The Fos wild-type leucine zipper is unable to support homodimerization. This finding is generally explained by the negative net charge of the Fos zipper leading to the electrostatic repulsion of two monomers. Using a LexA-dependent in vivo assay in Escherichia coli, we show here that additional antideterminants for Fos zipper association are the residues in position a within the Fos zipper interface. If the wild-type Fos zipper is fused to the DNA binding domain of the LexA repressor (LexA-DBD), no excess repression is observed as compared with the LexA-DBD alone, in agreement with the incapacity of the wild-type Fos zipper to promote homodimerization. If hydrophobic amino acids (Ile, Leu, Val, Phe, Met) are inserted into the five a positions of a LexA-Fos zipper fusion protein, substantial transcriptional repression is recovered showing that Fos zipper homodimerization is not only limited by the repulsion of negatively charged residues but also by the nonhydrophobic nature of the a residues. The most efficient variants (harboring Ile or Leu in the five a positions) show an about 80-fold increase in transcriptional repression as compared with the wild-type Fos zipper fusion protein. In the case of multiple identical substitutions, the overall improvement is correlated with the hydrophobicity of the inserted side chains, i.e. Ile > Leu > Val > Phe > Met. However at least for Val, Phe, and Met the impact of a given residue type on the association efficiency depends strongly on the heptad, i.e. on the local environment of the a residue. This is particularly striking for the second heptad of the Fos zipper, where Val is less well tolerated than Phe and Met. Most likely the a2 residue modulates the interhelical repulsion between two glutamic acid side chains in positions g1 and e2.

Most of the hydrophobic Fos zipper variants are also improved in heteroassociation with a Jun leucine zipper, such that roughly half of the additional free energy of homodimerization is imported into the heterodimer. A few candidates (including the Fos wild-type zipper) deviate from this correlation, showing considerable excess heteroassociation.

Many transcription factors associate with their DNA binding site as either homodimers or heterodimers, making use of the so-called bZIP DNA binding motif. These proteins share the common feature of a basic region adjacent to a “leucine zipper” which is essential for dimerization (for a recent review, see Ref. 1). Most of them can form homodimers, and a subset forms heterodimers with other leucine zipper proteins. Leucine zippers are attractive targets to interfere with different cellular functions or dysfunctions. Granger-Schnarr et al. (2) have shown recently that expression of a leucine zipper fused to the LexA DNA binding domain in transformed NIH 3T3 fibroblasts leads to the recovery of an untransformed phenotype. In this special case the target was a member of the AP-1 family of transcription factors.

These factors are expressed in a variety of cell types, usually at low basal levels, and can be induced in response to a wide set of stimuli, including growth factors, oncogenes, cytokines and UV irradiation (for reviews, see Refs. 3 and 4). AP-1 consists of two groups of bZIP proteins, the Fos-related proteins (c-Fos, Fos B, Fra1, Fra2), and the J un proteins (c-J un, J unB, J unD). A Fos protein has to dimerize with a J un protein in order to bind to DNA, whereas J un forms both homodimers and heterodimers with other J un and Fos proteins. J un and Fos proteins interact further with several other transcription factors, giving rise to a cross-talk between different signal transduction pathways by the formation of alternative dimeric species. J un and/or Fos interact via their leucine zippers with members of the ATF/CREB (5), C/EBP (6), Maf (7, 8), and Rel families (9, 10) as well as MyoD (11), FIP (12) and LRF-1 (13). Regions outside the leucine zipper seem to play a role in the interaction with the glucocorticoid receptor (14). The composition of these mixed oligomers may dictate an altered DNA binding specificity (5, 15–17) or lead to the repression of transcription (11).

The dimerization domains of J un and Fos proteins are a-helical structures characterized by a periodic repeat of leucine every 7th amino acid, i.e. every two helical turns, which forms a parallel coiled-coil structure (18, 19). The seven amino acids of each repeat are referred to by the letters a to g (a’to g’ referring to the opposite helix). The d positions are occupied by the highly conserved leucine residues and non-polar residues usually occur also at the a positions. Both a and d positions contribute to the formation of the hydrophobic interface which accounts for most of the free energy of dimerization. Additional stability can be provided by interhelical salt bridges between oppositely charged residues at positions e and g.

The preferential assembly of J un and Fos as heterodimers has been shown to be mostly specified by the residues occupying the g and e positions (20). Among these residues two pairs of oppositely charged amino acids in positions g2 and e2 account for most of the additional free energy of heterodimerization (21). The J un protein forms functional homodimers while the Fos protein does not. Besides electrostatic repulsion, it has been suggested that Fos homodimerization might also be hampered by a lack of hydrophobic residues in the a positions (22). In these positions the c-Fos protein, as well as the other members of the Fos family, harbors indeed mostly polar residues, i.e. two threonine, two lysine, and one isoleucine residues.

