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Genetics

Trans-dominant negative mutants of Fos and Jun

(oncoproteins/leucine zipper/transcription factor/trans-activation)

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ABSTRACT **Jun and Fos nuclear oncoproteins form a complex that regulates transcription from promoters containing activator protein AP-1 binding sites. The leucine-zipper and basic-region domains of both Fos and Jun are necessary for formation of the heterodimer that binds to DNA. Reciprocal mutations in the basic region of Fos or Jun can influence the binding of the heterodimer to DNA, implying a symmetrical binding site. DNA-binding mutants of Jun exhibit increased affinity for Fos and are capable of suppressing wild-type Fos–Jun DNA-binding activity. In contrast, mutations in the basic domain of Fos, which prevent binding to DNA in association with Jun, do not significantly diminish the ability of the wild-type heterodimer to bind to DNA. These dominant negative mutants are functional *in vivo* and can be exploited to study the role of Fos and Jun in normal and transformed cells.**

The products of nuclear protooncogenes Fos and Jun are members of the growing family of proteins containing leucine zippers (1). The two oncoproteins form a noncovalent association and can regulate the transcription of genes containing activator protein AP-1 binding sites (2–4). AP-1 was first described as a nuclear factor that recognized a specific symmetrical DNA recognition sequence (TGACTCA) found in the enhancer elements of simian virus 40, the human metallothionein IIA gene, and the control regions of genes containing TPA (phorbol 12-tetradecanoate 13-acetate)-responsive promoter elements (TREs) (5, 6). AP-1, biochemically purified via its specific DNA-binding activity, was shown to contain several polypeptides ranging in size from 35 to 50 kDa (7, 8). The major polypeptide species is antigenically and biochemically related to the product of the protooncogene c-*jun*, the cellular homolog of the transforming gene of avian sarcoma virus 17 (9–14). Studies by several groups have further identified Jun/AP-1 as the Fos-binding protein p39 (2–4) and have shown that cooperation between these two nuclear oncoproteins is required for full transactivation from TRE-containing promoters in transfected cells (2, 4).

The Jun/AP-1 nuclear protein contains a region of homology to the DNA-binding domain of the yeast transcription factor GCN4 (15), known to bind as a dimer to a target sequence similar to the AP-1 binding motif (16). Additionally, the DNA-binding domains of AP-1 and GCN4 are functionally interchangeable in yeast (17, 18). Analysis of the sequences of several DNA-binding proteins has revealed a conserved region that is similar in many transcription factors. Members of this family of transcription factors include nuclear oncoproteins Fos, Myc, and Jun; the yeast transcription factors GCN4 and yAP-1; CCAAT-binding protein/enhancer binding protein (C/EBP); and cyclic AMP-responsive element binding protein (CREB) (1, 16, 19–31). This conserved region is composed of two structures: (*i*) the basic motif, which is an arginine- and lysine-rich region; and (*ii*) a leucine zipper, which consists of four to five leucine residues regularly spaced at intervals of seven amino acids whose sequence is consistent with the formation of an amphipathic α-helix (1, 20, 21, 24–27, 30). Previously, we and other groups (20, 21, 24, 26, 27, 30) have demonstrated that, while the leucine-zipper domain of both Fos and Jun are necessary for heterodimer formation, the basic region is required for DNA binding (32).

We have carried out a systematic analysis of individual amino acids in the basic region of both Fos and Jun proteins and report that functionally crucial amino acids in the basic region are conserved between Fos and Jun. Furthermore, we show that mutations within the basic region of Jun can alter its affinity for Fos and are capable of suppressing wild-type Fos–Jun DNA-binding activity. The Jun DNA-binding mutants also can function in a trans-dominant negative manner to suppress transcriptional trans-activation by Fos–Jun heterodimers.

