Can the Monomer of the Leucine Zipper Proteins Recognize the Dimer Binding Site without Dimerization?

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Received February 27, 1995. Revised Manuscript Received October 12, 1995[⊗]

Abstract: It is generally believed that leucine zipper regulatory proteins for DNA transcription recognize their DNA binding sites as *dimers* preformed in solution (and that the monomers do not bind specifically to these sites). To test this idea, we synthesized the 31-residue peptide v-Jun-br, which contains *only* the DNA binding region of the v-Jun *monomer*. Footprinting assays show that v-Jun-br *monomers specifically protect the DNA binding site of v-Jun in almost identically the same way as dimers*. Thus, (i) the monomer recognizes the half-site of the dimer binding site and (ii) dimerization does not appreciably affect the bound conformation of each monomer. These results may have implications in the regulation of transcription by such proteins. Thus, two monomers of v-Jun might bind sequentially to the dimer binding site followed by dimerization of v-Jun while bound. This may allow binding at concentrations too low for dimerization in solution.

1. Introduction

The molecular mechanism by which cells adapt their phenotype in response to external stimuli is of great interest in modern biology. A crucial role in modulating gene expression is likely played by the products of proto-oncogenes, a number of which reside in the nucleus. Properties commonly exhibited by such nuclear oncogenes include (a) rapid (often transient) induction in respone to numerous agents, (b) messenger RNA with a short half-life, and (c) a short half-life for the proteins encoded by the nuclear oncogene.[1] Fos and Jun (both members of the leucine zipper protein family) have been observed as the products of immediate−early induced genes in response to external stimuli.[2−4]

Leucine zipper proteins bind to DNA as a dimer, and it is believed that the dimerization of leucine zipper protein is a prerequisite to specifically recognizing the binding sites.[5,6] However, the short lifetime of such nuclear oncogenes raises questions as to whether the concentrations are suitable for dimerization in solution.

We report herein evidence that the leucine zipper basic region of v-Jun can bind as monomers to the dimer binding site. We suggest that this may be the dominant process at low concentrations. Section 2 summarizes previous experiments and conclusions concerning the binding mechanism. Section 3 discusses details for the experiments reported herein, while section 4 reports the results. Section 5 covers kinetics issues relating to the mechanisms of binding, and section 6 contains further discussion.

2. DNA Binding Mechanism of Leucine Zipper Proteins

Leucine zipper proteins have about 60 residues with the C-terminus containing a leucine zipper region (4 or 5 leucines occurring every 7 residues) responsible for dimerization and the N-terminus containing a basic region (about 30 residues) responsible for DNA binding.[7,8] The leucine zipper proteins dimerize by using the leucine zipper region to form a coiled-coil structure for the dimer.[8,9] Most mutant leucine zipper proteins unable to carry out dimer formation fail to recognize the binding site.[10−12] Many leucine zipper proteins which have mutations on the basic region also fail to bind to the specific DNA site even though the mutants can form heterodimers with other wild-type leucine zipper monomers.[5,6] Therefore, it is believed that the dimerization of leucine zipper protein is a prerequisite to specific recognition of the binding sites. This idea is supported by the observation that the oxidized dimer of the GCN4 basic region specifically recognizes the GCN4 dimer binding site, but the monomer does not.[13,14]

While carrying out a project aimed at designing new long DNA binding proteins,[15−18] we observed that the monomer of

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⊗ Abstract published in *Advance ACS Abstracts,* April 1, 1996.

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S0002-7863(95)00653-6 CCC: $12.00

a Peptides

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v-Jun-br:       S  QERIKAERKR  MRNRIAASKS  RKRKLERIAR
V-Jun-N : CGG   S  QERIKAERKR  MRNRIAASKS  RKRKLERIAR
v-Jun-C :       S  QERIKAERKR  MRNRIAASKS  RKRKLERIAR  GGC
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b Oligonucleotides

