Leucine Repeats and an Adjacent DNA Binding Domain Mediate the Formation of Functional cFos-cJun Heterodimers

RICHARD TURNER AND ROBERT TJIAN*

The discovery that the AP-1 family of enhancer binding factors includes a complex of the cellular Fos (cFos) and cellular Jun (cJun) proteins established a direct and important link between oncogenesis and transcriptional regulation. Homodimeric cJun protein synthesized in vitro is capable of binding selectively to AP-1 recognition sites, whereas the cFos polypeptide is not. When cotranslated, the cFos and cJun proteins can form a stable, heterodimeric complex with the DNA binding properties of AP-1/cJun. The related proteins Jun B and vJun are also able to form DNA binding complexes with cFos. Directed mutagenesis of the cFos protein reveals that a leucine repeat structure is required for binding to cJun, in a manner consistent with the proposed function of the "leucine zipper." A novel domain adjacent to, but distinct from, the leucine repeat of cFos is required for DNA binding by cFos-cJun heterodimers. Thus experimental evidence is presented that leucine repeats can mediate complex formation between heterologous proteins and that promotes further understanding of the molecular mechanisms underlying the function of two proto-oncogene products.

THE STUDY OF TRANSCRIPTION FACTORS THAT BIND TO THE control regions of eukaryotic genes has begun to provide new insights into the mechanisms governing transcriptional initiation in response to physiological cues and extracellular signals (*1*). Transcription factor AP-1 was originally shown to bind to the DNA sequence TGACTCA (or close variants thereof) found in the enhancers of several viral and cellular genes including SV40 and human metallothionein IIa (*2*), and to selectively activate transcription of linked genes in vitro. The AP-1 binding sites are responsible for mediating induction by tumor promoters, and AP-1 activity in vivo is stimulated by phorbol esters (*3*). These findings provided the first clue that the AP-1 family of transcription factors may be a nuclear target for specific signal transduction events that are mediated by protein kinase C. Thus, AP-1 proteins are likely to be intermediaries in transmitting information from the cell surface to the nucleus and thus elicit ordered changes in gene expression.

A major component of purified AP-1 protein preparations from HeLa cells is the *c-jun* proto-oncogene product, a polypeptide

The authors are in the Howard Hughes Medical Institute, Department of Biochemistry, University of California, Berkeley, CA 94720.

*To whom correspondence should be addressed.

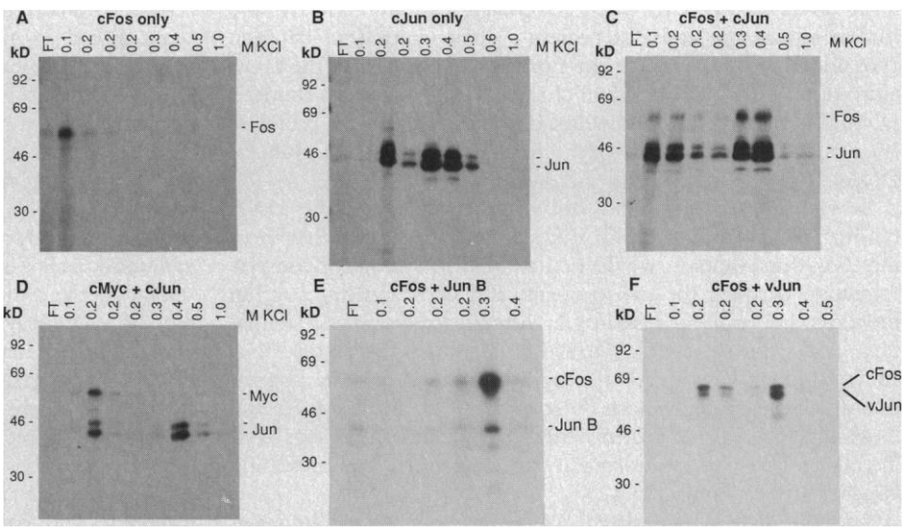

Fig. 1. AP-1 site DNA affinity chromatography assays. (**A**) cFos protein only, (**B**) cJun only, (**C**) cFos plus cJun, (**D**) cMyc plus cJun, (**E**) cFos plus Jun B, (**F**) cFos plus vJun. Plasmids were prepared and digested by standard procedures (*26*). In vitro transcripts were generated with either SP6, T3, or T7 RNA polymerase, and purified by phenol extraction and ethanol precipitation. Translations were effected in rabbit reticulocyte or wheat germ extracts (Promega) according to the manufacturers instructions, with varying amounts of [^{35}S]methionine. Extracts were diluted in Z buffer [25 m*M* Hepes-KOH, *p*H 7.7, 12.5 m*M* $MgCl_2$, 20 percent (v/v) glycerol, 0.1 percent (v/v) NP-40, 1 m*M* dithiothreitol (DTT)] to give a salt concentration of less than 0.1*M* KCl, then applied to 200-μl AP-1 site (SV40 sequence) DNA affinity columns (*27*). Proteins were eluted with washes (1 ml) of Z buffer at increasing KCl concentration (usually steps of 0.1*M*), precipitated with trichloroacetic acid, and fractionated by electrophoresis in 10 percent (w/v) polyacrylamide-SDS gels. The gels were then treated sequentially with 50 percent (v/v) methanol, 5 percent (v/v) methanol, and Amplify (Amersham), and then dried and subjected to fluorography.

