Construction, Purification, and Characterization of a Hybrid Protein Comprising the DNA Binding Domain of the LexA Repressor and the Jun Leucine Zipper: A Circular Dichroism and Mutagenesis Study[†,‡]

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ABSTRACT: An increasing number of eukaryotic transcription factors interacting specifically with DNA comprise a dimerization motif called the "leucine zipper". These leucine zipper proteins form homodimers and/or heterodimers with another protein containing a leucine zipper motif. The leucine zipper of the oncoprotein Jun is particular in that Jun may form homodimers as well as heterodimers with the oncoprotein Fos, which are however more stable than the Jun–Jun homodimers. Leucine zipper dimerization is thought to occur through a coiled-coil arrangement of parallel α-helices, but the rules governing the specificity of homo- and/or heterodimerization are still largely unknown. To address this question in the case of the Jun leucine zipper, we constructed a fusion protein containing the amino-terminal DNA binding domain of the LexA repressor from *Escherichia coli* fused to the Jun leucine zipper. This hybrid protein (LexA–JunZip) is stable in *E. coli* and confers much tighter repression in vivo than the DNA binding domain of LexA alone. DNA binding competition experiments with synthetic Jun and Fos leucine zipper peptides in vitro showed that the leucine zipper mediated dimerization of LexA–JunZip is essential for DNA binding of the fusion protein. The purified LexA–JunZip protein dimerizes in vitro with a dimerization constant of 2×10^7 M^{-1} at 5 °C. Dimerization is very sensitive to temperature, since the dimerization constant drops at 20 °C to 2×10^6 M^{-1} and at 30 °C to only 3×10^5 M^{-1}. The heptad repeat $(a,b,c,d,e,f,g)_n$ within leucine zipper proteins comprises leucine side chains in position d and generally another hydrophobic side chain in position a. This hydrophobic interface confers probably most of the dimerization free energy, but it has been suggested that the interaction between residues of opposite charge in positions e and g might define the specificity of leucine zipper assembly. To address this question, we have mutated simultaneously and randomly one g and two e codons. It turns out that the Jun leucine zipper prefers in fact hydrophobic side chains in the chosen positions and that the potential charge–charge interactions between positions e and g within the same heptad are not necessarily favorable.

An increasing number of eukaryotic transcription factors interacting specifically with DNA as dimers presents a dimerization interface termed the leucine zipper. The dimerization unit contains a repeat of leucines regularly spaced every seven residues and organized potentially in an amphipatic α-helix (Landschulz et al., 1988; Kouzarides & Ziff, 1988; Sassone-Corsi et al., 1988a; Schuermann et al., 1989; Gentz et al., 1989; Ransone et al., 1989; Neuberg et al., 1989a). Physicochemical studies of isolated zipper peptides indicate that this moiety adopts a coiled-coil structure of parallel α-helices (O'Shea et al., 1989a; Oas et al., 1990).

In the intact protein, dimerization of two leucine zipper motifs is thought to position the adjacent N-terminal basic regions of the protein to interact with a palindromic recognition site in DNA according to the "scissors grip" model (Oas et al., 1990; Vinson et al., 1989; Kouzarides & Ziff, 1989; Sellers et al., 1989; Oakley et al., 1990; Weiss, 1990).

Jun and Fos are two nuclear oncoproteins that belong to this family and regulate the transcription of genes containing AP-1 binding sites (Chiu et al., 1988; Rauscher et al., 1988a; Sassone-Corsi et al., 1988b). Although the DNA binding domains of each are highly homologous (Gentz et al., 1989; Neuberg et al., 1989a; Turner & Tijan, 1989), the two proteins possess different relative affinities for their symmetrical DNA binding site (TGACTCA). These differences are thought to be due to differences in the hetero- or homodimerization capacity of their leucine zipper domains (Kouzarides & Ziff, 1989; Neuberg et al., 1989b). Jun can form a homodimeric protein complex that binds to DNA, while Fos is unable to do so under similar experimental conditions. Furthermore, Fos–Jun heterodimers possess at least a 30-fold higher affinity for DNA than does the Jun–Jun homodimer (Gentz et al., 1989; Neuberg et al., 1989a; Turner & Tijan, 1989; Halazonetis et al., 1988; Rauscher et al., 1988b; Nakabeppu et al., 1988). Therefore, the affinity of these transcription factors for their target DNA is largely determined by the dimerization capacity of their leucine zipper domains.

We have constructed a chimeric protein between the DNA binding domain of the *Escherichia coli* LexA repressor and

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the Jun leucine zipper in order to develop a system in which the dimerization of leucine zippers may be studied in *E. coli* via the DNA binding properties of the fusion protein. A similar approach has been recently used to study the dimerization domain of the GCN4 protein fused to the DNA binding domain of phage λ repressor (Hu et al., 1990).

We show that the hybrid protein LexA–Jun leucine zipper (LexA–JunZip) is functional both in vivo and in vitro. The protein has been purified and further characterized. We show by circular dichroism measurements and by competition experiments with synthetic peptides that the protein dimerizes efficiently via its leucine zipper domain. Finally, we describe a set of dimerization LexA–JunZip mutant proteins obtained by random cassette mutagenesis. The amino acids within a heptad repeat of a coiled-coil are generaly labeled as a, b, c, d, e, f, and g, where a and d are thought to form the hydrophobic interface between the two helices. In the case of the leucine zippers the d position is nearly exclusively occupied by leucine side chains. The mutations reported in this work concern three positions in the carboxy-terminal part of the Jun leucine zipper, that is, two e and one g positions according to the coiled-coil structural model. These positions are generally occupied by charged or strongly polar amino acid side chains. Surprisingly, those mutant LexA–JunZip proteins that seem to dimerize as well or even better than the wild type protein harbor mostly hydrophobic amino acids in these positions. This result suggests that amino acids in these positions may contribute to the formation of the hydrophobic interface formed mainly by the leucine side chains (position d) and the hydrophobic side chains in position a. We show further that potential charge–charge interactions between e and g positions within the same heptad are not necessarily favorable.