MATERIALS AND METHODS

Mutagenesis. The isolation and characterization of the full-length clone of human *FOS* (ref. 31; W. W. Lamph, personal communication), murine Jun (12), all expression vectors, and details of oligonucleotide-directed mutagenesis have been described (21, 27). Hybridization of the JunΔRK or FosΔRK oligonucleotide (a 54-mer that contains sequences on either side of the basic region plus an additional *Bam*HI site) to the appropriate phage M13 DNA resulted in an in-frame deletion of the entire basic domain. The *in vitro* transcription, translation, Fos–Jun association, and gel shift assays were performed as described (21).

DNA Transfection and Transient Expression Assays. Mouse embryonal carcinoma F9 cells were plated in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal calf serum (FCS) at 5×10^5 cells per 10-cm tissue culture dish 24 hr before DNA transfection. Cells were transfected by the calcium phosphate coprecipitation technique (21) and exposed to the precipitate for 12 hr. After the cells were washed with phosphate-buffered saline, fresh medium containing 0.5% FCS was added, and the cells were harvested after 24 hr. When <20 μg of specific DNA was used per 10-cm culture dish, plBl31 plasmid DNA was added to give 20 μg of total DNA, and chloramphenicol acetyltransferase (CAT) activity was determined.

RESULTS

Influence of Point Mutations and Spacing Requirements in the Basic Region of Fos and Jun on DNA Binding. We asked

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Abbreviations: TPA, phorbol 12-tetradecanoate 13-acetate; TRE, TPA-responsive promoter element; TK, thymidine kinase; CAT, chloramphenicol acetyltransferase; JunLZ, leucine-zipper domain of Jun.

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whether specific changes, additions, or deletions in the highly charged basic region, amino-terminal to the leucine repeat in both Fos and Jun, would alter their DNA-binding properties (Fig. 1). Several groups have demonstrated the importance of this region with regard to DNA binding (20, 25, 26, 30); however, the relative contribution of specific residues within the domain has not been investigated. Removal of the entire arginine/lysine-rich (RK-rich in single letter code) basic region of Jun (amino acids 251–276; mutant JunΔRK) or Fos (amino acids 133–159; mutant FosΔRK) completely abolished the ability to bind to the TRE when complexed with their wild-type counterpart (Fig. 2*A*, compare lanes 2 and 3 with lane 1), although protein–protein interaction was not affected (data not shown). By utilizing site-directed mutagenesis, a *Bam*HI site was introduced in-frame into both the Fos and Jun cDNAs (Fig. 1) between the sequences coding for the basic region and the leucine zipper. This resulted in the addition of two amino acids at Fos position 164 and Jun position 282. These constructs, when cotranslated or mixed after translation, were unable to bind DNA in the presence of either their wild-type counterparts or each other (Fig. 2*A*, lane 4), despite the fact that a protein heterodimer was still formed. This suggests that there is an absolute spacing requirement between the two domains.

To examine the contribution of specific amino acids within the basic motif of both proteins, valine residues were substituted for the basic amino acids at positions 261, 262, 264, 273,275, and 276 in Jun and positions 143, 144, 146, 155, 158, and 159 in Fos as shown in Fig. 1.‡ *In vitro* translated proteins were assayed for TRE-specific DNA binding. Fig. 2*B* shows that DNA binding was reduced when [Val^{262}]Jun, [Val^{273}]-Jun, or [Val^{276}]Jun was mixed with Fos wild-type lysate (compare lanes 3, 5, and 7 with lane 1), whereas [Val^{261}]Jun, [Val^{264}]Jun, and [Val^{275}]Jun bound at nearly wild-type levels (lanes 2, 4, and 6). Similar results were observed with the Fos mutants: [Val^{144}]Fos, [Val^{155}]Fos, and [Val^{159}]Fos displayed reduced or no TRE binding (Fig. 2*C*, compare lanes 3, 5, and 7 to lane 1), whereas [Val^{143}]Fos, [Val^{146}]Fos, and [Val^{158}]-Fos bound to DNA at or near wild-type levels in the presence of Jun (lanes 2, 4, and 6). These results show that specific residues in both Fos and Jun play a role in creating stable DNA–protein interactions. Since several of the basic region mutants displayed normal DNA binding with their wild-type counterpart, a complete binding analysis among all of the mutants was undertaken. The results are summarized in Fig. 3.‡ We conclude that (*i*) all of the Jun and Fos mutants that show impaired DNA binding in association with their wild-type counterparts also were unable to bind to DNA with the corresponding mutants, and (*ii*) mutants [Val^{262}]Jun, [Val^{273}]-Jun, [Val^{144}]Fos, and [Val^{155}]Fos likely represent the residues involved in DNA contact.

