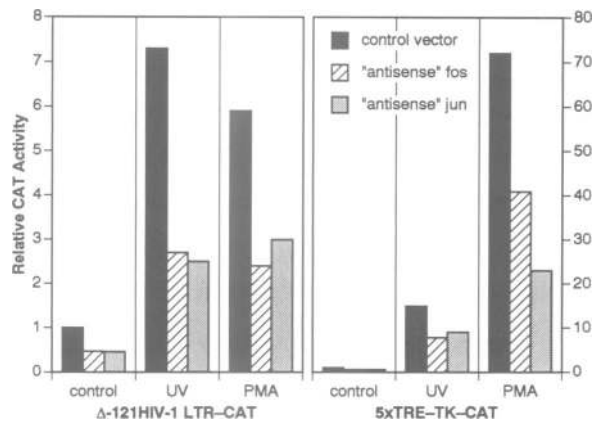

Fig. 2. 'Antisense' *fos* and 'antisense' *jun* inhibit κB enhancer-dependent gene expression. HeLa cells were transiently transfected with 5 μg of Δ-121HIV-1 LTR−CAT or 5×TRE−TK−CAT reporter plasmids and 10 μg of control vector (KSV10$^+$ or SV65, respectively), 'antisense' *fos* expression vector (SV*sof*$^+$) or 'antisense' *jun* expression vector (SVanti-*jun*) as indicated. Eight hours after transfection cells were treated with 20 J/m^2 UV (254 nm) or 60 ng/ml PMA or were left untreated (control). 42 h after transfection CAT activity was determined. Values shown represent the normalized mean inductive response from at least three independent experiments. The standard errors were consistently less than 20% of the means.

or in combination for transient transfections, efficient synthesis was obtained in several types of cells (Western blot of COS cells is shown in Figure 3). Further, the expression vectors did not greatly cross-influence each other. For instance, NF-κB p65 expressed from pCMV4T-p65 was not enhanced by cotransfection of pCMV4T-Fos or pCMV4T-Jun (compare lanes 2, 3 and 4 of Figure 3; normalize for the non-specific band migrating faster than p65 and present in all samples). Also the amounts of Jun and Fos expressed from pCMV4T-Jun and pCMV4T-Fos respectively were only slightly enhanced by cotransfection with pCMV4T-p65 (Figure 3, lanes 5–10).

Recent reports have described the activation of κB-dependent promoters in Jurkat T cells and COS cells by overexpression of NF-κB p65 (Schmitz and Baeuerle, 1991; Ballard *et al.*, 1992; Ruben *et al.*, 1992) while two other members of the NF-κB family, NF-κB p50 and c-Rel, were not able to transactivate the HIV-1 κB enhancer in mammalian cells by themselves (Schmitz and Baeuerle, 1991; Doerre *et al.*, 1993). We chose to transfect these NF-κB subunits into the mouse embryonal carcinoma cell line F9 because these cells exhibit exceedingly low levels of constitutive nuclear NF-κB, Fos and Jun (Chiu *et al.*, 1988; Israël *et al.*, 1989; B.Stein, unpublished data). The 50 kDa and 65 kDa subunits of NF-κB and c-Rel were separately overexpressed in F9 cells and their effect on the transcription of κB-TATA-CAT in the absence or presence of Fos or Jun was determined. NF-κB p65 alone was able to activate the κB enhancer in a dose-dependent manner in F9 cells, while NF-κB p50 and human c-Rel were not (Figure 4, and data not shown). Cotransfection of these F9 cells with NF-κB p65 and wild-type Fos or wild-type Jun expression vectors produced synergistic activation of the κB-TATA-CAT reporter plasmid compared with either vector alone (Figure 4, compare lines 1, 2 and 5). In contrast, NF-κB p50 and human c-Rel failed to synergize with either wild-type Fos or Jun (Figure 4, and data not shown). The enhancement by Fos or Jun alone (Figure 4, control vector column, lines 2 and 5) could either be due to a cryptic AP-1 site in the reporter plasmid or be due to synergism with low levels of endogenous NF-κB p65. Overexpression of NF-κB p50 did not alter the reporter expression (see last column, same lines). Parallel transfection studies performed with various deletion mutants of Fos and Jun confirmed our data shown in Figure 1B. The synergistic inductive response with NF-κB p65 required the basic region (ΔRK) and leucine zipper (ΔLZ) motifs of both proto-oncogene products and the N-terminal activation domains of Jun (Δ1–194) (Figure 4).

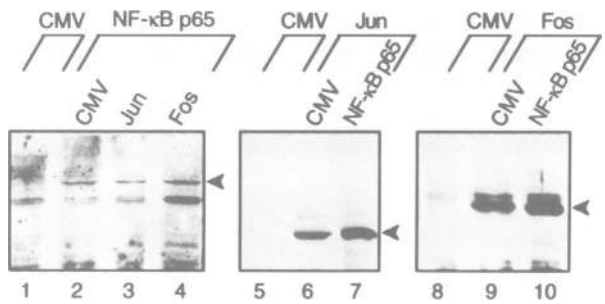

Fig. 3. Western blot analyses. Monkey COS cells were transiently transfected with 12.5 μg of pCMV4T (lanes 1, 5 and 8) or combinations of 2.5 μg pCMV4T–p65 with 10 μg pCMV4T–Jun or pCMV4T–Fos as indicated. The total amount of DNA was held constant in all transfections by the addition of varying amounts of parental pCMV4T vector DNA. Nuclear extracts prepared from cells 3 days after transfection were fractionated by SDS–PAGE, blotted and immunostained with antibodies specific for NF-κB p65 (lanes 1–4), Jun (lanes 5–7) and Fos (lanes 8–10). The arrows indicate the position of the proteins (visualized by antibody) encoded by the transfected expression vectors.

κB-TATA-CAT	Control vector	NF-κB p65	NF-κB p50
Control vector	1.0	48.7 ± 19.1	1.2 ± 0.2
Fos wt	3.0 ± 1.5	209.9 ± 16.3	2.1 ± 0.2
FosΔRK	1.1 ± 0.3	33.6 ± 8.7	n.d.
FosΔLZ	0.8 ± 0.1	42.9 ± 15.1	n.d.
Jun wt	4.6 ± 0.8	226.5 ± 34.1	4.5 ± 0.3
JunΔRK	0.9 ± 0.1	25.8 ± 4.9	n.d.
JunΔLZ	1.0 ± 0.1	53.6 ± 9.7	n.d.
JunΔ1–194	1.2 ± 0.1	28.7 ± 7.3	n.d.
JunB	1.6	39.5	n.d.
JunD	1.1 ± 0.2	39.6 ± 8.3	n.d.

Fig. 4. Synergistic activation of the κB enhancer by coexpression of NF-κB p65 with Fos or Jun. F9 embryonal carcinoma cells were transiently transfected with 8 μg κB-TATA-CAT reporter plasmid together with 1 μg of pCMV4T–Fos expression vectors (Fos wt, FosΔRK or FosΔLZ) or pCMV4T–Jun expression vectors (Jun wt, JunΔRK, JunΔLZ, JunΔ1–194, JunB or JunD) in the presence of 250 ng pCMV4T-p65 or pCMV4T-p50 expression vector as indicated. The total amount of DNA was held constant in all transfections by the addition of varying amounts of parental pCMV4T vector DNA. Six hours after transfection, CAT activity was determined. The mean levels of CAT activity with standard error determined in at least three independent experiments are presented as fold induction relative to activity obtained following transfection of the pCMV4T control plasmid alone. All Jun, Fos and NF-κB proteins were expressed at similar levels (data not shown).

