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Addition of Positively Charged Tripeptide to the N-Terminus of Fos bZIP Domain; Implications on DNA Bending, Affinity and Specificity

by

Tokameh Mahmoudi

A thesis submitted in conformity with the requirements for the degree of Master’s of Science Graduate Department of Biochemistry University of Toronto

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Abstract

GKH-Fos/Jun is a hybrid protein designed to contain a metal binding motif in the form of a GKH tripeptide at the N-terminus of Fos bZIP domain dimerized with the Jun bZIP domain. We examined the effect of the addition of positively charged GKH motif to Fos on the DNA binding characteristics of the Fos/Jun heterodimer. Comparison of the apparent dissociation constants of the unmodified control Fos/Jun (cFos/Jun) and GKH-Fos/Jun heterodimers reveals that GKH-Fos/Jun binds the AP-1 site with a 6 fold decreased affinity than does cFos/Jun. In addition, GKH-Fos/Jun exhibits a 4 fold increased affinity for nonspecific DNA in comparison to cFos/Jun. Furthermore, helical phasing analysis indicates that GKH-Fos/Jun and cFos/Jun both bend the DNA at the AP-1 site. However, the degree of the induced bend by GKH-Fos/Jun is greater than that induced by cFos/Jun. Our results suggest that the unfavorable energetic cost of increased DNA bending by GKH-Fos/Jun results in a decrease in specificity and affinity of binding of the heterodimer to the AP-1 site.
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<tr>
<td>A</td>
<td>adenosine</td>
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<td>AP-1</td>
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<td>ATCUN</td>
<td>amino terminus copper(II) and nickel(II) binding domain</td>
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<td>ATF</td>
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<td>bp</td>
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<td>basic region leucine zipper</td>
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<td>bovine serum albumin</td>
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<tr>
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<td>dTTP</td>
<td>2'- deoxythymidine - 5'-triphasate</td>
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<td>DNA</td>
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<td>DNaseI</td>
<td>deoxyribonuclease I</td>
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<tr>
<td>DR-2</td>
<td>direct repeat - two base pair spacer</td>
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<td>DTT</td>
<td>dithiothreitol</td>
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<td>E. coli</td>
<td><em>Escherichia coli</em></td>
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<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
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<td>ESR</td>
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<td>Fra</td>
<td>Fos related antigen</td>
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<td>Abbreviation</td>
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<tr>
<td>G</td>
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<td>NH₂-glycine-lysine-histidine-Fos</td>
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<td>IPTG</td>
<td>isopropyl-1-thio-β-D-galactoside</td>
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<tr>
<td>kD</td>
<td>kilo Dalton</td>
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<td>JNK</td>
<td>Jun amino-terminal kinase</td>
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<tr>
<td>JunD</td>
<td>Drosophila Jun</td>
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<tr>
<td>KOAc</td>
<td>potassium acetate</td>
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<tr>
<td>LB</td>
<td>Luria-Bertani media</td>
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<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<td>polyacrylamide gel electrophoresis</td>
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<td>PKA</td>
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<td>PMSF</td>
<td>α-toluene-sulfonyl-flouride</td>
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<tr>
<td>poly(dI.dC)</td>
<td>polydeoxyinosinicdeoxyctidylic acid</td>
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<td>RNA</td>
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<td>S</td>
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<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<td>T</td>
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<tr>
<td>TBE</td>
<td>tris borate EDTA</td>
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<tr>
<td>v-jun</td>
<td>viral jun (gene)</td>
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Chapter One

Introduction
1.1 Design of GKH-Fos/Jun

The design of sequence specific DNA cleaving proteins is a novel area of study, accomplished by the generation of hybrid proteins consisting of a sequence specific DNA binding protein attached to a DNA cleavage moiety. The selective binding and cleavage of DNA by affinity cleaving hybrid proteins can be used to examine the site and orientation of protein-DNA interactions. As well, further advance in this field may lead to the targeting of affinity cleaving proteins to cut specific sequences on genomic DNA and perhaps their use in cancer therapy.

The covalent attachment of an Fe-EDTA moiety to a sequence specific DNA binding protein has been used extensively to generate DNA cleaving proteins (Sluka et al., 1990; Oakley & Dervan, 1990; Dumoulin et al., 1996; Erlanson et al., 1996). Chemical synthesis is required to create this class of affinity cleaving proteins through the covalent linking of EDTA to a specific amino acid on the DNA binding protein of interest. One structural domain that has also been used in the design of DNA cleaving proteins is the Amino Terminus Cu(II) and Ni(II) binding (ATCUN) motif, which consists of naturally occurring amino acids. Ligation of metal by this motif requires an amino terminal nitrogen, two peptide nitrogens and the imidazole nitrogen from a histidine residue in the third position (Laussac & Sarkar, 1984).