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To address the question if the nonhydrophobic nature of
these residues is indeed a major antitdeterminant for Fos zipper dimerization, we have generated a restricted random collection of Fos zipper mutants. LexA proteins were inserted into Leu, Val, Phe, or Met in the five a positions. The dimerization capacities of these Fos zipper variants were assayed in a bacterial in vivo test using protein fusions between these zippers and the LexA DNA binding domain (23, 24), which is devoid of intrinsic dimerization activity both free in solution (25, 26) and bound to operator DNA (27). We have used this assay previously to study J un zipper variants mutated in the e and g positions (28). Similar approaches made, respectively, use of the phage λ (29) and the phage 434 (30) repressor DNA binding domain. Here we show that a Fos leucine zipper can considerably improve the repressor activity of the LexA DNA binding domain, if the a positions of the coiled-coil are occupied by hydrophobic residues. The observed improvement is correlated with the hydrophobicity of the inserted side chain (Ile $>$ Leu $>$ Val $>$ Phe $>$ Met) and a Fos zipper variant can reach the efficiency of the J un leucine zipper when all the a positions are occupied by leucine and isoleucine residues. However the effect of a given residue depends strongly on the heptad where it has been inserted. For example, Val is more penalizing than Phe or Met if inserted in the a position of the second heptad of the Fos zipper. Most likely the a residue in this heptad modulates the interhelical repulsion between the two glutamic acid side chains in positions g and e (21). Finally, we show that the most efficient homoassociating Fos zipper variants are also improved in heteroassociation with the wild-type J un zipper. For most of the hydrophobic Fos zipper variants, one observes a correlation between homoassociation and heteroassociation such that roughly half of the additional free energy of homodimerization is conserved upon interaction with the wild-type J un zipper.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—Escherichia coli strains J L806 (lexA71::Tn5/sulA11/lexAop-lacZ d ind) and J L1436 (J L806 but $>$ sulA1::lacZ d ind and $>$ lacI instead of $>$ $>$ Tn3) and the plasmid pJLW70 bearing the lexA gene were kindly provided by J ohn Little (31, 32). Plasmid pMS504 bearing the wild-type Lea-c-Fos zipper fusion was constructed by insertion of a Bst III restriction site into the Xmn I site of pJLW70 using a short oligonucleotide. The C-terminal domain of Lea was deleted by Bst I/Pst I digestion and the remaining domain (residues 3–87) was fused to the Fos zipper. The DNA coding for this peptide (see Fig. 1A) was chemically synthesized and fused to the N-terminal part of Lea via the Bst E1 site, which contributes a valine in position 88. Plasmid pDP107 bearing the LexA-c-jun zipper fusion was obtained by insertion of a Xho I restriction site within the Xmn I site of pJLW70. The C-terminal part of Lea was deleted by a Xho I/Pst I digestion, and the remaining N-terminal domain was fused to the c-jun zipper sequence shown in Fig. 1B. The Xho I site contributes a serine residue in position 88.

Mutagenesis of the a Positions of the c-Fos Zipper—Hydrophobic amino acids were introduced into the a positions of the c-Fos leucine zipper using degenerated synthetic oligodeoxynucleotides. One oligonucleotide corresponds to the coding strand and the second to the noncoding strand of the c-Fos zipper sequence in order to allow for double strand DNA synthesis by annealing and extending the complementary 3’ ends of the oligos. The coding strand contains an Age I restriction site close to the 5’ end, the noncoding strand bears a Dra I restriction site (see Fig. 1A). Degenerated complementary oligodeoxynucleotides were introduced at the five a positions (using NNN or NNN for the oligodeoxynucleotides corresponding, respectively, to the coding or the complementary strand) which allows to introduce selectively the hydrophobic residues Leu, Ile, Val, Phe, or Met. The two oligodeoxynucleotide families (overlapping by 12 base pairs) were annealed, and the 3’ ends were extended with T7 polymerase essentially as described (33). A collection of Fos c-Fos zipper variants was constructed by cloning Age I/Xho I fragments into a similarly digested pMS504 plasmid resulting in a set of plasmids called pMS500. Recombinant DNA was isolated and the LexA-c-Fos variants were identified by sequencing the entire gene, including the promoter sequence.

The single mutations in an all-leucine context (Fig. 4) were obtained either by the insertion of a small synthetic oligodeoxynucleotide cassette into the gene of the randomly obtained mutant protein (FosL1L1L1L1) or via the recombination of several randomly obtained variants shown in Table I (series pMS500) using the appropriate restriction enzymes.

In Vivo Measurement of the Association Capacity of the Mutant Proteins—The homo- and heteroassociation capacities of the different fusion proteins were determined in vivo as the repressor activity that can be conferred to the DNA binding domain of the Lea protein. The repressor activity is determined as the amount of β-galactosidase units that can be measured in appropriate E. coli indicator strains (J L1346 or J L1436) in the absence or in the presence of various concentrations of IPTG (35) diluted 100-fold in Luria broth containing the same IPTG concentration. Assays and units were as described (34). The homoaasociation capacity of the mutant proteins was measured using the pMS500 pUC-like plasmids. In these constructs, the expression of the mutant proteins is under the control of a lambda promoter.

