

# Non-leucine residues in the leucine repeats of Fos and Jun contribute to the stability and determine the specificity of dimerization

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## ABSTRACT

Various transcription factors, including C/EBP, GCN4 and members of the Fos, Jun and Myc families have been shown to form highly specific complexes via  $\alpha$ -helical structures referred to as leucine zippers. Experimental evidence has suggested that dimerization involves the formation of hydrophobic bonds between leucine residues in laterally aligned coiled coil structures. However, the specificity of interaction between leucine zipper proteins is not understood. In this study, we show that amino acids, which are located in positions *a*, *e*, and *g* are instrumental in the formation of Fos/Jun heterodimers, presumably by establishing intermolecular electrostatic and hydrophobic interactions. These residues are highly conserved in proteins of the Fos or Jun families but completely different between Fos and Jun, suggesting that these residues determine the specificity of interaction. This conclusion is supported by the observation that the substitution of amino acids in position *a* or *g* in Fos with the corresponding Jun amino acids facilitates the association of two Fos leucine repeats. In addition, we show that a conserved histidine residue, located 7 amino acids (i.e., two  $\alpha$ -helical turns) C-terminally to the 5th leucine in Fos and Jun, is also important for complex formation.

## INTRODUCTION

A variety of transregulatory proteins are known to bind to distinct DNA elements as dimeric protein complexes. These consist of either protein homodimers, as is the case of the transcriptional activators GCN4 and C/EBP (1,2) or heterodimers consisting of two different proteins, such as Fos and Jun (for a review see 3,4) and some members of the ATF family (5). Heterodimeric complexes between Fos and Jun [referred to as the transcription factor AP-1; (6)] bind with high affinity to a specific DNA sequence, the TPA-response element [TRE; 7–14].

It has been proposed that the interaction of the two proteins is mediated by a heptad repeat of five leucines present in both

proteins, known as the 'leucine zipper' (2). This spacing of leucine residues within a putative  $\alpha$ -helix is conserved in various other proteins including GCN4, C/EBP, Fos-, Jun-, Myc-, and ATF family members (listed in Busch and Sassone-Corsi, 15). The leucine zipper model is supported by the results of mutagenesis experiments which showed that leucine residues are essential for the dimerization of Fos and Jun (10, 16–21). Moreover, we and others could demonstrate that dimer formation is a prerequisite for specific binding to a palindromic TRE (9, 11, 16, 18, 22), stimulation of AP-1 dependent transcription and induction of morphological transformation (19). Based on observations made with synthetic leucine zipper containing peptides, an alternative model was proposed by O'Shea *et al.* which postulates that the heptad leucine repeat is a coiled coil (23, 24). In this model, a stable interaction is mediated by a 4–3 repeat of hydrophobic amino acids, and the specificity of this interaction can be modulated by charged residues in adjacent helical positions.

The 'scissors grip' model addresses the interaction of zipper protein dimers with dyad-symmetric DNA recognition sequences (25). This model predicts that the helices of the two interacting proteins disengage beyond the leucine zipper due to the repulsion of the positively charged surfaces in the adjacent basic DNA binding domains. The  $\alpha$ -helices of the two interacting proteins are suggested to bifurcate and to form Y-like shaped structures. The bifurcation point is thought to meet the DNA at the center of the dyad-symmetric site, thus enabling the 'two arms of the Y' to interact with the two half sites of the DNA recognition sequence by tracking in opposite directions along the major groove of the DNA. Recently, experimental evidence supporting the 'scissors grip' model was obtained, based on the dimerization and DNA-binding characteristics of synthetic or purified leucine zipper containing protein fragments (26–28).

The interaction of leucine residues, however, cannot account for the observed selectivity in dimer formation among proteins sharing a leucine repeat structure. It seems likely that amino acids other than leucines confer the dimerization specificity. Support for this conclusion comes from 'domain swapping' experiments. Substitution of the leucine zipper of Fos with the corresponding

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domain from either Jun or GCN4 enabled the resulting chimeric protein to form homodimers, which specifically bind to the TRE (29–31). Although a few cases have been reported, where substitutions of single non-leucine residues in Jun affected its complex forming potential with Fos (32, 33), the random mutagenesis of amino acids in the Fos zipper did not have any significant effect on its binding to Jun (19, 20).

In the present study, we have performed a systematic mutational analysis of non-leucine residues in Fos and Jun and determined their contribution to the formation of heterodimers. Our results show that amino acids in those helical positions, which in a three-dimensional  $\alpha$ -helix are located close to the leucines (i.e., positions a, e and g), are crucial for the formation of heterodimers, presumably by establishing electrostatic or hydrophobic interactions. Since these residues are highly conserved among Fos and Jun family members but different between Fos and Jun, it is likely that these amino acids determine the specificity of interaction and thus prevent Fos-Fos homodimer formation due to the repulsion of equally charged amino acid side chains. In agreement with this conclusion, we find that the dimerization of Fos zippers is facilitated when the amino acids in position a or g are replaced with the corresponding Jun amino acids. Moreover, we show, by swapping wild-type or mutant leucine zippers between Fos and Jun, that the specificity of protein complex formation via leucine repeats is not dependent on the presence of other amino acid sequences.

## METHODS

### Fos zipper mutants

The parental Fos construct used in this study is E300, a C-terminally truncated Fos hybrid gene derived from the FBJ-MuSV and FBR-MuSV encoded oncogenes. The protein derived from this hybrid construct is identical to c-Fos except for the lack of 64 C-terminal amino acids and 3 FBJ-MuSV-specific point mutations (outside the leucine zipper; also described in (19)). All Fos zipper mutants are shown in Fig. 2.

