Parallel Association of Fos and Jun Leucine Zippers Juxtaposes DNA Binding Domains

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The protein products of the *fos* and *jun* proto-oncogenes form a heterodimeric complex that participates in a stable high affinity interaction with DNA elements containing AP-1 binding sites. The effects of deletions and point mutations in Fos and Jun on protein complex formation and DNA binding have been examined. The data suggest that Fos and Jun dimerize via a parallel interaction of helical domains containing a heptad repeat of leucine residues (the leucine zipper). Dimerization is required for DNA binding and results in the appropriate juxtaposition of basic amino acid regions from Fos and Jun, both of which are required for association with DNA.

THE REGULATION OF GENE EXPRESSION BY EXTRACELLULAR signals underlies the long-term phenotypic response of a cell to its environment. Insights into the molecular basis of this response are now being made as a result of an amalgamation of studies on oncogenes with those concerning proteins involved in transcriptional regulation (*1*). In particular, the protein products of two proto-oncogenes, Jun and Fos, have been shown to interact with a transcriptional regulatory element known as the AP-1 binding site (*2–4*). The presence of Fos and Jun in oligonucleotide affinity-purified preparations of AP-1 led to a series of studies that identified the Fos-binding protein p39 as the product of c-*jun* (*5*). While Jun can interact with AP-1 sites in the absence of Fos (*6*), Fos requires the presence of Jun for AP-1 binding activity (*7*). Moreover, the Fos-Jun complex binds to the AP-1 site with an approximately 30 times greater apparent affinity than Jun alone, primarily because of stabilization of the protein-DNA interaction (*7*).

Both *fos* and *jun* are members of gene families whose expression is induced by a great variety of extracellular stimuli (*3, 5, 8, 9, 10*). A common feature of these genes is a structural motif referred to as the leucine zipper (*11*). McKnight and colleagues identified this motif and proposed that it functions in protein dimerization (*12*). The leucine zipper consists of a heptad repetition of leucine residues which, when arranged on an idealized α helix, align on one face at every second turn.

We have undertaken a mutagenesis analysis of Fos and Jun to identify the domains involved in protein complex formation and DNA binding (*13*). Our results show that Fos and Jun do indeed dimerize via the leucine zipper domains. However, the association appears to occur in a parallel rather than in an antiparallel configuration, and is reminiscent of the coiled-coil structure. Dimerization is required for DNA binding that involves regions of both Fos and Jun that are rich in basic amino acids.

Deletion analysis of Fos. As a first step toward delineating the regions of Fos involved in protein-protein and protein-DNA interactions, a series of truncation mutations were constructed by exonuclease III digestion of a c-*fos*(rat) cDNA clone (*14, 15*). The extent of these deletions, with reference to the leucine heptad repeat, is indicated in Fig. 1A. Protein complex formation was assayed by co-immunoprecipitation of proteins translated in vitro with antibodies to Fos (anti-Fos) or to Jun (anti-Jun) (Fig. 1B), and DNA binding activity was measured by gel-shift assay (Fig. 1C). The anti-Fos used to detect complex formation was directed against Fos amino acids 127 to 152 (*16*), a region that is present in all of the truncations shown in Fig. 1B. The data show that the truncated Fos proteins containing amino acids 102 to 380, 1 to 258, 1 to 235, 1 to 223, 51 to 237, and, to a lesser extent, 1 to 199 all associate with Jun; whereas 1 to 172 and 1 to 156 do not. This demonstrates that Fos-Jun complex formation requires the presence of a complete zipper region. DNA binding activity of the Fos-Jun complex (Fig. 1C) was detected only with the truncations that associated with Jun (102 to 380, 1 to 258, 1 to 235, 1 to 223, 1 to 199, and 51 to 237). In the cases in which no DNA binding activity specific for the Fos-Jun complex was detected [Fos(1–172), Fos(1–156), (Fig. 1C)], Jun DNA binding activity (indicated by a small arrowhead) was still present. These data define a central region of Fos (amino acids 156 to 199) that is required for both DNA binding and Fos-Jun complex formation. In the case of the truncation Fos(1–199), which contains the complete zipper domain and extends six amino acids beyond the fifth leucine, association with Jun was not efficient even though high levels of DNA binding activity were detected (Fig. 1C). This suggests that amino acids, COOH-terminal to the zipper domain, play a role in complex formation. Alternatively, the reduced ability of Fos(1–199) to associate with Jun may be because the coprecipitation assay was carried out in the presence of detergents (0.1 percent SDS, 1 percent NP-40, and 1 percent sodium deoxycholate), whereas the gel-shift assay was carried out in the absence of detergents. It is possible that detergents cause a destabilization of the Fos protein structure when the end of the protein is very close to the zipper domain.