For the measurement of the heteroassociation capacity, a two-plasmid system was used allowing for a low expression of the proteins in order to minimize or to prevent the formation of homodimers. The expression of the lexA-c-junZIP gene was decreased by the introduction of a mutation within the ribosome binding site. This mutation (REG9) has been shown to reduce the level of expression by 4-fold (35) and has been produced by an exchange of the Cia I/Mlu I restriction fragment of pDP107 (see above) for the Cia I/Mlu I restriction fragment of REG9 giving rise to plasmid pDP204. The lexA-c-junZIP gene was further transferred to a plasmid with lower copy number (pACYC184) which enables it to coexist with vectors that carry the catE1 origin of replication. The Cia/I/Mlu restriction fragment of pDP204 (750 base pairs) was fused to the pACYC184 vector after Hind III digestion (2550 base pairs), giving rise to plasmid pDP301.

The expression of the lexA-c-FosZIP wild-type and mutant genes was also decreased by exchanging the Cia I/Mlu I restriction fragment of pMS504 and of 15 candidates of the pMS500 series for the Cia I/Mlu I restriction fragment of REG9 giving rise to plasmid pMS604 and to the pMS600 set of plasmids for the wild-type and the mutant proteins, respectively.

Assay of the State-level Value of the Lea-Fos Zipper Fusion Proteins—Crude extracts were prepared from the E. coli strain J L1436 in the presence of $>$ $>$ 5 × 10$^{-5}$ M IPTG according to (35). Lysates were subjected to electrophoresis in 12.5% SDS-polyacylamide gels and transferred to a poly(vinylidene difluoride) Immobilon-P membrane. The fusion proteins were detected with purified rabbit antibodies directed against the N-terminal domain of Lea using an enhanced chemiluminescence detection system (Amersham ECL, RPN2106), preflashed films for detection, and a Shimadzu CS-9000 densitometer for quantification.

Construction and Purification of the Full-length Fos Protein Mutant Proteins.—The wild-type Fos protein contains six additional hydrophobic residues in positions $>$ $>$ a2, a3, a4, a5, or a6, and finally pDFsos9 (Eco) harboring leucine residues in all five a positions.

The Fos proteins (385 amino acids) were purified by nickel affinity chromatography in the presence of guanidine hydrochloride (36).

Electrophoretic Mobility Shift Assays—10-μl samples of protein/DNA mixtures, containing 1.15 μg of Fos protein and about 5 × 10$^{-10}$ M of a 21-base pair DNA duplex harboring an AP-1 binding site, were incubated in a buffer containing 50 mM Tris-HCl, pH 8.0, 5 mM MgCl$_2$, 5 mM DTT, 1 mM EDTA, 10% glycerol, 0.1% Nonidet P-40, 2 μg of bovine serum albumin, and 0.1 μg of poly(dI-dC) and subsequently run on a 4% polyacrylamide gel (with 1:37bisacrylamide). The AP-1 oligonucleotide 5’-AGAARAGCTGGAATTCATCCTGC-3’ was synthesized as the 5’-end labeled probe. The bands corresponding to the specific complexes were excised from the gel, and the DNA was eluted and sequenced as described (33).

1 The abbreviation used is: IPTG, isopropyl-$eta$-D-thiogalactopyranoside.
Fos Leucine Zipper Variants with Increased Association Capacity

A

| LexA DBD | a₁ | Fos Zipper | a₂ | a₃ |
|----------|----|------------|----|----|
| E K L E | D  | L E D R K A S A L Q T | | |
| 5′-GAAACGAAGCTCTGACCGGACCGTACGCAAGGACGAGAGAAGAAGGCGCTCCAGACC | | | |

AP-1 oligonucleotide: 5′-TTCCGCTGACTCATCAAGCGC
3′-AAGGCGGACTGATAGTTGTCG

RESULTS

Association of J un and Fos Leucine Zippers Probed by Repressor Activity Conferred to the LexA DNA Binding Domain—The gene fragments coding for the Fos or the J un leucine zippers have been assembled from synthetic oligonucleotides (see Fig. 1) and were fused to the LexA DNA binding domain (LexA-DBD) as described under “Materials and Methods.” The LexA part corresponds to residues 1–87 of the E. coli LexA protein (37). The Fos leucine zipper comprises residues 162–200 of c-Fos, and the J un leucine zipper encompasses residues 277–315 of the human c-Jun protein. The fusion proteins are referred to as LexA-c-FosZIP and LexA-c-JunZIP, respectively. The expression of both proteins is driven by the same IPTG-inducible promoter (lacUV5) and, for this part of the results, both are cloned into identical expression vectors.

The ability of these domains to associate is tested as their capacity to restore the repressor activity of the truncated LexA protein (devoid of its own dimerization domain) in an in vivo test using appropriate E. coli indicator strains (32), one of which is schematized in Fig. 2A. The JL1436 host strain is deficient in the chromosomal recA gene and bears a lacZ gene driven by the LexA-dependent sulA promoter, whereas in the JL806 strain, the lacZ gene is under the control of the stronger (also LexA-regulated) recA promoter. The repressor activity of the fusion protein is determined as the residual β-galactosidase activity that can be measured at different levels of expression of the proteins obtained upon varying the IPTG concentration. Characteristic repression curves are shown in Fig. 2B. In the absence of repression (expression vector alone), we measured about 4200 β-galactosidase units regardless of the IPTG concentration. When the truncated form of LexA (DBD alone) was expressed, a significant repression of the lacZ gene was observed only upon overproduction of the protein, reaching about 2300 β-galactosidase units at 10⁻⁵ M IPTG. With the entire LexA protein, the lacZ gene is nearly completely turned off even in the absence of IPTG. In the absence of IPTG the c-Jun zipper is able to restore the DNA binding capacity of the truncated LexA protein at least partially, whereas the c-Fos zipper does not. Upon overproduction of the fusion protein, a level of repression identical to that obtained with LexA was reached with the LexA-c-Jun unZIP protein, whereas with its c-FosZIP counterpart the repression was the same as with the LexA DNA binding domain alone. These results are in agreement with previous results showing that (unlike the J un zipper) the Fos zipper is unable to promote homodimerization.