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o-CC: 5'-ctcagatccggatcctaggttaaacgATGAcgTCATcggtataggtcgagaattcggatcct-3'
      3'-gagtctaggcctaggatccaatttgcTACTgcAGTAgccatatccagctcttaagcctagga-5'

o-NN: 5'-ctcagatccggatcctaggttaaacgTCATcgATGAcggtataggtcgagaattcggatcct-3'
      3'-gagtctaggcctaggatccaatttgcAGTAgcTACGgccatatccagctcttaagcctagga-5'
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Figure 1. Sequences of the protein (a) and oligonucleotides (b) used in the gel retardation and footprinting studies. The total length of each oligonucleotide is 62. Peptide v-Jun-br contains the basic region of v-Jun (amino acids 214−244).[30] Peptides v-Jun-br and v-Jun-C were prepared as described previously.[15−17] Peptide v-Jun-br was chemically synthesized and purified, and the purity was checked by mass spectroscopy at the Biopolymer Synthesis Center at the California Institute of Technology:[15,17] calculated, 3822.3; experimental, 3824.6.

the basic region of v-Jun binds selectively to the dimer binding site. These results, reported herein, suggest that under appropriate conditions (low concentrations) the dimerization of v-Jun proteins might occur by (i) first binding one monomer to the DNA binding site and then (ii) binding of the second monomer, followed by (iii) coupling of the leucine zippers of the bound monomers to form the bound dimer. If so, this mechanism might be particularly relevant for binding of short-lived DNA binding proteins.

Leucine zipper proteins dimerize via the leucine zipper regions, leading to a Y-shaped dimer where each arm is basic and recognizes half of the dimer DNA binding site. The basic region has no fixed conformation in solution, but changes into an α-helix when bound to the specific site.[19−22] This model has been confirmed by a recent X-ray crystal structure for the complex of DNA with GCN4 (another leucine zipper protein) homodimer[8] and for the complex of DNA with Jun/Fos heterodimer.[23] The X-ray studies show that the DNA binding site and the α-helix of the basic region of these leucine zipper proteins are both linear. However, depending on the nature of the binding site, other systems may bend.[24] In the gel electrophoresis using Jun heterodimer, a bent α-helix was proposed for the basic region of Jun to explain the DNA bending induced by the binding of Jun.[25]

Experiments using only the basic region of GCN4[13] or v-Jun[15−17] (without the leucine zipper region), but dimerized at the carboxy termini (denoted as pCC) by an added linker, showed that the basic region alone will recognize the dimer binding site (denoted o-CC). In addition, dimerization at the amino termini to form a rearranged protein (denoted pNN) leads to recognition of the rearranged oNN binding site.[15−17] These studies suggested that the α-helices are bent when bound to DNA.[15−17]

It is widely believed that protein dimerization is essential for leucine zipper proteins to effect specific DNA recognition. Evidence in favor of this view are the following observations: (i) Most mutations that prevent dimerization also prevent DNA binding.[10−15] (ii) A normal Jun and a mutant Fos on its basic region cannot recognize specific DNA sites even though they can make a heterodimer together.[5,6] (iii) GCN4 makes a stable dimer in the absence of the specific DNA binding site.[26] (iv) The oxidized dimer of the GCN4 basis region specifically recognizes the dimer binding site, but the reduced monomer does not.[13]

On the other hand, consider the following: (v) NMR experiments show that, in the absence of the specific DNA binding site, the lifetime of the GCN4 homodimer is between 10 ms and 1 s.[22] This shows that, in the absence of specific DNA, the GCN4 dimer is not stable in solution. (vi) Competition experiments show that peptides containing only the basic region of Jun, Fos, and CREB retain their promoter selectivity.[6,27] (vii) LexA binds to DNA as a dimer, but the monomer of LexA also recognizes the half-site of the full dimer binding site.[28] (viii) Skn-1 which contains a basic region similar to those of leucine zipper proteins, but lacks a leucine zipper dimerization region, binds to specific DNA sequences as a monomer.[29]

3. Materials and Experiments

3.1. Peptides and Oligonucleotide Synthesis. In order to obtain a direct test of whether predimerization is essential for the binding of leucine zipper protein, we synthesized a peptide, v-Jun-br (Figure 1a), containing only the basic region of v-Jun monomer and carried out footprinting assays for oligonucleotides containing the dimer binding site.

Peptide monomers v-Jun-br, v-Jun-N, and v-Jun-C were chemically synthesized and purified as described previously[15,17] (see the caption for Figure 1). The automated stepwise syntheses were done on an Applied Biosystems Model 430A peptide synthesizer with an optimized synthetic protocol for the *N*-*tert*-butoxycarbonyl (*t*-Boc) chemistry. The peptides were purified by reversed-phase high-performance liquid chromatography (HPLC) on a Vydac C18 column. A linear gradient of 0−50% aqueous/acetonitrile/0.1% trifloroacetic acid was run over 120 min.