We have obtained evidence by glutaraldehyde cross-linking and gel-shift assays suggesting that Fos and Jun bind to DNA as a heterodimer. However, in that all these assays were performed with proteins synthesized in reticulocyte lysates, we cannot rule out the possibility that other factors, present in the lysate, contribute to DNA binding.

High-resolution mutagenesis of Fos. Site-specific mutagenesis of Fos was carried out to define precisely the regions of Fos involved in heterodimer formation and DNA binding. Mutations were made by oligonucleotide replacement in the critical central region of Fos (amino acids 122 to 200) (Fig. 2) (*17*). Conversion of individual leucines in the zipper to glycine residues reduced but did not block

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association with Jun or DNA binding. However, the leucine mutations were not all equivalent; for example, FosL4-G associated with Jun more efficiently than FosL5-G. A phenylalanine substitution for L2 (FosL2-F) was tolerated better than any of the glycine substitutions. FosL2-F showed no apparent reduction in Jun association or DNA binding activities. Unlike glycine, phenylalanine can participate in hydrophobic interactions. In contrast, conversion of L5 to proline (FosL5-P) completely abolished association with Jun and DNA binding. Proline is a much more drastic substitution than glycine as it cannot participate in hydrogen bonding and it will cause a bend in α helical regions (*18*). Whereas glycine is not often found in α helices (*19*) and it cannot contribute to helix stabilization, it can participate in hydrogen bonding and will not necessarily disrupt a stable α helix. These data are consistent with the proposal that the zipper region is helical in nature and that each of the leucines contributes to complex formation (*11*).

Amino acid deletions in the zipper motif of Fos completely abolished Jun association and DNA binding. Of particular significance were the deletions Fos(179) and Fos(188–192). While Jun association and DNA binding were detectable with FosL3-G, deletion of L3 [Fos(179)] completely blocked both of these activities. Fos(188–192) in which five amino acids between L4 and L5 were deleted did not associate with Jun nor did it bind to DNA. In contrast, substitution mutations in the zipper region that did not involve leucines, Fos168,K and Fos189,191,Q,Q, had no effect on protein complex formation or DNA binding. These data are consistent with a requirement for leucines along one ridge of an α helix.

DNA binding domain of Fos is adjacent to the zipper. Mutations were also made in the highly charged region of Fos, NH_2-terminal to the leucine zipper motif, which is conserved in Fra-1 (*20*) and exhibits a low level of sequence similarity with the DNA binding domain of the yeast transcription factor GCN4 (*21*). Deletion of the sequence KRRIRRE [Fos(139–145)] completely abolished DNA binding but did not affect Fos-Jun complex formation (Fig. 2). Interestingly, this sequence is within the major epitope recognized by an anti-Fos antibody to a synthetic peptide corresponding to amino acids 127 to 152 (*16*) that is a potent blocker of gel-shift activity (*4, 7*). (Immunoprecipitation of this mutated Fos protein was not very efficient and detection required a ten times longer autoradiographic exposure than the others.) Only a subset of the basic amino acids in the deleted sequence seems to be critical for DNA binding because a substitution in this region, Fos(139–141), NNN, still exhibited detectable DNA-binding activity. In contrast, the substitution mutation, Fos143,E in which a basic residue (R) was replaced with an acidic amino acid (E), severely reduced DNA binding activity (Fig. 2C). Replacement of two other arginine residues upstream from this region with alanines (Fos125–126, AA) had no effect on DNA binding. Thus, while it is formally possible that the basic region 139 to 145 of Fos affects the DNA binding properties of the Fos-Jun complex indirectly, it is more likely that this region constitutes part of a DNA-binding domain.

Fos150–152,KKK, in which three alanine residues were replaced with three lysines, exhibited reduced protein complex formation (Fig. 2B) and no DNA binding activity (Fig. 2C). These alanines are conserved in Fra-1 (*20*) and two alanines are conserved in Jun (*22*). 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 (*23*). However, questions such as these cannot be addressed by mutagenesis studies alone and await resolution by x-ray crystallographic analysis.