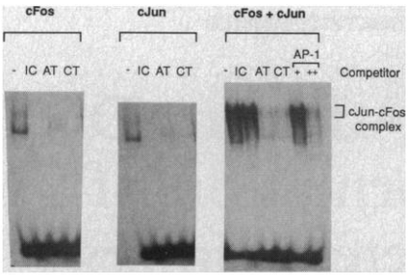

Fig. 2. Mobility shift analyses. The cJun and cFos proteins were translated (legend to Fig. 1), then mixed with a ^{32}P-labeled, double-stranded, blunt-ended oligonucleotide containing an AP-1 site (top strand sequence; 5′-GAGCCGCAAGTGACTCAGCGCGGGGCGTGTGCAGG-3′, derived from the human metallothionein IIa gene) in 20 μl of 20 m*M* Hepes-KOH, *p*H 7.9, 50 m*M* KCl, 4 m*M* $MgCl_2$, 4 m*M* spermidine, 0.2 m*M* EDTA, 0.5 m*M* DTT, 0.05 percent (v/v) NP-40, 20% (v/v) glycerol. Competitor DNA was also added to where indicated; 1 μg of poly(dI·dC), poly(dA·dT), or sonicated calf thymus DNA, 0.25 (3 ng, +) or 2.5 (30 ng, ++) pmol of unlabeled, double-stranded oligonucleotide containing one AP-1-site. Binding reactions were for 15 minutes at room temperature, and were then separated in 4 percent (w/v) polyacrylamide-gels containing half-strength TBE (tris-borate FDTA).

capable of binding specifically to the TGACTCA sequence and activating transcription in vitro (*4–6*). A second oncoprotein, cFos, that is also a putative transcription factor (*7*), is also associated with the same DNA sequence (*8*), and forms a complex with the cJun protein in nuclear extracts (*9*). The involvement of two oncoproteins in AP-1 activity emphasizes the role of these transcription factors in maintaining normal programs of gene expression (*10*). Also, AP-1 preparations contain at least one Jun-related protein, Jun B (*11*), and various Fos-related antigens of unknown function (*12*).

Inspection of the amino acid sequences of cJun, cFos, cMyc, the yeast transcriptional activator GCN4, and the mammalian enhancer binding protein C/EBP has revealed an interesting structural motif, the leucine repeat, consisting of four to five leucine residues regularly spaced at seven–amino acid intervals (*13*). This putative alpha helical "leucine zipper" region has been proposed to mediate homodimer formation of C/EBP through hydrophobic interactions, and to be involved in DNA binding by the resulting dimeric structures (*13*). In that cFos and cJun form a stable complex with each other, contacts between these two proteins may be mediated through the leucine repeats, resulting in a hetero-oligomeric complex.

We now describe in vitro studies undertaken to characterize the individual functions of cFos and cJun proteins, as well as those of potential multifactor combinations implicated in AP-1 activity. The following questions were considered. (i) Does cFos bind to the AP-1 site on its own or only when complexed with cJun? (ii) Are the leucine repeats the basis for protein-protein interactions between cFos and cJun? (iii) What is the subunit composition of the cFos-cJun complex? (iv) Do cFos and cJun both contribute to a chimeric DNA binding structure, and where does this reside in the proteins? (v) Does cFos binding alter the affinity or specificity of cJun for DNA?

In vitro–translated cFos and cJun form a specific DNA binding complex. In order to study the structure and behavior of the cFos-cJun complex, we devised an in vitro system for complex formation between the two proteins. Rat cFos and human cJun proteins were each generated by in vitro transcription and translation, and sequence-specific DNA binding was assayed on DNA affinity columns bearing AP-1 recognition sites. The entire population of labeled proteins, both those that bind to the AP-1 site and those that do not, were then eluted by sequential washings with increasing salt concentrations and subsequent electrophoresis and visualization by fluorography.

Typically, in vitro–translated cJun elutes from such columns at 0.3 to 0.4*M* KCl (with the SV40 AP-1 sequence) in (Fig. 1B), or 0.5 to 0.6*M* KCl (with the metallothionein AP-1 sequence as shown below). This behavior is characteristic of sequence-specific DNA binding and confirms our findings on the DNA binding affinity and specificity of purified HeLa AP-1 and bacterially expressed cJun (*3, 4*). In marked contrast, cFos protein synthesized alone in vitro fails to bind specifically to the AP-1 DNA binding site and is eluted at 0.1*M* salt (Fig. 1A). However, when cotranslated with cJun (Fig. 1C) or synthesized in the presence of *Escherichia coli* Jun protein, cFos now elutes with cJun in the higher salt DNA binding column fractions. Thus, it appears that cFos and cJun synthesized in vitro can form a functional complex that is required for the binding of cFos to the AP-1 DNA recognition site. The presence of cFos does not appear to affect the affinity of cJun binding to DNA as measured by the salt concentration required for elution from the AP-1 recognition sequence.