The procedure to synthesize homodimer pCC (and pNN) is done in oxidizing conditions (5 mM oxidized dithiothretiol). v-Jun-C (or v-Jun-N) dimerizes to form pCC (or pNN) which was purified by HPLC.

The oligonucleotides o-CC and o-NN (Figure 1b) were synthesized using the facilities at the Biopolymer Synthesis Center at Caltech and purified as described.[16,17] o-CC has the binding site (ATGAcgTCAT) of the v-Jun dimer while o-NN has a rearranged half-site (TCATcgATGA; see Figure 1b). The synthesized oligonucleotides were purified using 10% denaturing polyacrylamide gel, and duplexes were made between complementary oligonucleotides.

3.2. Footprinting Assays. The footprinting assay solution (in 50 μL) contained bovine serum albumin at 100 mg/mL, 5% glycerol, 20

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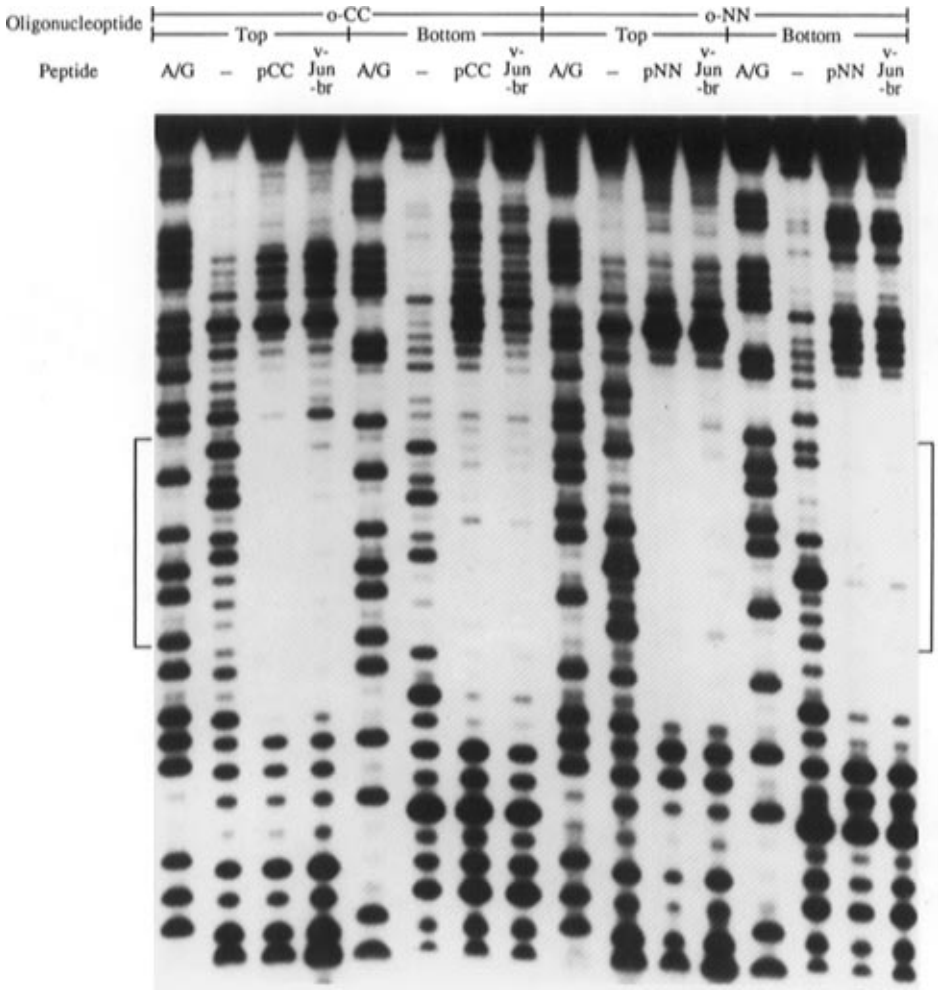