Transdominant suppression of DNA binding. The mutated proteins that reduced or abolished DNA binding activity while still participating in protein complex formation with Jun [Fos150–152,KKK; Fos(139–145); Fos143,E] also abolished the DNA

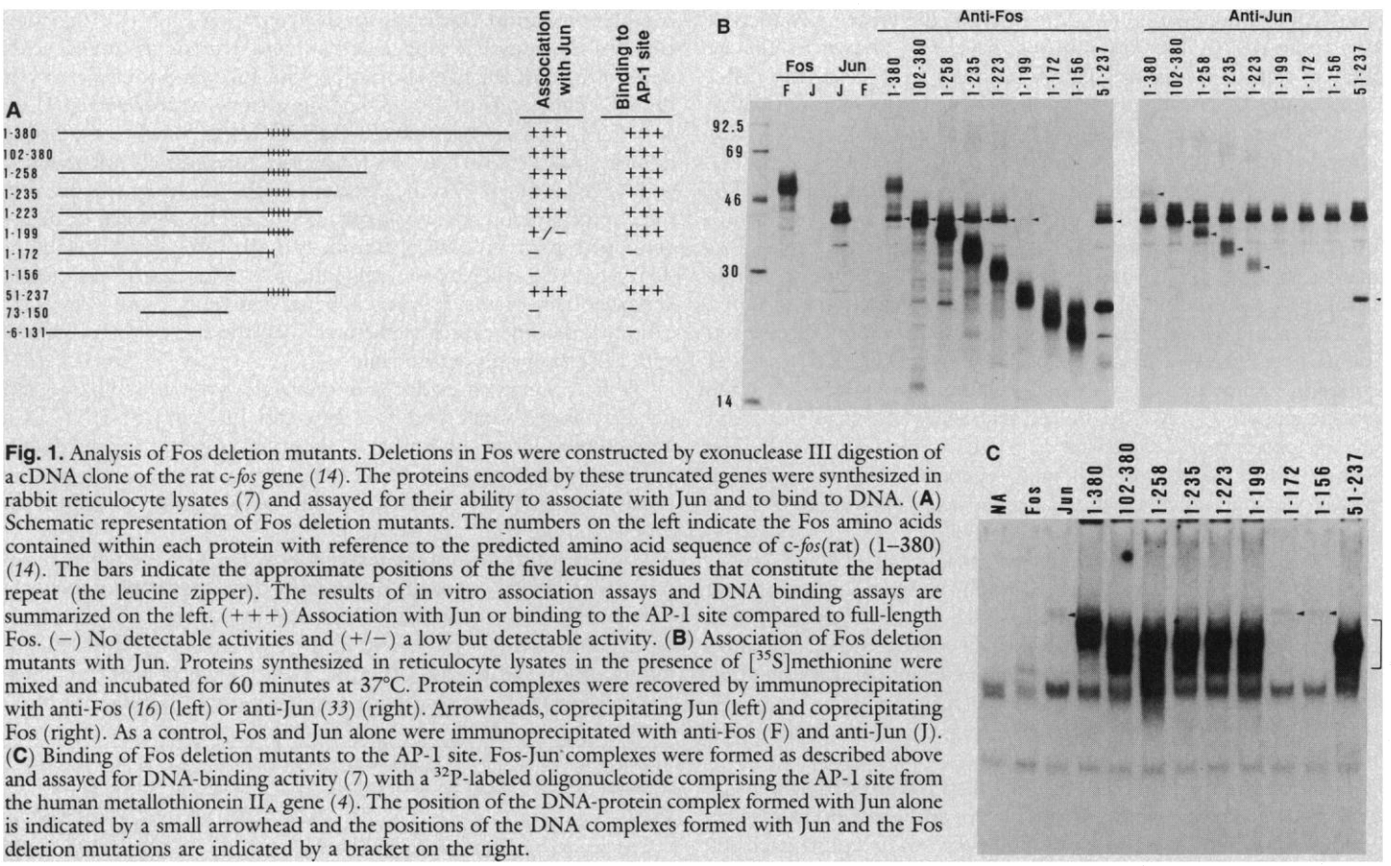

Fig. 1. Analysis of Fos deletion mutants. Deletions in Fos were constructed by exonuclease III digestion of a cDNA clone of the rat c-*fos* gene (*14*). The proteins encoded by these truncated genes were synthesized in rabbit reticulocyte lysates (*7*) and assayed for their ability to associate with Jun and to bind to DNA. (**A**) Schematic representation of Fos deletion mutants. The numbers on the left indicate the Fos amino acids contained within each protein with reference to the predicted amino acid sequence of c-*fos*(rat) (1–380) (*14*). The bars indicate the approximate positions of the five leucine residues that constitute the heptad repeat (the leucine zipper). The results of in vitro association assays and DNA binding assays are summarized on the left. (+++) Association with Jun or binding to the AP-1 site compared to full-length Fos. (−) No detectable activities and (+/−) a low but detectable activity. (**B**) Association of Fos deletion mutants with Jun. Proteins synthesized in reticulocyte lysates in the presence of [^{35}S]methionine were mixed and incubated for 60 minutes at 37°C. Protein complexes were recovered by immunoprecipitation with anti-Fos (*16*) (left) or anti-Jun (*33*) (right). Arrowheads, coprecipitating Jun (left) and coprecipitating Fos (right). As a control, Fos and Jun alone were immunoprecipitated with anti-Fos (F) and anti-Jun (J). (**C**) Binding of Fos deletion mutants to the AP-1 site. Fos-Jun complexes were formed as described above and assayed for DNA-binding activity (*7*) with a ^{32}P-labeled oligonucleotide comprising the AP-1 site from the human metallothionein II_A gene (*4*). The position of the DNA-protein complex formed with Jun alone is indicated by a small arrowhead and the positions of the DNA complexes formed with Jun and the Fos deletion mutations are indicated by a bracket on the right.