The data obtained with transfections in F9 cells and the data shown in Figure 3 indicate functional synergism between NF-κB and AP-1 subunits. Since F9 cells contain no c-Jun, and since the c-Fos protein requires a partner for transcriptional activity, one interpretation is that Fos either binds to an unknown AP-1 subunit, and then acts on NF-κB, or that Fos dimerizes with NF-κB p65. In the case of Jun overexpression, Jun could act as a homodimer or as a heterodimer with either an unknown AP-1 subunit or NF-κB p65. Further, the data show that the enhancement by Fos and Jun of the κB-dependent reporter requires at least one of the NF-κB subunits, namely NF-κB p65.

Fos and Jun are associated with the nuclear form of NF-κB

To assay directly whether Fos and/or Jun were physically associated with NF-κB, electrophoretic mobility shift assays (EMSAs) were performed. To examine the potential presence of NF-κB complexes containing Fos or Jun in cell extracts, anti-Fos and anti-Jun antibodies were preincubated with various NF-κB preparations and the resultant complexes were analyzed for κB-specific DNA binding activity in EMSAs. NF-κB present in HeLa cytosolic or nuclear extracts formed a specific single protein–DNA complex, which was identical in mobility with that formed by an NF-κB p50/p65 heterodimer of *in vitro* translated proteins. Both anti-Fos (Figure 5A, upper panel) and anti-Jun antibodies (Figure 5A, lower panel), but not the corresponding control reagents (protein G–Sepharose or normal

rabbit serum, respectively), partially inhibited *in vitro* binding of both basal level and PMA-induced nuclear NF-κB to the ^{32}P-radiolabeled HIV-1 κB enhancer (lanes 6, 8, 18 and 20) suggesting a role for Fos or Jun in the formation of the complexes. Similar results were obtained if DNA binding reactions were supplemented with antisera after the formation of the nucleoprotein complexes (not shown). Further, the anti-Jun antibodies consistently generated a new more rapidly migrating complex (Figure 5A, lanes 18 and 20). In contrast, neither of these antibodies altered the DNA binding activity of crude or purified preparations of cytoplasmic NF-κB (Figure 5A, lanes 1–4, 9, 10, 13–16, 21 and 22). These results not only exclude potential non-specific inhibitory effects of these antibodies but also suggest that the association of Fos or Jun with NF-κB may be limited to the nucleus, the subcellular location where Jun and Fos are most abundant. Finally, the anti-Fos and anti-Jun antibodies did not inhibit the control binding of cellular proteins to an unrelated SV40 probe (Figure 5A, lanes 11, 12, 23 and 24) and did not fortuitously cross-react with *in*

