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DNA binding and transactivation properties of Fos variants with homodimerization capacity

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ABSTRACT

The mammalian Fos and Fos-related proteins are unable to form homodimers and to bind DNA in the absence of a second protein, like c-Jun for example. In order to study the implications of hydrophobic point mutations in the c-Fos leucine zipper on DNA binding of the entire c-Fos protein, we have constructed and purified a set of Fos mutant proteins harboring one or several isoleucine or leucine residues in the five Fos zipper a positions. We show that a single point mutation in the hydrophobic interface of the c-Fos leucine zipper enables the c-Fos mutant protein to bind specifically to an oligonucleotide duplex harboring the TRE consensus sequence TGA(C/G)TCA. This point mutation ($Thr_{169}\rightarrow Ile$) is situated in the a position of the second heptade (a_2) of the Fos zipper. The introduction of additional isoleucine residues in the other a positions progressively increases the DNA binding affinity of these homodimerizing Fos zipper variants. Heterodimerization of these c-Fos variants with c-Jun reveals a complex behaviour, in that the DNA binding affinity of these heterodimers does not simply increase with the number of isoleucine side chains in position a. For example, a c-Fos variant harboring a wild-type Thr in position a1 and Ile in the four other a positions (c-Fos4I) interacts more tightly with c-Jun than a variant harboring Ile in all five a positions (c-Fos5I). The same holds true for the corresponding leucine variants, suggesting that the wild-type a1 residue of the Fos zipper (Thr_{162}) is thermodynamically relevant for Fos–Jun heterodimer formation and DNA binding. The c-Fos4I variant forms heterodimers with c-Jun slightly better than the wild-type zipper protein, suggesting that the driving force for Fos–Jun heterodimerization is not the simple fact that the Fos protein is unable to form homodimers. These c-Fos variants were further tested for their transactivation properties in F9 and NIH3T3 cells. At low expression levels the most efficiently homodimerizing variant (c-Fos5I) activates transcription in F9 cells about 6-fold. However part of this activation may be due to the formation of heterodimers with a member of the Jun family (like JunD for example), since a wild-type c-Fos expression vector confers a 3-fold activation under these conditions. In the case of the homodimerizing c-Fos variants however, this activation is abrogated at higher expression levels due to a strong inhibition of basal transcription activity.

INTRODUCTION

The oncoproteins c-Fos and c-Jun belong to the family of bZip proteins characterized by their DNA binding domain composed of the leucine zipper (promoting dimerization) and the basic region (promoting specific DNA binding) (1). The members of the Jun subgroup (c-Jun, JunB and JunD) can form homodimers or heterodimers between each other and with the Fos subgroup members (c-Fos, FosB, Fra-l and Fra-2), whereas the Fos members can interact with DNA only by forming heterodimers with one of the Jun proteins (2–4).

The leucine zippers of c-Jun and c-Fos are composed of five successive heptads where the seven residues of each heptad are referred to by letters from **a** to **g** (or **a′** to **g′** for the complementary helix). Two interacting leucine zippers form a parallel coiled-coil where residues at positions **a**, **d**, **e** and **g** form the dimerization interface. These residues are responsible for leucine zipper contacts and determine homo- and heterodimer stability and specificity (5–8). In the case of the c-Fos zipper, repulsion between negatively charged side chains is one of the reasons for weak homodimerization (6,7). The conserved Leu residues are located in position **d**. In general the **a** positions are also occupied by hydrophobic residues. However in the case of the c-Fos leucine zipper the five **a** positions are predominantly polar or charged, containing respectively Thr, Thr, Lys, Ile and Lys in the positions $\mathbf{a_1}$–$\mathbf{a_5}$.

Here we determine the DNA binding affinity and the transactivation properties of five c-Fos mutant proteins harboring one or several isoleucine or leucine residues in the **a** positions. c-Fos homodimer DNA binding affinity progressively increases with the number of additional isoleucine residues. A single isoleucine in position $\mathbf{a_2}$ is sufficient to confer measurable homodimerization capacity.

Heterodimerization of these isoleucine and leucine c-Fos **a** variants with a c-Jun DNA binding domain reveals a more complex behaviour. Replacement of the two Thr residues (especially that in

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position $\mathbf{a_1}$) by Ile is unfavorable for Fos–Jun heterodimerization, whereas the replacement of one or both Lys **a** residues by Ile is apparently favorable for heterodimerization with c-Jun.

We asked further if these c-Fos variants with acquired homodimerization capacity would be able to act as transcriptional activators. At low expression levels the most efficient variant activates transcription only ~6-fold in F9 cells, which is fairly low as compared to the activation by c-Jun. Additionally, the use of higher expression levels of these c-Fos variants leads to a strong and progressive inhibition of transcription even below the level confered by the reporter plasmid alone. This inhibition of basal promoter activity is observed in F9, but not in NIH3T3 cells. We argue that the potential intrinsic activation capacity of the hydrophobic c-Fos variants is rapidly overcompensated by this inhibitory effect. Since c-Fos is known to act as a negative transcriptional regulator under certain circumstances, it seems likely that the c-Fos leucine zipper mutations described here enhance the intrinsic inhibitory function of c-Fos.

MATERIALS AND METHODS

Construction of Fos proteins with variant leucine zippers

The pDS c-Fos *Escherichia coli* expression vector containing the rat *c-fos* gene was kindly provided by Tom Curran. It contains an N-terminal His-tag allowing for nickel–agarose affinity purification (9).The *c-fos* gene was excised using *Sph*I/*Bam*HI restriction sites and introduced into the same restriction sites in M13mp18 polylinker for subsequent site directed mutagenesis. To allow for leucine zipper subcloning, a silent *Eco*RI site was introduced after the fifth leucine of the c-Fos zipper using the following oligonucleotide: 5′-AGAAAAGCTGGAATTCATCCTGGCGG-3′. In a second round of mutagenesis, the Thr in position $\mathbf{a_1}$ was changed to Leu or Ile, using respectively the oligonucleotides: 5′-GAACTGCTGGACACC-3′ and 5′-GAACTGATCGACACC-3′. The Thr in the $\mathbf{a_2}$ position was also substitued by Ile using site directed mutagenesis with the oligonucleotide 5′-GCGGAAATCGACCAG-3′.