Materials and Methods

Bacterial Strains and Plasmids. *E. coli* strain JL806 [relevant markers: *lexA*71::Tn5(Def) Δ(*lac*IPOZYA)169 *sulA*11 (λ*rec*Aop–*lacZ* c*I ind*⁻)/F′::Tn3 lacI^q lacZΔM15] and the plasmid pJWL70 bearing the *lexA* gene were kindly provided by John Little (Little & Hill, 1985).

Plasmid pTS301 bearing the chimeric protein was constructed as follows. The N-terminal domain of LexA (amino acids 1–87) was fused to the Jun leucine zipper (amino acids 280–312) limited by the C-terminal six amino acids of the yeast transcriptional activator GNC4. We decided to terminate the Jun leucine zipper by the sequence found in the GCN4 protein since GCN4 naturally stops with this sequence giving rise to the following sequence for the zipper part of LexA–JunZip (Struhl, 1987, 1988):

...SIARLEEKVKTLKAQNSELAST-
ANMLREQVAQLKKLVGER-COOH

The DNA coding for this peptide was chemically synthesized, annealed, ligated, and purified on a polyacrylamide gel. The N-terminal domain of LexA, obtained from *Xmn*I/*Pst*I cleavage of plasmid pJWL70 was fused to the leucine zipper fragment via a *Xho*I linker, which contributes to a serine in position 88 of the hybrid protein (the first amino acid of the sequence shown above). Note that in this construction the chimeric protein is under the control of the inducible lacUV5 promoter. The plasmids that expressed LexA–JunZip were selected in the JL806 indicator strain as white lac⁻ colonies.

Plasmid pTS101 bears the first 87 amino acids of LexA plus a C-terminal SGNN tetrapeptide that is not part of the original LexA sequence.

Plasmid pΔRV harboring the *sulA* gene was from Stewart Cole (Freudl et al., 1987).

Plasmid pTS390 is like pTS301 but harbors to a G to T mutation in the ribosome binding site of the *lexA* mRNA leading to decreased expression levels of LexA–JunZip as compared to pTS301.

Purification of the LexA–Jun Leucine Zipper Hybrid Protein. LexA–JunZip expression in strain DH5α was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG)[1] 4 h before the cells were harvested by centrifugation. The cells were suspended in 4 volumes (w/v) of buffer A (50 mM Tris-HCl, pH 7.6, 5 mM EDTA, 10 mM $MgCl_2$, 100 mM NaCl, 7 mM ME, and 4% glycerol). Phenylmethanesulfonyl fluoride and lysozyme were added to a final concentration of 160 μg/mL and 0.4 mg/mL, respectively. Cell lysis was allowed to proceed for 15 min with stirring and was completed by sonication keeping the temperature always below 7 °C. After the addition of DNaseI followed by a 15 min incubation period with stirring at 4 °C, the lysate was centrifuged for 30 min at 27000g. To the resulting supernatant was added solid ammonium sulfate to 35% saturation at 4 °C, and the precipitate was removed by centrifugation. To this supernatant was added ammonium sulfate to 70% saturation. The precipitated proteins were collected by centrifugation and dissolved in buffer B (10 mM NaH_2PO_4/Na_2HPO_4, pH 7.2, 0.1 mM EDTA, 5 mM ME, and 5% glycerol) containing 0.1 M NaCl. The solution was then applied on a Fractogel EMD SO_3^- 650 (M) Merck column previously equilibrated with the same buffer. The loaded column was extensively washed with buffer B plus 0.3 M NaCl. The bound proteins were eluted with a linear NaCl gradient from 0.3 to 0.7 M. LexA–JunZip was identified by its large A_{235}/A_{280} absorption ratio due to the absence of tryptophan and tyrosine residues within the protein and was found to elute in the last one-third of the gradient. Fractions containing the protein were pooled and properly diluted to decrease the ionic strength of the buffer to about 0.2 M NaCl. This fraction was further purified on a phosphocellulose column. The column was extensively washed with buffer B + 0.3 M NaCl, and the LexA–JunZip chimeric protein was eluted within the second one-third of a linear NaCl gradient from 0.3 to 0.7 M. The protein was concentrated by centrifugation in Centricon tubes, washed twice with a storage buffer 2× (20 mM NaH_2PO_4/Na_2HPO_4, pH 7.2, 0.2 mM EDTA, 0.2 mM DTT, and 300 mM NaCl). The purified protein was stored at −20 °C in 50% glycerol. The final protein concentration was determined from absorption measurements at 205 nm according to the method of Scopes (1974). LexA has been purified as described (Schnarr et al., 1985).

Assay of Repressor Activity in Vivo. The repressor activity of the chimeric protein and of the mutant LexA–JunZip proteins was estimated by measuring the β-galactosidase activity as described by Miller (1972) on logarithmic phase cultures of JL806 bearing wild-type or mutant LexA–JunZip coding plasmids in the absence or in the presence of IPTG. Overnight precultures were grown at the indicated IPTG concentrations, and logarithmic phase cultures were obtained by 100-fold dilution in LB containing the same IPTG concentration.

Electrophoretic Mobility Shift Assays. A 262-bp fragment containing the *sulA* SOS operator was prepared by *Bam*HI digestion of the pΔRV plasmid, ^{32}P labeling, and subsequent digestion with *Hpa*II. The 5′ labeling of the fragment and the gel retardation assay were achieved as previously described (Granger-Schnarr et al., 1988). Binding reactions were carried

[1] Abbreviations: IPTG, isopropyl β-D-thiogalactopyranoside; CD, circular dichroism; PAGE, polyacrylamide gel electrophoresis.

out at room temperature for 20 min, followed by separation on a 5% polyacrylamide gel in 1× TBE buffer, pH 7.9 (TBE: 50 mM Tris, 145 mM boric acid, and 2 mM EDTA).

The peptides used in binding competition studies were chemically synthesized and purified by HPLC. Two of them correspond to the Fos leucine zipper: peptide Fos 1 comprises amino acids 160–199 of the c-Fos protein and stops by a tyrosine residue. Peptide Fos 2 is nine residues shorter than peptide 1 (three at the N-terminal and 6 at the C-terminal end of the peptide).