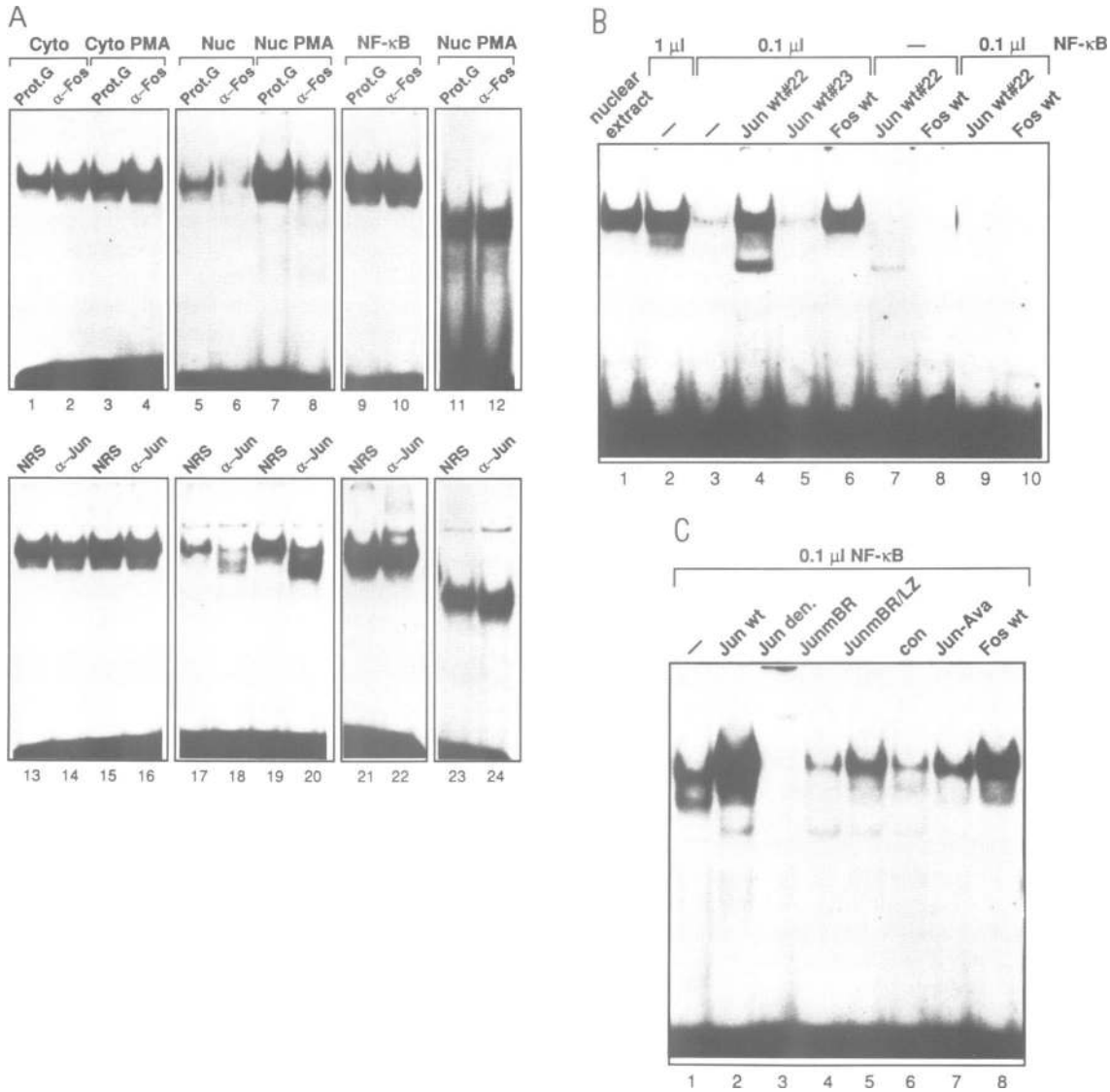

Fig. 5. Fos and Jun are associated with nuclear NF-κB and enhance the DNA binding activity of cytoplasmic NF-κB. **(A)** Cytoplasmic (Cyto) and nuclear (Nuc) extracts prepared from either untreated or PMA-induced (60 ng/ml for 1 h) HeLa tk$^-$ cells or purified cytoplasmic NF-κB isolated from human placenta (a gift from Dr P.Baeuerle) were incubated at 4°C for 3 h either with a monoclonal anti-Fos antibody 411 (α-Fos) bound to protein G–Sepharose or with protein G–Sepharose alone (Prot. G) (lanes 1–12) or rabbit anti-Jun specific antibodies (α-Jun) or control normal rabbit serum (NRS) (lanes 13–24) as indicated. After addition of ^{32}P-radiolabeled probes (HIV-1 κB, lanes 1–10 and 13–22; SV40, lanes 11, 12, 23 and 24) and incubation at room temperature for 30 min, DNA–protein complexes were analyzed by EMSAs using low ionic strength native gels. **(B)** Nuclear extracts (5 μg) prepared from PMA-induced (60 ng/ml for 1 h) HeLa tk$^-$ cells (lane 1) or purified cytoplasmic NF-κB (lanes 2–6 and 9–10) were incubated for 30 min at room temperature with ^{32}P-radiolabeled probes (HIV-1 κB, lanes 1–8; mutant HIV-1 κB, lanes 9 and 10) and the resultant reaction mixes were analyzed by EMSAs. Before the addition of ^{32}P-radiolabeled probes suboptimal amounts of NF-κB (lane 2 versus lane 3) were preincubated at 4°C for 10 min with 1 μl of heparin–Sepharose column eluates of bacterially expressed wild-type Jun (Jun wt#22, lanes 4, 7 and 9) or the following column fraction lacking Jun (Jun wt#23, lane 5) or affinity purified wild-type Fos (lanes 6, 8 and 10). **(C)** Suboptimal amounts of NF-κB were also preincubated at 4°C for 10 min with 1 μl of heparin–Sepharose column eluates of bacterially expressed wild-type Jun (Jun wt, lane 2), heat-denatured (10 min, 90°C) wild-type Jun (Jun den., lane 3), various mutants of Jun including JunmBR (lane 4), JunmBR/LZ (lane 5), Jun-Ava (lane 7) or wild-type Fos (lane 8). The control (con) sample represents the identical 22nd column fraction obtained from bacterial extracts prepared from bacteria not transformed with Jun or Fos expression vectors (lane 6). All shifted nucleoprotein complexes were shown to represent specific binding events by competition with a 100-fold molar excess of unlabeled oligonucleotides (data not shown). We consider slight differences in complex migration (lanes 1, 2, 4, etc.) to be artefacts caused by different concentrations of proteins and buffer.

vitro translated members of the NF-κB/Rel family of proteins including the 50 kDa and 65 kDa subunits of NF-κB and human c-Rel (data not shown). Of note, the cytoplasmic and nuclear NF-κB protein−DNA complexes displayed, within the limits of the resolution, identical electrophoretic mobilities. Either nuclear NF-κB preparations contain Fos or Jun complexes that migrate to the position of cytoplasmic NF-κB, or the interaction of Jun and/or Fos with NF-κB does not survive the electrophoretic procedure (see also Discussion). In this latter case, however, the association of Jun or Fos with NF-κB in solution results in increased affinity of NF-κB to its binding site (see below) and conversely the addition of anti-Jun and anti-Fos antibodies depletes extracts for Jun or Fos, respectively, which lowers the affinity of NF-κB to DNA.

Fos and Jun enhance the DNA binding activity of cytoplasmic NF-κB

Additional support for a direct physical interaction of Fos and Jun with NF-κB was obtained by *in vitro* protein mixing experiments (Figure 5B). Specifically, a heparin−Sepharose column fraction (#22) containing enriched recombinant Jun protein expressed in *Escherichia coli* was found to enhance markedly the DNA binding activity of limiting amounts of cytoplasmic NF-κB to the HIV-1 κB enhancer as assayed by EMSAs (Figure 5B, compare lanes 3 and 4). Of note, no change in mobility was observed upon addition of Jun. The faster migrating band in lane 4 was not observed in other experiments (see Figure 5C, lane 2). The next column fraction (#23), which lacked Jun protein activity, had no stimulatory effect on NF-κB binding (Figure 5B, lane 5). Similarly, purified recombinant Fos protein augmented NF-κB binding activity (Figure 5B, lane 6). The enhanced formation of these nucleoprotein complexes proved entirely dependent on the presence of NF-κB since Jun and Fos alone were not able to bind to the κB probe (Figure 5B, lanes 7 and 8). Further, neither wild-type Jun nor Fos proteins enhanced complex formation when a mutated κB enhancer site was employed as the probe (Figure 5B, lanes 9 and 10). The identical column fraction, #22, isolated from extracts of mock transfected bacteria ('con' in Figure 5C) exhibited no stimulatory activity indicating that the potentiation of NF-κB binding was not the result of a contaminating bacterial protein (Figure 5C, compare lanes 1 and 6). Further, these stimulatory effects on NF-κB binding were not obtained with Jun proteins that had either been denatured with heat or specifically mutated within either the basic region (JunmBR) or the bZIP domain (JunmBR/LZ) (Figure 5C, compare lanes 3−5 with 1). The decrease of NF-κB binding by the addition of denatured Jun is most probably caused by binding material not entering the gel. Interestingly, a miniature version of Jun, designated Jun-Ava, containing only the C-terminal 99 amino acids of this protein (which include the bZIP region), failed to augment NF-κB binding (Figure 5C, compare lane 7 with lane 1). However, this protein retained full capacity to bind specifically to the AP-1 enhancer (Smeal *et al.*, 1989; data not shown).

In vitro association of the Rel homology domain of NF-κB p65 with the bZIP regions of Jun and Fos

Our EMSA data suggest a physical interaction of NF-κB and Jun or Fos and the presence of Jun and Fos in the nuclear form of NF-κB. In order to determine whether the subunits of NF-κB and Fos or Jun can associate *in vitro* in the absence of DNA, two types of experiments were done: (i) co-immunoprecipitation after chemical cross-linking and (ii) affinity chromatography. For coprecipitation we expressed NF-κB p65 and NF-κB p50 in bacteria using the FLAG-HMK expression vector system (Blanar and Rutter, 1992), and partially purified and labeled these proteins with [γ-^{32}P]ATP *in vitro*. NF-κB p65 is remarkable for the presence of a potential 'mini-leucine zipper' motif composed of three leucines arranged in a heptad repeat (^{436}LSEALLQLQFDDEDL^{450}) (Schmitz and Baeuerle, 1991; Ruben *et al.*, 1992). To test the possible function of this NF-κB p65 'mini-leucine zipper' in the interactions with Fos or Jun, conservative alanine substitution mutations were introduced at each of the three leucine residues and the leucine at position 5, thus preserving the α-helical character of this subregion domain (NF-κB p65mLZ: ^{436}ASEAALQAQFDDEDA^{450}). This mutant was also expressed. Bacterially expressed NF-κB p65 and NF-κB p65mLZ are composed of two major species, full-length p65 or p65mLZ respectively and a ~46 kDa truncation (a degradation product or an internal translation stop). We identified this 46 kDa protein as a C-terminal truncation because it can be labeled with [γ-^{32}P]ATP, indicating the presence of the N-terminal HMK peptide, and because it interacted with antibodies directed against an N-terminal but not a C-terminal peptide of NF-κB p65 (data not shown). The radioactively labeled NF-κB p65, NF-κB p65mLZ and NF-κB p50 proteins were separately combined with bacterially expressed NF-κB p50, Jun, Fos or the bacterially expressed FLAG-HMK peptide. The potentially formed heteromeric protein complexes were chemically cross-linked using the reversible cross-linker dithio-bis(succinimidyl-propionate) (DSP) to stabilize complexes during immunoprecipitation. Before subjecting the samples to SDS−PAGE the cross-linked complexes were cleaved under reducing conditions. The full-length and C-terminally truncated ^{32}P-labeled NF-κB p65 proteins were efficiently co-immunoprecipitated with anti-p50 antibodies through their association with NF-κB p50 (Figure 6, compare lanes 1 and 2). Similarly, both forms of ^{32}P-labeled p65 protein could be co-immunoprecipitated with anti-Jun antibodies in the presence of bacterially expressed Jun or with anti-Fos antibodies in the presence of bacterially expressed Fos (Figure 6, compare lane 3 with 4 and 5 with 6). To determine whether the 'mini-leucine zipper' of NF-κB p65 is necessary for this type of physical interaction with Jun or Fos, we combined ^{32}P-labeled NF-κB p65mLZ with bacterially expressed Jun or Fos. Both proteins interacted efficiently with NF-κB p65mLZ as shown by co-immunoprecipitations with antibodies directed against Jun or Fos (Figure 6, lanes 7−10). As expected from our transfection analyses (Figure 4) we could not detect a specific interaction between NF-κB p50 (bacterially expressed NF-κB p50 contains two species) and Jun or Fos by co-immunoprecipitation (Figure 6, lanes 11−14). In similar experiments we demonstrated the interaction of NF-κB p50 with C/EBPβ (Stein *et al.*, 1993). Very small amounts of radiolabeled protein were visible in the presence of the control FLAG-HMK peptide (Figure 6, lanes 1, 3, 5, 7, 9, 11 and 13). This is most probably due to non-specific cross-linking, precipitation by the antibodies and/or binding to protein A−Sepharose.