The mutagenized *6his–fos* genes were excised out of the M13mp18-*fos* polylinker through *Sma*I–*Hin*dIII digestion. Cohesive ends were filled-in to become blunt and the different *fos* fragments were introduced into a blunt *Eco*RI site of the eukaryotic expression vector pSG5 (10). In pSG5 constructs, *fos* leucine zipper exchange could be done with unique *Pst*I/*Eco*RI restriction sites, since the *Pst*I site is localized between the $\mathbf{a_1}$ and $\mathbf{a_2}$ positions and the *Eco*RI site after the fifth conserved leucine. The variant leucine zippers were excised from pMS500 5L and pMS500 5I, bearing respectively five leucines or five isoleucines in each of the five **a** positions (11). Different c-Fos proteins were obtained in this way for eukaryotic expression: pSG c-FosWT (bearing only the silent *Eco*RI site at the end of the leucine zipper), pSG c-FosI_2 (having only a Thr → Ile substitution in the $\mathbf{a_2}$ position), pSG c-Fos5L, pSG c-Fos5I, pSG c-Fos4L and pSG c-Fos4I (harboring several Leu or Ile substitutions in the **a** positions).

The different *c-fos* genes were excised from pSG5 using *Sph*I–*Bam*HI restriction sites and reintroduced into the pDS56 *E.coli* expression vector giving rise to bacterial expression vectors of His-tagged c-Fos with variant leucine zippers, i.e. pDS c-FosWT, pDS c-FosI_2, pDS c-Fos5L, pDS c-Fos5I, pDS c-Fos4L and pDS c-Fos4I.

Purification of His-tagged c-Fos proteins with variant leucine zipper

pDS Fos expression vectors were introduced in *E.coli* strain RB791 (W3110 *lacI^{q}L8*). One litre of LB medium was inoculated with 100 ml of a saturated overnight culture. At an OD_{600} of 0.8, protein expression was induced upon addition of 1 mM IPTG and cells were grown for another 3 h. Cells were harvested and sonicated in lysis buffer (25 mM Na_2HPO_4/NaH_2PO_4 pH 7.9; 200 mM NaCl) complemented with several protease inhibitors. The following steps were perfomed at 4°C essentially as described (12). The lysate was centrifuged for 1 h at 27 500 *g* and the pellet containing insoluble Fos proteins trapped in inclusion bodies was solubilised in 40 ml buffer A (25 mM Na_2HPO_4/NaH_2PO_4 pH 8; 6 M guanidine–HCl; 10 mM β-mercaptoethanol; 1 mM PMSF) with overnight agitation. Solubilized proteins were cleared by an additional centrifugation for 1 h at 27 500 *g*. The supernatant was added to 2 ml of 50% nickel–NTA–agarose (Qiagene) equilibrated in buffer A. The batch incubation was agitated 2 h and centrifuged 2 min at 1000 *g*. The pellet was washed with 40 ml of buffer A, and loaded on a 5 ml disposable column (Qiagene). The column was washed with 20 ml of Buffer B (25 mM Na_2HPO_4–NaH_2PO_4 pH 6.5; 6 M guanidine–HCl; 10 mM β-mercaptoethanol; 1 mM PMSF). The Fos protein was eluted with 10 ml of buffer C (25 mM Na_2HPO_4/NaH_2PO_4 pH 5; 6 M guanidine–HCl; 10 mM β-mercaptoethanol; 1 mM PMSF). The purified protein was dialyzed overnight against several changes of buffer D (25 mM Na_2HPO_4/NaH_2PO_4 pH 7; 10 mM β-mercaptoethanol) until guanidine concentration reached 0.5 M (for guanidine–HCl concentrations <0.5 M protein precipitation is observed). At each step the dialyzed sample was cleared by centrifugation at 15 000 *g*. A last dialysis was performed against storage buffer S (25 mM Na_2HPO_4/NaH_2PO_4 pH 7; 0.5 M guanidine–HCl; 1 mM EDTA; 0.01% Nonidet P-40; 1 mM DTT, 5% glycerol). Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Cat No 500-0006). The protein was divided into aliquots and stored frozen at –80°C. The purity of the protein was analysed by SDS–PAGE (12% acrylamide, 1/30).

Electrophoretic mobility shift assay (EMSA)

DNA binding experiments were performed in 10 µl reaction mixtures harboring variable amounts of protein. Protein stock solutions were adjusted to a concentration of 1.85×10^{-5} M and submitted to successive 2-fold dilutions in storage buffer S (see above), giving rise to a range of final protein concentrations between 4.5×10^{-10} and 9.2×10^{-7} M for homodimer shift assays. For heterodimer shift assays, serial dilutions were done with an equimolar mixture of one of the Fos variants and the c-Jun DNA binding domain ($Jun_{247–324}$). The 8 µl reaction mixtures contained 2 µl of 5× binding buffer (250 mM Tris–HCl pH 8; 5 mM EDTA; 50% glycerol; 0.5% Nonidet-P40; 1 mg/ml BSA), 1 µl of 50 mM DTT and the appropriate amount of purified protein. The binding mixtures were incubated for 10 min at 37°C. An aliquot of 1 µl of 0.1 µg/ml poly(dI–dC) was added, incubated for another 5 min at room temperature, and specific DNA binding was performed by adding 1 µl of a ^{32}P-labelled TRE-21 DNA fragment (~3000 c.p.m.) containing the TRE binding site TGA(C/G)TCA in the context of a 21 base pair (bp) DNA duplex of the following sequence: 5′-TTCCGGC **TGACTCA** TCAAGCG (13). After 15 min incubation at room temperature the sample was loaded on

a native 5% acrylamide (1:30) gel. The protein–DNA complexes were visualized by autoradiography of the dried gel at –80°C. The specificity of the interaction was tested by adding either excess amounts of unlabelled TRE-21 or of the non-specific competitor duplex GEM-21 (13) of the following sequence: 5-TTCCGGC CCGAATT TCAAGCG. GEM-21 contains the same lateral sequences as TRE-21, but a completely scrambled TGACTCA-motif maintaining, however, the overall base pair composition to assure the same overall stability of the duplex.