To establish the specificity of the protein-protein interaction between cFos and cJun, we tested four other leucine repeat-containing proteins (*13*), cMyc, GCN4, Jun B, and vJun (*14*), for their ability to form DNA binding complexes with either protein (Fig. 1, D to F). As expected, cMyc was unable to bind to cJun, and cFos was unable to bind to GCN4. In vitro–translated human Jun B and vJun are each able to bind to the AP-1 DNA site as measured by DNA affinity chromatography, and cotranslated cFos is able to form DNA binding complexes with both Jun B and vJun proteins (Fig. 1). These experiments show that binding between cFos and cJun, vJun, or Jun B is specific, and may reflect important interactions in vivo.

A second convenient DNA binding assay is mobility shift analysis using a ^{32}P-labeled DNA probe (Fig. 2). With either cFos or cJun proteins translated individually, the gels show weak retarded bands that are sensitive to competition by low levels of nonspecific competitor DNA. With cotranslated cFos and cJun proteins, a more prominent, slower migrating group of bands is present, which resists challenge by an excess of poly(dI-dC) competitor. The formation of this complex is efficiently inhibited by oligonucleotides containing AP-1 sites, suggesting that it represents a specific protein-DNA aggregate. These results confirm the ability of the cFos-cJun complex to bind DNA sequence specifically. However, the complex is not particularly resistant to nonspecific competitor DNA's such as poly(dA·dT) or calf thymus DNA.

Deoxyribonuclease I (DNase I) footprinting experiments have shown that various purified, bacterially expressed cJun proteins are alone capable of efficient, sequence-specific DNA binding to the AP-1 site (*4, 6*). Furthermore, bacterially expressed cJun protein activates transcription from promoters containing AP-1 sites in reconstituted in vitro transcription reactions, in the absence of detectable cFos protein (*6*). We have now confirmed the ability of in vitro–translated cJun alone to bind sequence specifically to AP-1 sites by affinity column chromatography (Fig. 1B). A discrepancy therefore exists with the results of mobility shift experiments, which suggest that cJun alone cannot associate efficiently with AP-1 DNA sites. One possible explanation of the data is that the cJun-DNA complex is unstable under gel mobility shift conditions, and that the addition of cFos significantly extends the half-life and apparent stability of the complex with DNA. However, it is also possible that mobility shift analyses disrupt the complex between cJun and DNA in an artifactual manner as a result of the application of an electric field under nonphysiological ionic conditions or the presence of a gel matrix, which could nonspecifically adsorb cJun protein. We therefore interpret such mobility shift experiments with caution until it is possible to investigate the DNA binding properties of purified cJun, cFos, and cJun/cFos aggregates by multiple assay techniques with consistent results.

The development of an in vitro model for the interaction between

cFos and cJun allows us to identify the regions of each protein which mediate complex formation. A series of deletion mutants of cFos maps the region necessary for complex formation with cJun to 125 amino acids in the center of the molecule (amino acids 102 to 227). Similarly experiments with a series of bacterially expressed truncated cJun proteins, indicate that the COOH-terminal 137–amino acid DNA binding domain (p19/cJun) (*4*) is sufficient to form a DNA binding complex with full length cFos. Therefore, in both cFos and cJun the leucine repeat forms part of the domain found to be necessary for complex formation (Fig. 3A).

Mutagenesis of the cFos leucine repeat abrogates binding to cJun. In order to dissect further the structural basis for cFos binding to cJun, we introduced single and multiple amino acid substitutions in the leucine repeat region of cFos (Fig. 3B). The effects of these mutations on the binding of full-length cFos to cJun, and thereby to the AP-1 DNA recognition sequence, were assayed by DNA affinity chromatography, mobility shift analysis, and immunoprecipitation with antibodies to cFos (anti-cFos). Mutant cFos proteins are denoted below by a superscript number which refers to Fig. 3B or 4B.

The introduction of proline residues would be expected to distort the putative alpha helix of the leucine repeat (*15*). Three single substitutions of prolines for leucines in this region indeed destroy the ability of cFos to bind to cJun and thereby to DNA [mutants cFos(1–3)]. In contrast, the substitution mutant cFos(4), in which a proline residue has been introduced immediately to the carboxyl-terminal side of the leucine repeat, is unperturbed with respect to binding to cJun and to DNA. These results suggest that disruption of the leucine repeat structure interferes with binding of cFos to cJun, which is required for sequence-specific binding of cFos to the AP-1 site.

The substitution of single alanine or valine residues in place of leucines in the repeat [cFOS(5–6)] does not significantly impair binding of the mutant proteins to cJun. However, double substitutions of valine or alanine for leucines [cFos(8–10)] abrogate binding of the cFos mutants to cJun and to DNA. In contrast to prolines, alanines and valines are strong helix-forming residues (*15*) and are therefore highly unlikely to block cFos-cJun complex formation merely through gross structural perturbation. Therefore, the leucine repeat of cFos is an essential component of the protein binding site for cJun.