Figure 2. DNase I footprinting assays of v-Jun-br with oligonucleotides oCC and oNN. In order to compare the results of protection between monomer and dimer, DNase I footprinting assays of pCC and pNN were also carried out together with oCC and oNN, respectively. The brackets show the expected dimer binding sites (see Figure 1b). Peptide concentrations were determined as described previously.[15,17] A 50 000 cpm sample of each 5′-^{32}P-labeled probe DNA, bovine serum albumin (BSA) at 0.1%, poly(dI·dC) at 2 μg/mL, and 600 nM of pCC (or pNN) or 3 μM v-Jun-br (where indicated) were used in 50 μL of footprinting reaction solution as described previously.[15,17]

mM Tris-HCl (pH 7.5), 4 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, poly(dI·dC) at 2 μg/mL, 50 000 cpm of each 5′-^{32}P-labeled probe DNA (about 20 fmol), and 0.6 μM pCC (or pNN) or 3.0 μM v-Jun-br where indicated. This solution was stored at 4 °C for 1 h. After adding 5 μL of DNase I diluted in 1× footprinting assay buffer, the solutions were stored for 1 min more at 4 °C. The DNase I digestion was stopped by addition of 100 μL of DNase I stop solution containing 15 mM EDTA (pH 8.0), 100 mM NaCl, 25 μg/mL sonicated salmon sperm DNA, and 25 μg/mL yeast tRNA. This was phenol/chloroform extracted, ethanol precipitated, and washed with 70% ethanol. The pallet was resuspended in 5 μL of formamide loading buffer, denatured at 90 °C for 4 min, and analyzed on 10% denaturing polyacrylamide sequencing gel (50% urea).

4. Results

The footprinting assays (Figure 2) show that the monomer v-Jun-br protects identically the same site as the dimer pCC (and pNN). (a) Columns 3 and 7 show that, for o-CC (top and bottom), the dimer pCC leads to recognition of the pCC binding site (marked with brackets). (b) Columns 4 and 8 show that monomer v-Jun-br also protects the complete pCC dimer binding site. (c) Columns 11 and 15 show that, for o-NN (top and bottom), the dimer pNN leads to recognition of the pNN binding site. (d) Columns 12 and 16 show that the monomer v-Jun-br also protects the complete pNN dimer binding site.

Because v-Jun-br contains only the basic region, there is no possibility of dimerization. Since the C-termini become positioned near each other when two monomers bind to the pCC binding site while the N-termini of both monomers are positioned near each other when two monomers bind to the pNN binding site, the similarity in the results between monomers and dimers shows that there are no specific interactions between the two monomers when bound to the site.

These results also indicate that the added linkers (Gly-Gly-Cys or Cys-Gly-Gly) when oxidized to form the dimer do not appreciably change the bound conformations of the monomers on the binding site of pCC (and pNN). Thus, each monomer retains the same contacts with DNA on both sites.[16,17]

These results also suggest that oxidization and covalent bonding of the thiol groups of the linkers to make the pCC and pNN dimers do not cause sufficient tension to change the contacts between the monomer and DNA.

5. Comparison between Dimer Formation in Solution and Dimer Formation on DNA

Figure 3 shows the relevant steps for two processes of forming bound DNA dimer: (a) Figure 3a considers that the dimer forms in solution, leading to an equilibrium constant of

$$K_D = k_{fMM}/k_{rMM} = [D]/([M_1][M_2]) \quad (1)$$

and the dimer binds to DNA, leading to an equilibrium constant of

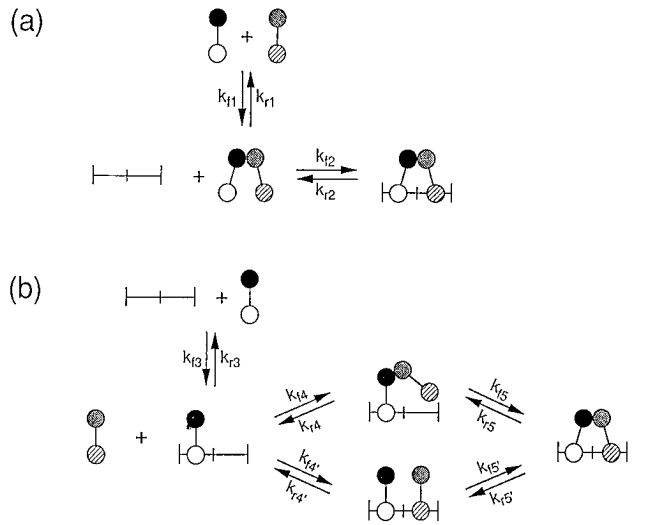

Figure 3. Two pathways for DNA binding of protein dimers: (a) dimer-only binding to the DNA binding site and (b) sequencial binding of two monomers to the DNA binding site. The darker (black and checked) circles represent dimerization regions and the brighter (white and striped) circles represent DNA binding regions (modeled after Figure 1 of Kim et al.[28]). k_f indicates the forward rate constant, and k_r indicates the reverse rate constant.