### Insertion of new restriction sites by site-directed mutagenesis

In order to facilitate the production of a large number of mutants, new unique restriction sites were introduced by oligonucleotide-directed mutagenesis, according to the gapped duplex DNA method based on the pMa/c 5–8 vector system [Stanssens, Mc Keown, Friedrich, and Fritz, unpublished data; for a detailed description see (19)]. In E300, two appropriate restriction sites had already been inserted 5' and 3' of the zipper [*Sst*I at nucleotide position 2,418 and *Sph*I at 2,547 as described in (29)]. The leucine zipper region could thus be excised with *Sst*I and *Sph*I and replaced by synthetic oligonucleotides harbouring single or multiple amino acid changes. In addition, a *Xba*I site at position 2,606 was created with the following oligonucleotide (non-coding strand of the FBR-MuSV *fos* gene):

(2,620) 5'-CCAGTCAAATCTAGAGAGGCCACAGACATCTC-3' (2,589)  
*Xba*I

All changes and substitutions by synthetic oligonucleotides were subsequently confirmed by sequence analysis. The modified E300 inserts were then subcloned into the *in vitro* transcription/translation vector pTZ18R.

### Mutants FZ1-6, FZ2-5, FZ3-5, FZ3-5J, FZ4-4/1, FZ4-6, FZ4-6J, FZ5-6, FZ6-6, and L4H

To mutate multiple amino acids in the same construct, four overlapping oligonucleotides (82 and 47bp for the coding strand,

71 and 58 bp for the non-coding strand) were treated with T4 Polynucleotide kinase, allowed to rehybridize, digested with *Sst*I and *Sph*I, isolated by gel electrophoresis, recovered on glass beads (GeneClean, Dianova) and inserted into the *Sst*I/*Sph*I sites of E300. Wherever possible, a new restriction site which did not alter the amino acid sequence was also introduced to facilitate the identification of mutants.

### Mutants FZ1-2 and FZ3-2

In a first cloning step we inserted 4 new unique restriction sites into the Fos zipper region without changing the amino acid sequence (construct FZ0, not listed in Fig. 2), using the procedure described above. These were *Xho*I at position 2,450, *Afl*III at 2,495, *Eco*RI at 2,516 and *Hind*III at 2,522 (35). Mutants FZ1-2 and FZ3-2 were then generated by cloning a synthetic, double-stranded oligonucleotide into the *Xho*I (2,450)/*Eco*RI (2,516) sites or *Xho*I (2,450)/*Hind*III(2,522) sites, respectively.

### Mutants FZ4-4/2, FZ4-3, FZ4-2, FZ4-1, FZFZ3-3/4-2J, and FZ4-4/3-2J

The internal *Afl*III (2,495) site in most of the mutants described allowed the exchange of the N-terminal or C-terminal halves of the Fos zipper. Mutants FZ4-4/2 and FZ4-3 were generated by replacing the C-terminal leucine zipper sequences (*Afl*III to *Sph*I) of FZ4-6 and 4-4/1 with wild-type sequences present in FZ0. FZ4-2 and 4-1 were obtained by replacing the N-terminal sequences (*Sst*I to *Afl*III) with those of FZ0. Constructs FZ3-3/4-2J and 4-4/3-2J were generated by mutual exchange of the C-terminal halves of FZ4-6J and FZ3-5J (via the *Afl*III site).

### Mutants L2, L4, and L5

These constructs were generated by site-directed mutagenesis of individual leucine residues in the Fos zipper and have been described earlier (19).

### Mutants 206-AA and EGGSD

For construct 206-AA, a double-stranded oligonucleotide spanning a *Sph*I (2,547)/*Xba*I (2,606) fragment was cloned into the corresponding restriction sites of FZ0. EGGSD was generated by cloning a double-stranded oligonucleotide with *Eco*RI (2,516)-*Sly*I (2,562) termini into the corresponding restriction sites of 206-AA (providing the new *Sly*I site).

### Ψ-Fos

This construct represents E300 with a Jun leucine zipper (see Fig. 3) and has been described in (29).

### Jun zipper mutants

A full length cDNA clone of *c-jun* (37) served as template for all mutagenesis experiments. In analogy to E300, two unique restriction sites were introduced 5' and 3' to the leucine zipper by site-directed mutagenesis, a *Hind*III site at nucleotide position 1,180 and an *Eco*RI site at position 1,311 [numbering according to (37)] using the following oligonucleotides (non-coding strand):

(1,190) 5'-CCGAGCGATCCGCTCAAGCTTCCTTTCCGG-3' (1,170)  
*Hind*III

(1,333) 5'-GCATGAGTTGGCACCCAGAATTCACGTGGTTCATGAC-3' (1,297)  
*Eco*RI

### Mutants JZ3-4, JZ5-5, JZ4-6, and JZ3-2/1

These were obtained by cloning four overlapping oligonucleotides encoding the respective amino acid changes (see Fig. 2) into the new *Hind*III and *Eco*RI sites in *c-jun*, essentially as described

for some of the Fos mutants above. Construct JZ3-2/1 contains an additional *Sst*I site at position 1,244 located 5' to the two amino acid changes in helical position 3.

#### Mutants JZ1-2, JZ3-2, JZ4-1, JZ4-2, and JZ7-1 (L4)

Based on construct JZ3-2/1, oligonucleotides with *Sst*I (1,244) and *Eco*RI (1,311) termini containing the respective mutations were inserted into the corresponding vector sites replacing the 3-2/1 mutation.

#### Ψ-Jun

Four synthetic overlapping oligonucleotides encoding the entire region of the c-Fos leucine zipper (aa 160–201, see Fig. 3) were inserted into the *Hind*III–*Eco*RI sites of *c-jun* as described above. This cloning step did not regenerate the original sites since the palindromic recognition sequences were altered in the insert. In addition, the oligonucleotides harbored *Sst*I, *Xho*I, *Afl*III, *Eco*RI, and *Hind*III sites in the same positions as described for FZ0. This enabled the transfer of certain Fos zipper mutants into a Jun background.