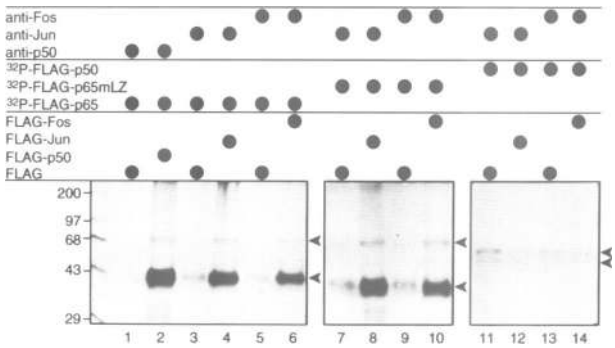

Fig. 6. *In vitro* association of NF-κB p65 with Jun or Fos. Bacterially expressed, purified proteins were incubated as indicated with ^{32}P-labeled bacterial NF-κB p65 (lanes 1–6), NF-κB p65mLZ (lanes 7–10) and NF-κB p50 (lanes 11–14). After cross-linking with DSP the protein complexes were immunoprecipitated with antibodies directed against NF-κB p50 (lanes 1 and 2), Jun (lanes 3, 4, 7, 8, 11 and 12) and Fos (lanes 5, 6, 9, 10, 13 and 14). The immunocomplexes were reduced and subjected to SDS–PAGE. The arrows indicate the positions of the ^{32}P-labeled proteins.

In the alternative approach to demonstrate a physical interaction between NF-κB and Fos or Jun, the coding regions of NF-κB p65 and c-Jun were fused in-frame to the glutathione S-transferase (GST) gene. GST fusion proteins were expressed in bacteria and bound to glutathione–Sepharose beads. Several mutant cDNAs of NF-κB p65 and NF-κB p50 as well as mutant cDNAs of Jun and Fos were transcribed *in vitro* and translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine or [^{35}S]cysteine. Equivalent amounts of radiolabeled proteins were incubated separately with control GST beads, GST-Jun or GST-p65 beads. After extensive washing the bound proteins were eluted and analyzed by SDS–PAGE. As expected, wild-type Fos and a mutant form of Fos with internal deletion of the DNA binding region (FosΔRK) bound efficiently to GST-Jun while FosΔLZ, missing the leucine zipper, did not bind at all (Figure 7A, compare lanes 4–6 with lanes 1–3). Further, wild-type Jun was bound to GST-Jun, while JunΔLZ was unable to bind (Figure 7A, lanes 12–14). JunΔRK showed background binding to GST and only slightly elevated binding to GST-Jun. This may be a peculiarity of this construct. All other data clearly demonstrate that the assay indicates specific protein–protein interactions. Confirming our previous results (Figure 6), NF-κB p65 showed a strong specific physical association with GST-Jun (Figure 7: panel A, lane 15 and panel B, lane 10). This interaction seems to be of similar strength to the Jun–GST-Jun interaction (Figure 7: panel A, compare lane 15 with lane 12; panel B, compare lane 10 with lane 17). Non-specific binding to GST beads was low (Figure 7, panel A, lane 10 and panel B, lane 1). Further, we were unable to detect the interaction of a 50 kDa protein in our NF-κB p50 expressing rabbit reticulocyte lysate with GST-Jun (Figure 7A, lane 16) confirming our previous results shown in Figure 6 (although we have not explored the origin of the low molecular weight bands in Figure 7B, lane 16).

To determine which region in NF-κB p65 was required for interaction with Jun, several mutant forms of NF-κB p65 were combined with GST-Jun (Figure 7B, lanes 10–15). Mutation of the 'mini-leucine zipper' motif (NF-κB p65mLZ) or an internal deletion of 10 amino acids inside the Rel homology domain (NF-κB p65Δ10) did not prevent the physical interaction with GST-Jun. Similarly, a C-terminally truncated form of NF-κB p65 encompassing only the Rel homology domain (NF-κB p65Eco) still bound efficiently to GST-Jun. A further truncation of the Rel homology domain (NF-κB p65Bgl) resulted in the complete loss of interaction with Jun. Since this mutant cannot dimerize (B.Stein, unpublished data) we constructed a chimeric protein containing the GAL4 DNA binding and dimerization domain fused in-frame to p65Bgl (GAL4/p65Bgl). Although this chimeric protein is able to form homodimers through the GAL4 domain (data not shown) it shows only marginal affinity for GST-Jun (Figure 7B, lane 15). The low binding activity to GST-Jun is most likely caused by the GAL4 domain (data not shown). As is frequently the case in such fusion protein studies, a small amount of the *in vitro* translated NF-κB p65 and Jun proteins bound non-specifically to the control GST beads. This binding was not observed in all experiments and was always significantly lower than the comparable specific binding. We also confirmed the interaction of NF-κB p65 and NF-κB p65mLZ with GST-Jun by using ^{32}P-labeled bacterially expressed FLAG-HMK fusion proteins of NF-κB p65 and NF-κB p65mLZ (Figure 7B, lanes 19–22). From the data in Figures 6 and 7 we conclude that NF-κB p65 but not NF-κB p50 interacts with Jun through its N-terminal Rel homology domain.

We then tried to define the region within Jun and Fos that is necessary for the physical interaction with NF-κB p65. Unfortunately, in the reverse setting, with NF-κB p65 fused to GST, the GST fusion protein interaction system was less sensitive than the co-immunoprecipitation system. As shown in Figure 7C, ^{35}S-labeled *in vitro* translated Jun and Fos showed only very weak specific binding or even no specific binding to GST-p65 (lanes 10–15). While in previous experiments we have demonstrated the existence of an interaction between p65 and the homodimerization-deficient Fos (Figure 6, lane 6), the interaction with GST-p65 is improved if a combination of radiolabeled Fos and Jun is added. This resulted in binding of both Fos and Jun to GST-p65 (Figure 7C, compare lane 16 with lane 7). We further tested mutant forms of Fos (FosΔLZ and FosΔRK) in combination with wild-type Jun relative to binding to GST-p65. Confirming the results obtained with Fos mutants *in vivo* (Figures 1B and 4), the bZIP region of Fos is necessary for the interaction with NF-κB p65. It thus appears that the cross-coupling of NF-κB p65 with Jun or Fos involves the Rel homology domain and the bZIP region.