Since cMyc and GCN4 contain leucine residues at seven–amino acid intervals but are unable to bind cJun or cFos respectively, the leucine residues cannot alone account for the observed specificity of the interaction between cFos and cJun. We therefore introduced multiple alanine substitutions in the cFos repeat between the leucine residues in an attempt to locate other amino acid residues involved in cJun binding [Fig. 3B, mutants cFos(11–16)]. Surprisingly, none of these mutations affects cFos binding to cJun, or the binding of the complex to DNA, in DNA affinity chromatography or mobility shift analyses. Furthermore, immunoprecipitation experiments show that in none of these mutants is the strength of binding to cJun detectably affected [solution conditions of 1*M* KCl and 0.1 percent (w/v) SDS are insufficient to separate wild-type cFos or mutants cFos(11–16) from cJun]. These results provide useful controls for the effects of mutations in the leucine positions of the repeat on cFos/cJun binding. They also suggest that the amino acid sequences responsible for conferring specificity on the cFos/cJun interaction are either located outside the leucine repeat of cFos, or are encoded redundantly within the repeat.

Effect of a charged region adjacent to the leucine repeat on DNA binding. Inspection of the cFos amino acid sequence to the

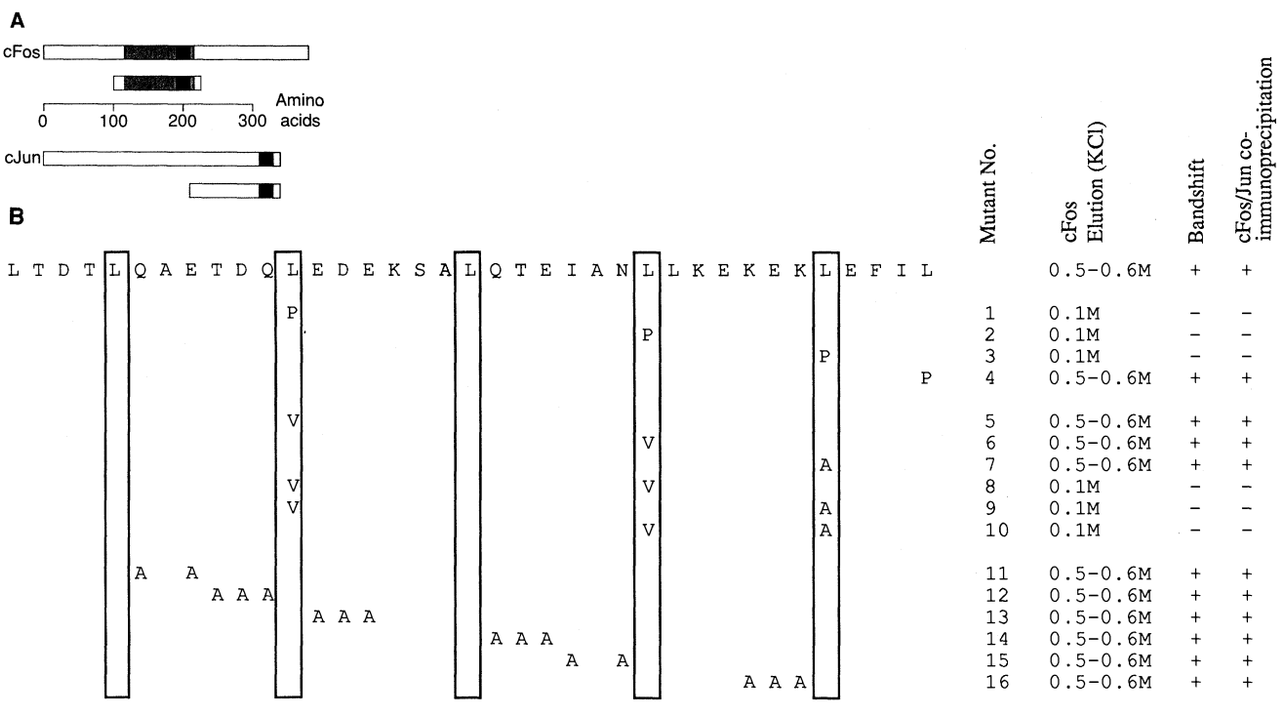

Fig. 3. (**A**) Deletion analysis of cFos and cJun with respect to complex formation. The minimal segment that can interact with the other protein in vitro is shown below the full-length sequence. Cross-hatching denotes the region of vFos that is essential for transformation (*24*). Black boxes mark the leucine repeats. (**B**) Mutations of the cFos leucine repeat region. The sequence shown consists of residues 161 to 197 of the rat cFos protein. All mutants were repeatedly assayed for binding of full-length cFos to full-length cJun protein in DNA affinity chromatography (with the metallothionein AP-1 sequence), mobility shift, and immunoprecipitation assays. Mutations were created by oligonucleotide-directed mutagenesis (*28*) of a clone extending from the Sal I site (base 440) to the Sau I site (base 820) of *c-fos* cDNA (*29*). The mutated regions were completely sequenced by chain termination, then subcloned into a recipient plasmid containing the remainder of the *c-fos* open reading frame and sequenced on the complementary strand. Plasmids were transcribed, translated, and assayed as described in the legend to Fig. 1. All *c-fos* mutants produced full-length, immunoreactive proteins, as measured by Western blotting.