#### JFZ3-3, JFZ3-4, JFZ4-4, JFZ4-5, JFZ3-3/4-1, and JFZ4-4/3-1

JFZ3-4 and JFZ4-5 were obtained by cloning the *Sst*I–*Eco*RI fragments of mutant FZ3-5J and the *Sst*I–*Hind*III fragment of FZ4-6J into the corresponding sites of Ψ-Jun. From this construct, a 210 bp C-terminal *Afl*III–*Sal*I fragment was excised in both mutants and replaced with the corresponding fragment of Ψ-Jun to yield constructs JFZ3-3 and JFZ4-4. Likewise, the mutual exchange of the same C-terminal DNA fragments between JFZ3-4 and 4-5 yielded constructs JFZ3-3/4-1 and 4-4/3-1.

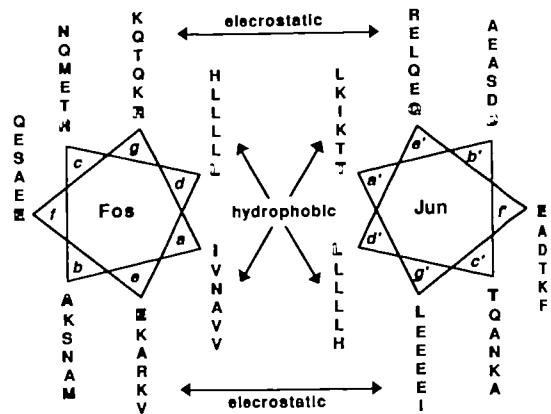
#### *In vitro* reconstitution of Fos/Jun complexes

DNA constructs were linearized and transcribed *in vitro* as described before (19). The RNA was then translated using rabbit reticulocyte lysate (Promega Biotech). The translation efficiency was checked by co-translating an aliquot of each construct in the presence of <sup>35</sup>S-methionine followed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, gels were fixed and processed in Amplify (Amersham), dried and exposed to Fuji RX film. Translation efficiency was determined by scanning the autoradiographs and identical amounts of each protein were used in reconstitution assays. All proteins were synthesized with similar efficiencies. Reconstitution experiments were performed as described before (19). <sup>35</sup>S-methionine labeled Jun was incubated with unlabeled Fos and immunoprecipitated with Fos-specific antibodies (antisera 455 or KX, the latter kindly provided by Dr. H. Rahmsdorf). Proteins were then analyzed by SDS-PAGE and processed as described above.

## RESULTS

### Mutational analysis of non-leucine residues in the Fos and Jun leucine repeat

A helical wheel representation of the Fos and Jun leucine repeats and the interactions predicted by the coiled-coil model are shown in Fig. 1 (24). We first investigated whether amino acids other than leucines may participate in Fos-Jun heterodimer formation. Mutant proteins were generated in which parts of the leucine repeats in Fos and Jun were replaced with synthetic oligonucleotides via restriction sites introduced by site-directed mutagenesis (see Methods for details). We systematically changed the amino acids located at a distance of 1, 2, 3, 4, 5 or 6 residues relative to the preceding leucine (i. e. in the helical positions *e*,



**Figure 1.** Helical wheel representation of the Fos and Jun leucine repeats according to O'Shea *et al.* (24), including the conserved histidines in position *d*. The view is from the N-terminus towards the C-terminus. The most N-terminally located amino acids in each helical position are shown in outlined fonts. Numbers refer to the position of the corresponding amino acid relative to the preceding leucine. Potential interactions between residues in helical positions *d* and *d'* (leucines), *a* and *a'* (hydrophobic), and *e* and *g*, respectively (electrostatic), are indicated by arrows.

*f*, *g*, *a*, *b*, and *c*; see Fig. 1). In a first series of mutants, all residues in a given position in Fos (which in an ideal  $\alpha$ -helix are located on top of each other as shown in Fig. 1) were altered. To preserve the  $\alpha$ -helical conformation of the leucine repeat, amino acids in the Fos zipper were replaced either with the corresponding residues from Jun or with other non-leucine amino acids that are likely to be found within  $\alpha$ -helical domains [according to Richardson and Richardson (34), e.g., alanine, valine, aspartic acid, glutamic acid or lysine] in positions *a* and *g*. A summary of all Fos mutants used in this study is shown in Fig. 2. The leucines in position *d* were excluded from the mutagenesis since their role in Fos-Jun association has already been established (19). After *in vitro* transcription/translation the mutated Fos proteins were analyzed for heterodimer formation with Jun. As shown in Fig. 4a, alterations of 5 or 6 amino acids in position *b*, *c* or *f* (lanes 6, 2 and 7) had no detectable effect, while mutations in positions *a*, *e* or *g* (lanes 4, 5, and 3) practically abolished binding (> 10% of E300, i. e., a FBJ/FBR hybrid protein containing a wild-type leucine repeat). Two leucine mutants which have previously been described were included in this analysis (for details see Methods) and showed a ~60% decrease in binding as reported in (19).

We then analyzed whether amino acids in similar positions in the leucine zipper in Jun may also be crucial for complex formation. Positions *a*, *b* and *g* in Jun were replaced either with the corresponding residues from Fos or with other  $\alpha$ -helix-forming amino acids (see Fig. 2). The results shown in Fig. 4b strongly suggest that changes in positions *a* and *g* dramatically affect binding to Fos: the substitution of 6 and 4 amino acids in these positions decreased the association with Fos by >95% and 76%, respectively (constructs JZ4-6 and JZ3-4, lanes 2 and 3), while no decrease in binding was detected when 5 amino acids in position *b* were changed (construct JZ5-5, lane 4).