Peptide Fos 1 :

Ac-ELTDTLQAETDQLEDEKSALQTEIANLLKEKEKLEFILAAY

165 172 179 186 193

Peptide Fos 2 :

DTLQAETDQLEDEKSALQTEIANLLKEKEKLE

165 172 179 186 193

Peptide Jun 1 corresponds to the Jun leucine zipper; it comprises amino acids 278–317 of the c-Jun protein and is also terminated by a tyrosine residue. Peptide Jun 2 is nine residues shorter than peptide 1.

Peptide Jun 1 :

Ac-ERIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVMNY

283 290 297 304 311

Peptide Jun 2 :

ARLEEKVKTLKAQNSELASTANMLREQVAQLK

283 290 297 304 311

All peptide concentrations were determined from absorption measurements at 205 nm according to the method of Scopes (1974).

Circular Dichroism (CD) Measurements. CD measurements were done on a Jobin-Yvon Mark III dichrograph using water-jacketed cylindrical cells of a path length of either 1 or 10 cm. The dichrograph was interfaced with an Advanced Information computer. Spectral resolution was 2 nm. Data were acquired every 0.2 nm. Melting curves were monitored on a x–y drawing table. The temperature within the cell (x-axis) was directly measured by a platinum resistance immersed into the cell. The temperature was automatically raised with use of a programmable circulating water bath and a heating rate of about 1 deg/min.

Cassette Mutagenesis. Positions e4, g4, and e5 are bordered by two unique enzymatic restriction sites, *Sac*I and *Pst*I, allowing the replacement of this segment by synthetic oligonucleotides. Therefore, plasmid pTS390, bearing the LexA–JunZip gene was linearized by a *Sac*I/*Pst*I double digestion. The large fragment was purified on a 0.7% agarose gel and used as a cloning vector for a family of synthetic oligonucleotides degenerated in the three codons corresponding to amino acids in positions e4, g4, and e5. The following oligonucleotides were used, with N being the positions where an equimolar amount of the four bases was added simultaneously during DNA synthesis:

5'-TCAGCTGNNNAAACTGGTTGGCGAACGCTAACTGCA

CGACNNNTTTGACCAACCGCTTGCGATTG-5'

5'-CGCATCCACGGCCAACATGCTGNNNGAANNNGTTGC

TCGAGCGTAGGTGCCGGTTGTACGACNNNCTTNNNCAACGAGT-5'

The two double-stranded oligonucleotides were phosphorylated and ligated. The efficiency of the ligation was found to be around 70% by PAGE. The ligated oligonucleotides were then introduced into the pTS390 *Sac*I/*Pst*I large fragment, and the ligation mixture was transfected into JL806. Tetracycline-resistant cells were grown in liquid medium overnight, and small preparations of plasmid DNA were performed. This plasmid DNA was used to retransfect JL806. Tetracycline-resistant colonies were then isolated and tested for their repressor activity (a phenotype screening could be achieved by use of MacConkey lactose indicator plates supplemented with 1 mM IPTG), and their plasmid DNA was sequenced by use of a USB Sequenase kit.

Results

The LexA–JunZip Fusion Protein Functions in Vivo as a Repressor. We constructed a chimeric protein, designated LexA–JunZip, containing the DNA binding domain and part of the hinge region of LexA (residues 1–87) fused to the leucine zipper of the c-Jun protein (residues 280–312) as described under Materials and Methods. Note that the expression of the protein is under control of the IPTG-inducible promoter lacUV5.

The ability of the hybrid protein to function in vivo as a transcriptional repressor was determined by introducing the plasmid bearing the gene for LexA–JunZip (pTS301) in the indicator strain JL806. This host strain is deficient in the chromosomal *lexA* allele and bears a *recA–lacZ* fusion, giving rise to a LexA control of the *lacZ* gene. Introduction of pTS301 in this host strain gives rise to a lac$^-$ phenotype on MacConkey lactose plates (white colonies), suggesting that the LexA–JunZip protein interacts with the *recA* operator.

Furthermore, we compared the in vivo operator binding efficiency of the chimeric protein to that of LexA or to that of its isolated DNA binding domain. This was done by measurements of the β-galactosidase activity under different levels of expression of the proteins obtained by varying the concentration of IPTG. As a negative controle we used the JL806 strain transformed with pBR322. In this case, we measured about 10 000 β-galactosidase units regardless of the IPTG concentration (Figure 1). When the isolated DNA binding domain of LexA was expressed (pTS101 in Figure 1), a significant repression of the *lacZ* gene was observed only upon overexpression of the truncated protein reaching about 2500 β-galactosidase units at 10^{-3} M IPTG. In the case of LexA, we measured 920 β-galactosidase units in the absence of IPTG and 230 in the presence of 10^{-3} M IPTG. In this assay, the LexA–JunZip protein appears far more efficient than the isolated DNA binding domain of LexA (1400 and 7800 units measured in the absence of IPTG and 230 and 2600 units measured in the presence of 10^{-3} M IPTG, respectively) and slightly less efficient than the LexA protein (1400 and 900 units, respectively, measured in the absence of IPTG and comparable values measured in the presence of 10^{-3} M IPTG).

These in vivo results suggest that the Jun leucine zipper can efficiently replace the natural dimerization domain of LexA.