In the presence of Cu(II) or Ni(II) and a reducing agent such as ascorbate, the ATCUN motif has been shown to cleave DNA through the generation of a reactive oxygen species which is hypothesized to be the hydroxyl radical (Chiou,
1983). In one design, specific DNA cleavage was achieved by the attachment of the ATCUN motif GGH to the N-Terminus of the DNA binding region of a helix turn helix protein, Hin recombinase (Mack & Dervan, 1990). In another design using GGH, the DNA binding protein chosen was SP-1. (Nagaoka et al., 1994). In this case the hybrid metallo-protein generated consists of a zinc finger motif as well as the ATCUN motif. In addition, a variation of the ATCUN motif, using the amino R group of the amino acid ornithine as one of the ligands for coordination of the metal has been used in protein design with the advantage that the cleavage moiety does not necessarily have to be placed at the amino terminus (Shullenberger et al., 1993).

GKH-Fos/Jun is a hybrid protein previously designed in our laboratory to contain the ATCUN motif in the form of a GlyLysHis (GKH) tripeptide at the amino terminus of the Fos bZIP domain dimerized with the Jun bZIP domain (Harford et al., 1996). Lysine was chosen in the design as the second residue of the ATCUN motif in order to increase the affinity of the heterodimer for the DNA. Lysine has a high propensity of \( \alpha \) helicity (Johnson et al., 1994), and the DNA binding domain of bZIP proteins becomes highly \( \alpha \) helical upon binding to the DNA. In addition, the positively charged side chain of lysine was originally predicted to enhance DNA binding due to favorable electrostatic interactions with the DNA phosphodiester back bone. The ATCUN motif was attached to the amino terminus of Fos and not Jun because Fos does not form stable homodimers and this would ensure the presence of one cleavage moiety per heterodimer bound.
The designed Cu(II)-GKH-Fos/Jun dimer, in the presence of ascorbate and \( \text{H}_2\text{O}_2 \) was shown to cleave AP-1 site containing DNA. However, the incubation time required to achieve substantial cleavage of the DNA was between 16 to 20 hours, indicating low efficiency of DNA cleavage by the designed heterodimer. In addition, the nature of DNA cleavage appeared to be a single stranded cut approximately 35 bp 5' of the AP-1 site on each strand.

1.2 Project aims

Our aims in this project were twofold. First we were interested to improve the cleavage reaction conditions and increase the efficiency of cleavage by the designed GKH-Fos/Jun heterodimer. The cleavage reaction conditions were improved by reducing the concentration of glycerol, a known hydroxyl radical scavenger, from the cleavage reaction in order to increase the efficiency of DNA cleavage. 10% glycerol was originally included in the reaction buffer in order to stabilize the protein. In addition, DTT was removed from the reaction buffer because it is thought to interfere with the OH radical mediated mechanism of the cleavage reaction.

Another factor thought to contribute to the low cleavage efficiency by GKH-Fos/Jun is the presence of a conserved cysteine residue (C#154 Fos and C#272 Jun) in the DNA binding domains of Fos and Jun, shown in bold in Figure 1.3, which is the target of redox mediated regulation of binding of the dimer to the AP-1 site. Under the oxidizing nature of the cleavage reaction these cysteine residues are thought to become oxidized and thereby render the heterodimer
unable to bind to the AP-1 site (Abate et al., 1990a). In order to obtain the best crystals for solving the structure of the Jun/Fos/AP-1 complex, Glover and Harrison mutated these cysteine residues into serines in order to generate a stable protein-DNA complex (Glover & Harrison, 1995). We therefore mutated these cysteine residues into serines in both Jun (C272-S) and GKH-Fos (C154-S) in an attempt to improve the affinity of DNA binding and efficiency of cleavage by the designed GKH-Fos/Jun heterodimer.

Second, we were also interested in better characterizing the nature of this heterodimer-DNA interaction with regards to how specific DNA cleavage occurs 3.5 turns of the DNA helix away from the AP-1 site. We postulated that some type of DNA bending is induced by the ATCUN motif present in the modified heterodimer. We also examined whether the addition of the positively charged GKH motif to Fos alters the DNA binding affinity and specificity of the Jun/Fos heterodimer.

Having characterized the nature of GKH-Fos/Jun-AP-1 interaction and having optimized cleavage efficiency, it would be possible to use the designed protein as a tool to study various factors that influence the orientation of binding of bZIP heterodimers to the palindromic AP-1 site. Recently it has been shown that several factors influence the orientation of binding of the Jun/Fos heterodimer to the AP-1 site. The interaction between the central guanine in the AP-1 site and a conserved arginine residue in the basic domain of Fos as well as the nature of the sequences flanking the AP-1 site have been shown to have an effect on the orientation of binding of the Jun/Fos heterodimer to the AP-1 site.
(Leonard et al., 1997 and Rajaram & Kerppola, 1997). In addition, cross talk between other transcription factors such as NFAT and the AP-1 proteins is thought to confer a preferred orientation of binding of the Jun/Fos heterodimer to the AP-1 site (Erlanson et al., 1996).