### Substitution of individual amino acids located in the leucine zipper

With a second series of mutants the relevance of positions *a*, *e* and *g* in Fos and Jun was further investigated. To make the effects obtained with Fos and Jun zipper mutants comparable, single and

Hel. pos.	165	172	179	186	193	200	Binding to Jun <sup>a</sup>
Fos (E300)	ELTDT L QAEYDQ L EDEKSA L QTEIAN L LKEKEK L EFLILAA H RNSG						100
FZ1-6 <sup>b</sup>	E	K	A	R	K	V	5
FZ1-2				A	K		56
FZ2-5 <sup>b</sup>	E	A	S	E	Q		102
FZ3-5	A	A	A	A	E		7
FZ3-5 <sup>b</sup>	K	Q	T	Q	K		18
FZ3-3	A	A	A				2
FZ3-3/4-2 <sup>b</sup>	K	Q	T	V	V		20
FZ3-2				A	E		60
FZ4-6	A	A	E	K	A	D	0
FZ4-6 <sup>b</sup>	I	V	M	A	V	V	123
FZ4-4/3-2 <sup>b</sup>	I	V	M	A	Q	K	40
FZ4-4/1	A	A	A	E	A		4
FZ4-4/2	A	A	E	K			8
FZ4-3	A	A	E			D	18
FZ4-2				A			12
FZ4-1				A			94
FZ5-6 <sup>b</sup>	A	K	A	N	A	H	93
FZ6-6 <sup>b</sup>	R	T	E	H	Q	H	106
L2		V					34
L4				A			45
L5					V		73
L4H				A		A	3
EGGSD						EGGSD	35
206-AA						AA	92

Hel. pos.	283	290	297	304	311	318	Binding to Fos <sup>a</sup>
Jun	ERLAR L EEEVKT L KAQNSE L ASTAMH I REQVAQ L KQKVDH H RNSG						100
JZ1-2				A	A		68
JZ3-2/1				K	E		78
JZ3-2/2				A	A	A	68
JZ3-4	E	A	K	E			24
JZ4-6	D	E	A	E	D	K	4
JZ4-2					A	A	16
JZ4-1					A		100
JZ5-5 <sup>c</sup>	D	D		A	E	A	110
JZ7-1				V			125

**Figure 2. Top panel:** Structure of the Fos leucine zipper mutants and binding to Jun. Numbers at the top indicate amino acid positions in the Fos protein; the numbers below refer to the helical position of the respective amino acid relative to the preceding leucine (see helical wheel model in Fig. 1). The wild-type Fos (E300) sequence is shown below. The mutant sequences are identical to E300 except for the positions shown. **Bottom panel:** Structure of the Jun leucine zipper mutants and binding to E300 (Fos). Numbers at the top indicate amino acid positions in the Jun protein; the numbers below refer to the helical position of the respective amino acid (see helical wheel model in Fig. 1). The wild-type Jun sequence is shown below. The mutant proteins are identical to the wild-type Jun sequence, except for the changes indicated.

Nomenclature: FZ: Fos proteins with mutations in the zipper; the first number indicates the position of the residue(s) changed, the number following the slash is the total number of residues changed. Constructs L2, L4, and L5 have been published previously. JZ: Jun proteins with mutations in the zipper; numbers have the same meaning as described for FZ mutants.

<sup>a</sup>: percentage of binding to *in vitro* translated Jun. Values were determined by densitometry and normalized to 100% binding for E300. Each value represents a mean value of at least two reconstitution experiments.

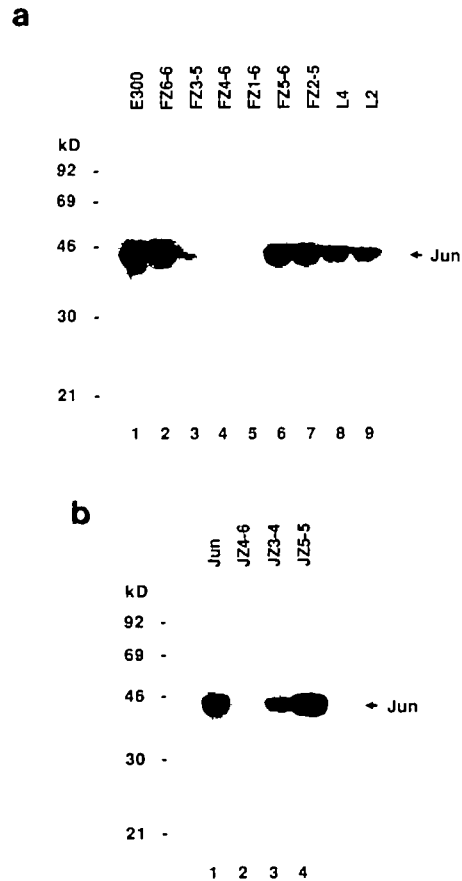
<sup>b</sup>: Amino acids substituted in FZ constructs derived from Jun.

<sup>c</sup>: Amino acids substituted in JZ constructs derived from Fos.

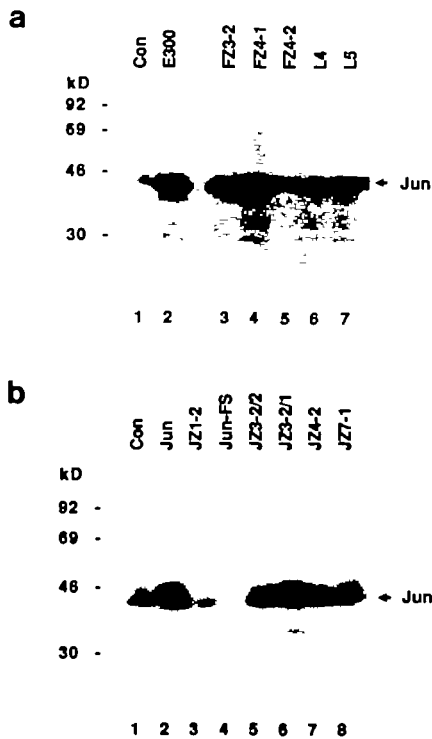
double mutations were introduced at corresponding positions using alanine for substitutions wherever possible (Fig. 2). The ability of the Fos mutants to form heterodimers with Jun was analyzed in the experiment shown in Fig. 5a. The data suggest that, apart from the known crucial role of leucine residues in position *d*, amino acids in position 4 appear to be of particular importance. The exchange of 2 amino acids in FZ4-2 (lane 5) reduced binding to 12% (as determined by scanning the autoradiograph; see legend to Fig. 2). Substitutions in positions *e* and *g* (FZ1-2 and FZ3-2), which are directly adjacent, have comparably moderate effects, reducing the binding to Jun by about 40% relative to E300. Alterations of 3 or more amino acids in positions *a* and *e* in Fos practically abolished binding (FZ3-3, FZ4-3, FZ4-4/1 and FZ4-4/2 in Fig. 2). In contrast, a single