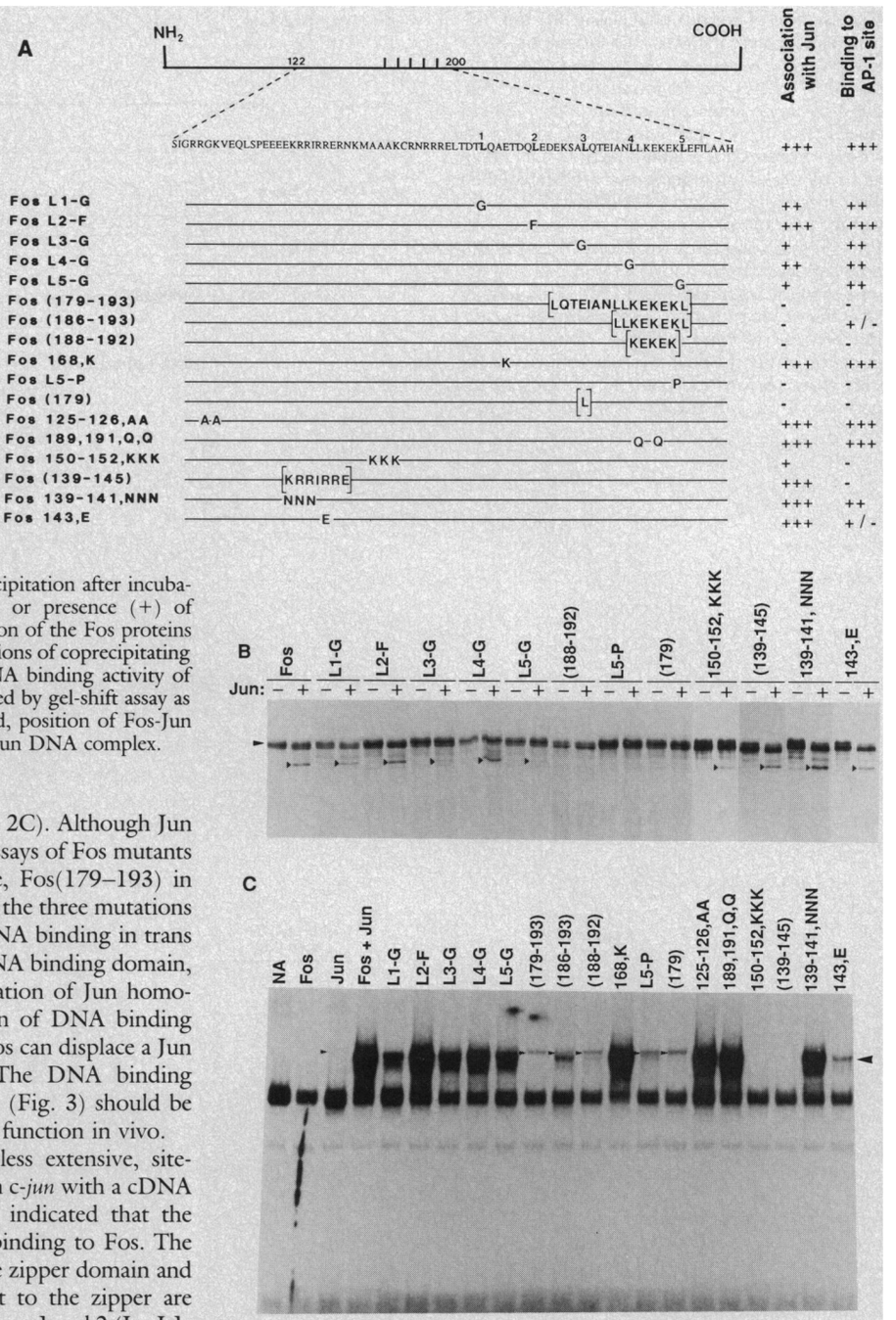

Fig. 2. Site-directed mutagenesis of Fos. The full-length c-*fos*(rat) cDNA was cloned into a plasmid vector pDS6 (*34*) containing an *f*1 origin of replication and a strong T5 promoter for synthesis of RNA in vitro. Mutations were constructed by oligonucleotide replacement with the Muta-Gene Phagemid Kit (Bio-Rad Laboratories). The structure of each mutant was confirmed by dideoxynucleotide sequencing. (**A**) Representation of the Fos mutants. The amino acid sequence of the central region of Fos (amino acids 122 to 200) encompassing the leucine zipper (*17*). The leucines of the zipper are numbered 1 to 5 with 1 being nearest the amino terminus. The mutants are named according to the position of the mutation and the substituted amino acids [see (*17*) for nomenclature of mutants]. Deletions are illustrated by indicating the numbers of the deleted amino acids in parentheses (the Fos initiator methionine is given the number 1). The results of Jun association assays and DNA binding assays are indicated on the left. The relative activities of the mutants are designated (+++), (++), (+), (+/−) or (−) by comparison with the activities of the wild-type Fos-Jun complex (+++). (**B**) The ability of Fos mutants to bind Jun was determined by immunoprecipitation after incubation for 60 minutes at 37°C in the absence (−) or presence (+) of [^{35}S]methionine-labeled Jun. The approximate position of the Fos proteins is indicated by a large arrowhead at the left. The positions of coprecipitating Jun are indicated by small arrowheads. (**C**) The DNA binding activity of mutant Fos proteins in the presence of Jun was assayed by gel-shift assay as described in the legend to Fig. 1C. Large arrowhead, position of Fos-Jun DNA complexes; small arrowheads, position of the Jun DNA complex.