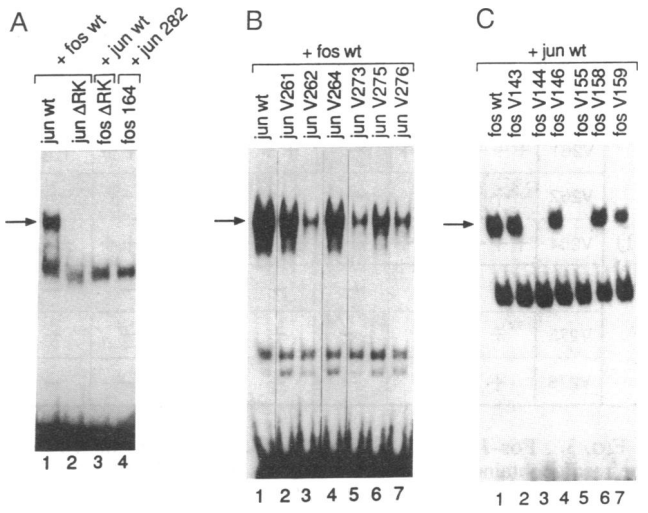

FIG. 2. Gel shift analysis of basic-region mutants. ^{32}P-labeled TRE was incubated prior to gel electrophoresis with unlabeled translation products of wild-type and basic-region deletion mutants (lanes 1–3) and Jun and Fos *Bam*HI insertion mutants (lane 4) (*A*), of wild-type Fos plus Jun basic-region point mutants (*B*), and of wild-type Jun plus Fos basic-region point mutants (*C*).

Trans-Dominant Suppression of Fos–Jun DNA Binding. Since deletion of the basic domain of either Fos or Jun abolished DNA binding but had no apparent effect on protein complex formation, we asked whether cotranslating wild-type Jun and Fos RNAs with increasing concentrations of either FosΔRK or JunΔRK RNAs would create inactive DNA-binding complexes, thus reducing the amount of TRE-binding observed when compared with control reactions containing only wild-type RNAs. Cotranslation of FosΔRK RNA had no effect on the ability of wild-type Jun and Fos proteins to bind to DNA until the mutant protein was expressed at levels 2-fold above its wild-type counterpart (Fig. 4*A*, compare lanes 1–3 with control lane 7). However, JunΔRK was able to prevent TRE binding when cotranslated at only half the level of either wild-type protein (compare lanes 4–6 with control lane 7). These results suggest that both Fos and Jun mutants have the ability to interfere with wild-type DNA binding; however, JunΔRK appears to act in a more dominant fashion than FosΔRK. Similar results were obtained when point mutations of Jun ([Val^{262}]Jun, lanes 8–10) or Fos ([Val^{144}]Fos, lanes 11–13) were used. Only Jun DNA-binding mutants showed a trans-dominant negative effect.

‡IUPAC-IUB nomenclature for named peptides modified by replacement residues is used in text; in figures, the single-letter code is used for designations. Thus [Val^{262}]Jun becomes jun V262 in figures, etc.