Eukaryotic cells and transfections

F9 cells were grown in DMEM (Sigma, Cat. No. D-5796) supplemented with 15% foetal bovine serum (BioWhittaker, Cat. No. 14501E). Cells (3.5×10^5) were seeded in 60 mm plates for transfection assays. NIH3T3 cells were grown in DMEM (GIBCO BRL, Cat. No. 41965-039) supplemented with 10% newborn calf serum (BioWhittaker, Cat. No. 14.416.E). Cells (2.5×10^5) were seeded in 60 mm plates for transfection assays. Plates were placed at 37°C in a humidified atmosphere of 10% CO_2. Six hours later transfections were carried out using the calcium phosphate procedure (14): 2 µg of 3× TRE-tk-Luc plasmid were co-transfected with 0.1, 0.5, 2, 5 and 10 µg of pSG Fos variants for homodimer transactivation assays. pSK was used to complete a total of 15 µg plasmid DNA. Cells were grown for another 24 h. Before harvesting the cells, the plates were washed twice and luciferase assays were performed as described in the Luciferase Assay System (Promega Kit, Cat. No. E4030). The 3× TRE-tk-Luc plasmid was constructed as follows: a 5′-AGCTTGATGAGTC-AGACCG and a 5′-GATCCGGTCTGACTCATCA oligonucleotide was annealed and ligated. The 3× TRE ligation product was purified from a 5% (1:30) polyacrylamide gel and cloned into a *Bam*HI/*Hin*dIII digested tk-Luc plasmid (15) kindly provided by B. Binétruy. ΔTRE-tk-Luc was obtained by *Bam*HI/*Hin*dIII digestion of the tk-Luc plasmid, fill-in and re-ligation. The data presented in Figures 4 and 5 are the average of two independent transfection experiments.

RESULTS

In order to study the implications of hydrophobic point mutations in the c-Fos leucine zipper on DNA binding of the entire c-Fos protein, we have constructed a set of *c-fos* mutant genes harboring one to five point mutations in the leucine zipper **a** positions. The polar residues T, T, K and K in positions $\mathbf{a_1}$, $\mathbf{a_2}$, $\mathbf{a_3}$ and $\mathbf{a_4}$ were partly or entirely replaced by Ile or Leu, since these residues were shown previously to be most efficient in promoting homodimerization of the isolated c-Fos leucine zipper fused to the LexA DNA binding domain (11). Five c-Fos variants as well as the wild-type c-Fos protein were expressed in *E.coli* and purified using nickel–agarose chromatography. The final purity was at least 95% as determined by SDS polyacrylamide gel electrophoresis. The DNA binding affinity of six purified c-Fos variants has been determined using EMSA in the presence of non-specific competitor DNA (Table 1) under low salt conditions (see Materials and Methods). DNA binding is also observed in the presence of 50 mM NaCl in the binding buffer, however binding is somewhat weaker under these conditions making the determination of complete binding isotherms more difficult.

DNA binding affinity of the c-Fos5I and c-Fos4I variants

Figure 1A shows EMSA results for the c-Fos5I homodimer harboring exclusively isoleucine residues in the five **a** positions. Half of the TRE-21 DNA duplex (13) is shifted for a total protein concentration of only ~2×10^{-8} M. Since the protein concentration is in large excess over the DNA concentration, we may assume that free and total protein concentration are approximately the same. In this case the equilibrium association constant for DNA binding is the reciprocal of the protein concentration required for binding half of the DNA (see Table 1 for the corresponding K_A values). This interaction is specific, since addition of an excess of unlabelled specific TRE-21 duplex competes for DNA binding, whereas an unspecific control duplex (GEM-21) of the same length and the same overall base pair composition is unable to abolish binding of the ^{32}P-labelled TRE-duplex by the c-Fos5I homodimer and the other Fos variants tested in this study (data not shown). Under the same experimental conditions the c-Fos protein harboring a wild-type leucine zipper sequence is unable to interact specifically with the TRE probe.

In an attempt to reduce the number of point mutations within the c-Fos zipper necessary to confer homodimerization, we studied next the c-Fos4I variant harboring the wild-type Thr residue in position $\mathbf{a_1}$ and Ile in $\mathbf{a_2}$, $\mathbf{a_3}$, $\mathbf{a_4}$ and $\mathbf{a_5}$. The equilibrium association constant for DNA binding of the c-Fos4I homodimer is only about three times smaller than that of the c-Fos5I variant (Table 1). In the context of a Fos–Jun heterodimer the wild-type Thr in position $\mathbf{a_1}$ is even more favorable than an isoleucine (see below).

The c-FosI_2 variant: a single point mutation in the hydrophobic interface is sufficient to confer DNA binding as a homodimer

The c-Fos4I variant contains three point mutations as compared to the wild-type Fos zipper, i.e. threonine in position $\mathbf{a_2}$ and the two lysine residues in positions $\mathbf{a_3}$ and $\mathbf{a_5}$ are replaced by isoleucine. We wondered if a single isoleucine in one of these positions could be sufficient to confer homodimerization and thus DNA binding to the c-Fos protein. The most promising single point mutation seemed to be the replacement of threonine in $\mathbf{a_2}$, since the two lysine residues in $\mathbf{a_3}$ and $\mathbf{a_5}$ are potentially involved in hydrogen bonds and/or salt bridges with two glutamic acid residues in the preceding $\mathbf{g'}$ positions of the opposite Fos leucine zipper (see Fig. 1D for illustration). This kind of contact has been observed in the Fos–Jun DNA complex between these lysine side chains and two glutamine side chains in Jun (8).