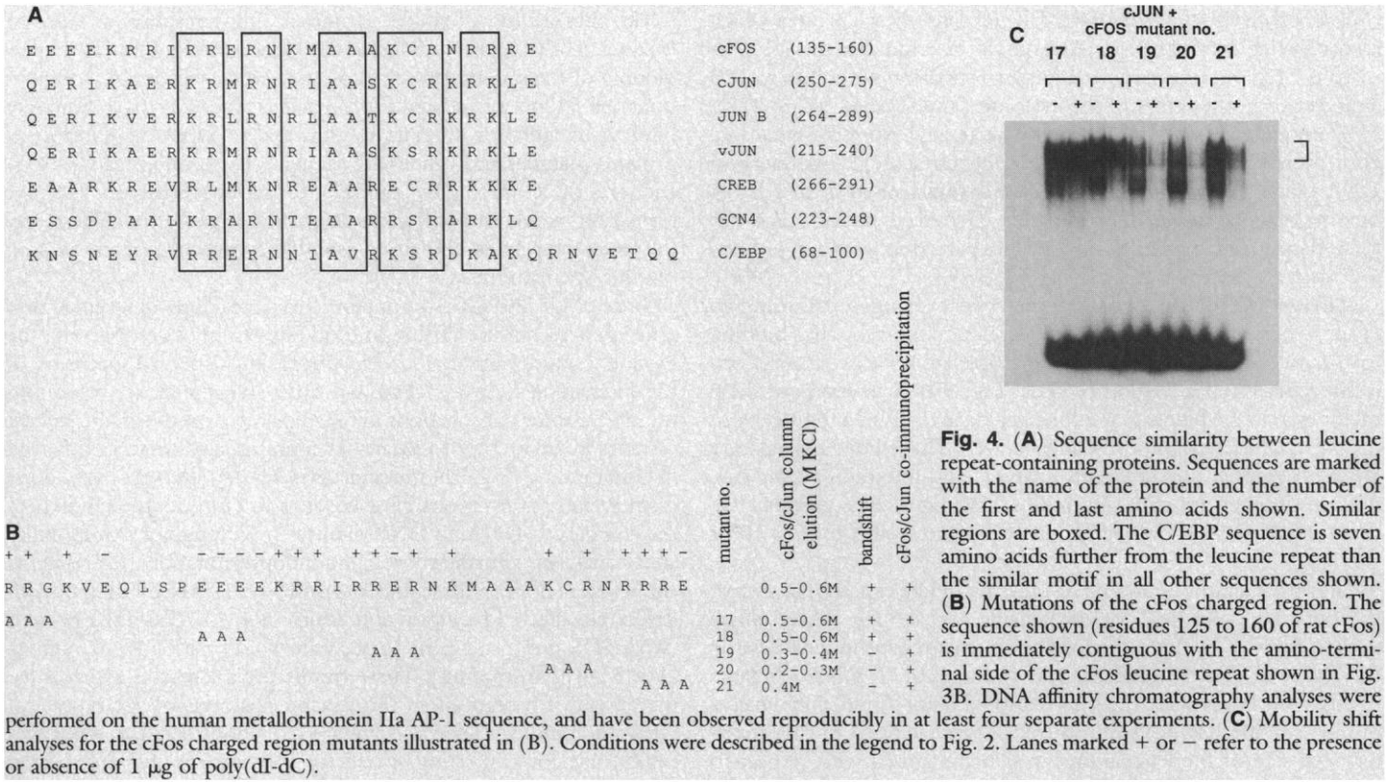

Fig. 4. (**A**) Sequence similarity between leucine repeat-containing proteins. Sequences are marked with the name of the protein and the number of the first and last amino acids shown. Similar regions are boxed. The C/EBP sequence is seven amino acids further from the leucine repeat than the similar motif in all other sequences shown. (**B**) Mutations of the cFos charged region. The sequence shown (residues 125 to 160 of rat cFos) is immediately contiguous with the amino-terminal side of the cFos leucine repeat shown in Fig. 3B. DNA affinity chromatography analyses were performed on the human metallothionein IIa AP-1 sequence, and have been observed reproducibly in at least four separate experiments. (**C**) Mobility shift analyses for the cFos charged region mutants illustrated in (B). Conditions were described in the legend to Fig. 2. Lanes marked + or − refer to the presence or absence of 1 μg of poly(dI-dC).

amino-terminal side of the leucine repeat identifies a region of the protein that is rich in basic residues. Since this sequence shares significant similarity with short stretches of amino acids in the other leucine repeat-containing proteins cJun, JunB, vJun, a CREB isolate (*16*), GCN4, and C/EBP (*17*) (Fig. 4A), this region of cFos may be involved in specific DNA binding.