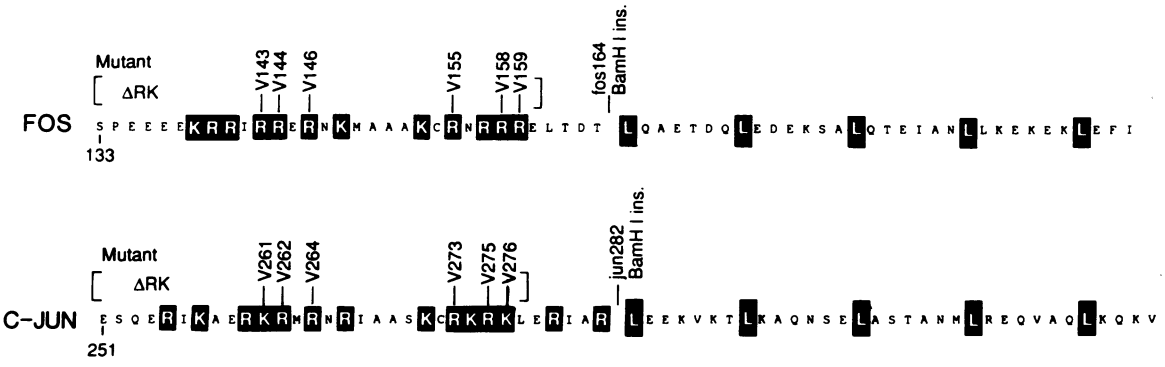

FIG. 1. DNA–binding mutants. The amino acid sequence encompassing the basic-region and leucine-zipper domains of Fos and Jun is shown. The positions of point mutations are indicated above the amino acid sequences (in single letter code) of Fos and Jun. The deleted regions (ΔRK) are designated by brackets. The *Bam*HI insertion sites at the junction of the DNA-binding and leucine-zipper domains are indicated.

		FOS						
		WT	V143	**V144**	V146	**V155**	V158	V159
JUN	WT	+++	++	−	++	−	+++	++
	V261	+++	+++	−	++	−	+++	++
	V262	+	−	−	−	−	−	−
	V264	+++	+++	−	+++	−	+++	++
	V273	+	−	−	−	−	−	−
	V275	+	+	−	+	−	−	−
	V276	+	+	−	+	−	−	−

FIG. 3. Fos–Jun DNA-binding matrix. Data are compiled from the results obtained with gel shift analysis of individual Fos and Jun basic-region point mutants with wild type (WT) and each other. +++, DNA binding by wild-type protein; ++, 25% of wild-type binding; +, 2–5% of wild-type binding; −, no binding.

Order-of-addition experiments were conducted to further test this hypothesis. Combinations of wild-type and mutant RNAs were cotranslated and then supplemented with lysate containing either Fos, FosΔRK, Jun, or JunΔRK *in vitro* synthesized proteins. DNA binding was assayed by gel shift analysis (Fig. 4*B*). When Jun and Fos wild-type RNAs were cotranslated (thus the heterodimeric complex is preformed) and supplemented with up to a 2-fold excess of FosΔRK lysate (lanes 2–4), there was no significant change in DNA-binding activity. When the order of addition was altered so that Jun and FosΔRK were cotranslated and later supplemented with either control lysate (lane 1) or wild-type Fos protein (lanes 6–8), we saw a restoration of DNA binding as the levels of added Fos protein increased. This suggests that the wild-type Fos protein is dominant to its mutant counterpart (FosΔRK) in forming a Fos–Jun–DNA complex. Identical experiments were performed with JunΔRK (Fig. 4*B*, lanes 10–12), which, unlike its Fos counterpart (FosΔRK), abolished DNA binding as increasing concentrations of the mutant protein were added to cotranslated Jun and Fos proteins (compare lanes 10–12 to control lane 9). In fact, when the JunΔRK mutant was cotranslated with Fos prior to the addition of wild-type Jun, the ability to bind to DNA was completely lost even when a 2-fold excess of Jun wild-type protein (6 μl) was added (lane 16). These data suggest that JunΔRK is a functional dominant negative mutant (33), capable of interacting with wild-type Fos and Jun proteins, thus causing the formation of nonfunctional heterodimeric complexes.

Trans-Dominant Negative Suppression of Transcriptional Trans-Activation by JunΔRK. Having demonstrated that JunΔRK can successfully suppress DNA binding of the wild-type Fos–Jun heterodimer (Fig. 4), we next asked whether this construct would display the same properties *in vivo* in a transient transfection assay. Fos, Jun, and JunΔRK expression vectors were cotransfected with TRE/TK-CAT (TK=thymidine kinase) as described (32). JunΔRK failed to cooperate with Fos in transcriptional trans-activation (Fig. 5, lane 8) as compared with wild-type Jun (lanes 6 and 7). Additionally, JunΔRK suppressed trans-activation almost 4-fold when cotransfected at either a 1:1 (lane 9) or a 2:1 (lane 10) wild-type/mutant ratio. Thus, JunΔRK is a functional dominant negative mutant *in vivo* as well as *in vitro*.