Having a protein which can selectively cleave the DNA on one side of the AP-1 site (on the side of GKH-Fos) depending on the orientation of binding of the dimer, enables us to use cleavage experiments in order to examine the effect of such factors on the orientation of binding to the AP-1 site. In addition, aside from heterodimerization with members of the Jun family of bZIP proteins, Fos has been reported to dimerize with other members of the bZIP family such as the ATF protein family (Hai & Curran 1991; Kerppola & Curran, 1993; Chatton et al., 1994 and De Cesare et al., 1995), the human retina specific gene nrl, MAF and MAF-related bZIP proteins (Fujiwara et al., 1993; Kerppola & Curran 1994a,b; Kataoka et al., 1994; Igarashi et al., 1995 and Kataoka et al., 1995). Using cleavage reactions, it will also be possible to determine the effect of different dimerization partners of Fos on the orientation of binding of the different combinations of dimers to the palindromic AP-1 site.

1.3 The Jun/Fos heterodimer

Jun and Fos are proto-oncogenes and members of the bZIP family of eukaryotic transcription factors. Jun (c-jun) and Fos (c-fos) are cellular homologues of the oncogenes necessary for the induction of tumors by the avian sarcoma virus 17 and the FBJ murine sarcoma virus respectively (reviewed in
Curran, 1992). They modulate the transcription of genes by binding as either Jun homodimers or Jun/Fos heterodimers to the AP-1 consensus site present in the promoter regions of many genes (Angel et al., 1987; Rauscher III et al., 1988; Curran &Franza, 1988; Sassone-Corsi et al., 1988). They contain several activation domains, which mediate transcriptional regulation (Figure 1.1). Jun contains activation domains A1, the stronger determinant, located near its N-terminus, and A2, which is located further toward the C-terminus of the protein (Bohmann & Tjian, 1989 and Baichwal & Tjian, 1990). Fos also contains several transactivation domains located C-terminus to its leucine zipper region (Abate et al., 1991 and Sutherland et al., 1992). In addition, Fos and Jun contain two conserved adjacent motifs, homology box 1 (HOB1) and homology box 2 (HOB2), which lie within their activation domains and appear to activate transcription cooperatively (Sutherland et al., 1992 and Bannister et al., 1994).

Jun and Fos are generally expressed at low basal levels but are transiently induced in response to a variety of extracellular stimuli (reviewed in Curran & Franza, 1988). The activity of Jun and Fos is regulated at different levels. Both Jun and Fos are subject to post translational modification by phosphorylation; multiple protein kinases including PKA, CKII, cdc2, and MAPK are capable of phosphorylating Jun or Fos on several sites in vitro (Abate et al., 1991) and some in vivo (reviewed in Karin et al., 1997). Phosphorylation of serine and threonine residues at the amino terminal activation domain of Jun by MAP kinase has been shown to increase its stability by decreasing its degradation via ubiquitination (Musti et al., 1997). On the other hand, phosphorylation of serine residues near
Figure 1.1 Schematic representation of the Jun and Fos proteins showing alignment of the bZIP domains. The dimerization interactions of the two proteins mediated through their leucine zipper domains are indicated by vertical lines. The positions of the transactivation domains including the HOB1 and HOB2 regions are indicated.
the carboxy terminus of Jun by MAP kinase (Pulverer et al., 1991), JNK and PKA (Smeal et al., 1994) enhances its transcriptional activation activity. The degradation of Fos, mediated through its carboxy terminal region was also shown to be enhanced in the presence of multiple protein kinases (and Jun), suggesting that phosphorylation in this region decreases that stability of Fos (Papavassiliou et al., 1992; Tsurumi et al., 1995).