Interaction of the Purified LexA–JunZip Fusion Protein with Operator DNA. The chimeric protein LexA–JunZip was purified as described under Materials and Methods and characterized for its ability to recognize an SOS operator in vitro. The protein was first compared to LexA in its binding behavior to a strong SOS operator, *sulA*. The data obtained from gel retardation asays (Figure 2) show that LexA–JunZip can form a specific complex with a DNA fragment bearing the *sulA* operator. The protein monomer concentration necessary to bind half of the operator DNA ($R_{50\%}$) has been determined by densitometry and found to be 2.4×10^{-8} M for LexA–JunZip as compared to 2×10^{-9} M for LexA. A comparison with the isolated DNA binding domain of LexA (amino acids 1–84) is hampered by the fact that this domain alone does not give rise to a well-defined specific complex in

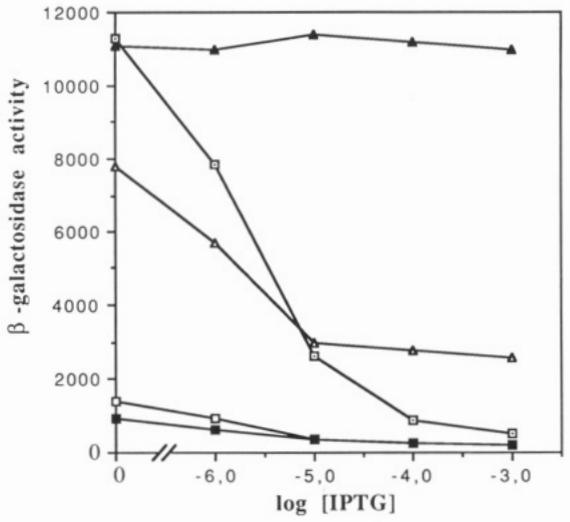

FIGURE 1: Repression of transcription of the *lacZ* gene in vivo as monitored by the β-galactosidase activity of strain JL806 containing either pTS301 (□, LexA–JunZip 1), harboring the gene for LexA–JunZip under the control of the lacUV5 promoter and the normal ribosome binding site of the *lexA* gene, or pTS390 (⊡, LexA–JunZip 2), harboring the same coding region with a less efficient ribosome binding site. As controls, JL806 has been transformed with pBR322 (▲), pJWL70 (■), harboring the entire *lexA* gene, and pTS101 (△), harboring a truncated *lexA* gene coding for the DNA binding domain of LexA only. Since LexA and the fusion protein are under the control of the lac repressor, addition of IPTG leads to increased levels of these proteins and thus to an increased repression of the β-galactosidase activity.

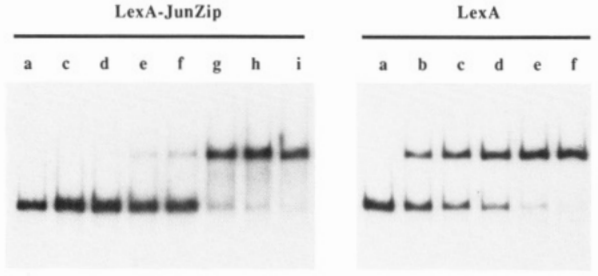

FIGURE 2: Electrophoretic mobility shift assays for the binding of purified LexA–JunZip (left panel) and LexA (right panel) to a 262-bp DNA fragment (harboring the *sulA* operator) as a function of repressor concentration. Concentrations used: no repressor (a), 10^{-9} M (b), 2×10^{-9} M (c), 4×10^{-9} M (d), 8×10^{-9} M (e), 1.6×10^{-8} M (f), 3.2×10^{-8} M (g), 6.4×10^{-8} M (h), and 1.28×10^{-7} M (i). The concentrations necessary to bind half of the DNA are about 2.4×10^{-8} M LexA–JunZip and 2×10^{-9} M LexA, respectively. Binding conditions were 20 °C, 1 × TBE, pH 7.9, 200 mM KCl, 5 mM NAH_2PO_4/Na_2HPO_4, and 50 mM NaF.

gel retardation assays (present work; Hurstel, 1989).

As compared to the entire LexA repressor, LexA–JunZip binds to the *sulA* operator with about 10-fold lower affinity at 20 °C. If similar assays are done in the cold (4 °C), the LexA–JunZip/operator complex is about as stable as the LexA/operator complex as expected from the CD measurements described below showing that low temperature favors the leucine zipper mediated dimerization.

The finding that LexA–JunZip gives rise to a well-defined complex in gel retardation assays, whereas the LexA DNA binding domain alone does not, shows that the addition of the leucine zipper dimerization domain substantially improves DNA binding, suggesting that the addition of the leucine zipper moiety to the LexA DNA binding domain allows its dimerization either in a free state or on operator DNA.

To test this assumption further, we designed competition experiments. In a first series of experiments, increasing

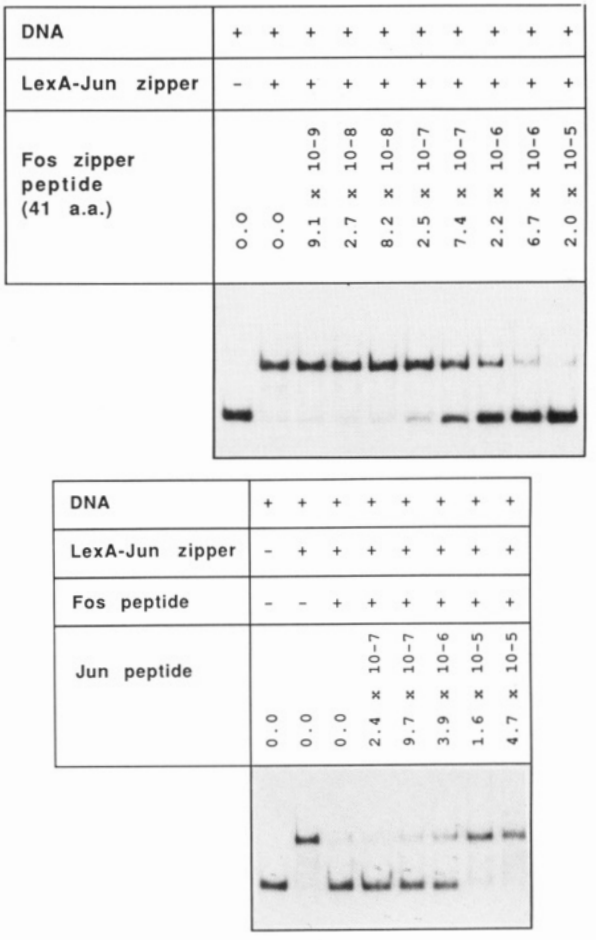

FIGURE 3: (Top) Gel showing that increasing amounts of the synthetic peptide Fos 1 inhibit the formation of the specific complex between LexA–JunZip and the *sulA* operator DNA. A concentration of about 1 μM is necessary to inhibit the binding of half of the DNA molecules to LexA–JunZip (binding conditions as in Figure 2). No competition was observed with the peptide Jun 1 for concentrations as high as 70 μM. The LexA–JunZip concentration was kept constant at 6.4×10^{-8} M. Peptide Fos 1 does not inhibit the formation of a complex between the entire LexA repressor and the *sulA* operator (data not shown). (Bottom) Gel showing that the peptide Jun 1 may however suppress the inhibitory effect of peptide Fos 1. About 5 μM Jun 1 is sufficient to revert half of the inhibitory effect of Fos 1 (the concentration of Fos 1 was kept constant at 4.5 μM, LexA–JunZip concentration 4.3×10^{-8} M; binding conditions were as in Figure 2).