Mutations in the Basic Region of Jun Alter Its Affinity for Fos Protein. Dominant suppression of DNA binding by a mutant Jun protein would suggest that it, compared with the wild-type Jun polypeptide, has a higher binding affinity for Fos. To test this hypothesis at the protein level, [^{35}S]-

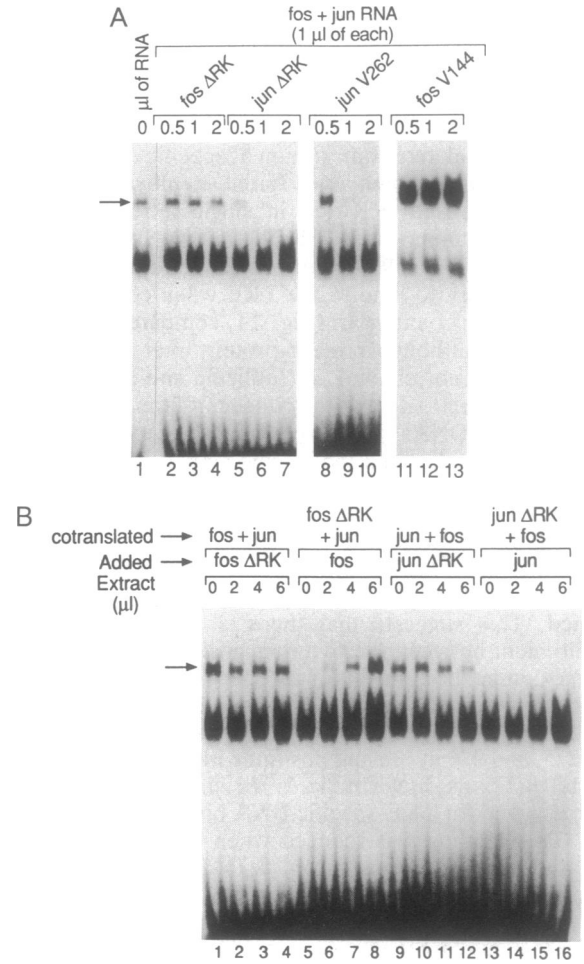

FIG. 4. Trans-dominant suppression of DNA binding. (*A*) Cotranslated products of Fos and Jun RNAs (lane 7) plus the indicated amounts of FosΔRK (lanes 1–3), JunΔRK RNA (lanes 4–6), [Val^{261}]Jun (lanes 8–10), or [Val^{144}]Fos (lanes 11–13) were assayed for binding to DNA as described (21). (*B*) Increasing concentrations of FosΔRK, Fos wild-type, JunΔRK, or Jun wild-type lysate were mixed with cotranslated products of Fos and Jun (lanes 1–4), FosΔRK and Jun (lanes 5–8), Jun and Fos (lanes 9–12), or JunΔRK and Fos (lanes 13–16) RNAs as indicated. The lysates were then assayed for DNA binding by gel shift analysis. The specific protein–DNA complex is indicated. The lower band is related to nonspecific binding of proteins from the reticulocyte lysate (27).