Accordingly, we generated a series of triple alanine substitution mutants throughout the region (Fig. 4B), and the mutant cFos proteins were tested for their ability to bind to cJun and to the AP-1 DNA recognition sequence. Three cFos mutants, cFos(19–21), each involving the removal of two basic amino acids, show reduced avidity of specific DNA binding by the cFos-cJun complex. None of the three mutants give a reproducible mobility shift of an AP-1 site containing DNA (Fig. 5C). Most significantly, DNA affinity chromatography analysis reveals that these three mutants vary in their strength of binding to DNA, as measured by the KCl concentration required to elute cFos and associated cJun from DNA affinity columns. The cFos(20) protein has the strongest mutant phenotype with respect to DNA binding, and cFos(21) has the weakest. Immunoprecipitation analyses show that cFos(19–21) are not measurably perturbed in their ability to form complexes with cJun.

In contrast, two control mutant proteins, cFos(17–18) are not affected either in their ability to bind to cJun or to the AP-1 DNA site. These results localize amino acids involved in DNA binding to the region immediately amino-terminal to the leucine repeat of cFos, and reveal relative contributions to the DNA binding affinity of the cFos-cJun complex. We have therefore identified a region of the cFos protein that is required for sequence-specific DNA binding by the cFos-cJun complex, but is apparently not involved in the protein-protein interaction. We refer to this basic region of cFos as a DNA binding domain, which appears to be distinct from the leucine repeat.

The cFos-cJun complex binds to DNA as a heterodimer. At this point, we investigated the structure of the cFos/cJun DNA binding complex. Since the AP-1 recognition sequence has twofold symmetry, and GCN4 is thought to bind as a dimer to the same DNA sequence (*18*), it seemed likely that cJun would also bind to DNA as a dimer. Therefore, cFos might bind to a preformed cJun dimer to generate a heterologous trimer or tetramer, in which case cFos would presumably not itself be in contact with DNA. Alternatively, cFos and cJun could form a heterodimer, in which case cFos would directly contribute part of the chimeric DNA binding domain. The latter possibility appeared more likely as the cFos-cJun complex is closest in sedimentation behavior to a dimer both in nuclear extracts (*19*) and synthesized in vitro. Most important, the finding that mutagenesis of certain basic amino acids in cFos interferes with DNA binding by both cFos and associated cJun protein strongly favors the latter model.

To unambiguously determine the protomeric structure of the cJun-cFos complex, we carried out glutaraldehyde cross-linking experiments (in the absence of DNA) with cJun and cJun-cFos complexes after purification on AP-1 DNA affinity columns, and fractionated the resulting covalently linked species in denaturing gels. Such experiments show that the 40-kD cJun subunit binds to its recognition sequence as a homodimer with an apparent molecular mass of 80 to 90 kD (Fig. 5A). Similarly, cross-linking of in vitro synthesized, labeled cJun with unlabeled p19/cJun (an *Escherichia coli*–synthesized cJun product of ~20 kD truncated at the amino-terminus (*4*) results in the formation of cJun species of ~60 and ~80 kD, respectively.

We next generated labeled cFos protein in the presence of unlabeled p19/cJun. The resulting cFos-cJun complex was purified on a DNA affinity column, then subjected to glutaraldehyde cross-linking. The cross-linking rapidly converts the major cFos species of around 60 kD into a new form of ~80 kD. This is precisely the molecular mass expected for a heterodimer of cFos and p19/cJun (Fig. 5A). Since cFos alone is incapable of binding to AP-1 DNA affinity resin, the observed band cannot represent a cFos homodimer. Moreover, the 80-kD band is immunoprecipitated by antibodies to cFos (anti-cFos) and to Jun (anti-cJun) antibodies,