Hel. pos.	283	290	297	304	311	318	Binding to Fos <sup>a</sup>
Ψ-Jun	RKIELTDT L QAEYDQ L EDEKSA L QTEIAN L LKEKEK L EFLILAA H RNSG						9
JFZ3-4		K	Q	T	Q		32
JFZ3-3		K	Q	T			15
JFZ3-3/4-1		K	Q	T	V		31
JFZ4-5	I	V	N	A	V		16
JFZ4-4	I	V	N	A	A		6
JFZ4-4/3-1	I	V	N	A	Q		28
Ψ-Fos	RURELRLAR L EEEVKT L KAQNSE L ASTAMH I REQVAQ L KQKVDH H RNSG						Binding to Ψ-Fos <sup>a</sup>
Jun							103
JFZ3-4							67
JFZ4-5							192

**Figure 3. Structure of the Ψ-Jun zipper mutants and of Ψ-Fos, and binding to E300 (Fos) and Ψ-Fos, respectively.** Numbers at the top of the two panels indicate amino acid positions in the Jun and Fos protein, respectively; the numbers below refer to the helical position of the respective amino acid relative to the preceding leucine (see helical wheel model in Fig. 1). The sequences below are Ψ-Jun and Ψ-Fos, as indicated. Amino acids of Fos transferred into Jun, and Jun residues transferred into Fos are double underlined. The Ψ-Jun mutants (termed JFZ) are identical to the parental protein shown at the top, except for the changes indicated. <sup>a</sup> percentage of binding to *in vitro* translated Fos or Ψ-Fos. Calculations were performed as described in Fig. 2 and wild-type binding normalized to 100%.



**Figure 4. Complex formation between Fos proteins mutated in different helical positions and wild-type Jun (panel a), and between Jun proteins mutated in analogous positions and wild-type Fos (panel b).** The structure of the mutants and the nomenclature is described in Fig. 2. The *in vitro* reconstitutions and immunoprecipitations were carried out as described in Methods. Wild-type Jun and Jun mutants were translated in the presence of <sup>35</sup>S-methionine and complexed to unlabeled Fos. Quantitation was done as explained in Fig. 2. Mutants L4 and L2 were included for comparison.



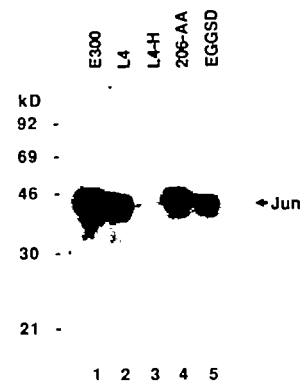
**Figure 5.** Effect of point mutations and two-amino acid changes in the putative interaction surface of Fos and Jun. Mutated proteins were allowed to bind to the respective wild-type counterpart, immunoprecipitated and analyzed as described in Fig. 4. Panel a: Fos zipper mutants complexed with Jun; panel b: Jun mutants complexed with Fos (E300). Nomenclature as in Fig. 2. Jun-FS: frame shift mutant starting at position 299 in c-Jun. Con:  $^{35}$ S-labeled Jun precipitated with Fos antiserum KX (no Fos added).

point mutation of Lys-190 to alanine (position *a*) in Fos had practically no effect on dimer formation ( Figs. 2 and 5a).

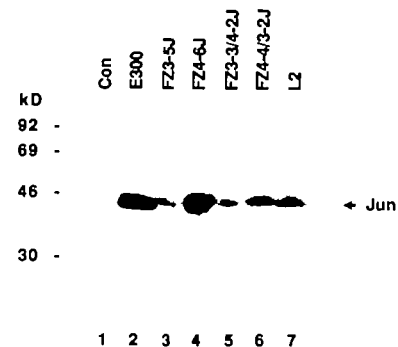
A similar set of leucine zipper mutants was also generated with Jun. Reconstitution of the Jun mutants with E300 protein yielded binding activities which were clearly position dependent and were largely analogous to the results described above for Fos (compare relative binding values in Fig. 2; reconstitutions shown in Figs. 5a and b). In analogy to construct FZ4-1, the mutation of Val-308 to alanine in JZ4-1 did not affect its binding to Fos (Fig. 5b). The JZ4-2 mutant, on the other hand, which carries an additional mutation (Val-315 → Ala) showed a considerable decrease in binding (16% binding relative to Jun); its binding activity is thus comparable to that of the corresponding Fos mutant FZ4-2 (12% binding). Constructs JZ3-2/1, JZ3-2/2 and JZ1-2 showed moderately reduced binding (60–70%), similar to the analogous Fos constructs (FZ mutants). Interestingly, mutant JZ7-1 (Leu-304 → Val) showed enhanced binding (125%), unlike its Fos counterpart L4 (Leu-186 → Ala; 45% binding).