$$K_{DS} = k_{fDS}/k_{rDS} = [DS]/([D][S]) \tag{2}$$

(b) Figure 3b considers that the monomer binds to the dimer binding site, leading to an equilibrium constant of

$$K_{MS} = k_{fMS}/k_{rMS} = [M_1S]/([M_1][S]) \tag{3}$$

which is followed by binding of the second monomer. This second step may occur by two pathways: (b1) dimerization of the leucine zipper of the free monomer to bound monomer, followed by binding of the second basic region to DNA, and (b2) binding of the second monomer to the dimer binding site, followed by dimerization of the leucine zipper regions.

In order to compare the two pathways a and b, consider the following kinetic scheme where M_i denotes the monomer, D denotes the dimer, and S denotes the DNA dimer binding site (the brackets indicate concentration). For pathway a we have

$$d[D]/dt = k_{fMM}[M_1][M_2] \tag{4}$$

$$d[D-S]_a/dt = k_{fDS}[S][D] \tag{5}$$

$$= K_D k_{fDS}[S][M_1][M_2] \tag{6}$$

where eq 1 was used. For pathway b1, we consider

$$d[M-S]/dt = k_{fMS}[S][M_1] \tag{7}$$

$$d[D-S]_b/dt = k_{fM-MS}[M_1S][M_2] \tag{8}$$

$$= K_{MS} k_{fM-MS}[S][M_1][M_2] \tag{9}$$

In each case the forward rate constant is much greater that the backward rate constant. For example,

$$K_D = k_{fMM}/k_{rMM} \approx 5 \times 10^4\ M^{-1} \tag{10}$$

based on the results of NMR experiments for GCN4.[22]

Thus, for conditions in which the concentration of a product is not too high compared to the concentration of the reactants, the backward reactions can be ignored in deriving equilibrium equations.

Equations 4−9 lead to the following relative rate constants:

$$\frac{(d[D]/dt)}{(d[D-S]_a/dt)} = \frac{k_{fMM}}{K_D k_{fDS}[S]} \tag{11a}$$

$$\approx 1/(K_D[S]) \tag{11b}$$

$$\frac{(d[D-S]_a/dt)}{(d[M-S]/dt)} = \frac{K_D k_{fDS}[M_2]}{k_{fMS}} \tag{12a}$$

$$\approx K_D[M_2] \tag{12b}$$

$$\frac{(d[D-S]_a/dt)}{(d[D-S]_b/dt)} = \frac{K_D k_{fDS}}{K_{MS} k_{fM-MS}} \tag{13a}$$

$$\approx K_D/K_{MS} \tag{13b}$$

$$\frac{(d[M-S]/dt)}{(d[D-S]_b/dt)} = \frac{k_{fMS}}{K_{MS} k_{fM-MS}[M_2]} \tag{14a}$$

$$\approx 1/(K_{MS}[M_2]) \tag{14b}$$

where eqs 11b, 12b, 13b, and 14b assume that the forward rate constants are similar (binding a monomer or a dimer to the DNA binding site).

If it is assumed that the dimerization rate constant of the monomers (k_{fMM}) is fast enough to provide dimers whenever they are needed, the binding of a dimer to the DNA binding site will be the rate-determining step in pathway a. From eq 14b, the rate-determining step for path b depends on the product of the concentration of monomer M_2 and the equilibrium constant of monomer binding to the DNA binding site. Thus eqs 7 and 9 becomes equal when $[M_2] = 1/K_{MS}$. From eq 13b, the relative rate constant for forming a complex between the dimer and the dimer binding site for path a to that for path b is equal to K_D/K_{MS}.

These equations allow an estimate to be made for the time to form the DNA bound dimer. At low concentration of monomers M_1 and M_2 ($<10^{-7}$ M), the DNA binding reaction for path a depends on the dimer binding reaction, while for path b the monomer binding to the monomer bound DNA binding site is the rate-determining step (assuming $K_{MS} \approx 10^6\ M^{-1}$ from ref 28).