Construction of Hydrophobic Combinatorial Fos Zipper Variants—In order to introduce randomly hydrophobic residues within the five a positions of the Fos leucine zipper, we used two complementary and partially overlapping synthetic oligonucleotides spanning the zipper part of the LexA-c-FosZIP protein. The codons corresponding to each a position of the five heptads were degenerated at the first and the third base of the codon, i.e. NTN or NAN for the coding strand and the complementary strand, respectively. As described earlier (29), this approach allows the random introduction of Leu, Ile, Val, Phe, or Met. After annealing and synthesis of the complementary strand, the mutated c-Fos zippers were ligated to the LexA DNA binding domain. The recombinant plasmids were isolated, and the fusion genes were entirely sequenced in order to check for additional mutations within the lexA part or the regulatory sequence. A total of 250 mutants was sequenced giving rise to 57 different Fos zipper variants. The other mutants represent duplicates (58) and variants (135) harboring additional mutations.

Indicator Strain JL1436 versus JL806—The 57 different LexA-c-FosZIP mutant proteins were characterized for their homoassociation capacities within the JL1436 and JL806 indicator strains. The difference between the two strains resides both in the strength of the promoter and the operator in front of the lacZ gene. The recA promoter (J L806) is stronger than the sulA promoter (J L1436), giving rise to 12,000 instead of 4200 β-galactosidase units in the unrepressed state. Fig. 3 shows that transcriptional repression by the different LexA-c-FosZIP variants in the two indicator strains is reasonably well

B

| LexA DBD | a₁ | Jun Zipper | a₂ | a₃ |
|----------|----|------------|----|----|
| E K L E | D  | L E D R K A S A L Q T | | |
| 5′-GAAACGAAGCTCTGACCGGACCGTACGCAAGGACGAGAGAAGAAGGCGCTCCAGACC | | | |

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pressed state (as a function of IPTG concentration, using strain JL1436. The unrepressed control of the promoter/operator region of the sulA gene is essentially identical except that the lacZ gene is under the control of the LexA repressor. The strain is deficient in the chromosomal lexA gene and transcription of the lacZ gene will be repressed only, if the LexA deficiency is complemented by a plasmid producing a functional LexA1–87-leucine zipper fusion protein. The production of only, if the LexA deficiency is complemented by a plasmid producing a LexA1–87-c-FosZip (see Table I), LexA-DBD alone allows already for a repression of 80% at 5 M IPTG in JL806, but only of 45% in JL1436. Second, it is virtually impossible to repress the recA promoter completely with TTKIK being the wild-type amino acids. For each protein, the β-galactosidase units given are those measured at $10^{-3}$ M IPTG. Except one (VVFVF), all the other combinatorial mutant proteins increase the repressor capacity of the truncated LexA protein, although to various extents. As shown previously in the case of the jun leucine zipper (28), Fos zipper variants with an improved hydrophobic interface can thus efficiently replace the LexA dimerization domain.

Given that for all the fusion proteins the LexA-DBD is unchanged, the increased repressor activity is likely to reflect the efficiency with which the different leucine zippers are able to associate, provided that the proteins are produced in similar amounts. Therefore for each mutant protein, the relative amount was measured (at least in duplicate) by an immunoblot analysis using antibodies against the LexA part of the chimeric proteins as described under “Materials and Methods.” The intracellular steady-state level of all the mutant proteins were found similar or identical to the parental LexA-c-FosZIP protein.

Table I shows the collection of Fos zipper variants classified according to the amount of β-galactosidase units measured in strain JL1436. The smaller the number of units, the tighter the transcription of the indicator gene is repressed by the LexA-Fos zipper fusion proteins. Under these conditions the LexA-DBD alone and the LexA-Fos wild-type zipper fusion (TTKIK in Table I) give rise to about 2300 units. The most strongly improved variants contain mostly Ile or Leu side chains in the five a positions, giving rise to only 28 units in the case of five Ile residues, i.e. an 82-fold improvement in repressor efficiency as compared with the Fos wild-type zipper fusion protein.

The different hydrophobic side chains are not equivalent in promoting Fos zipper association. Phenylalanine and methionine residues, especially, are mostly found within weakly improved Fos zipper variants, suggesting that these amino acids are not as efficient as leucine, isoleucine, or valine side chains (see Table I). In order to establish this hierarchy more precisely Fos zipper variants were created bearing the same hydrophobic amino acid within the five a positions (Table I, bottom right). As anticipated from the random collection we find the following hierarchy: Ile ≥ Leu > Val > Phe > Met. This order may be correlated ($R^2 = 0.9$). In the following most of the experiments were done with the JL1436 strain, because in this strain, the useful repression range is broader for two reasons. First, the LexA-DBD alone allows already for a repression of 80% at $10^{-3}$ M IPTG in JL806, but only of 45% in JL1436. Second, it is virtually impossible to repress the recA promoter completely even with very high amounts of LexA wild-type repressor.