amounts of peptide Fos 1 corresponding to the c-Fos leucine zipper (amino acids 160–200) were progressively added to a LexA–JunZip/operator complex. The results presented in the top panel of Figure 3 show that this peptide is able to dissociate the preformed complex. Half-dissociation is observed for a peptide concentration of about 1 μM. On the other hand, no competition was observed upon the addition of up to 70 μM synthetic peptide corresponding to the Jun leucine zipper (Jun 1) (data not shown).

In a second set of experiments (bottom panel of Figure 3), we show that the dissociation of the LexA–JunZip/operator complex by peptide Fos 1 can be reverted upon addition of the Jun leucine zipper peptide. The experiment was performed under conditions where the operator DNA is completely engaged in specific complexes (lane 2) but where the presence of 4.5 μM Fos peptide prevents its formation (lane 3). Increasing amounts of peptide Jun 1 were then added (lanes 4–8), leading to the reappearance of the specific LexA–JunZip/operator complex.

These experiments can be easily understood in terms of the specificity of the Jun–Fos dimerization. It is well-known that

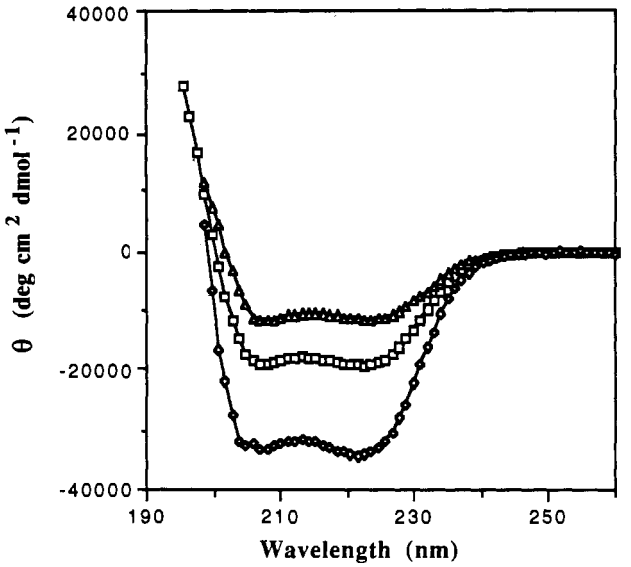

FIGURE 4: Circular dichroism spectra of LexA–JunZip (□) and $LexA_{1-84}$ (△) at 5 °C (10 mM NAH_2PO_4/Na_2HPO_4, pH 7.2, and 100 mM NaF) expressed as ellipticity per residue. The third spectrum is the ponderated difference spectrum (see text) of the two preceding spectra and corresponds to the contribution of the last 43 amino acids comprising the Jun zipper part of LexA–JunZip.

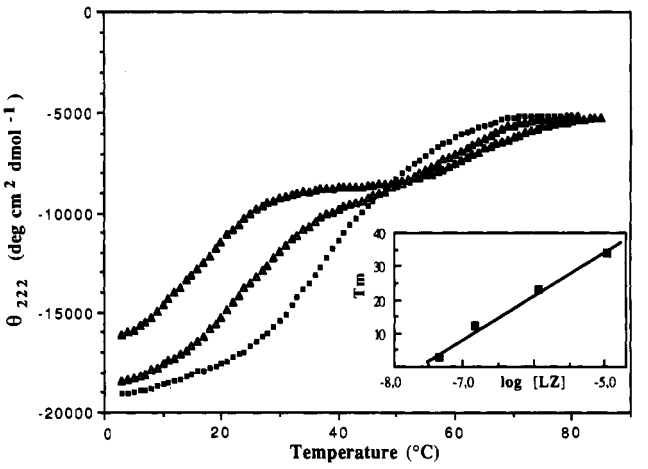

FIGURE 5: Thermal denaturation curves of LexA–JunZip at different concentrations [0.145 μM (▲), 1.14 μM (△), and 10.8 μM (■) in 10 mM NAH_2PO_4/Na_2HPO_4, pH 7.2, 100 mM NaF] using either 1- or 10-cm cylindrical water-jacketed cuvettes. The heating rate was about 1 deg/min, and about 90% of the initial CD signal was recovered upon slow cooling. The insert shows the dependence of the melting temperature of the first transition on the LexA–JunZip concentration [LZ] after correction for the second (minor) transition around 65 °C, which is essentially concentration independent in this concentration range.

Jun dimerizes far more efficiently with the Fos protein than with itself. The observation that the Fos leucine zipper peptide, but not the Jun leucine zipper peptide, interferes efficiently with the binding of LexA–JunZip to operator DNA is thus strongly in favor of a leucine zipper mediated dimeric binding mode of the chimeric protein to operator DNA. The chimeric protein is likely to be trapped by the Fos leucine zipper peptide in a heterodimeric complex (LexA–JunZip/Fos leucine zipper peptide) that either does not bind to the palindromic operator DNA or possesses a DNA binding constant too weak to be visualized by this technique. About 5 μM peptide Jun 1 is sufficient to revert half of the inhibitory effect produced by a similar concentration of peptide Fos 1 (4.5 μM). Under these conditions the Fos leucine zipper peptide is likely to be preferentially involved in a heterodimeric complex (Fos peptide–Jun peptide), allowing the specific LexA–JunZip/operator complex to be formed again. With a shorter Jun leucine zipper peptide (Jun 2) the peptide concentration necessary to revert half of the inhibitory effect produced by peptide Fos 1 is about 10-fold higher than with peptide Jun 1 (data not shown).