#### Mutation of a conserved histidine C-terminal to the leucine repeat affects Fos/Jun complex formation

We next extended our studies to amino acids outside the leucine zipper. We focussed our attention particularly on a histidine located 7 amino acids C-terminally to the 5th leucine, because this residue is completely conserved in all proteins of the Fos and Jun families (Fig. 2). Interestingly, His-200 turned out to be crucial for the interaction of Fos and Jun. While L4, a mutant of Fos where the 4th leucine is exchanged with alanine (Fig. 2)



**Figure 6.** Complex forming potential of Fos proteins mutated in sequences C-terminal to the leucine repeat (amino acids 194–207) with Jun. The structure of the mutants and a quantitation of the results is shown in Fig. 2. Mutant L4 (lane 2) was included as a reference for the double mutant L4-H.

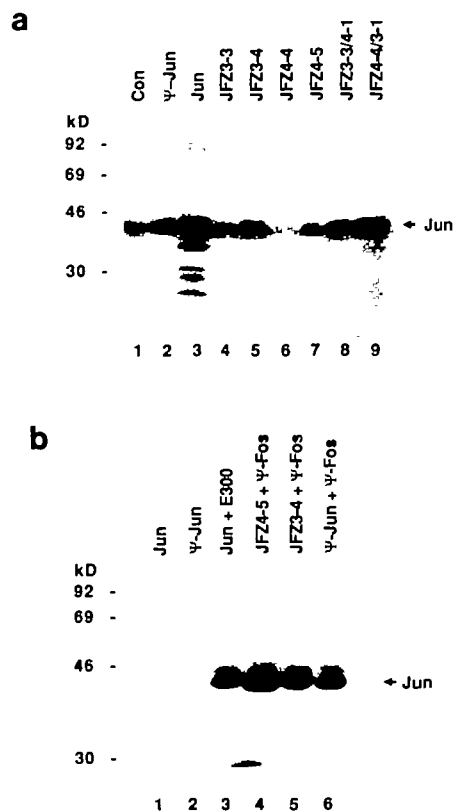


**Figure 7.** Effect of Jun sequences in positions 3 and 4 in Fos. The Fos mutants were complexed with wild-type Jun. Nomenclature as in Fig. 2, reconstitution and quantitative evaluation as indicated in Fig. 4. Con:  $^{35}$ S-labeled Jun precipitated with Fos antiserum KX (no Fos added).

showed only slightly reduced binding properties compared to E300, the additional mutation of His-200 (construct L4H) completely destroyed binding to Jun (Fig. 6, lanes 1–3). Another mutant with changes adjacent to His-200 also showed reduced binding (EGGSD; lane 5; 35% binding). Mutation of 2 adjacent amino acids located closer to the C-terminus had no detectable effect (206-AA; lane 4).

#### Exchange of amino acids between Fos and Jun leucine zippers

We next attempted to analyse whether the non-leucine residues also confer the specific character of the Fos/Jun association. Since it could not be excluded that sequences outside the leucine repeat might contribute to a significant extent to the binding specificity, we first analysed hybrid constructs (referred to as  $\Psi$ -Jun and  $\Psi$ -Fos) in which the leucine repeat in Jun was replaced with the zipper from Fos and vice versa. In the case of  $\Psi$ -Jun, this was achieved by first introducing appropriate restriction sites into the *c-jun* cDNA by site-directed mutagenesis and the replacement of the leucine zipper with a synthetic oligonucleotide (see Methods for details). The  $\Psi$ -Fos construct has previously been described (29). As shown in Fig. 8a (lane 2), the  $\Psi$ -Jun protein did not show any significant binding to Fos, but associated efficiently with  $\Psi$ -Fos. Complex formation between  $\Psi$ -Fos and  $\Psi$ -Jun occurred with similar efficiency as the association between Fos



**Figure 8.** Specificity of dimerization determined by testing the dimer forming potential of  $\Psi$ -Jun (Fos zipper in Jun) and  $\Psi$ -Jun mutants (substituted in positions 3 and 4 by Jun sequences). For nomenclature and quantitation see Fig. 3. Panel a: reconstitution with Fos protein (E300); panel b: reconstitution with  $\Psi$ -Fos (Jun zipper in Fos) or E300 as indicated above the lanes. All Jun proteins were labeled in the presence of  $^{35}\text{S}$ -methionine. Con:  $^{35}\text{S}$ -labeled Jun precipitated with Fos antiserum KX (no Fos added).

and Jun (Fig. 8b, lanes 3 and 6). This shows that the binding specificity is an integral part of the leucine zipper and is not dependent on other sequences in the protein.

We then introduced specific mutations into the Fos zipper, replacing amino acids in position *a* or *g* with corresponding residues from Jun (see Fig. 2). In contrast to substitutions with random helix-forming amino acids (FZ4-6), the presence of Jun residues in position *a* enabled the mutant Fos protein (construct FZ4-6J) to bind with even higher affinity to Jun than E300 (125% binding; Fig. 7). Substitutions in position *g* had adverse effects. Construct FZ3-5J showed strongly reduced affinity (18% binding; Fig. 7). As seen with two other mutants (FZ3-3/4-2J and FZ4-4/3-2J), changes in position *g* are dominant over those in position *a* (Figs. 2 and 7).

To analyse whether amino acids in these positions may determine the specificity of the interaction and thus prevent Fos homodimer formation, we next transferred the respective Fos zipper mutations into the  $\Psi$ -Jun construct and analyzed the encoded proteins for complex formation with E300. While the  $\Psi$ -Jun construct, which contains the wild-type Fos zipper did not show any significant binding to E300, the introduction of 4 Jun amino acids into position *g* in the Fos zipper of  $\Psi$ -Jun (constructs JFZ3-4) increased the binding to E300 to 32% (Figs. 3 and 8a). This mutation eliminates the 4 glutamic acid residues in position *g* which may exert repulsive forces in Fos homodimers. Construct JFZ3-3, which leaves the last glutamic acid residue intact, shows lower binding (15%).