**Fos Zipper Mutants with Hydrophobic Residues in the Five a Positions**—In Table I each mutant protein is identified according to the amino acid found in the five successive a positions, corresponding to the amino acid found in the five successive a positions.
understood in terms of the hydrophobicity of these side chains (see Discussion).

The Different a Positions Are Not Equivalent—The random mutations in Table I suggest further that the five a positions of the Fos zipper are not equivalent. For example a comparison of the mutant proteins LIVLL (52 β-galactosidase units) with the nearly identical LVLLL (198 β-galactosidase units) suggests that a valine residue in the second heptad may be less favorable than in the third heptad. To investigate the positional effects of a given type of residue more systematically, a set of 20 single mutations has been created in the context of an all-leucine hydrophobic interface. These variants contain a single Ile, Val, Phe, or Met in any of the five heptads.

As shown in Fig. 4, Ile is at least as efficient as Leu, since in none of the five a positions it influences the dimerization capacity of the FosLLLLL host zipper. This is not the case for the other three amino acids, which all, to various degrees, diminish the repressor activity of the LexA-FosLLLLL protein.

As suggested from the random combinatorial variants, we observe pronounced position-dependent effects. The a position of the first heptad is less sensitive to mutations than the four others, since only minor changes are observed even when a1 is occupied by the relatively unfavorable residues Phe and Met. This may be understood by the fact that a1 is not packed between two leucine layers as the other a residues (see Fig. 8B).

In position a2, the hierarchy (Ile > Leu > Val > Phe > Met) mentioned above is inverted, i.e. in this position Val is less favorable than Phe and Met. This atypical behavior is most likely linked to the two glutamic acid residues in positions g1 and e2, which tend to pack over the a2 side chain as discussed below. In a2 and a6, Val is well accepted, whereas in a6 it is again unfavorable, albeit less than in a2. Phe and Met are always unfavorable as compared with the parental FosLLLLL zipper, albeit with some modulations. Phe is particularly unfavorable in a3 and Met in a5. Hu et al. (29) also observed positional effects in a and d positions in a study of the GCN4 leucine zipper.

The Effect of Multiple Mutations Is Essentially Additive—The interpretation of the in vivo repression data in terms of dimerization efficiency is potentially complicated by the fact that the nature of the a (and d) position residues may modulate the number of strands within an α-helical coiled-coil. Ile in position a and Leu in d favors a classical two-stranded geometry (38), and Ile in both a and d leads to the formation of a tetrameric coiled-coil (39), whereas Leu in a and Ile in d leads to the formation of a tetrameric coiled-coil (38).

No crystal structure is available for a leucine zipper harboring Leu in both a and d. Zhu et al. (40) have shown by size exclusion chromatography for a coiled-coil with Leu residues at positions a and d that in the reduced form these peptides adopt a dimeric (two-stranded) coiled-coil structure. Harbury et al. (38) showed (also by size exclusion chromatography) that a GCN4 leucine zipper variant harboring Leu at positions a and d apparently forms trimers in the reduced state, but dimers and higher oligomers in the oxidized (disulfide-bonded) state. This behavior is not easily understood in comparison with an isoleucine zipper (Ile in a and d), which forms also trimers in the reduced state, but only hexamers in the oxidized state (38).

To address the question if a Fos leucine zipper variant harboring Leu at positions a and d would have a strong tendency to form higher order species, we have constructed and purified Fos protein variants harboring mostly or exclusively leucine or isoleucine in position a (for details see “Materials and Methods”). Fig. 5 shows that contrary to the Fos protein harboring a wild-type leucine zipper (lane 4), the Fos variants Fos5L (lane 1), Fos4L (lane 2), and Fos4 (lane 3), harboring a hydrophobic interface, are able to interact with a 21-base pair AP-1 binding site (TGACTCA).

Relevant for the question we ask here is the finding that the gel mobility of the two complexes with a leucine interface (Fos5L and Fos4L) is the same as that with an isoleucine interface (Fos4L). This behavior strongly suggests that the dominant species for both proteins should be a protein dimer, since Ile in position a (with Leu in position d) is known to favor the formation of leucine zipper dimers as shown by equilibrium ultracentrifugation (38). Gel retardation is in fact very sensitive to the oligomeric state of a protein bound to a small DNA duplex. For example, the migration of a dimeric variant of lac repressor (a protein of 360 amino acids, i.e. of similar size as the Fos protein) is different from that of the tetrameric repressor in a complex with a 40-base pair operator fragment (41).

For the following reason the data in Table I also argue in favor of the dimer as the dominant oligomerization state of the LexA-C-FosZip variants in vivo; if the repression effect of the multiple combinatorial mutations in Table I could be understood in terms of the sum of the corresponding single mutations...
(placed in a Leu hydrophobic interface, see Fig. 4), we may argue that multiple oligomerization states should not play a major role in the establishment of the repressor efficiency (Table I), since (contrary to the single mutations) the combinatorial mutations are not particularly leucine-rich, but contain also Ile (a dimer-driving residue in position $a_3$), as well as Val, Met, and Phe.