On the contrary to what we observed with the Fos 1 peptide, we were unable to detect any competition between the chimeric protein and a shorter peptide (Fos 2) lacking three and six amino acids at the N-terminal and the C-terminal end, respectively, as compared to Fos 1. This observation previously made by Busch and Sassone-Corsi (1990) with HeLa cell nuclear lysates prompted us to study the structural behavior of the different peptides and of the chimeric protein LexA–JunZip.

Circular Dichroism Measurements

The Leucine Zipper Domain of LexA–JunZip Is α-Helical. Figure 4 shows the circular dichroism (CD) spectra of the isolated amino-terminal domain of LexA ($LexA_{1-84}$) and the LexA–JunZip fusion protein. Both spectra show the double-minimum characteristic for proteins containing substantial amounts of residues in an α-helical configuration. At fairly high concentrations (10 μM) the ellipticity per residue is, however, much higher in the case of LexA–JunZip (θ_{222} = −19 000 deg cm^2 dmol^{-1} instead of −11 300 deg cm^2 dmol^{-1} for $LexA_{1-84}$), suggesting that the Jun leucine zipper domain 85–127 (43 amino acids) within LexA–JunZip contains high amounts of α-helical residues at this concentration. The bottom spectrum in Figure 4 represents the weighted difference spectrum of the LexA–JunZip and the $LexA_{1-84}$ spectra according to

$$\theta_{85-127} = (\theta_{1-127} - 84/127\theta_{1-84}) \times 127/43$$

This difference spectrum (θ_{222} = −34 000 deg cm^2 dmol^{-1}) should represent the ellipticity per residue of the leucine zipper part of the LexA–JunZip fusion protein. The high θ value suggests that most, if not all, of the residues 85–127 should be in an α-helical configuration under these conditions (Woody, 1985; Chen et al., 1974).

The Stability of the Leucine Zipper Domain of LexA–JunZip Is Concentration Dependent. Since the LexA amino-terminal domain and the leucine zipper domain originate from different proteins, we expect that the two domains will not significantly interact within LexA–JunZip. Thermal denaturation shows in fact two distinct transitions at low LexA–JunZip concentrations (0.145 μM) (see Figure 5): a major transition that is strongly concentration dependent varying from about 12 °C at 0.145 μM to about 34 °C at 10.8 μM and a second transition at about 65 °C that is fairly concentration independent. The melting point of $LexA_{1-84}$ is found close to this temperature, suggesting that the first transition corresponds to the melting of the leucine zipper domain and the second transition to the DNA binding domain $LexA_{1-84}$. About 90% of the initial signal is recovered upon slow cooling, suggesting that the thermal denaturation of LexA–JunZip is largely reversible.

The finding that the first transition depends on concentration (the melting temperature increases approximately linearly with log [LexA–JunZip], see insert of Figure 5) indicates that LexA–JunZip undergoes dimerization via the leucine zipper part of the protein.

The Leucine Zipper Domain of LexA–JunZip Is Unfolded in the Monomeric State. Figure 6 shows that the ellipticity per residue of LexA-JunZip depends strongly on the concentration of the protein. The θ_{222} values vary between about −10 000 deg cm^2 dmol^{-1} at low and −19 000 deg cm^2 dmol^{-1}

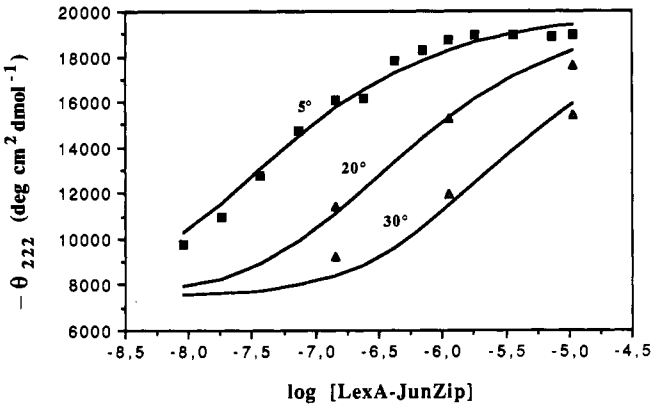

FIGURE 6: Concentration dependence of the experimental CD signal of LexA–JunZip and theoretical binding isotherms calculated from a simple monomer–dimer equilibrium. The best fits (full lines) of the experimental data were obtained by use of $\theta = -7500$ deg cm^2 dmol^{-1} for the monomer (in good agreement with the expected contribution of $LexA_{1-84}$ within the 127 amino acid long LexA–JunZip) and $\theta = -20000$ deg cm^2 dmol^{-1} for fully dimeric LexA–JunZip. The equilibrium association constants fitting the data best are 2×10^7 M^{-1} at 5 °C, 2×10^6 M^{-1} at 20 °C, and 3×10^5 M^{-1} at 30 °C. The buffer contained 10 mM NAH_2PO_4/Na_2HPO_4, pH 7.2, and 100 mM NaF, and most measurements were done in a cylindrical cuvette with a path length of 10 cm. These measurements were facilitated by the fact that LexA–JunZip does not adsorb to the cuvette walls even at very low concentrations, as judged from optical absorption measurements.

at high LexA–JunZip concentration. The experimental data may be reasonably well fitted assuming a monomer–dimer equilibrium model, for which in the monomeric state only the DNA binding domain of LexA–JunZip significantly contributes to the CD spectrum, whereas the leucine zipper is supposed to be unfolded and where only in the dimeric state the leucine zipper part of the fusion protein becomes α-helical. On the basis of this model the association constant for LexA–JunZip dimerization at 5 °C is found to be 2×10^7 M^{-1}. From the thermal denaturation curves in Figure 5 we may extract θ values at higher temperatures. Fitting of these values leads to dimerization constants of about 2×10^6 M^{-1} at 20 °C and of only 3×10^5 M^{-1} at 30 °C.