binding activity associated with Jun alone (Fig. 2C). Although Jun DNA binding activity was readily detected in assays of Fos mutants that failed to associate with Jun—for example, Fos(179–193) in Fig. 2C—it was not observed in the presence of the three mutations mentioned above. Dominant suppression of DNA binding in trans occurs when a mutant Fos protein, lacking a DNA binding domain, associates with Jun, thus preventing the formation of Jun homodimers that would bind to DNA. Suppression of DNA binding activity occurs in a dominant fashion because Fos can displace a Jun molecule from a homomeric complex (*7*). The DNA binding mutations in Fos and similar mutations in Jun (Fig. 3) should be useful biological probes for investigating AP-1 function in vivo.

Mutagenesis of Jun. A similar, although less extensive, site-directed mutagenesis analysis was carried out on c-*jun* with a cDNA clone of c-*jun*(rat) (*7*). Deletion studies (*24*) indicated that the leucine zipper region of Jun was involved in binding to Fos. The properties of a series of mutations in the leucine zipper domain and those of a deletion of basic amino acids next to the zipper are summarized in Fig. 3A. The substitution of leucines 1 and 2 (JunL1, L2-V) or leucines 4 and 5 (JunL4,L5-V) with valine residues reduced both DNA binding activity and complex formation with Fos (Fig. 3, B and C). A single valine substitution for leucine 5 had a similar though less pronounced effect. A glycine substitution for leucine 5 had a more dramatic effect than a valine substitution. Valine, unlike glycine, can contribute to hydrophobic interactions as well as helix stabilization. The original description of the leucine zipper model predicted valine to be one of the least acceptable substitutions for leucine because it might block interdigitation of leucines (*11*). Although the above-mentioned Jun mutations exhibited reduced DNA binding activity in the presence of Fos, none exhibited AP-1 gel-shift activity in the absence of Fos, an indication that homodimer formation of Jun is required for DNA binding. These mutations had less of an effect on Fos-Jun heterodimer formation, suggesting that the Fos-Jun interaction is of greater affinity than the Jun-Jun interaction.