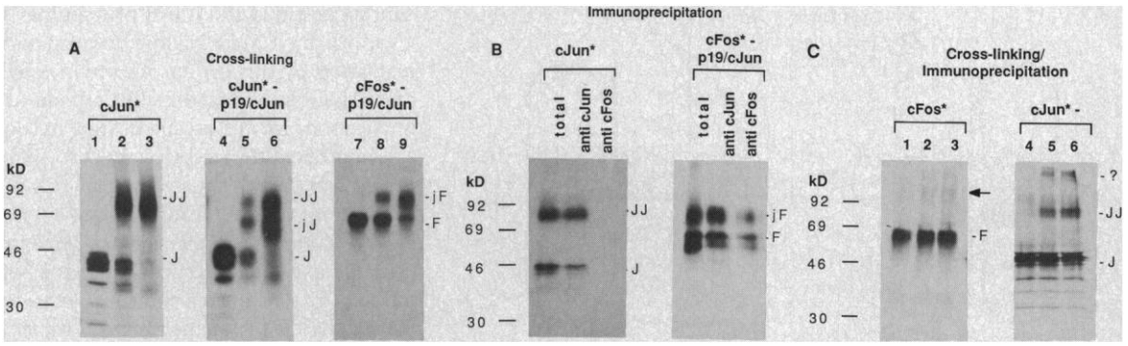

Fig. 5. (**A**) Glutaraldehyde cross-linking of cJun and cFos proteins. (Lanes 1 to 3) In vitro–translated cJun only; (lanes 4 to 6) cJun translated in vitro in the presence of affinity-purified p19/cJun (*4*); (lanes 7 to 9), cFos translated in vitro in the presence of p19/cJun. Proteins were purified by DNA affinity chromatography and eluted with buffer Z to which 1*M* KCl was added. Equal portions were treated with nothing (lanes 1, 4, and 7); 0.001 percent glutaraldehyde for 20 minutes (lanes 2, 5, and 8); or 0.001 percent glutaraldehyde for 1 hour (lanes 3, 6, and 9). Samples were then precipitated with trichloroacetic acid and subjected to electrophoresis in 10 percent (w/v) polyacrylamide-SDS gels, and the gels were fluorographed. Labeled species are denoted with asterisks. Bands are labeled as follows; cJun (J), p19/cJun (j), and cFos (F), and dimeric combinations thereof. (**B**) Immunoprecipitation of cross-linked proteins. Glutaraldehyde cross-linked cJun and cFos-p19/cJun were split into three equal portions, which were respectively trichloroacetic acid precipitated (total), or immunoprecipitated (*29*) with antiserum to cJun (anti-cJun), or antiserum to cFos (anti-cFos). (**C**) Cross-linking and subsequent immunoprecipitation of cFos and cJun proteins. In vitro–translated cFos and cJun were treated with nothing (lanes 1 and 4), 0.001 percent glutaraldehyde for 1 hour (lanes 2 and 5) or 3 hours (lanes 3 and 6). Proteins were then immunoprecipitated with anti-cFos or anti-cJun. An arrow denotes the expected position of the absent cFos homodimer. An additional band appearing in the cJun panel after cross-linking is also marked (?). This species is not observed when cJun aggregates have been selected for their ability to bind DNA.

confirming the presence of both proteins in the complex (Fig. 5B). We conclude from these direct cross-linking studies that functionally active cFos-cJun complexes exist, free in solution, predominantly as heterodimers.

cJun and potential cFos homodimers were also characterized by cross-linking in unfractionated in vitro translation extracts, with subsequent immunoprecipitation. The results (Fig. 5C) indicate that a cJun homodimer is formed efficiently after cross-linking, despite the presence of rabbit reticulocyte lysate. Surprisingly, however, cFos is unable to form homodimers to any detectable extent.

Function of the cFos leucine repeat. Our in vitro experiments on the cFos-cJun complex establish that regularly spaced leucine residues in an alpha helical structure indeed participate in heteromeric complex formation, and substituting valine or alanine residues for the relevant leucines of cFos (in pairs) is sufficient to disrupt the interaction. It therefore appears that the steric characteristics of leucines are important in the function of the repeat (*13*). However, complex formation remains unperturbed by mutation of single leucines, suggesting that the contacts between cFos and cJun are relatively stable, and most likely involve multiple additional contacts between various other residues in the two leucine repeats. Similar studies show that mutation of single leucine residues in the C/EBP leucine repeat prevents homodimerization and DNA binding; in this case, however, the effect will be doubled by the presence of two mutant subunits per dimer (*20*).

Extensive mutagenesis of the residues between the leucines in the cFos repeat shows no detectable effect on the cFos-cJun interaction. This highlights the importance of the leucine residues in complex formation, but raises questions about the molecular basis of the observed specificity of complex formation between cFos and cJun. It is possible that specific recognition between different proteins is encoded (at least in part) outside the repeat region, or that it is encoded redundantly within the region between leucines. Alternatively, our use of relatively hydrophobic alanine substitutions might not disrupt the cFos-cJun interaction because any hydrophobic residue will be compatible with close contact between the proteins (*21*). Whereas further work is required to resolve these possibilities, we suggest at least that the highly charged character of the cFos leucine repeat is not required for binding to cJun. This conclusion also implies that the leucine repeat of cFos is unlikely to be directly involved in sequence-specific DNA binding by the cFos-cJun complex.

A novel DNA binding domain shared by a family of transcription factors and oncogenes. Inspection of sequences found to the amino terminal side of the leucine repeat in cFos, cJun, and related proteins reveals a region of similarity involving predominantly basic amino acids. Mutagenesis of this region of cFos shows that these sequences are required for DNA binding by the cFos-cJun complex, and therefore defines a novel DNA binding motif in this important family of transcriptional regulatory proteins. Secondary structure predictions (*15*) show that this region is probably alpha helical in nature; however, detailed understanding of its DNA binding specificity will require further mutagenesis experiments and direct structural investigation. We note that cFos DNA binding domain mutants are "trans-dominant" in that they interfere with DNA binding by associated cJun protein, providing strong evidence that cFos contributes directly to a chimeric DNA binding domain.

Other investigators have reported that cFos stabilizes the binding of cJun to the AP-1 recognition sequence (*22*), as determined by mobility shift experiments. Using analytical DNA affinity chromatography, we find no evidence that cFos affects the affinity of cJun for the AP-1 recognition sequence, in agreement with our previous finding (with DNase I footprinting) that cJun is capable of efficient sequence-specific DNA binding in the absence of cFos (*4*). It is not clear, at the present time, which assay reflects the true behavior of cJun with respect to DNA binding in vivo.