methionine-labeled Fos, Jun, and JunΔRK proteins were synthesized *in vitro* and utilized in competitive protein binding assays containing increasing concentrations of a synthetic peptide corresponding to the leucine-zipper domain of Jun (JunLZ). The amount of Jun protein coimmunoprecipitated with Fos decreased as the concentration of JunLZ peptide was increased (Fig. 6*A*), demonstrating that the peptide can act as a specific inhibitor of heterodimer formation. Identical concentrations of an amino-terminal Jun peptide (amino acids 6–24) had no effect on the ability of Fos and Jun to form heterodimers (data not shown). At a concentration of 0.02 mM JunLZ peptide, Fos–Jun complex formation is completely blocked (in Fig. 6*A*, compare control lane 1 to lane 3); in contrast, Fos–JunΔRK complexes were able to form at a peptide concentration of 0.03 mM (lanes 7–9). Approximately 2-fold more JunLZ peptide compared with the wild-type Fos–Jun dimer, was needed to reduce maximal heterodimer formation of Fos and JunΔRK to 50% (Fig. 6*B*). When binding experiments were carried out with two of the Jun basic-region point mutants ([Val^{264}]Jun and [Val^{275}]Jun; Fig. 1), there was a 1.5-fold increase over wild type in the affinity

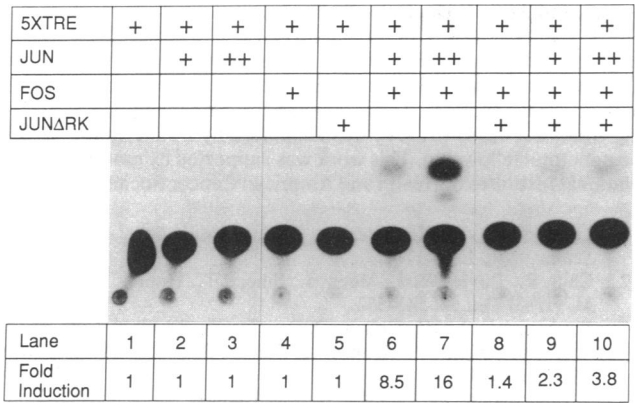

FIG. 5. Trans-dominant negative suppression of transcriptional trans-activation by JunΔRK. Cotransfection experiments using TRE/TK-CAT as a reporter plasmid were carried out in mouse embryonal carcinoma F9 cells with different ratios of wild-type and mutant DNA as follows: TRE/TK-CAT, 2 μg (lanes 1–10: Jun, 2 μg (lanes 2, 6, and 9), 4 μg (lanes 3, 7, and 10), and 6 μg (lane 4); Fos, 10 μg (lanes 4 and 6–10); and JunΔRK, 2 μg (lanes 5 and 8–10). The fold induction of trans-activation is indicated at the bottom.

for Fos protein (Fig. 6*C*). Thus, the affinity of Jun protein for Fos is doubled when the basic domain is removed or altered by specific amino acid substitutions. These results also point out that regions outside of the leucine zipper contribute to stable heterodimer formation.

DISCUSSION

Gene transcription requires the binding of specific proteins to specific DNA sequences. Modes of specific protein–DNA interaction include: (*i*) the helix-turn-helix motif (34); (*ii*) the zinc-finger-binding structure, which has been found in a large number of putative and established transcription factors (35); (*iii*) the homeodomain, first recognized in homeotic genes responsible for regulating *Drosophila* development and now also found in a variety of other transcriptional control proteins (36); (*iv*) an amphipathic helix-loop-helix motif identified recently in the immunoglobin enhancer-binding, *Drosophila* daughterless, Myc, and MyoD proteins (37); and (*v*) the leucine zipper, which mediates dimer formation through hydrophobic interactions and is thought to be involved in DNA-binding by the resulting dimeric structures (1). Fos and Jun oncoproteins form heterodimers via their leucine-zipper domain, while the basic region contributes to DNA binding (20, 21, 24–27, 30). In this manuscript we have explored the role of the basic region of Fos and Jun in DNA binding with a view towards generating trans-dominant negative–negative mutants that can suppress trans-activation by Fos–Jun heterodimers.