To test this hypothesis we have proceeded in the following four steps. 1) The $\beta$-galactosidase units of the 20 different single mutations were normalized to the all-leucine zipper (FosLLLLL) to obtain a relative dissociation constant $K_{\text{rel single}}$ for each single mutation in this context. In the case of the single mutations these relative dissociation constants may be considered as a penalty index $p_{\text{single}}$, associated with the mutation of a given leucine to isoleucine, valine, phenylalanine, or methionine.

Relative Dissociation Constants May Be Determined from the $\beta$-Galactosidase Units (42) According to the following equation,

$$p_{\text{single}} = K_{\text{rel single}} = \frac{\theta_{\text{L}} \cdot (1 - \theta_{\text{single}})}{\theta_{\text{single}} \cdot (1 - \theta_{\text{L}})} \cdot \frac{Z_{\text{single}}}{Z_{\text{L}}} \cdot Z_{\text{L}}$$

(Eq. 1)

where $\theta_{\text{single}}$ is the fractional occupancy of the sulA operator by one of the LexA-mutant Fos zipper hybrid proteins, $\theta_{\text{L}}$, the

![FIG. 4. Positional effects of single mutations (Ile, Val, Phe, or Met) in the context of an all-leucine hydrophobic interface. The repression of the lacZ gene (strain J L1436, 10$^{-5}$ M IPTG) conferred by LexA$_{1–87}$-Fos zipper proteins varies according to the $a$ position, where the mutated residue has been incorporated. The penalty index $p_{\text{single}}$ of each mutant protein relative to the parental FosLLLLL zipper (see Equation 1) is given in brackets behind the number of the experimentally determined $\beta$-galactosidase units (black bars). The gray bars are adjusted penalty values obtained upon optimization of the fits of log($K_{\text{rel exp}}$) versus log($K_{\text{rel theo}}$) plots. For the LLMLL and LLLML variants such an adjustment was not possible because the combinatorial mutant set (Table I) contains no methionine in positions $a_3$ and $a_4$.](http://www.jbc.org/)

![FIG. 5. Electrophoretic mobility shift assay showing that full-length Fos protein variants harboring a hydrophobic leucine zipper interface are able to interact with a 21-mbase pair DNA duplex harboring an AP-1 binding site (TGACTCA): Fos$_{5L}$ (lane 1), Fos$_{4L}$ (lane 2), Fos$_{4I}$ (lane 3); whereas a Fos protein harboring the wild-type leucine zipper (lane 4) does not bind to this oligonucleotide. The protein concentration was 1.15 × 10$^{-7}$ M and the DNA concentration about 5 × 10$^{-10}$ M. For the assay conditions, see “Materials and Methods.”]
multiple leucine zipper mutations may thus be explained by the additive effect of the individual mutations in the context of an all-leucine hydrophobic interface. This behavior suggests indeed that the repressor efficiency of the Lex A-Fos zipper fusion proteins in vivo is dominated by a single oligomerization state. This dominant state is most likely a Fos zipper dimer, since those leucine zippers which are rich in Ile in position a have been shown to form exclusively dimers (38, 39).

The correlation between $K_{\text{rel}}^{\text{theo}}$ and $K_{\text{rel}}^{\text{exp}}$ can be further improved by adjusting the $K_{\text{rel}}^{\text{exp}}$ values. Adjustment was achieved by a stepwise variation of each $K_{\text{rel}}^{\text{single}}$ value and subsequent recalculation of the correlation between $K_{\text{rel}}^{\text{theo}}$ and $K_{\text{rel}}^{\text{exp}}$. Using an adjusted $K_{\text{rel}}^{\text{single}}$ data set (see gray bars in Fig. 4), the correlation (not shown) can be increased to $R^2 = 0.90$. Major improvement is achieved upon increasing the $K_{\text{rel}}^{\text{single}}$ values of MLLLL and LLLLL and diminishing that of LLLFL. Most of the other $K_{\text{rel}}^{\text{single}}$ values (especially those of the valine single mutations) are close to their optimal values in terms of their fitting efficiency.

Improving the Heteroassociation Capacity of a Fos Leucine Zipper Can Improve Its Heteroassociation Capacity with a Jun Leucine Zipper—It has been suggested that one of the decisive parameters for a leucine zipper to heterodimerize rather than to homodimerize resides in its incapacity or in its poor capacity to homodimerize (20). Since the Fos zipper variants shown in Table I have acquired the capacity to associate forming most likely dimeric coiled-coil structures, it was interesting to study their heteroassociation capacity with a Jun leucine zipper using the LexA-mediated repression system described above. In a first step it was necessary to reduce the expression of the Lex A-Fos zipper fusion proteins such that transcriptional repression due to homoa ssociation was negligible thus allowing the measurement of heteroa ssociation only. This was achieved by using a down-mutation of the lacUV5 promoter (35) for both LexA-c-FosZip and LexA-c-JunZip. LexA-c-JunZip was additionally subcloned into a compatible plasmid having a lower copy number and finally, transcriptional repression was measured in the absence of IPTG to assure maximal repression of the fusion genes by the lac repressor (see Fig. 2).