Figure 1B shows that the single point mutation of Thr_{169} to Ile in position $\mathbf{a_2}$ is indeed sufficient to confer DNA binding to the Fos homodimer. About 3×10^{-7} M of the c-FosI_2 variant are required to bind half of the DNA. No specific DNA binding is observed for the c-Fos wild-type leucine zipper protein in this concentration range (i.e. from 10^{-9} to 10^{-6} M). Typical isotherms as those shown in Figure 1C require at least a 5- to 10-fold increase in protein concentration to move from 0% DNA binding to 50%, i.e. theoretically at least 5×10^{-6} M of the c-Fos wild-type leucine zipper protein would be required to achieve 50% specific binding. We may thus estimate that the c-FosI_2 variant binds the TRE-21 duplex at least 17-fold better than the c-Fos wild-type leucine zipper protein.

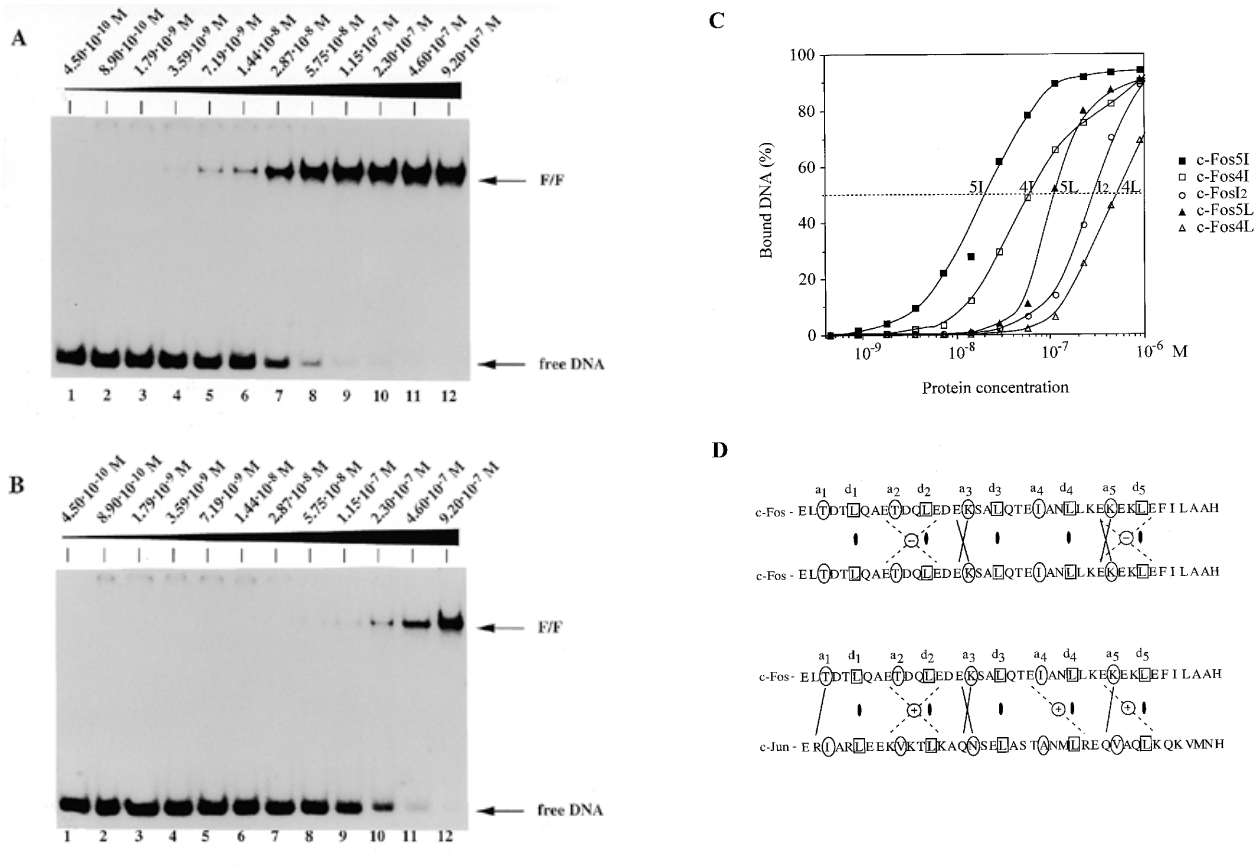

Figure 1. Homodimeric electrophoretic mobility shift assays with the c-Fos5I variant (**A**) harboring five isoleucine side chains in the five **a** positions and the c-FosI_2 variant (**B**) harboring a single isoleucine in position $\mathbf{a_2}$ using a ^{32}P-labelled TRE-containing oligonucleotide (Tre-21, 34). The homodimeric complexes are indicated as F–F. Protein concentrations are indicated on top. The corresponding equilibrium assosciation constants are summarized in Table 1. (**C**) Homodimeric DNA binding isotherms for the c-Fos5I, 4I, 5L, I_2 and 4L variants as a function of the total protein concentration. The c-Fos wild-type leucine zipper protein does not give rise to specific band shifts under these conditions. The EMSA gels were quantified using a Fuji phosphoimager. (**D**) Illustration of hypothetical homodimeric configuration of a c-Fos leucine zipper (top) and the heterodimeric configuration of a c-Fos–c-Jun complex (bottom) (8). Electrostatic repulsions (–) and attractions (+) between **e** and **g′** residues are represented as dashed lines, potential interactions between **a** and **g′** residues are reprented as full lines.