Fos							Jun						
<i>l</i>	<i>c</i>	<i>g</i>	<i>d</i>	<i>a</i>	<i>e</i>	<i>b</i>	<i>r</i>	<i>c'</i>	<i>g'</i>	<i>d'</i>	<i>a'</i>	<i>e'</i>	<i>b'</i>
E	T	L	L	T	Q	D	E	R	R	L	I	E	A
A	Q	E	L	T	E	D	E	T	K	L	V	K	K
D	A	E	L	K	Q	S	A	E	Q	L	M	A	S
T	N	E	L	I	L	A	S	M	T	L	A	R	N
K	K	E	L	K	E	E	E	Q	Q	L	V	K	A
F	A	I	H	L	R	A	Q	N	K	H	V	V	M

**Figure 9.** Amino acid conservation in the leucine repeats of Fos and Jun proteins. The sequence between Glu-160 and Arg-201 in c-Fos was aligned with the corresponding sequence in Fra-1, Fra-2 and FosB (taken from 38–40). Likewise, amino acids between Glu-278 and Val-319 in c-Jun were aligned with the corresponding residues in JunB and JunD (according to 6, 37, 41–43). Amino acids are displayed according to their topological position in the heptad repeat (see helical wheel model in Fig. 1) from N-terminal (top) to C-terminal (bottom); conserved amino acids among all Fos or Jun proteins are shown in outlined font. Numbers above refer to the helical position relative to the preceding leucine.

Elimination of an additional positive charge in position *a* (construct JFZ3-3/4-1), however, has a similar effect as the substitution of the 4th glutamic acid in helical position *g* (compare to JFZ3-4). This result indicates that the charged amino acids in helical positions *a* and *g* are at least in part responsible for the lack of Fos to form homodimers. This conclusion is supported by the second series of constructs analyzed in this experiment. Five Jun residues introduced into position *a* also increased complex formation with Fos (16%; JFZ4-5; Figs. 3 and 8a). In contrast, construct JFZ4-4, in which the last lysine was left unchanged, did not show enhanced binding relative to  $\Psi$ -Jun (6% versus 9%). Elimination in this construct of an additional negative charge (glutamic acid) in helical position *g*, however, significantly enhanced the association with Fos (JFZ4-4/3-1; 28%).

An important role for amino acids in helical positions *a* and *g* in the specificity of protein-protein association is also suggested by the ability of  $\Psi$ -Jun proteins with mutations in their Fos zipper to form complexes with  $\Psi$ -Fos. Mutant JFZ4-5 showed strongly enhanced binding to  $\Psi$ -Fos (approximately two-fold; Figs. 3 and 8b, lane 4), similar to the binding of FZ4-6J to Jun (Fig. 7, lane 4). In analogy to the reduced binding of FZ3-5J to Jun (18%; Fig. 7, lane 3), JFZ3-4 and  $\Psi$ -Fos also showed a weaker interaction, although the effect was less pronounced (67%; Fig. 8b, lane 5). These data confirm the conclusion that the binding characteristics of zipper mutants can be preserved when transferred into a different protein 'background'.

## DISCUSSION

bZip proteins constitute a class of structurally distinct transcriptional regulators, whose hallmark is the presence of a leucine zipper, serving as a protein dimerization interface, adjacent to an  $\alpha$ -helical basic region, which is instrumental in DNA binding. The invariant spacing between both regions and the conservation of specific residues in most, if not all bZip proteins have led Vinson *et al.* (25) to propose the 'scissors grip' model (see Introduction for details). According to this model, two bZip proteins form homodimeric or heterodimeric complexes via the leucine zippers, and establish specific contacts to a dyad-symmetric DNA sequence with the basic regions of both proteins binding to one half site of the palindromic DNA element. The structural features of the bipartite DNA binding site and the conformational changes upon binding to DNA have

recently been elucidated in some detail (26–28). In addition, these and other studies performed with purified proteins or synthetic peptides (23, 24, 28) have provided sufficient evidence to conclude that the interaction of bZip proteins is brought about by the parallel, non-covalent association of laterally aligned coils.

Despite this structural information, the mechanism determining the dimerization of either identical or non-identical protein subunits is poorly understood and experimental evidence in this direction is limited. A convincing model was suggested by O'Shea *et al.*, which postulates that two heptad repeats of hydrophobic residues in each leucine zipper are likely to interact in a coiled coil. The specificity of interaction would be determined by amino acids in adjacent positions, predominantly charged residues (23, 24). In this study, we have performed a comprehensive structure-function analysis to characterize some aspects of the Fos/Jun complex formation. The analysis of the mutant proteins was performed in an *in vitro* reconstitution assay, which has previously been shown to allow for a quantitative analysis of complex forming properties (19). To exclude the possibility that mutation-related structural changes affected the results, we tested three different polyclonal antisera with the same set of mutants [Fos antiserum 455 and KX; Jun antiserum K31(35), Adamkiewicz, unpublished results]. In these experiments, we could not find any significant differences regardless of which antiserum was chosen (data not shown).

#### The interaction of Fos and Jun is dependent on amino acids in four different helical positions

Our results clearly indicate that non-leucine residues are instrumental in the formation of heterodimers between Fos and Jun. In Fos, amino acids in positions *a*, *e* and *g* are crucial, and in Jun, at least residues in positions *a* and *g*. The importance of position 1 in Jun, however, is suggested by the effects seen with construct JZ1-2. A more detailed analysis of single or double mutants within these positions revealed that changes in corresponding positions in either Fos or Jun had similar effects on the complex formation. Substitutions of 2 or more residues in position *a* had a more pronounced effect on the binding than those in positions *e* or *g*, resulting in a similar loss in binding as the elimination of 2 leucines (19). In contrast, single mutations of amino acids in positions *a*, *e* and *g* in Fos did not show any noticeable reduction in binding to Jun, similar to the substitution of some of the leucines (19).