At high concentration of monomers ($>10^{-5}$ M) path a (which involves formation of a dimer complex followed by binding of the complex to the dimer binding site) becomes faster than path b (from eq 12) because of the high population of protein dimers in solution. However, for a low concentration of monomers, the monomer binding mechanism (path b) leads to a net rate increase of 10−100 times [depending on the ratio of K_D and K_{MS} (see Figure 3b)] for forming a complex of two monomers at the DNA binding site compared to the dimer-only binding mechanism (path a). (In the case of LexA, a rate increase of about 75 times is proposed under their experimental conditions.[28]) Because the rate constant of binding the dimer complex to the dimer binding site depends on the concentration of both monomers (as in eqs 2 and 4), reaction through path b leads to a larger rate for complex formation when the concentration of

either monomer is very low (as in the case of Jun and Fos where heterodimers are made between them).

6. Discussion

It has been believed that leucine zipper proteins recognize their DNA binding sites as dimers which are preformed in solution and that monomers do not bind selectively to the DNA binding sites.[13,26] However, our current results (Figure 2) show that the monomer of the v-Jun basic region (v-Jun-br) specifically binds to both halves of both dimer binding sites o-CC and o-NN. Because v-Jun-br has no functional motif to become a dimer and because it recognizes both the o-CC and o-NN binding sites, we conclude that v-Jun-br recognizes the half-site of the dimer binding site as a monomer even though it has much weaker binding affinity to specific DNA sites compared to a dimer. These results are consistent with competition experiments which show that peptides including only the basic region of Jun, Fos, and CREB compete with the intrinsic Jun/Fos and CREB in DNA binding.[27]

These results contrast with the situation for GCN4 where only the dimer binds. This difference could be because v-Jun binds to DNA in a conformation different from that of GCN4.

Indeed residues on the carboxy terminus of the basic region of various leucine zipper proteins differ greatly from each other while the residues of the rest of the basic region are highly conserved.[7,30] Thus, mutations on the terminal residues of Fos substantially reduced the DNA binding affinity.[31] In contrast, the terminal residues of GCN4 do not show any direct involvement in DNA binding.[8,23] Therefore, the terminal residues may be responsible for the difference in behavior among leucine zipper proteins (as proposed by refs 18 and 32).

Experiment shows that the basic region of Jun competes with the Jun/Fos heterodimer in DNA binding.[6] This suggests that the Jun basic region recognizes the specific DNA site. Experimental results on the heterodimer formed between a wild-type Jun and a mutant Fos might seem inconsistent. This mutant Fos lacks the ability to bind to specific DNA sites but is still able to form a heterodimer with a Jun monomer that cannot recognize the specific DNA site.[5,6] This apparent discripancy can be rationalized because the much weaker DNA binding affinity of a monomer as compared to a dimer might prevent detection of the monomer during gel retardation asseys at the concentrations used.

Our results[17] are consistent with a recent study[28] on the DNA binding protein LexA, which as a dimer recognizes a site having dyad symmetry. Kim et al.[29] showed that the standard dimer binding mechanism does not explain the fast binding rates of DNA binding proteins when equilibrium constants of dimerization of monomers are too low to provide appropriate concentrations of dimers in solution. Kim et al. proposed the mechanism in Figure 3b for the binding of LexA proteins to their DNA binding sites. In this proposed DNA binding mechanism, a monomer first binds to the binding site and dimerization with a second LexA occurs on the DNA binding site. The dissociation constant of LexA is similar to that of leucine zipper protein for both complex formation[28,33] between protein and DNA and protein dimerization.[22,34] Our results[17] are also consistent with experimental results[29] which show that the Skn-1 basic region binds to DNA as a monomer. The basic region of Skn-1 shows greater homology with Jun than ours to GCN4.

7. Summary

For both pCC and pNN binding sites, the monomer and dimer of v-Jun-br both lead to complete protection of the binding site with the same length of protected region. This suggests that v-Jun might dimerize on the binding site, removing the prerequisite of dimerization before binding. This could have profound implications in the regulatory mechanisms involving leucine zipper proteins. For example, it could allow binding at concentrations too low for dimerization in solution.

Acknowledgment. This research was supported by a grant from the Biological and Chemical Technology Research (BCTR) Program (David Boron) of the Department of Energy. The facilities of the Materials and Molecular Simulation Center (MSC) are also supported by grants from the National Science Foundation (CHE 94-13930 and ASC 92-17368), Asahi Chemical, Asahi Glass, Chevron Petroleum Technology Co., BF Goodrich, BP Chemical, Vestar, Hughes Research Laboratories, Xerox, and Beckman Institute.

JA950653T

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