Table 1. Association constants (K_A) for homodimerization (and heterodimerization with a c-$Jun_{247–324}$ peptide) of different c-Fos mutant proteins carrying hydrophobic Ile or Leu substitutions at one or several **a** positions of their leucine zippers

	a_1	a_2	a_3	a_4	a_5
c-Fos wild-type:	T	T	K	I	K
c-FosI_2:	T	**I**	K	I	K
c-Fos4I:	T	**I**	**I**	I	**I**
c-Fos5I:	**I**	**I**	**I**	I	**I**
c-Fos4L:	T	**L**	**L**	**L**	**L**
c-Fos5L:	**L**	**L**	**L**	**L**	**L**
	Homodimer K_A		Heterodimer K_A		
c-Fos5I	4.4×10^7 M^{-1}		3.8×10^7 M^{-1}		
c-Fos4I	1.6×10^7 M^{-1}		1.2×10^8 M^{-1}		
c-Fos5L	8.7×10^6 M^{-1}		1.4×10^6 M^{-1}		
c-FosI2	3.4×10^6 M^{-1}		4.4×10^7 M^{-1}		
c-Fos4L	2.0×10^6 M^{-1}		8.7×10^6 M^{-1}		
c-FosWT	$< 2.0 \times 10^5$ M^{-1}		7.6×10^7 M^{-1}		
c-JunWT	1.1×10^7 M^{-1}		–		

The c-$Jun_{247–324}$ peptide (13) contains the basic region and the leucine zipper of c-Jun. The K_A of the full length c-Jun protein (c-JunWT) has been determined for comparison. The concentrations of the various c-Fos proteins required to achieve 50% DNA binding were determined from EMSA binding isotherms (Figs 1C and 3).

Perkins *et al*. (16) have shown that the Fos protein from *Drosophila* is able to form homodimers. The five **a** residues of this protein are TVGIK instead of TTKIK for the mammalian proteins. It seems likely that the hydrophobic valine in position $\mathbf{a_2}$ (instead of threonine) is at least in part responsible for this homodimerization activity of *Drosophila* Fos, since we show here that an isoleucine in this position confers homodimerization to the mammalian Fos protein.

The c-Fos4L and c-Fos5L variants

Similar studies have been done with leucine substitutions in four or five **a** positions (c-Fos4L and c-Fos5L) respectively. The possible drawback of leucine residues in position **a** is that such zippers are able to form not only dimers, but also parallel coiled-coil trimers, whereas isoleucine in position **a** favors dimer formation (17). As judged from gel migration the c-Fos4L and c-Fos5L variants bind the TRE-duplex however as dimers. Figure 1C shows the binding isotherms for these variants together with the isoleucine derivatives. The c-Fos5L derivative binds the TRE-duplex about four times more tightly than the c-Fos4L derivative in good agreement with the ~3-fold difference between c-Fos5I and c-Fos4I (see above). Again this loss in binding affinity may be attributed to the threonine in position $\mathbf{a_1}$, which is moderately unfavorable for Fos homodimerization, but favorable for Fos–Jun heterodimerization (see below). As compared to the isoleucine derivatives we observe the following order of homodimer DNA binding affinity (Table 1):

Homodimer DNA binding: Fos5I > Fos4I > Fos5L > $FosI_2$ > Fos4L >> FosWT.

As compared to these c-Fos variants, the full-length c-Jun homodimer binds the TRE-duplex ~4-fold less tightly than c-Fos5I with an association constant intermediate between those of c-Fos4I and c-Fos5L (Table 1).

Heterodimeric DNA binding of c-Fos hydrophobic variants with the c-Jun bZip domain

In the following we have studied the heterodimeric DNA binding of these c-Fos variants with a purified c-Jun DNA binding domain ($c\text{-}Jun_{247–324}$) comprising essentially the basic region and the leucine zipper of c-Jun (13). The use of the c-Jun bZip domain (instead of full-length c-Jun) as a partner for the different Fos variants allows us to distinguish between the heterodimer and the two possible homodimer complexes in EMSA experiments. Figure 2 shows that the Fos homodimer complexes (F–F) migrate more slowly than the $Fos–Jun_{247–324}$ heterodimer complexes (F–J), which in turn migrate more slowly than the $Jun_{247–324}–Jun_{247–324}$ homodimer complexes (J–J). The EMSA titrations shown in Figure 2 were obtained with equimolar mixtures of one of the six c-Fos variants and $c\text{-}Jun_{247–324}$. The concentrations indicated on top of the gels represent the total protein concentration, i.e. $[c_{Fos}]+[c_{Jun}]$. The corresponding binding isotherms are shown in Figure 3 and the K_A values are listed in Table 1. The EMSA titrations (Fig. 2A–D) reveal the following order of heterodimer affinity for the TRE-21 duplex:

Heterodimer DNA binding: Fos4I > FosWT > $FosI_2$ > Fos5I > Fos4L > Fos5L.

This order is different from that observed in the case of TRE-binding by the homodimers. As expected, the c-FosWT variant has strong heterodimerization capacity, albeit its affinity remains slightly weaker than that of the c-Fos4I variant. c-Fos4I is also one of the tightly homodimerizing Fos variants, suggesting that at least under our experimental conditions the driving force for Fos–Jun heterodimerization is not simply the fact that the Fos protein is unable to form homodimers.

In the case of Fos–Jun heterodimerization we observe further that the Fos variants harboring the wild-type Thr residue in position $\mathbf{a_1}$ are more efficient than those harboring either an isoleucine or a leucine in this position (i.e. Fos4I > Fos5I and Fos4L > Fos5L). The opposite order was observed in the case of homodimer binding (see above). This observation is most likely explained by the fact that according to Glover and Harrison (8) this threonine side chain could form a hydrogen bond with an arginine in the preceding **g** position of the Jun zipper (see Fig. 1D for illustration). This interaction apparently leads to a 3-fold higher DNA binding affinity of Fos4I–Jun as compared to Fos5I–Jun (Table 1). To our knowledge this is the first evidence that the Thr–Arg contact observed in the cocrystal is thermodynamically relevant.

The Thr residue in position $\mathbf{a_2}$ seems also favorable for Jun–Fos heterodimerization since its replacement by Ile leads to a slight decrease of Fos–Jun DNA binding (~1.7-fold, compare c-FosWT and $c\text{-}FosI_2$ in Table 1).