A similar titration has been done with peptide Jun 1. This peptide is able to adopt an α-helical configuration just as Fos 1. On the contrary, peptide Fos 2 has a relatively small and concentration-independent CD signal (θ = −9600 dec cm^2 dmol^{-1} between 2 and 9 μM), suggesting that this latter peptide does not readily form a homodimer. The fact that Fos 2 does not efficiently inhibit the formation of the LexA–JunZip/operator complex (see above) suggests that this peptide is also fairly deficient in Fos–Jun heterodimerization.

Mutational Study of Two e and One g Positions

It is now fairly well established that leucine zippers dimerize as parallel α-helices in a coiled-coil arrangement (O'Shea et al., 1989; Oas et al., 1990). The most highly conserved feature of this structure is the preferential occurrence of apolar residues in positions a and d within a heptad repeat as in the case of numerous fibrous proteins [for a review see Cohen and Parry (1990)]. Another feature of coiled-coil structures deduced from the study of fibrous protein is interchain ionic interactions and, more particularly, interactions between charged residues in positions e and g. According to this view a g residue within one monomer may make an electrostatic contact with the e residue within the same heptad and potentially with the more distant e position in the following heptad of another monomer. In order to address the question of which side-chain combinations in a given e,g,e triangle (Figure 7) are favorable,

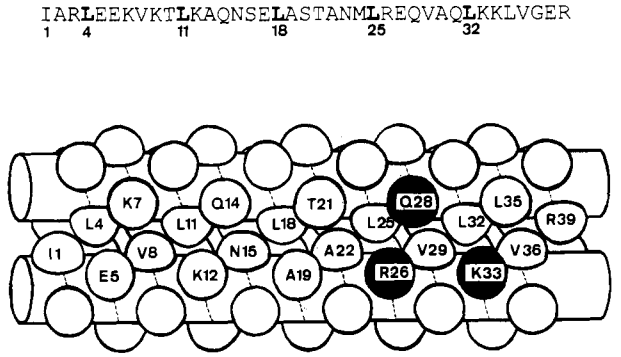

FIGURE 7: Coiled-coil model for LexA–JunZip homodimerization. The three amino acids shown in black have been subjected to simultaneous random mutagenesis.

Table I: Mutant LexA–JunZip Proteins Generated by Random Cassette Mutagenesis of the Codons Corresponding to R26, Q28, and K33 within the Wild-Type LexA–JunZip Protein Using the Numbering Scheme of Figure 7[a]

mutant protein	β-galactosidase activity	mutant protein	β-galactosidase activity
FML	330	REL	1440
ILI	340	IHL	2450
SLL	340	LSW	2920
FLI	390	LRN	3610
RQK (wt)	550	LQN	3750
TLL	580	GSM	8500
RLE	660	SGY	8640
SCF	670	VNG	9000
RQV	790	TNS	9650
CRI	1150	KSG	10500
LEL	1300		

[a] The β-galactosidase activity of each mutant protein was determined in strain JL806. Tight repression of transcription by a LexA–JunZip variant leads to a small β-galactosidase activity.

neutral, or unfavorable for dimerization of the LexA–JunZip protein, we have simultaneously replaced three such residues using a bacterial test that we previously used to study the wild-type LexA protein (Oertel-Buchheit et al., 1990).

To this purpose, three positions (e4, g4, and e5) were mutagenized by multiple random changes (see Materials and Methods). The sequence of the zipper part of the protein and a schematic representation of the dimer are shown in Figure 7. In the wild-type protein, these positions are occupied by three polar residues (R, Q, and K) harboring no opposite charges, suggesting that one could potentially obtain homodimerization up mutations since the R, Q, K combination does not allow the formation of electrostatic bonds.

As shown above the chimeric protein is nearly as active as the entire LexA protein with respect to its repressor activity toward a *recAop–lacZ* fusion (Figure 1). In order to facilitate the identification of dimerization up mutant proteins we decreased the intracellular content of the chimeric protein by a translational down mutation of the initial construction. This plasmid (pTS390) is a derivative of pTS301 bearing a G to T mutation within the Shine–Dalgarno sequence. The β-galactosidase activity that can be measured with this plasmid under different conditions of induction indicates that with this construction the amplitude of the response should facilitate the identification of dimerization up mutant proteins on MacConkey lactose indicator plates and the subsequent assay of the β-galactosidase activity. A total of 24 LexA–JunZip mutant proteins were generated and tested for their ability to function as repressors (Table I). Since we have shown that the repressor activity of LexA–JunZip depends on its dimerization capability and that only the leucine zipper moiety of

the protein has been mutated, we will discuss the results in terms of the dimerization capacity of the different mutant proteins.

Four mutant proteins bear a stop codon in either position g4 or e5 and were totally inactive, suggesting that these truncated Jun leucine zippers are either unable to form stable homodimers or are unstable within the cell or both.

Further, no repression was observed with five other mutant proteins (KSG, TNS, VNG, SGY, and GSM). Four of them contain a change to a glycine residue and are likely to be structural mutant proteins since glycine is known as a helix breaker.

Ten mutant proteins were found partially active, seven of them being strongly affected (LQN, LRN, LSW, IHL, REL, LEL, and CRI) and three reproducibly slightly affected (RQV, SCF, and RLE). Five mutant proteins were fully active (TLL, FLI, SLL, ILI, and FML), four of them being even better repressors than the wild-type protein. The analysis of these functional mutations reveals an unexpected result, in that the three polar residues found in the wild-type protein can apparently be replaced by hydrophobic residues leading to an enhanced dimerization capacity of the leucine zipper. Furthermore, the occurrence of residues of opposite charge in positions e and g (as in the mutant protein REL) does not necessarily enhance the dimerization capacity of the leucine zipper since a potential electrostatic interaction between residues located in e4 and g4 does not contribute significant additional energy (REL is not a better repressor than LEL).

Discussion

The chimeric protein LexA–JunZip appears as an efficient tool to study the dimerization specificity of the Jun protein. Replacement of the genuine dimerization domain of LexA by the Jun leucine zipper does not interfere with the correct docking of the LexA DNA binding domains to the DNA double helix despite the fact that so far no efforts have been undertaken to optimize the length of the spacer between the DNA binding domain and the zipper part of the hybrid protein. The native leucine zipper DNA binding proteins require on the contrary a strict phasing between the DNA binding and the dimerization domain (Agre et al., 1989; Vinson et al., 1989; Schuermann et al., 1989).