NF-κB and AP-1 cooperate in stimulating an AP-1-dependent promoter

In the negative interaction between the glucocorticoid receptor and AP-1 we have discovered that the interference is mutual: AP-1 blocks receptor action at the hormone-dependent promoter and the steroid receptor inhibits AP-1 transactivating function at the AP-1-dependent promoter (Jonat *et al.*, 1990). This precedent stimulated us to ask whether NF-κB and Fos/Jun would not only synergize in activating a κB enhancer, but NF-κB would potentiate transcription from an AP-1-dependent promoter. Indeed, co-transfection of NF-κB p65 but not NF-κB p50 with Fos or Jun produced synergistic activation of a reporter CAT

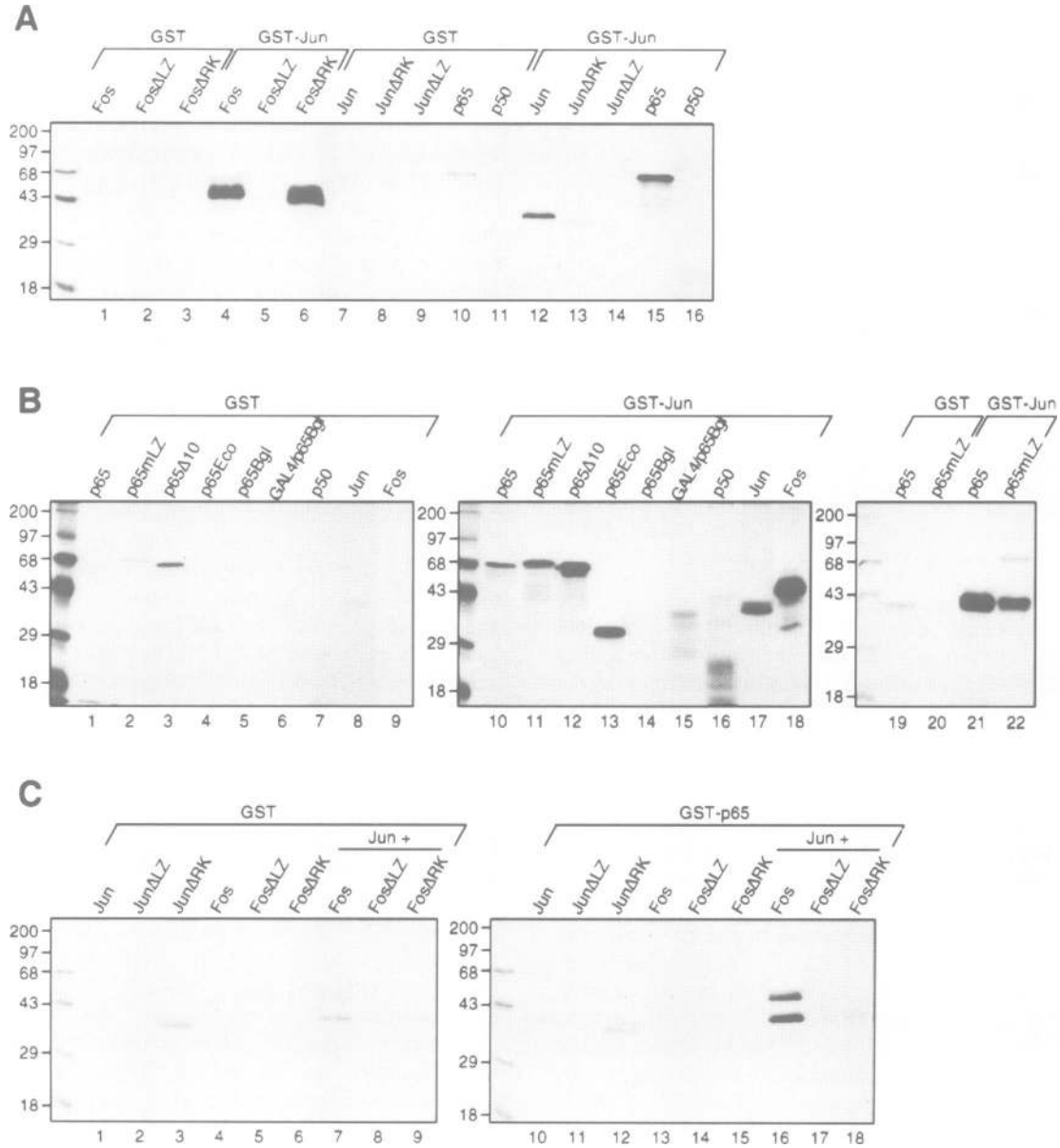

Fig. 7. The Rel homology domain of NF-$\varkappa$B p65 interacts with the bZIP region of Jun or Fos *in vitro*. Radiolabeled proteins, translated in rabbit reticulocyte lysate, were incubated as indicated with bacterially expressed GST (panel A, lanes 1–3 and 7–11; panel B, lanes 1–9, 19 and 20; panel C, lanes 1–9), GST-Jun (panel A, lanes 4–6 and 12–16; panel B, lanes 10–18, 21 and 22) and GST-p65 (panel C, lanes 10–18) immobilized on glutathione–Sepharose beads. After extensive washing the bound proteins were eluted and analyzed by SDS–PAGE. All radiolabeled proteins were expressed in rabbit reticulocyte lysate at similar levels (data not shown).

plasmid containing a pentameric repeat of the human collagenase gene AP-1 binding site (5×TRE-TATA-CAT) (Figure 8A, compare lines 1, 2 and 6). This synergism was used to examine the structural requirements of NF-$\varkappa$B p65 that are necessary for the observed cross-coupling. The expectation was that the functional requirements would match those of the physical interaction. We tested various mutant forms of NF-$\varkappa$B p65 for the synergism with Jun and Fos. Analyses of the 'mini-leucine zipper' mutant, NF-$\varkappa$B p65mLZ, revealed both reduced $\varkappa$B enhancer-dependent transcriptional activity alone in F9 and Jurkat T cells (data not shown) and a reduced but not absent functional synergy with wild-type Jun and Fos (Figure 8A, line 3). NF-$\varkappa$B p65Δ10 is an alternatively spliced form of NF-$\varkappa$B p65 with an internal deletion of 10 amino acids inside the Rel homology domain. This mutant dimerizes only weakly (Narayanan *et al.*, 1992; B.Stein, unpublished data) and is unable to synergize with Jun or Fos (Figure 8A, line 4). NF-$\varkappa$B p65Eco is a C-terminal truncation encompassing only the Rel homology domain without the C-terminal transactivation domain. This mutant is also unable to synergize with Jun or Fos (Figure 8A, line 5). This indicates that the functional synergy between NF-$\varkappa$B and Jun or Fos requires the transcriptional activation domains of NF-$\varkappa$B p65 and of Jun (see JunΔ1–194 in Figure 4). In addition Jun and Fos need their bZIP regions for this type of synergism. The reciprocal cross-coupling of these transcription factor family members represents a reasonable support for the existence of this novel complex of transcription factors.