On the contrary, the replacement of Lys in positon $\mathbf{a_3}$ and $\mathbf{a_5}$ by Ile seems to be favorable for heterodimerization with Jun, since the c-Fos4I–Jun heterodimer has a slightly higher DNA binding affinity (~1.6-fold) than the corresponding c-FosWT heterodimer, despite the fact that the c-Fos4I variant harbors also the unfavorable Thr_{169}→Ile mutation in position $\mathbf{a_2}$. The Lys→Ile mutations in positions $\mathbf{a_3}$ and $\mathbf{a_5}$ apparently (over)compensate the negative effect of the Thr→Ile mutation in position $\mathbf{a_2}$.

Transactivation properties of the homodimerizing c-Fos proteins in F9 cells

We asked further if these Fos variants with acquired homodimerization capacity would act as transcriptional activators in transient transfection assays. These assays were performed in mouse teratocarcinoma F9 cells which have a very low level of AP1 DNA binding activity and consequently a low background of TRE-dependent reporter gene expression (18). c-Fos expression vectors were used in co-transfection with a luciferase reporter gene controlled by three TRE sites fused to the minimal thymidine kinase promoter. Figure 4A shows that at low concentrations of expression vector (0.1 μg/dish) an ~6-fold activation is observed for the c-Fos5I variant, whereas the other variants (including the c-Fos wild-type protein) activate luciferase expression only ~3-fold under these experimental conditions. However, we cannot exclude that transactivation at low levels of expression may be due to the formation of heterodimers with a member of the Jun family, in particular with endogenous JunD, which is present in F9 cells (19). The fact that wild-type c-Fos also induces a 3-fold stimulation suggests that heterodimerization may indeed play a role.

However the use of higher amounts of expression vector leads to a strong and progressive decrease of luciferase activity even below the basal level obtained with the 3× TRE-tk-Luc indicator plasmid alone. Figure 4A shows that this inhibition of basal promoter activity is observed mostly for the multiple leucine zipper mutations (5I, 4I, 5L and 4L), whereas the wild-type and the I_2 variant show a smaller decrease in luciferase activity. Throughout the entire range the I_2 variant is somewhat more

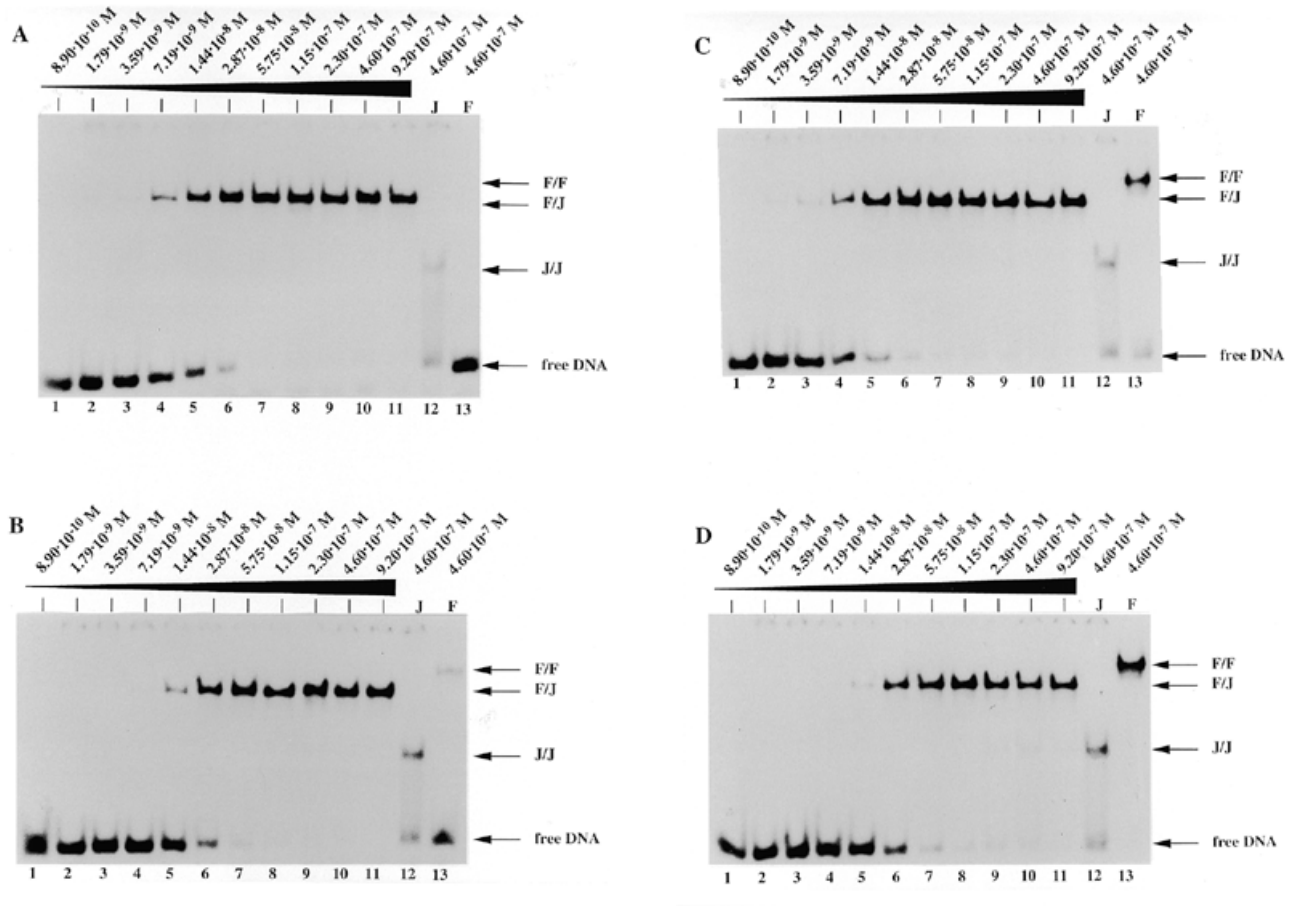

Figure 2. Heterodimeric electrophoretic mobility shift assays with several c-Fos variants in the presence of a purified c-Jun bZip domain. (**A**) c-FosWT, (**B**) c-FosI_2, (**C**) c-Fos4I and (**D**) c-Fos5I were assayed for heterodimerization with a c-$Jun_{247-324}$ bZip peptide (34). Protein concentrations are indicated on top. These concentrations correspond to additive and equimolar concentrations of c-Fos protein and c-Jun peptide (lanes 1–11). Homodimer c-$Jun_{247–324}$ (lane 12) and c-Fos variant (lane 13) control assays were performed at 4.6×10^{-7}M. The c-$Jun_{247–324}$ and c-Fos homodimer–DNA complexes (J/J and F/F respectively) and the heterodimer complexes (F/J) are indicated with arrows.