Fig. 7 shows the results obtained with a representative subset of the combinatorial Fos zipper variants of Table I. Under these conditions the LexA-c-JunZip protein is not able to repress the lacZ gene in JL1436 and also most of the LexA-c-FosZip variants fall into the range of the unpressed state, i.e. 4183 units $\pm 10$. Only two c-FosZip variants (LLLLL and ILLLL) show a slight residual repression due to homodimerization.

The coexpression of LexA-c-JunZip and LexA-c-FosZip leads to the recovery of transcriptional repression. The two wild-type zipper sequences together produce an about 3-fold repression, whereas coexpression of LexA-c-JunZip with the FosZip-variant leads to a 10-fold repression under these conditions. Interestingly the four Fos zipper variants in Fig. 7 being most improved in heteroassociation with the Jun zipper (i.e. LLLLL, IILL, LIVVL, and ILLLL) are also the most efficient homoassociating species of this set of Fos zipper variants.

Improved Heteroassociation and Heteroassociation Are Correlated—We asked further if it is possible to establish a quantitative correlation between improved heteroassociation and improved homoassociation. One may expect that the change in free energy of a Fos zipper variant within a heterodimer with the Jun wild-type zipper should be about half of the change in free energy of the same variant within the corresponding homodimer, because in the heterodimer only one of the partners is mutated. If this holds true, one might expect a linear relationship between the square root of the $K_{\text{rel}}^{\text{exp}}$ values for ho-
modimerization (derived from Equation 3) and the corresponding \( K_{rel}^{homo} \) values for heterodimerization (derived from the same equation, using this time the heterodimerization of the FosLLLฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟฟффfffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffb

**DISCUSSION**

The Fos and Jun leucine zippers have been extensively studied in the past using both genetic and biophysical approaches. Most of the mutagenesis work has been focused on loss-of-function mutations, which established the importance of the conserved leucine side chains for dimerization and DNA binding using either single or multiple hydrophobic (valine, phenylalanine) or helix breaking substituents (22, 43–45).

A gain-of-function mutation has been described in the case of the Fos zipper (46) where the replacement of Glu-168 (which is the g residue of the first heptad) by lysine enables a truncated Fos protein to interact with DNA as a homodimer. For technical reasons the interaction of this variant with Jun could not be evaluated. It seems, however, unlikely that Fos E168K would be also improved in Jun binding, since in the Fos-Jun heterodimer this mutation would juxtapose two lysine residues.

**Homoeasociation of Improved Hydrophobic Fos Zipper Variants—**Here we describe Fos zipper variants with a modified hydrophobic interface, which are improved both in homoasociation and in heteroasociation with the Jun zipper as inferred from the recovery of repressor activity in the context of LexA-Fos zipper fusion proteins. We may conclude from these studies that the homoeasociation of the wild-type Fos zipper is not only limited by the repulsion of the negatively charged residues in positions g and e (20, 21, 46, 47) but also by the nonhydrophobic nature of the a positions. Just as the Fos E168K variant (46) is able to overcome the handicap of the poor hydrophobic interface, the hydrophobic Fos zipper variants described here are able to overcome the repulsion of the negatively charged g and e residues. The most efficient Fos zipper variants are those harboring only Ile and Leu in the a positions (see Table I). One may wonder if such a rather uniform hydrophobic interface is sufficiently specific to hold the two leucine zippers in register. Apparently slippage is not a major problem for these variants, since the full-length Fos proteins harboring leucine or isoleucine in position a bind specifically to DNA. Slippage would almost certainly prevent DNA binding, since the two basic
domains would be out of frame.

The degree of improvement of these variants in homoa ssociation is, however, not uniform and depends both on the nature of the hydrophobic amino acid substituents and on the position within the Fos zipper. If one compares Fos zipper variants harboring only Leu or Ile or Val or Phe or Met in the five a positions, then the improvement may be correlated with the hydrophobicity of the side chains (see Fig. 9A). No correlation exists between homoa ssociation improvement and a helix propensity of these residues, since all relevant helicity scales (compiled in Ref. 48) attribute a higher a helix propensity to Met (the least efficient homoa ssociation inducing residue in our experiments) as compared with Ile (the most efficient residue in our experiments).

The hydrophobicity scale used for the plot in Fig. 9A is from Radzicka and Wolfenden (49) and corresponds to the free energies of transfer of the amino acid side chains from cyclohexane to water (45). B, representation of a Fos homodimer (with variable a positions) as two parallel helical cylinders as adapted from Hu et al. (29). Coupling between g residues in one helix with e residues in the opposite helix is indicated by white bars.

Fig. 9. A, correlation between the homoa ssociation capacity of Fos zipper variants harboring only either leucine (L), isoleucine (I), valine (V), phenylalanine (F), or methionine (M) in the five a positions with the hydrophobicity of these side chains as inferred from the free energy of transfer from cyclohexane to water (45). B, representation of a Fos homodimer (with variable a positions) as two parallel helical cylinders as adapted from Hu et al. (29). Coupling between g residues in one helix with e residues in the opposite helix is indicated by white bars.