The specificity of the cross-coupling between NF-$\varkappa$B p65 and Jun or Fos is evident from several lines of experiments. Three closely related members of these families, JunB, JunD

A

5×TRE-TATA-CAT	Control vector	Jun wt	Fos wt	JunB	JunD
Control vector	1.0	11.8 ± 2.7	1.2 ± 0.1	1.1	1.0 ± 0.1
NF-κB p65	4.6 ± 0.9	142.8 ± 21.6	29.2 ± 9.2	4.9	7.5 ± 0.3
NF-κB p65mLZ	1.1 ± 0.7	42.9 ± 0.4	5.0 ± 1.5		
NF-κB p65Δ10	0.9 ± 0.1	7.5 ± 0.4	1.3 ± 0.3		
NF-κB p65Eco	0.9 ± 0.3	8.2 ± 0.9	0.8 ± 0.3		
NF-κB p50	0.9 ± 0.4	18.1 ± 2.2	1.2 ± 0.6		

B

		Control vector	Jun wt	Fos wt
SRE-TATA-CAT	control vector	1.0	1.6 ± 0.1	0.7 ± 0.1
	NF-κB p65	1.3 ± 0.2	2.9 ± 0.2	0.8 ± 0.1
TATA-CAT	control vector	1.0	1.1 ± 0.2	0.9 ± 0.1
	NF-κB p65	1.1 ± 0.1	1.2 ± 0.2	1.0 ± 0.1
Δ-121HIV-1 LTR-CAT	control vector	1.0	4.4 ± 0.6	1.7 ± 0.4
	NF-κB p65	22.0 ± 7.9	92.9 ± 39.9	135.9 ± 1.7

Fig. 8. Synergistic activation of the AP-1 enhancer by coexpression of NF-κB p65 with Fos or Jun. F9 embryonal carcinoma cells were transiently transfected with 8 μg of CAT reporter plasmids [5×TRE-TATA-CAT (**A**), SRE−TATA−CAT, TATA−CAT, Δ-121HIV-1 LTR CAT (**B**)] together with 1 μg of pCMV4T−Fos expression vector (Fos wt) or pCMV4T−Jun expression vector (Jun wt) in the presence of 250 ng pCMV4T−NF-κB expression vectors (p65, p65mLZ, p65Δ10, p65Eco, p50) as indicated. The total amount of DNA was held constant in all transfections by the addition of varying amounts of parental pCMV4T vector DNA. Six hours after transfection, CAT activity was determined. The mean levels of CAT activity with standard error determined in at least three independent experiments are presented as fold induction relative to activity obtained following transfection of the pCMV4T control plasmid alone. The relative basal levels of expression of 5×AP-1−TATA and SRE−TATA compared with TATA were 2:25:1. All Jun, Fos and NF-κB mutant proteins were expressed at similar levels (data not shown).

and NF-κB p50, did not show any synergistic activation when cotransfected in F9 cells (Figures 4 and 8A). Further, combinations of NF-κB p65 and Fos or Jun produced no stimulatory effects on a reporter plasmid containing the serum-response element (SRE) of the human c-*fos* promoter linked to the same TATA box (SRE-TATA-CAT) or on the parental TATA-CAT plasmid alone (Figure 8B). In addition NF-κB p65 is not able to synergize with CREB on a CRE-TK-CAT reporter or with Myb on a Myb-TK-CAT reporter (Stein *et al.*, 1993).

Discussion

This study provides evidence for an unexpected functional and molecular interplay between the NF-κB transcription factor complex and the Fos/Jun family of enhancer binding proteins. This interaction appears to be confined to the nucleus *in vivo* and leads to enhanced transcriptional activity at both κB and AP-1 enhancer-dependent promoters. These synergistic effects depend on the transactivation domains of NF-κB p65 and Jun. In addition, the functional and physical interaction of these distinct transcription factor families requires the presence of the bZIP regions of Fos and Jun and the Rel homology domain of NF-κB p65, which speaks for a link between protein−protein interaction and functional cooperation.

The functional synergism between two members of the AP-1 family, Jun and Fos, and NF-κB p65 is characterized by an increase of transcriptional activity of promoters containing κB or AP-1 enhancer motifs. Synergism is seen with minimal promoters, thus depending only on factors that can bind to the NF-κB and AP-1 binding sites. In principle, synergism could be produced by: (i) mutual increase of the other factor's synthesis, (ii) an indirect effect on the other factor's activity level, e.g. by affecting post-translational modification, or (iii) by direct physical interaction. Western blot analyses have suggested that one factor does not significantly influence the expression of the other factor (Figure 3). Moreover, we have excluded mutual synthesis control by expression of proteins from CMV enhancer-driven vectors and by expression in cells with low endogenous NF-κB and AP-1 activities (Figures 4 and 8). Various data speak for a role of direct physical interaction in the generation of synergism between NF-κB and Jun/Fos. Mutual synergism at two different elements, at κB and at the AP-1 motif, is best explained by direct association of the synergizing factors that must possess transactivation and interaction domains. Deletion of the N-terminal activation domains of Jun is associated with a complete loss of transcriptional enhancement via the κB enhancer (JunΔ1−119: Figure 1B; JunΔ1−194: Figure 4). Further, a mutation in a leucine zipper-like structure of NF-κB p65 that is part of the transactivation domain (NF-κB p65mLZ) or deletion of the C-terminal transactivation domain (NF-κB p65Eco) leads to reduction or loss of functional synergy with Fos and Jun (Figure 8A). The importance of this 'mini-leucine zipper' motif for maximal transcriptional activity of NF-κB p65 has also been noted by others (Schmitz and Baeuerle, 1991; Ruben *et al.*, 1992). This motif is lacking in other members of the Rel/Dorsal family of transcription factors, including NF-κB p50, human c-Rel and *Drosophila* Dorsal (Gilmore, 1990). The importance of the transactivation domains of Jun and Fos is also evident from our antisense experiments (Figure 2). In these experiments the induced activity of NF-κB is inhibited by antisense c-*jun* or antisense c-*fos* RNA suggesting that Jun and/or Fos provided NF-κB with their transactivation domain(s). The functional synergy is limited to NF-κB p65 and Jun or Fos since closely related members

of both transcription factor families like NF-κB p50, c-Rel, JunB and JunD as well as other factors of the bZIP family like CREB and Myb (not shown) were not able to synergize. Thus, small differences in the Rel homology domains or the transactivation and bZIP regions among these members might account for the observed lack of synergizing interaction.

Our *in vivo* data have been complemented by DNA binding studies showing that Fos and Jun proteins enhance the DNA binding activity of NF-κB *in vitro* (Figure 5B and C). The stimulatory effects on NF-κB DNA binding required the presence of the bZIP region of Jun. Interestingly, a miniature version of Jun, termed Jun-Ava, containing only the bZIP region failed to augment NF-κB DNA binding (Figure 5C) suggesting that additional sequences outside the bZIP region are necessary to stabilize this type of DNA–protein interaction. Of note, it has been shown that DNA binding of some transcription factors is increased by small basic polypeptides, such as poly-L-lysine (Bannister and Kouzarides, 1992). Since Jun-Ava is not able to increase NF-κB p65 binding through its basic DNA binding region we believe that this mechanism does not apply here. Antibodies to Fos and to Jun as well as a large excess of a competing oligonucleotide that constitutes the AP-1 binding site (Kaina *et al.*, 1989; Herrlich *et al.*, 1990) reduce the binding of NF-κB to its site. Thus Fos or Jun must be required for the formation of the NF-κB–DNA complex or be present within the complex. The identical migration of DNA complexes with IκB-free cytoplasmic NF-κB, which does not contain Fos or Jun, and those with NF-κB/Fos or Jun in EMSAs suggests that subunit exchange occurs. Band-shift position is, however, not a particularly reliable criterion. NF-κB as well as Jun/Fos are known to induce DNA bending (Schreck *et al.*, 1990; Kerppola and Curran, 1991). It is therefore possible that in a multimeric complex the DNA bending induced by NF-κB p65 will be compensated by the Jun/Fos-induced DNA bending. Thus a change in mobility by increase in complex size might not be seen.