active than the c-Fos wild-type zipper protein. The degree of transcriptional repression is not obviously related to the homo- or heterodimerization capacity of these variants *in vitro*. The two variants which show no repression of basal transcription activity (wild-type c-Fos and c-FosI_2) can be both weak homo- and strong heterodimerizing species. The association of these two properties might be the reason why these variants do not repress basal transcription.

These data suggest that at least c-Fos5I (the most strongly homodimerizing variant as shown above) may act as a weak transcriptional activator at low expression rates, but that this activation is rapidly overcompensated by an inhibitory effect which becomes dominant at higher expression rates. Transfection with c-Jun does not show this kind of inhibition and gives rise to ~13-fold induction at 0.5 µg and to 39-fold induction at 5 µg of expression vector.

To establish further if the repression of basal promoter activity is due to DNA-binding to the three TRE elements, the same experiments were done with a ΔTRE-tk-Luc reporter plasmid lacking these TRE binding sites. Figure 4B shows that with this reporter plasmid the degree of inhibition is even more pronounced, reaching a 9-fold repression (for the 4I and 4L variants) as compared to a 5-fold repression with the TRE-reporter plasmid.

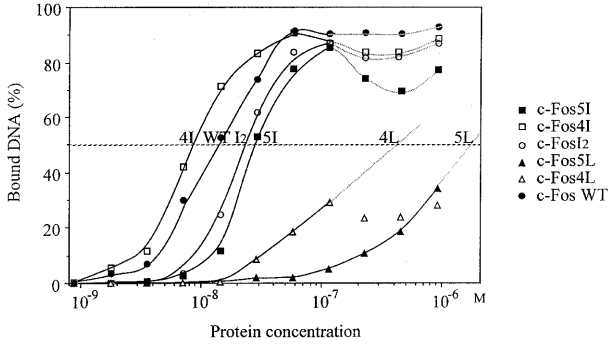

Figure 3. Heterodimerization isotherms of the different c-Fos variants. The relative amount of bound DNA involved in a heterodimeric c-Fos–c-$Jun_{247–324}$ complex (F–J in Fig. 2) is shown as a function of the total protein concentration. The decrease of the F–J complex for some of the variants (especially c-Fos5I and c-Fos4L) at concentrations $>10^{-7}$ M is due to an increase of the J–J homodimer complex.

We may conclude from these data that inhibition by the Fos4I, 4L, 5I and 5L variants is not due to binding to these TRE-elements.

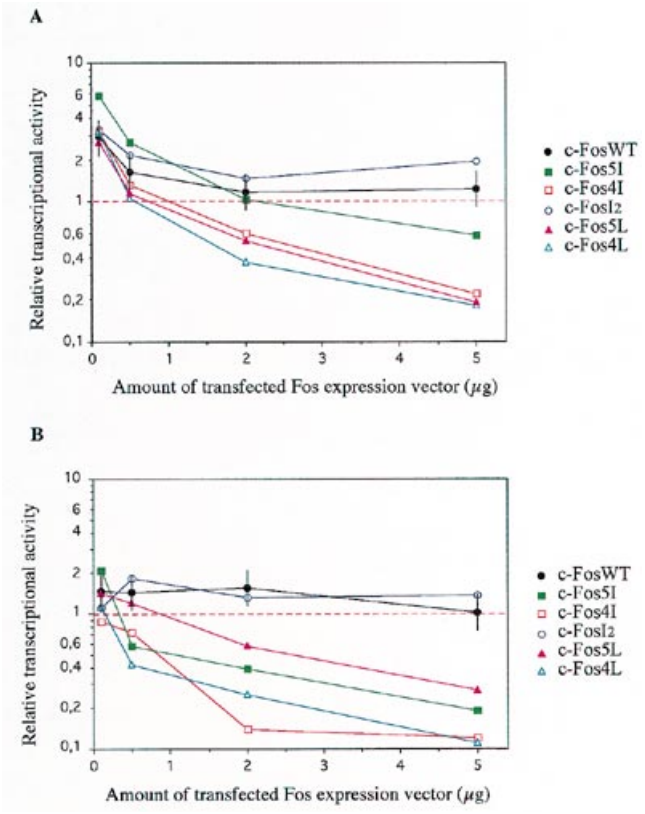

Figure 4. Transactivation assays in F9 cells. Variable amounts of pSG5 derived expression vectors (10) coding for one of the c-Fos variants were co-transfected with 2 μg of a 3× TRE-tk-Luc reporter plasmid (**A**) or with a ΔTRE-tk-Luc reporter plasmid lacking the TRE binding sites (**B**). Luciferase activities were normalized with respect to the number of luciferase units obtained with the reporter plasmids in the absence of c-Fos expression vectors. A logarithmic scale was chosen in order to visualize both transcriptional activation and repression. The data are the average of two independent transfections.

To make sure that the pronounced inhibitory effect of the Fos5I, Fos4I, Fos5L and Fos4L variants in F9 cells is not due to strikingly different expression levels of these proteins as compared to c-FosWT and c-FosI_2, we have checked the steady state protein levels using western blotting of crude extracts. No significant differences in protein expression levels could be detected (data not shown).

Transactivation properties of the homodimerizing c-Fos proteins in NIH3T3 cells

As compared to F9 cells, serum-stimulated NIH3T3 cells have a relatively high intrinsic AP1 DNA binding activity (20). Figure 5 shows that in this cell line the multiple mutations c-Fos5I, 4I, 5L and 4L do not give rise to the phenomenon of transcriptional inhibition below the basal promoter activity.