Results from mobility shift analyses have been used (*22*) to suggest that the cFos-cJun complex may be a heterodimer. However, since the mobility of protein-DNA complexes in nondenaturing gels may not directly reflect their protein subunit composition, it is not clear from the data (*22*) whether the cFos-cJun complex binds as a dimer or higher order multimer, with significantly different implications for the role of cFos in the complex. To circumvent this problem, we have purified active cFos-cJun complexes by DNA affinity chromatography and then cross-linked heterologous subunits in the absence of DNA. These experiments allow direct molecular mass measurements of the oligomeric complex, and provide definitive evidence that the cFos-cJun complex is a heterodimer.

cFos and cJun: A chimeric oncoprotein complex with specific DNA binding properties. Our data show that cJun homodimers and cJun-cFos heterodimers are capable of sequence-specific DNA

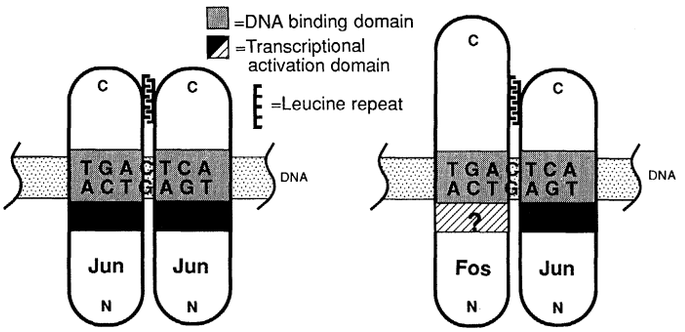

Fig. 6. Model for DNA binding and transcriptional activation by the heterodimeric cFos-cJun complex. We illustrate cFos and cJun interacting through their leucine repeats in a parallel fashion (*21*). Additional contacts between the two proteins are not implied or precluded. cFos and cJun are shown each to contact half of the palindromic TGACTCA DNA recognition sequence; the possibility that each contacts the entire sequence on opposite strands is also possible, although perhaps less likely. A transcriptional activating domain immediately adjacent on the amino-terminal side to the DNA binding domain and leucine repeat of cJun (*6*) is illustrated by shading.

binding, but that cFos alone is neither able to dimerize nor to bind DNA. This is in agreement with experiments in which purified HeLa cFos protein was unable to bind the AP-1 recognition sequence in the absence of cJun, whereas cJun was fully able to bind alone (*23*). Our finding that mutagenesis of the cFos DNA binding domain prevents DNA binding by cFos-cJun complexes suggests that two functional subunits are required for the dimer to bind DNA. We propose, therefore, that cFos is unable to bind DNA alone due to its inability to homodimerize. Further work will be required to account for the inability of cFos to form homodimers, since its leucine repeat and associated DNA binding motif are clearly functionally competent in concert with cJun, and deletion experiments have failed to identify a domain of cFos that might inhibit homodimerization.

A model (Fig. 6) summarizes our present concept of the structure and action of the cFos-cJun complex. The leucine repeats of cFos and cJun are diagrammed in a parallel arrangement, by analogy with contractile proteins and GCN4 (*21*). We depict the DNA binding element as a chimeric structure, separate from the leucine repeats, contributed by both cFos and cJun polypeptides. Finally, transcriptional activation by cJun appears to involve amino acid sequences located on the amino-terminal side of the DNA binding domain (*6*). A similar region of the cFos protein may also participate in this function (*7, 24*). The central portion of the cFos protein, which includes both the leucine repeat and the DNA binding domain, is required for its neoplastic transforming activity in vivo (*24*).

The discovery that leucine repeats can mediate contacts between different proto-oncogene products and that the complexes formed can act as promoter-selective transcription factors raises new possibilities for the differential regulation of gene expression by combinations of physically associated transcription factors. Perhaps cMyc, a putative DNA binding protein of unknown specificity, likewise requires the coordinate action of an as yet uncharacterized leucine repeat–containing protein.

Implications for transcriptional regulation and oncogenesis. A plethora of proteins have now been implicated in AP-1 DNA binding and transcriptional activation functions. The heterodimeric cFos-cJun complex, a likely transcriptional activator, represents the best characterized member of this family of proteins. It may be that cFos subtly alters the DNA binding specificity or transcriptional activity of cJun in as yet uncharacterized ways, perhaps involving interactions with other transcription factors or with chromatin. Our data suggest that the Jun B protein may be associated with cFos in vivo, which clearly expands the combinatorial possibilities for gene regulation by this group of proteins. The finding that cFos is also capable of binding to the vJun protein in vitro may have important implications for the mechanism of oncogenesis by *v-jun*.

It appears to be necessary either to synthesize cFos in the presence of cJun, or to cotranslate the proteins in order to observe efficient complex formation. How may we rationalize this observation given the requirement of the cell to generate biologically active cFos protein rapidly after serum induction? Since cJun also appears to be induced by serum with similar kinetics to cFos (*25*), it may be that the two nascent proteins form a complex immediately after stimulation, and that this is then transported into the nucleus to exert its biological effects. The precise mechanisms by which cellular signalling events such as phorbol ester induction modulate gene expression through changes in the transcriptional activity of cFos and cJun remain to be elucidated.

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