The cross-coupling of NF-κB p65 with Jun or Fos appears to involve the formation of one or several novel heteromeric transcription factor complexes. Our *in vitro* data support the existence of NF-κB p65–Jun and NF-κB p65–Fos complexes (Figures 5, 6 and 7). This physical interaction depends both on the Rel homology domain of NF-κB p65 and the bZIP region of Jun or Fos (Figure 7). A truncated form of NF-κB p65 encompassing only the Rel homology domain (NF-κB p65Eco) interacts at least as well as wild-type NF-κB p65 with GST-Jun while a further truncation of the Rel homology domain (NF-κB p65Bgl) obliterates this interaction. Since NF-κB p65Eco lacks the transactivation domains it is not surprising that it cannot synergize with Jun and/or Fos *in vivo* (Figure 8), confirming our hypothesis that the novel heteromeric complex is more active because both transcription factor families provide their own transactivation domains. NF-κB p65Δ10, a predominantly monomeric protein, seems to interact with GST-Jun (Figure 7) and also the monomeric Fos protein interacts with NF-κB p65 (Figure 6) and Fos showed synergistic effects comparable to those of Jun (Figure 8A). However, we were unable to detect any functional synergism with NF-κB p65Δ10 *in vivo*.

What is the stoichiometric nature of the complex between NF-κB and Fos/Jun? There are two possible configurations that could be supported by the data: a monomeric association of Fos or Jun with either NF-κB p65 or NF-κB p65/p50, or a more complex multimeric configuration. The monomeric association is the best interpretation for the data of Figure 8A, column 3: Fos, in the absence of Jun in F9 cells, most probably cooperates directly with NF-κB p65 or with NF-κB p65 under participation of endogenous NF-κB p50. Bacterially expressed Fos improves the binding of limiting amounts of purified cytoplasmic NF-κB to the κB sequence (Figure 5B and C). Further, bacterially expressed FLAG-Fos co-immunoprecitates with labeled FLAG-p65 (Figure 6). These experiments rule out any potential role of other members of the AP-1 family. The suggestion that a multimeric factor complex, e.g. of NF-κB p65/p50 coupling to Fos/Jun, is formed is favored by the reciprocity of synergism at either κB or AP-1 sites. One factor would bind to the proper sequence; synergism would result from the combined usage of all transactivation domains. The formation of the multimer also seems to be suggested by the observation that the binding of Fos plus Jun to GST-p65 is more effective than that of either protein alone (Figure 7C). A precedent for the dimer–dimer interaction is set by the observation that the nuclear receptors for glucocorticoids (Diamond *et al.*, 1990; Jonat *et al.*, 1990; Lucibello *et al.*, 1990; Schüle *et al.*, 1990; Yang-Yen *et al.*, 1990) and retinoic acid (Schüle *et al.*, 1991) directly interact with Fos and Jun, which leads to suppressed rather than enhanced function of Fos and Jun.

Irrespective of the exact molecular complex configuration, the synergism seems biologically relevant. We have shown that the response of κB-driven genes to phorbol esters or ultraviolet irradiation is reduced in the absence of Fos or Jun (antisense experiments of Figure 2). Further, antisera against Jun and Fos diminished binding of nuclear NF-κB to a κB binding site (Figure 4). Thus conditions seem to exist where both AP-1 and NF-κB are activated and can synergize without artificially overexpressing one or the other factor by transfection. Additional experiments are necessary to show whether antisense p65 inhibits AP-1-dependent promoters although we would expect this reciprocity from our cotransfection experiments (Figure 8A). Both NF-κB and Jun/Fos are activated by cytokines like interleukin-1 and tumor necrosis factor-α (Brenner *et al.*, 1989; Duh *et al.*, 1989; Lowenthal *et al.*, 1989; Muegge *et al.*, 1989, 1993; Osborn *et al.*, 1989; Shirakawa *et al.*, 1989; Lafyatis *et al.*, 1990; Haliday *et al.*, 1991; Beg *et al.*, 1993). Thus the positive synergy between NF-κB p65 and Jun/Fos might have important implications for both immune and inflammatory responses. Further, both transcription factor families belong to the class of immediate early genes that are activated by T-cell mitogens and serum growth factors (Treisman, 1985, 1986; Lamph *et al.*, 1988; Baldwin *et al.*, 1991; Baker *et al.*, 1992; Han *et al.*, 1992). Therefore the cross-coupling between NF-κB p65 and Jun/Fos might also be important for the G_0/G_1 transition during cell cycle progression. One is tempted to propose that there must be conditions when cells are stimulated only for either AP-1 or NF-κB activity. For example, it has recently been reported that anti-oxidants strongly induced AP-1 but inhibited NF-κB activation (Meyer *et al.*, 1993). Under such inhibitory conditions, NF-κB would be an ideal target for synergistic activation by AP-1. In addition, since NF-κB is thought to be the more potent transcriptional activator, the

AP-1 binding site might be the preferred target of this synergistic activation. The data presented here on the cross-coupling of NF-κB with Fos and Jun highlight an additional mechanism of gene regulation, involving direct interaction of NF-κB p65 with members of a structurally distinct transcription factor family, which appears to integrate and potentiate their individual biological activities.

Materials and methods

Plasmid constructs

All promoter−CAT reporter plasmids (Stein *et al.*, 1989a; Jonat *et al.*, 1992) and most of the Jun cDNA expression vectors (Angel *et al.*, 1988b, 1989; Offringa *et al.*, 1990) have been previously described. The v-JunmBR expression plasmid was generated by PCR-assisted mutagenesis of the wild-type v-*jun* gene, replacing amino acids ^{224}KSRKRK^{229} of the basic region with ^{224}QSQKQQ^{229}. v-JunmBR/LZ encodes a 225 amino acid v-*jun* variant, containing the sequence VHDLLLKLCNQFSEHVERGCML between positions 204 and 225, which both alters the basic region and deletes the C-terminal 62 amino acids within the leucine zipper domain. T7v-Jun wt was prepared by digestion of RSVv-Jun with *Pst*I, addition of an octamer *Nde*I linker, digestion with *Nde*I and *Bam*HI and cloning of the resulting 1.8 kb fragment into the pET-3a expression plasmid (Studier *et al.*, 1990). The various Fos expression vectors E300 (wt, wild-type), D4 (mBR, mutation in basic region), FA183 (mLZ, mutation in leucine zipper), and the parental vector, RΔXneo, have been previously described (Jenuwein and Müller, 1987; Schürmann *et al.*, 1989). The pCMV4T plasmid was derived from pCMV4 (Andersson *et al.*, 1989) by inserting an oligonucleotide with stop codons in all three reading frames into the *Sma*I site. The pCMV4T-p65 and pCMV4T-p50 plasmids contain cDNAs encoding human NF-κB p65 (Ruben *et al.*, 1991) and NF-κB p50 [amino acids 1−462; (Kieran *et al.*, 1990)], respectively. The plasmid pCMV4T-p65mLZ was constructed by PCR-assisted site-directed mutagenesis of NF-κB p65, resulting in the change of amino acids ^{436}LSEALLQLQFDDEDL^{450} to ^{436}ASEAALQAQFDDEDA^{450}. The plasmid pCMV4T-p65Δ10 encodes an alternatively spliced form of NF-κB p65 with an internal deletion of amino acid residues 222−231 (Narayanan *et al.*, 1992; Ruben *et al.*, 1992). The plasmid pCMV4T-p65Eco contains a cDNA encoding the Rel homology domain of NF-κB p65 (amino acids 1−282). The mouse c-Jun and human c-Fos pCMV4T expression vectors contain full-length coding sequences of the corresponding T7 polymerase-driven vectors (Ransone *et al.*, 1989, 1990) cloned downstream of the CMV immediate early region promoter. The pCMV4T−mouse c-JunΔ1−194 plasmid was constructed by PCR-assisted deletion of the first 194 amino acids and insertion of an artificial translation initiation sequence. Expression vectors for mouse JunB and mouse JunD were generated by cloning the cDNAs for mouse JunB (Ryder *et al.*, 1988) and mouse JunD (Ryder *et al.*, 1989), respectively, into pCMV4T. The 'antisense' *fos* (SV*sof*$^+$) and 'antisense' *jun* (SVanti-*jun*) expression vectors and their corresponding control vectors (KSV10$^+$ and SV65) are as described by Schönthal *et al.* (1988a,b). The bacterial FLAG-HMK fusion protein expression vectors were constructed by cloning the cDNAs for NF-κB p65, NF-κB p65mLZ, NF-κB p50 (amino acids 1−462), mouse c-Jun and human c-Fos as PCR-amplified fragments into the *Eco*RI site of the T7 polymerase-driven FLAG-HMK vector (Blanar and Rutter, 1992). The bacterial GST fusion protein expression vectors were constructed by cloning the cDNAs for NF-κB p65 and mouse c-Jun as PCR-amplified fragments in-frame into the *Eco*RI site of pGEX-1N (Amrad). Expression vectors for *in vitro* transcription were generated by cloning the cDNAs for NF-κB p65, NF-κB p65mLZ, NF-κB p65Δ10 and NF-κB p50 (amino acids 1−462) into pGEM4 (Promega). *In vitro* translated proteins NF-κB p65Eco (amino acids 1−282) and NF-κB p65Bgl (amino acids 1−194) were generated by run-off transcription terminated at an internal *Eco*RI site or *Bgl*II site, respectively. The chimeric protein GAL4/p65Bgl was synthesized from a pGEM4 vector encoding the GAL4 DNA binding domain (amino acids 1−147) fused in-frame to NF-κB p65 (amino acids 1−194). The T7 polymerase driven expression vectors for mouse c-Jun and human c-Fos have been described (Ransone *et al.*, 1989, 1990).