However the activation response of these multiple mutant proteins is again rather different as compared to the I_2 and the wild-type zipper variants, which show a progressive increase in activation with increasing amounts of expression vector. On the contary, the 5I and 4I variants activate between 4- and 3-fold at a low plasmid concentration (i.e. slightly better than the I_2 and wild-type zipper variants), but higher expression rates only marginally increase (4I) or again reduce the level of activation (5I).

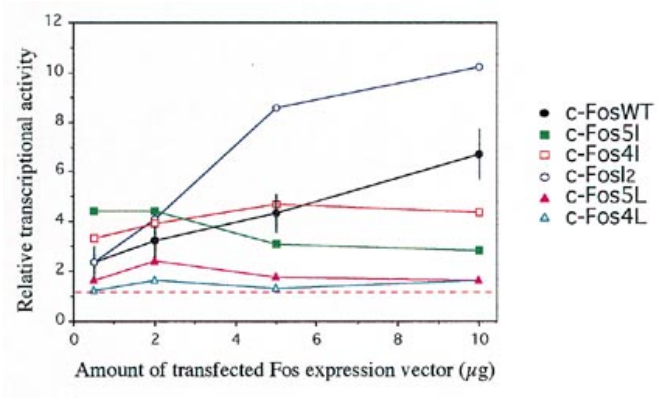

Figure 5. Transactivation assays in NIH3T3 cells. Variable amounts of pSG-derived expression vectors coding for one of the c-Fos variants were co-transfected with 2 μg of a 3× TRE-tk-Luc reporter plasmid. Luciferase activities were normalized with respect to the number of luciferase units obtained with the reporter plasmids in the absence of c-Fos expression vectors. The data are the average of two independent transfections.

The 5L and 4L variants neither activate nor inhibit the transcription of the reporter gene in NIH3T3 cells. More pronounced than in F9 cells, the I_2-variant is again more active than the Fos wild-type zipper protein.

DISCUSSION

The c-Fos wild-type leucine zipper fails to promote homodimer formation for both electrostatic and hydrophobic reasons (6,7,11). The protein contains many negative charges in the **e** and **g** positions leading to electrostatic repulsion and most of the **a** residues supposed to be hydrophobic are in fact polar or charged.

Despite this accumulation of molecular handicaps, a single point mutation in the hydrophobic interface confers DNA binding and thus homodimerization capacity to the c-FosI_2 variant harboring isoleucine instead of threonine in position $\mathbf{a_2}$. This residue is in a key position, since situated between the glutamic acid residues in positions $\mathbf{g_1}$ and $\mathbf{e_2}$ (Fig. 1D). Host–guest studies have shown that electrostatic repulsion between these residues is particularly destabilizing for the host leucine zipper (7). Since the nature of the $\mathbf{a_2}$ residue modulates the severity of the electrostatic repulsion (11), the Thr_{169}→Ile mutation in position $\mathbf{a_2}$ is probably beneficial for c-Fos homodimerization not only because a polar residue is replaced by a hydrophobic side chain, but also because a bulky isoleucine side chain reduces electrostatic repulsion between the glutamic acid side chains in positions $\mathbf{g_1}$ and $\mathbf{e'_2}$.

The fact that single point mutations in the $\mathbf{g_1}$ position (21) and in the $\mathbf{a_2}$ position (this work) of the c-Fos zipper are sufficient to induce specific DNA binding of c-Fos homodimers suggests that the Fos leucine zipper is not that deficient and that only small changes are required to allow Fos homodimer formation. In this context it is interesting to notice that a Fos wild-type leucine zipper peptide is indeed able to form homodimers with a dissociation constant of ~6 μM (22), whereas the Jun leucine zipper promotes coiled-coil formation with a dissociation constant of ~0.5 μM (5).

If not only the $\mathbf{a_2}$ position, but also additional **a** positions of the Fos zipper are replaced by isoleucine, the DNA binding capacity of the homodimer is progressively increased, i. e.:

TTKIK << T**I**KIK < T**III** < **IIII**
(<17×) (5×) (3×)

The crystal structure of the bZip Jun/Fos–DNA complex (8) suggests the existence of hydrogen bonds between several c-Fos **a** residues with the preceeding **g′** residue of the Jun zipper. Host–guest studies in solution had shown previously the thermodynamic relevance of electrostatic forces between **g′** and **e** residues in the Fos/Jun system (6,7), but the possible importance of **g′–a** interactions had not been addressed in these experiments. A comparison of the heterodimer DNA binding affinity of the Fos variants used here, shows that the interaction between the threonine side chain in position $\mathbf{a_1}$ of Fos and the arginine side chain $\mathbf{g_{0'}}$ of Jun (Fig. 1D) is indeed thermodynamically relevant for Fos–Jun heterodimerization.

The *in vitro* DNA binding affinity of the most strongly improved c-Fos homodimerizing variants examined in this study is superior to that determined for a c-Jun homodimer. As a consequence we expected that these Fos variants should be able to activate transcription, since c-Fos has been shown to contain several activation domains which are functional as fusion proteins with a heterologous DNA binding domain (23,24). Additionally these c-Fos activation domains are also functional if c-Fos is involved in a complex with a truncated c-Jun protein lacking its own activation domain (25,26).

However, none of the homodimerizing c-Fos variants may be considered as a strong transcriptional activator. The activation observed at low expression levels is rapidly abrogated and finally overcompensated by a strong inhibition of basal promoter activity at higher expresssion levels in F9 cells. This inhibition is independent of binding to the three TRE elements, since it is also observed with a reporter construct lacking these elements. Inhibition of basal transcription activity is further cell-type specific, since this effect is not observed in NIH3T3 cells. In this context it is worthwhile to remember that in addition to its role as a transcriptional activator, c-Fos is known to act also as a negative transcriptional regulator (27–33). Conceivably, the c-Fos leucine zipper mutations described here enhance the intrinsic inhibitory function of c-Fos.

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