A single proline in place of L3 (JunL3-P) completely abolished Fos-Jun association and all DNA binding activity. As previously noted for the Fos mutation, FosL5-P, this result supports the proposal that the zipper region is helical in nature. Deletion of the basic amino acid region RKRMRNR [Jun(260–266)] resulted in a complete loss of DNA binding activity and a slight reduction in complexing with Fos. In summary, Jun forms homodimers and heterodimers via the leucine zipper motif and has a region of basic amino acids adjacent to the zipper that is required for DNA binding.

Fos and Jun leucine zippers associate in parallel. In the original description of the leucine zipper model, Landschulz *et al*. favored an antiparallel conformation for the leucine zipper (*11*). They argued that interdigitation of leucines, predicted by the zipper model, is more permissible for antiparallel helices. The availability of muta-

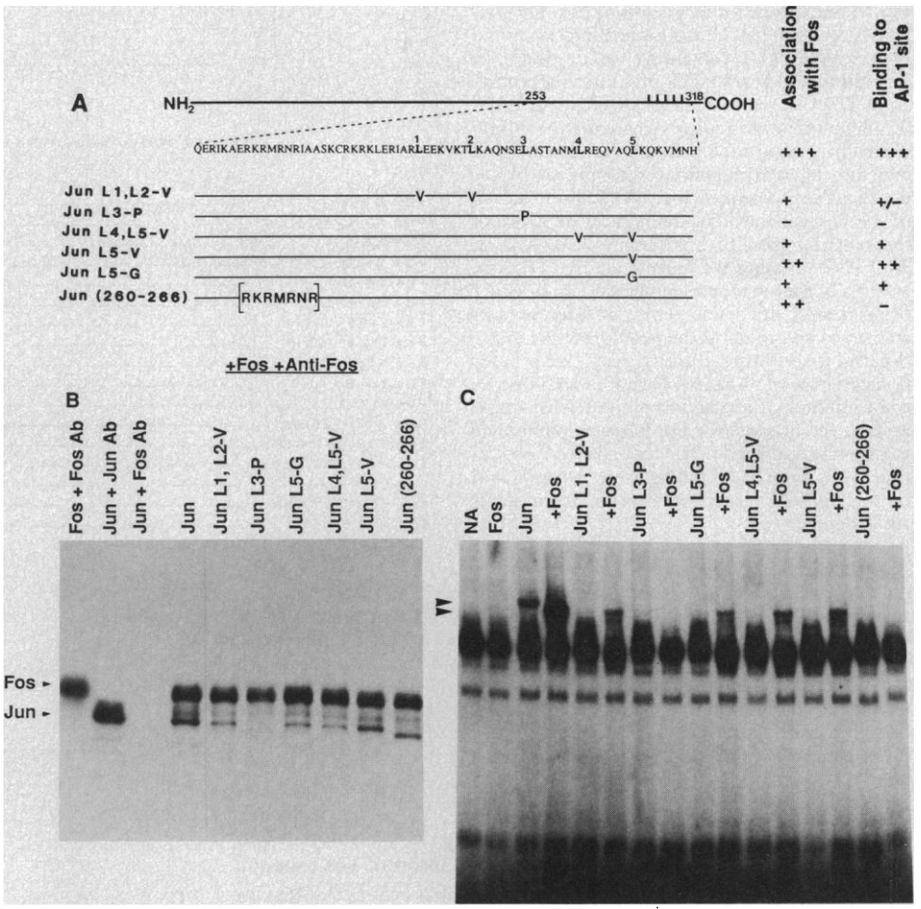

Fig. 3. Site-directed mutagenesis of Jun. (**A**) Schematic representation of Jun mutations. Mutations were made in a c-*jun*(rat) cDNA (7) as described for Fos. Jun amino acids 253 to 318 are listed in single letter amino acid code. The leucines of the zipper are numbered 1 to 5 in the amino-to-carboxyl-terminal direction. The nature and activities of Jun mutations are indicated in the same manner as the Fos mutations in Fig. 2. (**B**) Coprecipitation of mutated Jun proteins with Fos. Protein complexes were formed and immunoprecipitated with anti-Fos antibodies as described in the legend to Fig. 1. (**C**) DNA binding activities of Jun mutations. The ability of mutated Jun proteins to bind to DNA alone or in the presence of Fos (+Fos) was determined by gel-shift assay. Arrowheads on the left indicate the position of the Jun and the Fos-Jun DNA complexes.