Transfection of cells and analysis of CAT activity

HeLa tk$^-$ and human T47D mammary carcinoma cells were transiently transfected using DEAE−dextran (Kawai and Nishizawa, 1984) while murine NIH 3T3 fibroblasts, human GM637 fibroblasts and mouse F9 embryonal carcinoma cells were transiently transfected using calcium phosphate (Graham and van der Eb, 1973; Chen and Okayama, 1987). CAT enzymatic activity was assayed as previously described (Gorman *et al.*, 1982; Neumann *et al.*, 1987) and normalized for protein recovery (Bradford, 1976).

Nuclear extracts and electrophoretic mobility shift assays

Nuclear extracts were prepared and used in EMSAs as previously described (Stein *et al.*, 1989a). Binding buffer was 12 mM HEPES−NaOH pH 7.9, 4 mM Tris pH 7.9, 60 mM KCl, 5 mM $MgCl_2$, 0.6 mM EDTA, 12% glycerol, 5 mM dithiothreitol. The HIV-1 κB, HIV-1 mκB and the SV40 minimal origin probes were as previously described (Lücke-Huhle *et al.*, 1989; Stein *et al.*, 1989a). Polyclonal anti-Jun antibodies were produced by immunizing rabbits with a TrpE−v-Jun fusion protein prepared with the pTEv-*jun* plasmid (Angel *et al.*, 1988a). Monoclonal anti-Fos antibodies 411 were obtained from Microbiological Associates (Bethesda, MD).

Expression, purification and ^{32}P-labeling of bacterial proteins

Jun wild-type and mutant proteins mBR, mBR/LZ and Ava were expressed in *E.coli* BL21 using a pET-3a based expression vector (Studier *et al.*, 1990) with the corresponding cDNAs placed under control of a T7 polymerase promoter (Angel *et al.*, 1988a; Smeal *et al.*, 1989). After stimulation of the bacterial culture with 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 3 h, the proteins were partially purified from *E.coli* lysates by ammonium sulfate (0−40%) precipitation. The 20−40% ammonium sulfate fractions were then subjected to heparin−Sepharose chromatography and eluted with a step gradient of NaCl (0.3, 0.5 and 0.7 M). The eluted samples were dialyzed against the EMSA buffer prior to use. Fos was expressed in bacteria using a *lacZ*/FBJv-*fos* expression vector and affinity purified (Risse *et al.*, 1990). All Jun and Fos bacterial proteins were expressed at similar levels as assessed by Western blotting analysis (data not shown).

The FLAG-HMK fusion proteins were expressed in *E.coli* BL21(lysS). The GST fusion proteins were expressed in *E.coli* JM109. Bacteria were grown overnight, diluted 1:10 and, after another hour of growth, stimulated with 0.4 mM IPTG for 3 h at 32°C. Bacteria were then harvested, resuspended in 0.1 vol of PBS and sonicated for 1 min in PBS and the extracts were cleared by centrifugation.

Bacterially expressed FLAG-HMK fusion proteins were purified by low pH elution from the M2 anti-FLAG antibody column (IBI-Kodak, New Haven, CT) according to the manufacturer's instructions. FLAG-HMK fusion proteins were labeled *in vitro* with [γ-^{32}P]ATP using heart muscle kinase (Sigma) in buffer with 20 mM Tris pH 7.6, 100 mM NaCl, 12 mM $MgCl_2$, 1 mM dithiothreitol for 60 min at 37°C (Blanar and Rutter, 1992).

Co-immunoprecipitation analysis

For co-immunoprecipitation analyses 0.6 μl of ^{32}P-labeled FLAG-HMK fusion protein were combined with 5 μl of unlabeled bacterial protein in EMSA binding buffer. After 15 min incubation at room temperature the chemical cross-linker dithio-bis(succinimidylpropionate) (DSP; Pierce) was added at a final concentration of 2 mM for 30 min at room temperature. The cross-linking reaction was squelched by the addition of 100 mM ethanolamine. After increasing the volume with RIPA buffer (10 mM Tris pH 8.0, 140 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS), normal rabbit serum and protein A−Sepharose were added for 1 h at 4°C. Then the immunoreaction was cleared and specific antibody and protein A−Sepharose were added for 2 h at 4°C. After washing the immunocomplexes, the cross-linked complexes were reduced with Laemmli SDS−PAGE loading buffer (Laemmli, 1970) containing 15% β-mercaptoethanol, boiled and subjected to SDS−PAGE.

Glutathione S-transferase fusion protein interaction assay

Glutathione−Sepharose 8A beads (Pharmacia) were equilibrated in PBS, 1% Triton X-100 and then mixed with 20 vol of bacterial GST fusion proteins on a rotary shaker for 20 min at room temperature. The beads were washed three times with PBS and then equilibrated in LSBT (20 mM HEPES−NaOH, pH 7.9, 100 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.05% NP-40, 1.5% Triton X-100). Five microlitres of a 1:1 (v:v) bead slurry in PBS were combined with 5 μl of ^{35}S-labeled reticulocyte lysate in a final volume of 200 μl LSBT, 1 mM DTT, 1 mM PMSF on a rotary shaker for 10 min at 37°C and then for 40 min at room temperature. The beads were washed four times with LSBT containing 500 mM NaCl and once with 50 mM Tris, pH 6.8. The bound proteins were eluted by boiling in Laemmli SDS−PAGE loading buffer (Laemmli, 1970) and subjected to SDS−PAGE.

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