Fos and Jun DNA-Binding Site Is Symmetrical. The spacing between the basic region and the leucine zipper is invariant and essential for maintaining the ability to bind to DNA. Even when the spacing is altered in both proteins at identical places with identical residues, they do not compensate for each other in binding to DNA (Fig. 2*A*, lane 4), although heterodimer formation is not affected. An insertion of two additional amino acids (*Bam*HI linker corresponding to amino acids glycine and serine) might disrupt the integrity of the heterodimer such that the contact points for the DNA are misaligned in the α-helix, as there are normally 3.5 residues per turn in a coiled coil (38). Alternatively, if the Fos–Jun polypeptides criss-cross at the junction of the leucine zipper and basic region, analogous to the "scissor-grip model" (39), any alteration of spacing in this region will not be functionally tolerated. A number of groups (25, 26, 30) have demonstrated

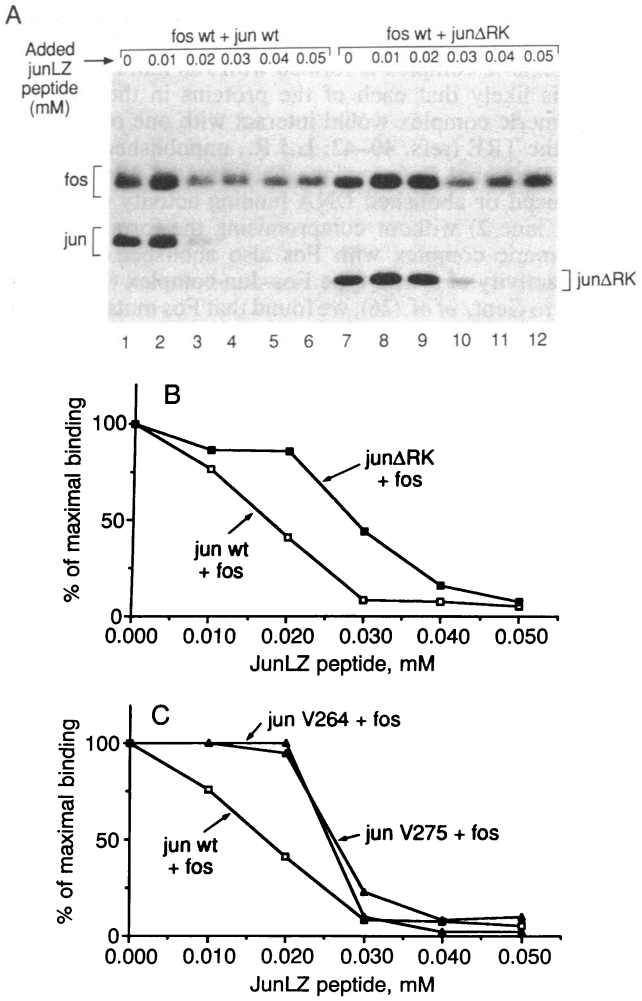

FIG. 6. Effect of a JunLZ peptide on the ability of Jun and JunΔRK to bind to Fos. (*A*) Increasing concentrations of JunLZ peptide were incubated with [^{35}S]methionine-labeled Fos and Jun (lanes 1–6) or JunΔRK and Fos (lanes 7–12) at 30°C for 30 min and were immunoprecipitated with anti-Fos monoclonal antibody. The positions of both the Fos and Jun proteins are indicated.(*B*) Binding curves for Jun or JunΔRK. The autoradiograms in *A* were photometrically scanned, and the percent of maximal binding was calculated for each concentration of JunLZ peptide. □, Jun wild type (wt) + Fos; ■, JunΔRK + Fos. (*C*) Binding curves for [Val^{264}]Jun and [Val^{275}]Jun. Autoradiograms (data not shown) were photometrically scanned, and the percent of maximal binding was calculated for each concentration of JunLZ peptide. □, Jun wild type (wt) + Fos; ▲, [Val^{264}]Jun + Fos; and △, [Val^{275}]Jun + Fos.

that the basic motif is critical in DNA binding; however, these studies involved drastic amino acid substitutions to glutamic acid and deletions that completely disrupt the local structure in that region. Site-specific mutagenesis of highly charged amino acids to valine in the basic region of Fos and Jun presented here identifies specific residues that may be critical contact points in protein–DNA interactions (Fig. 2 *B* and *C* and Fig. 3). Interestingly, the mutations made in the Jun protein did not affect its ability to homodimerize, although DNA binding and *in vivo* trans-activation was greatly reduced (L.J.R., unpublished observation).