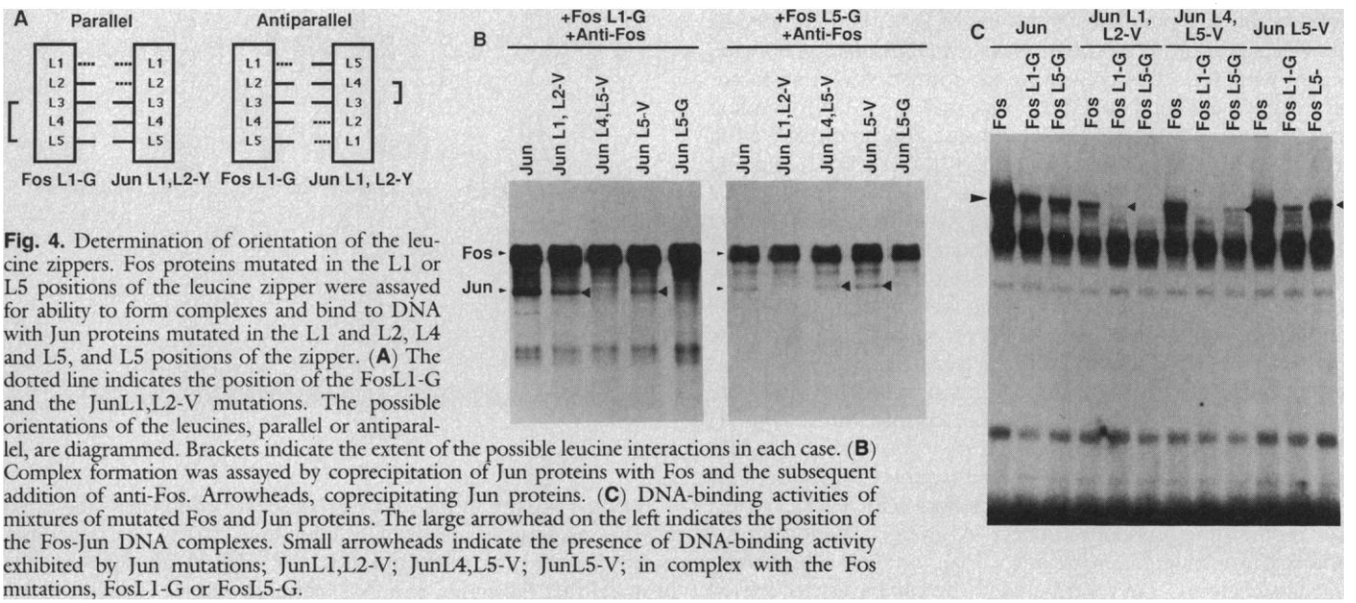

Fig. 4. Determination of orientation of the leucine zippers. Fos proteins mutated in the L1 or L5 positions of the leucine zipper were assayed for ability to form complexes and bind to DNA with Jun proteins mutated in the L1 and L2, L4 and L5, and L5 positions of the zipper. (**A**) The dotted line indicates the position of the FosL1-G and the JunL1,L2-V mutations. The possible orientations of the leucines, parallel or antiparallel, are diagrammed. Brackets indicate the extent of the possible leucine interactions in each case. (**B**) Complex formation was assayed by coprecipitation of Jun proteins with Fos and the subsequent addition of anti-Fos. Arrowheads, coprecipitating Jun proteins. (**C**) DNA-binding activities of mixtures of mutated Fos and Jun proteins. The large arrowhead on the left indicates the position of the Fos-Jun DNA complexes. Small arrowheads indicate the presence of DNA-binding activity exhibited by Jun mutations; JunL1,L2-V; JunL4,L5-V; JunL5-V; in complex with the Fos mutations, FosL1-G or FosL5-G.

tions in the leucine zippers of both Fos and Jun permitted an analysis of this proposal. The rationale behind this "orientation test" is illustrated schematically in Fig. 4A. Two possible orientations for association of the FosL1-G protein with JunL1,L2-V are indicated. In the parallel configuration, FosL1-G and JunL1,L2-V can still interact over a large region of the zipper; whereas in the antiparallel configuration a more reduced region is available for appropriate interaction (Fig. 4A). In essence, the effects of the mutations in each protein would be additive in only one orientation. This analysis was carried out with several different combinations of Fos and Jun mutations, and in all cases the results obtained were consistent with a parallel orientation of the leucine zipper.