LexA–JunZip seems also to be more versatile in its DNA recognition properties as compared to LexA in that the fusion protein recognizes SOS consensus operators with one or two additional base pairs in the center, while LexA recognizes these operators only weakly (P. Oertel-Buchheit, M. Granger-Schnarr, and M. Schnarr, unpublished results).

Three lines of evidence indicate that dimerization of the LexA–JunZip protein occurs via its leucine zipper moiety: (1) In vivo experiments show that the chimeric protein is far more efficient in controlling the expression of a reporter gene than the DNA binding domain alone. (2) In vitro experiments with the purified protein indicate that the presence of the leucine zipper moiety strengthens the DNA binding capacity of the amino-terminal domain of LexA. Additionally, we observed in competition experiments that a Fos leucine zipper peptide prevents the protein to bind to DNA and that this phenomenon may be reversed by the addition of a similar amount of a Jun leucine zipper peptide. (3) Circular dichroism measurements showed that both the stability and the ellipticity per residue of the LexA–JunZip protein strongly depend upon its concentration, indicating that the protein oligomerizes and that the leucine zipper part of the protein is unfolded in the monomeric state. Assuming a simple monomer–dimer equilibrium, we determined dimerization constants of 2×10^7 M^{-1} at 5 °C, 2×10^6 M^{-1} at 20 °C, and only 3×10^5 M^{-1} at 30 °C.

The competition experiments showed that while a Fos synthetic peptide spanning residues 160–200 can efficiently compete with the dimerization of the chimeric protein, a shorter peptide (Fos 2) corresponding to residues 163–194 cannot, in agreement with a similar observation of Busch and Sassone-Corsi (1990) using HeLa cell nuclear lysates. Kouzarides and Ziff (1989) had previously shown that all the information necessary for Fos to heterodimerize with Jun is located within residues 163–200 and that ending the Fos leucine zipper domain immediately after the last leucine (residue 193) prevents an efficient heterodimerization. An explanation could have been that the charged residue in position 194 (glutamic acid in position e5) stabilizes the heterodimer by ionic interactions. Given that Fos 2 contains this amino acid, it seems that in fact some of the residues between positions 195 and 200 seem to be important for the stabilization of the heterodimer. Recently, Schuermann et al. (1991) have shown in fact that the mutation of His-200 to alanine within the Fos zipper prevents Fos–Jun heterodimerization at least if a second mutation is present (fourth leucine changed to alanine). However, the absence of the last six amino acids does not prevent completely heterodimerization to occur since a short Jun leucine zipper peptide (Jun 2 residues 281–312), which lacks the corresponding six amino acids, can apparently heterodimerize with a full-length Fos peptide albeit with about 10-fold smaller affinity as judged from DNA binding competition experiments.

So far the specificity of homo- and heterodimerization of the different leucine zipper transcription factors is only poorly understood. Since leucine side chains in position d are a common feature of these factors, the specificity of interaction should arise from the other residues within the dimerization interface, that is, those found in positions a, e, and g, which are found (just as d) on one half-side of an idealized α-helical cylinder with 3.5 amino acid residues per helical turn (Figure 7). A comparison of the most frequently occurring amino acids in positions e and g within leucine zipper proteins reveals that they are preferentially occupied by charged or strongly polar residues. This observation suggests that ionic interactions could play a role in dimer association. As seen from Figure 7, a g residue may form intermolecular electrostatic bonds preferentially with two e residues, one in the same heptad, the other in the following heptad. To address the question of which amino acid side chain combinations in such an e,g,e triangle (in the case of a homodimer the same geometry is found on the opposite side of the two α-helical cylinders) are favorable, neutral, or unfavorable for dimerization, we have randomly and simultaneously mutagenized the three codons corresponding to R26, Q28, and K33. We expected that opposite charges for example in position 26 and 28 should enhance the strength of dimerization and thus DNA binding of LexA–JunZip. However, an Arg-Glu combination in these positions is apparently not more favorable than Leu-Glu, a combination that allows obviously no charge–charge interactions, suggesting that potential charge–charge interactions between positions e and g within the same heptad are not necessarily favorable.

Those LexA–JunZip proteins that appear to be improved in dimerization and DNA binding contain surprisingly mostly hydrophobic side chains in the three mutated positions, whereas the wild-type protein contains Arg, Gln, and Lys. In fact, as seen from Figure 7, the nearest neighbors for these residues within the opposite helix are likely to be hydrophobic side chains in the positions a and d (L25 for R26, V29 for Q28,

and L32 for K33, according to the numbering scheme in Figure 7). It is thus conceivable that the introduction of hydrophobic side chains in these e and g positions may contribute to the hydrophobic interface between the two α-helices. This assumption is not necessarily in conflict with the wild-type configuration, since two of the three polar wild-type residues (Arg and Lys) contain long aliphatic side chains, which may potentially contribute to the hydrophobic interface. This seems to be the case for the antiparallel leucine zipper within *E. coli* seryl-tRNA synthetase (Cusack et al., 1990) that contains several Arg side chains in the a and d positions of one of the interacting helices. However, if hydrophobic side chains in the e and g positions may be favorable for the formation of coiled-coil dimers, one may wonder why the naturally occurring coiled-coils contain mostly polar side chains in this position. One reason for this may be that a protein that would contain mostly hydrophobic side chains in positions a, d, e, and g would hardly be soluble in water.

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Registry No. Ac-ELTDTLQAETDQLEDEKSALQTEIANLLKEKEKLEFILAAY, 135339-21-8; DTLQAETDQLEDEKSALQTEIANLLKEKEKLE, 135339-25-2; Ac-ERIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVMNY, 135339-22-9; ARLEEKVKTLKAQNSELASTANMLREQVAQLK, 135339-24-1; SIARLEEKVKTLKAQNSELASTANMLREQVAQLKKLVGER, 135339-23-0.

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