The symmetry between the DNA binding domains of the two proteins became evident when all of the Jun mutants were tested for their ability to cooperate in DNA binding with each of the Fos mutants (see Fig. 3). The reciprocity between Fos and Jun DNA-binding mutants may also be attributed to the dyad symmetry of the TRE sequence **TGACTCA**, whereby Fos and Jun recognize similar nucleotides in the

TRE. Although Fos does not bind to DNA in a sequence-specific manner alone, it can bind specifically to the TRE when a dimeric complex is formed with Jun (20, 21). Therefore, it is likely that each of the proteins in the Fos–Jun heterodimeric complex would interact with one of the half-sites of the TRE (refs. 40–42; L.J.R., unpublished results).

Trans-Dominant Suppression. The mutated Jun proteins that reduced or abolished DNA binding activity (JunΔRK; Fig. 2*A*, lane 2) without compromising the formation of a heterodimeric complex with Fos also abolished the DNA binding activity of a wild-type Fos–Jun complex (Fig. 4). In contrast to Gentz *et al.* (26), we found that Fos mutants do not function as a dominant negative mutant even when expressed at high levels as compared with the wild-type components (Fig. 4*B*, lanes 2–4). The parameters of the experiment were important in determining the extent of FosΔRK suppression of wild-type DNA binding. Cotranslation of all RNAs prior to gel shift analysis (Fig. 4*A*, lanes 1–3) resulted in a more substantial inhibition of DNA binding than when FosΔRK was added after wild-type heterodimer formation (Fig. 4*B*, lanes 2–4). In contrast, all Jun basic-region mutants significantly suppressed DNA binding under all conditions tested. The functionality of the Jun dominant negative mutants was demonstrated by the ability of JunΔRK to suppress transcriptional trans-activation by the wild-type Fos–Jun heterodimer (Fig. 5). The reduction of trans-activation by a factor of 4 seen with equal amounts of mutant and wild-type DNA (Fig. 5, lanes 9 and 10) suggests that these constructs can be efficiently used to block the normal function of Jun, and consequently Fos protein, in normal and transformed cells.

Protein–Protein Affinities Can Be Dictated by Changes in the DNA-Binding Domain. Why can Jun mutants actively suppress wild-type DNA binding, while parallel mutations in Fos have virtually no effect? Our data suggest that the mutant Jun proteins display a higher affinity for Fos, thus acting as successful competitors for wild-type Jun protein (Fig. 6). JunΔRK is capable of disrupting the ability of preformed Jun–Fos heterodimers to bind to DNA (Fig. 4*B*, lanes 9–12); however, with increasing concentrations of protein, wild-type Jun has no effect on a preformed JunΔRK–wild-type Fos heterodimer (Fig. 4*B*, lanes 13–16). Implications of the peptide competition studies (Fig. 6) are that residues within the basic motif may also influence protein–protein interaction. This notion is further supported by Gentz *et al.* (26), who demonstrated that the conversion of three hydrophobic alanine residues present in the basic region of Fos (residues 150–152; see Fig. 1) to basic lysine residues diminished protein complex formation and abolished DNA-binding activity. Two of these alanines are conserved in Jun (Fig. 1; ref. 12), and it is conceivable that they may play a role in interchain association of Fos and Jun, as alanines are involved in interchain association of other proteins. We have also observed that mutation of the four–three repeat in the leucine-zipper domains of Fos and Jun influences their DNA binding to TRE (L.J.R., J.V., K. Morley, P.W., and I.M.V., unpublished data). Higher order structure of these regions will better address these questions. However, it is clear that the leucine zipper and basic domain do not act as totally separate moieties but are interdependent on each other to form an optimal functional unit.

The dominant negative mutants of Jun and Fos could provide information on the *in vivo* function of these transcription factors in normal and transformed cells. Understanding the association of proteins such as Fos and Jun in transcription complexes may shed light on their role in oncogenesis as well as normal cell growth and differentiation. The availability of suppressors of DNA binding and consequently transcriptional activation will enable us to further delineate the role of Fos and Jun in pathways of gene activation in normal and transformed cells.

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