As all the amino acids in Fos and Jun which were identified to contribute to complex formation are located close to leucine residues, it is likely that they form one single dimerization surface. In agreement with the results of our structure-function analysis, positions *a* and *d* show total conservation, positions *e* and *g* show 67% identity (16 out of 24 residues) among the known members of each family (see Fig. 9). In this context it is noteworthy, that amino acids C-terminal to the 5th leucine, like His-200 in Fos, are also well conserved and important for protein dimerization. We therefore conclude that the leucine zipper may comprise at least one additional helical turn. It seems unlikely, however, that the dimerization domain extends much further since residues located more C-terminally in Fos are rich in prolines and presumably disrupt the  $\alpha$ -helical configuration.

#### The leucine repeats in Fos and Jun are exchangeable functional domains

In a previous study, we and others have demonstrated that, by transferring the leucine zipper of Jun or GCN4 into Fos, the dimerization specificity of the acceptor protein was changed to

that of the donor protein (29–31). In this study, we have extended these analyses by replacing the leucine repeat in Jun by that of Fos (Fig. 3). The resulting  $\Psi$ -Jun protein was shown to bind to  $\Psi$ -Fos, a Fos protein containing the Jun leucine zipper. This binding was stable under the stringent conditions of the immunoprecipitation (RIPA buffer), similar to the wild-type Fos-Jun complex.  $\Psi$ -Jun was, however, unable to form dimers with wild-type Fos (Fig. 8b, lanes 3 and 6; Fig. 8a, lane 2). It could be argued that the low increase in the amount of  $\Psi$ -Jun visible in Fig. 8a was due to residual dimer formation with Fos. However, this increase was also obtained in the absence of Fos protein (Fig. 8b, lane 2) and therefore most likely represents immunological cross-reactions. This is conceivable, since the 42 Fos residues in  $\Psi$ -Jun are also present in the antigen used to generate the polyclonal 455 and KX antisera. Based on these data, we conclude that (i) the zipper region in each protein can function as an independent unit and (ii) can confer binding specificity to a heterologous protein.

#### Determinants of preferential dimer formation

Based on the findings discussed above, we have attempted to localize residues in Fos and Jun that are crucial in determining the binding specificity. The net charges of the Fos and Jun leucine zippers are equal but of opposite polarity (24). It could thus be speculated that electrostatic interactions play an important role in the specificity of dimerization by favoring the formation of heterodimers and weakening or preventing the interaction of homodimers. This notion is supported by our observation that the incorporation of 5 Jun amino acids into Fos in position *g* (with a change in the net charge from  $-4$  to  $+2$ ; construct FZ3-5J) or 6 residues in position *e* (with a net charge changing from  $+2$  to  $-1$ ; construct FZ1-6) results in practically no binding to Jun. This conclusion is substantiated by the fact that these amino acids (helical positions *e* and *g*) are relatively well conserved among members of the same family (especially in Fos proteins) but are not conserved across the Fos and Jun family. In agreement with this interpretation, a similar alteration in position *g* of  $\Psi$ -Jun (with the net charge changing from  $-3$  to  $+1$ ; construct JFZ3-4) leads to an increased association with Fos. Furthermore, a single amino acid change affecting the last glutamic acid residue in helical position *g* has a clear positive effect on complex formation with Fos (compare JFZ3-4 and JFZ3-3 or JFZ4-4 and JFZ4-4/3-1). Thus, substitutions in position *g* and probably in position *e* in Fos do not only affect the stability of the Fos-Jun complex but in addition change the protein's binding specificity.

Different conclusions can be drawn from the results obtained with proteins where amino acids were exchanged in position *a* between Fos and Jun. When introduced into a Fos zipper, the Jun amino acids seem to facilitate both the Fos/Jun as well as the Fos/ $\Psi$ -Jun association. Our current view is that, as suggested by O'Shea *et al.*, the hydrophobic Jun repeat in position *a* confers a higher degree of stability due to an increased number of potential hydrophobic interactions. This notion is supported by the binding behavior of construct JFZ4-5 in which the nearly perfect hydrophobic repeat of Jun residues allows dimer formation with Fos, despite the putative repulsion effect of some charged residues in both zippers (in positions *e* and *g*). In Fos, the corresponding repeat is markedly less hydrophobic and contains two lysines. While the hydrophobic character of the leucine side chains may support the formation of stable Fos-Jun complexes (24), their positive charge is likely to contribute to the prevention of a Fos-Fos homodimer formation. The latter hypothesis is in agreement with the results described in Figs. 3 and 8 which show

that the elimination of a single positive charge (lysine) in helical position *a* can lead to a clearly enhanced association of Fos zippers (compare JFZ4-5 and JFZ4-4 or JFZ3-3 and JFZ3-3/4-1).

### Compatibility with the coiled coil model

Two of the features predicted by the coiled coil model of O'Shea *et al.* (23, 24) were of particular relevance for our study: (i) A repeat of hydrophobic non-leucine residues builds the 4-3 heptadic repeat together with the heptad leucine repeat, similar to the parallel  $\alpha$ -helical coiled coils of fibrous proteins. (ii) Charged amino acids in the laterally aligned sequences effect the specificity of interactions and prevent 'promiscuous' dimer formation. As discussed above, the experimental data obtained in this study can indeed be best explained by this concept. Our observations are also in agreement with the observations of Smeal *et al.* (32), who analyzed the effect of substituting single or multiple amino acids in the leucine zipper of Jun.

Based on the available evidence we come to the following conclusions: (i) the preferential heterodimer formation between Fos and Jun is due to the synergistic effect of both interhelical hydrophobic interactions and mutual electrostatic attraction. (ii) The relatively weak formation of Jun homodimers could be explained by the repulsion of positively charged arginines and lysines (net charge of + 5 in positions *e* and *g*) counteracting the hydrophobic interactions of the 4-3 repeat. (iii) Fos-Fos homodimer formation is completely prevented due to the additive effect of both the lower number of hydrophobic interactions and the repulsion of lysines in position 4 on the one hand, and the repulsion of 6 glutamic acid residues in the two lateral positions *e* and *g*.

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