The hydrophobicity scale used for the plot in Fig. 9A is from Radzicka and Wolfenden (49) and corresponds to the free energies of transfer of the amino acid side chains from cyclohexane to water. On this scale Leu, Ile, Val, Phe, and Met are the five most hydrophobic amino acid side chains. The compilation of 61 leucine zipper-containing transcription factors (1) reveals that 24% of the a positions are occupied by Val, 21% by Asn, 11% by Leu, and 10% by Ile. The residues being on average most efficient in promoting Fos zipper homodimerization (Ile > Leu > Val) are thus also beside the most common a residues in this leucine zipper compilation.

The correlation between hydrophobicity and repressor efficiency shown in Fig. 9A corresponds to five simultaneous mutations in position a. Since the five heptadepths of the Fos zipper are not identical these data correspond to an average over different local environments.

Residues in Position a Are Likely to Modulate g → e Coupling—However at least for Val, Phe, and Met the impact of a given residue type on the repressor efficiency depends strongly on the heptad, i.e. on the local environment of the different a residues (see Fig. 4). This is particularly striking for the a residue in heptad 2, where Val (233 units) is clearly more penalizing than Phe (70 units) and Met (139 units). This conclusion may be drawn both from single mutations in the context of an all-leucine interface and from the random mutations in Table I.

What are the possible reasons for this inversion from the general Val > Phe > Met hierarchy to Phe > Met > Val in heptad 2? The most likely explanation is that in heptad 2 the a residue modulates the interhelical repulsion of the two glutamic acid side chains in positions g1 and e2 (where the primed residue corresponds to the opposite helix, see Fig. 9B), since in the case of the Fos zipper mutants, the cavity into which an a1 residue has to fit is the same for a2, a3, a4, and a5. In a parallel coiled-coil the packing of the side chains in the dimer interface is such that an a residue is surrounded by four amino acid side chains of the opposite helix (50, 51). In the Fos zipper an a1 residue is always surrounded by a glutamic acid side chain (in position g1), two leucines (in d1 and d1'), and the opposite a' residue, which is conserved in the case of a homodimer (see Fig. 9B).

Whereas the cavity is the same (including the g residues), the e residues are variable from one heptad to the other. Host-guest studies have shown that electrostatic repulsion between glutamic acid residues in positions g1 and e2 is particularly destabilizing for the host leucine zippe r (21). The atypically behaving g1 residue lies just between these residues and may conceivably modulate the negative interaction between them. Valine might bring the two glutamic acid side chains close together and thus increase electrostatic repulsion. This hypothesis might also explain a puzzling result of Krylov et al. (52) who showed that the g → e coupling energy for glutamine pairs is surprisingly favorable if packed over valine residues in position a. Physical model building based on the co-ordinates of the GCN4 leucine zipper (50) showed us that two glutamine residues (or two protonated glutamic acid side chains) in g and e of the next heptad might be brought sufficiently close together to form potentially two interhelical hydrogen bonds. Independently of the precise molecular mechanism, our results strongly suggest that a residues, especially valine, may modulate interhelical g → e coupling.

It is worthwhile to notice that a mutation of valine in position a2 of the J un leucine zipper to phenylalanine gave rise to a more active J un protein (53), whereas the substitution of other a residues (Asn in a3, Ala in a4, and Val in a5) with phenylalanine was penalizing. In the J un leucine zipper, a2 lies also between equally charged residues (two lysines in g and e) which may also give rise to electrostatic repulsion, albeit with a smaller thermodynamic cost as in the case of two glutamic acids (21, 52).

Heteroassociation of Improved Fos Zipper Variants with the J un Leucine Zipper—A representative subset of hydrophobic Fos zipper variants has been further tested for heteroassociation with a wild-type J un zipper under conditions where neither protein alone conferred significant transcriptional repression. The rule that emerges from a correlation between homo- and heteroassociation data is that for most tested variants (13 of 15) improved homoassociation is correlated with heteroassociation such that roughly half of the additional free energy of homoassociation may be imported into the Fos/J un zipper complex (Fig. 8).

For these Fos zipper variants, one may conclude that the general stability rather than the specificity of Fos/J un zipper assembly is affected. However we would not expect that these Fos zipper variants would be able to dimerize efficiently with
any leucine zipper, since the major specificity determinants for Fos/Jun zipper assembly in positions e and g are conserved (20, 21).

However there are a few interesting exceptions from this correlation. Especially the wild-type Fos zipper does not fit to this correlation. It has an intermediate heteroassociation activity in this assay, but confers no homoassociation at all, suggesting that the Fos wild-type positions (TTKIK) are extremely unfavorable for homoassociation, but acceptable or favorable in the context of oppositely charged glutamic acid residues in positions gi, ei, and g, ei (see Fig. 9B), but acceptable or favorable in the context of oppositely charged g and e residues in the Fos/J un zipper complex (22).

Several canonical Fos zipper a variants (i.e. those for which homo- and heteroassociation are correlated) and the "excess heteroassociation" variants are currently under study for their transformation suppressor activity in ras-transformed fibroblasts.

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