In co-immunoprecipitation assays, FosL1-G interacted with JunL1,L2-V but not with JunL4,L5-V (Fig. 4B). Reduced binding of FosL1-G to JunL5-V was evident, although JunL5-V was a well-tolerated mutation when assayed with wild-type Fos. No binding of FosL1-G to JunL5-G was detected. The FosL5-G substitution bound to JunL4,L5-V but not to JunL1,L2-V. It associated with

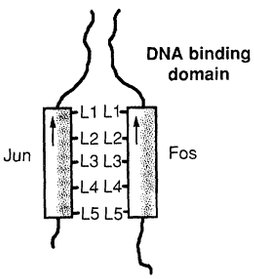

Fig. 5. Orientation of the Fos-Jun zipper region and DNA binding domains; the orientations of leucines 1 to 5 in the zipper region of Fos relative to those of Jun in a Fos-Jun complex. The approximate, relative positioning of the DNA binding regions of Fos and Jun are indicated. This diagram is intended to predict an orientation of these regions but is not intended to dictate a structural model for the proteins.

JunL5-V as well as it did to wild-type Jun. These data are compatible with a parallel configuration of the zippers. The results obtained from DNA binding assays (Fig. 5C) also imply a parallel association of the Fos and Jun zippers. The most telling example was the JunL5-V mutation, which gave levels of DNA binding with FosL5-G approaching that of wild-type Jun, but which gave a much reduced DNA binding activity with FosL1-G.

The leucine zipper as a coiled-coil. The observations described above are consistent with a parallel association of α helical regions of Fos and Jun. Recent elegant studies on synthetic peptides corresponding to the leucine zipper region of GCN4 also suggest a parallel association of α helices in the GCN4 leucine zipper (*25*). A parallel configuration associated with a heptad repeat of hydrophobic amino acids (particularly leucines) is reminiscent of the coiled-coil structure described by Crick (*26*). In the typical coiled-coil structure, hydrophobic interactions occur between amphipathic α helices at the 4,3 positions, which form a ridge of hydrophobicity along one face that can interact with a hydrophobic ridge of another α helix (*26, 27*). This "knobs in holes" association is distinct from the interdigitation model proposed for the leucine zipper (*11*). In the case of Jun, the zipper region can form a typical amphipathic helix as each of the amino acids along the 4,3 ridge are hydrophobic. In the case of Fos, the leucine residues align on a ridge with two lysines. Although lysines are charged amino acids, their side chain has a four-carbon backbone that is capable of participating in hydrophobic interactions (*25, 28*).

It is possible that the unique feature of the Fos zipper region, the lysine residues within the hydrophobic ridge that are conserved in Fra-1 (*20*), contributes to the specificity of the zipper interactions. That is, lysines reduce homodimeric association of Fos or Fra-1 (*29*) as they would be present on both interacting α helices.

Deletion and substitution mutations in regions of Fos and Jun containing basic amino acids indicate that both proteins contribute to the formation of a DNA-binding domain (Fig. 5). By analogy to other DNA binding proteins (*30*), we suggest that regions from each protein recognize a half-site of the AP-1 recognition sequence. This requires that the DNA-binding domains of Fos and Jun must be brought into close proximity with each other as the core recognition sequence consists of seven base pairs (*31*). The apparent affinity of the Fos-Jun heterodimer for the AP-1 site is 30-fold greater than that of the Jun-Jun homodimer (*7*). It is plausible that the affinity for DNA is, in part, a reflection of the affinities of the respective leucine zippers for dimerization. This is supported by the observation that mutations in the Jun leucine zipper abolished the ability of Jun homodimers to bind to DNA although several of the mutated Jun proteins could still bind to DNA in association with Fos (Fig. 3C).

The proteins encoded by several members of the *fos* and *jun* gene families can participate in heterodimeric complexes that bind to the AP-1 site. Each of these proteins is induced by a diverse array of extracellular stimuli although the time course of expression of each protein is variable (*19*). In fact, we have observed induction of an overlapping cascade of Fos-related proteins that participate in DNA binding complexes up to 8 hours after cell stimulation (*32*). We suggest that these proteins act in a coordinate manner to regulate patterns of gene expression in response to environmental cues. There are several ways in which they may do so. Distinct protein complexes may bind to the same regulatory elements of a subset of genes at different times post-stimulation. In this scenario, each complex may elicit different effects on gene transcription. Alternatively, the protein complexes may have unique site-specificity governed by flanking nucleotide sequences, interactions with other proteins, or availability of binding sites. In any event, the ability of these inducible proteins to form homo- and heterodimeric complexes expands the possible level of information transmission in the system. Thus, protein complexes that bind to AP-1 sites may provide a nuclear third messenger role in the biology of signal transduction.

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35. We thank S. McKnight, P. Kim, J. Anderson, and B. Franza for helpful discussions and communicating results prior to publication.

11 January 1989; accepted 3 March 1989