The activity of Jun and Fos is also regulated through the reduction/oxidation (redox) of a conserved cysteine residue located in the DNA binding domain of these proteins (Abate et al., 1990a). When the cysteines are oxidized, the affinity of binding of the Jun/Fos heterodimer to the AP-1 site is lowered substantially. In fact, these cysteine residues appear to be the target of a signaling cascade of redox regulation of DNA binding activity. The Jun/Fos heterodimer is activated through the reduction of these cysteine residues by nuclear redox factor (REF-1), also a DNA repair enzyme. REF-1 is an apurinic/apyrimidinic endonuclease whose redox and DNA repair activities are encoded by different regions. The redox activity of REF-1 is itself stimulated by thioredoxin (Xanthoudakis et al., 1992; Xanthoudakis & Curran, 1992; Yao et al., 1994). One of the differences between v-jun (the oncogenic homologue of c-jun) and c-jun that is thought to contribute to its oncogenic properties is that v-jun contains a serine residue in place of the conserved cysteine. v-jun is thus thought to bypass redox regulation of DNA binding. In fact, replacement of this cysteine residue in Fos by serine results in an increase in its ability to induce cellular transformation (Okuno et al., 1993).
Jun and Fos are also composed of a leucine zipper region, consisting of a repeat of leucine residues every 7 amino acids which mediates dimerization through the formation of an α helical coiled coil hydrophobic interface (Kouzarides & Ziff, 1988; Gentz et al., 1989; Glover & Harrison, 1995), as well as a region rich in basic amino acids, which is responsible for contacting the DNA in the major groove (Gentz et al., 1989; Glover & Harrison, 1995) (Figure 1.2). The sequence of basic and leucine zipper regions is highly conserved within the bZIP family. The sequence alignment of the DNA binding (basic) domains of various bZIP proteins is shown in Figure 1.3.

There is a conserved spacing of seven amino acid residues between the N-terminal leucine residue and a pair of C-terminal arginine residues in all bZIP proteins which comprises the fork region between the basic and leucine zipper domains. In the case of the GCN4 dimer, another member of the bZIP family, this seven amino acid spacing has been shown to be critical for DNA binding but can be changed by the insertion of an integral number of α helical turns without loss of GCN4 DNA binding (Pu and Struhl, 1991a). This demonstrates the requirement for a continuous α-helix spanning the leucine zipper domain and diverging into the basic region, which is positioned for contact with the DNA.

Different bZIP multigene family members have distinct DNA binding preferences. The asymmetric palindromic AP-1 site TGA(C/G)TCA is recognized by the Jun and Fos group of proteins, while the closely related symmetric palindromic ATF/CRE site, TGACGTCA is recognized by the ATF/CREB group. Cross group dimerization generates proteins with distinct
Figure 1.2 Schematic diagram of the X-ray crystal structure of the Fos and Jun bZIP domains bound to a 20 bp AP-1 site containing DNA fragment (Glover & Harrison, 1995).
<table>
<thead>
<tr>
<th>cFos</th>
<th>EEEKKRVRRERNKMAAAKCRNRRRELTK</th>
</tr>
</thead>
<tbody>
<tr>
<td>FosB</td>
<td>EEEKKRVRRERNKLAAAACRNRRRELTK</td>
</tr>
<tr>
<td>Fra1</td>
<td>EEEKKRVRRERNKLAAAACRNRRKELT</td>
</tr>
<tr>
<td>GCN4</td>
<td>SSDPAALKRARNTAARRRSRARKLQRM</td>
</tr>
<tr>
<td>cJun</td>
<td>ERIKAERKRMNRIAASKCRKRKLERI</td>
</tr>
<tr>
<td>JunB</td>
<td>ERIKAERKRLRNRLAATKCRKRKLERI</td>
</tr>
<tr>
<td>DJRA</td>
<td>EKIKLERKRQRNRVAASKCRKRKLERI</td>
</tr>
<tr>
<td>CREB</td>
<td>ATRKRELRLMKNEAARECRRKKKEYV</td>
</tr>
<tr>
<td>CEBP/G</td>
<td>NSDEYRQRERRNNMAVKKSKLSKQKA</td>
</tr>
<tr>
<td>ATF3</td>
<td>EDERKKRRERNKRIAACRQKRKVWV</td>
</tr>
<tr>
<td>GKH-Fos</td>
<td>GKHKKRVRRERNKMAAAKCRNRRRELTK</td>
</tr>
<tr>
<td>cFos</td>
<td>GKKRVRRERNKMAAAKCRNRRRELTK</td>
</tr>
</tbody>
</table>

**Figure 1.3**  Sequence alignment of the basic domain in several bZIP transcription factors. A conserved cysteine residue located in this region, which is thought to be sensitive to oxidation is shown in bold. The three amino acids located adjacent to the N-terminus of bZIP proteins, shown to be critical in DNA bending are underlined. The position of the GKH motif at the N-terminus of GKH-Fos (in italic) is shown relative to the position of the three critical amino acids involved in DNA bending.
affinities and preferences for the AP-1 or CRE sites (Hai & Curran 1991; Kerppola & Curran, 1993; Chatton et al., 1994 and De Cesare et al., 1995). In addition, another subgroup of bZIP proteins, the nrl, MAF and MAF-related proteins also form cross group dimers within the bZIP family and bind to AP-1, CRE, as well as asymmetric hybrid consensus sites composed of the AP-1 or CRE and MAF half sites (Fujiwara et al., 1993; Kerppola & Curran 1994a,b; Kataoka et al., 1994; Igarashi et al., 1995 and Kataoka et al., 1995; Kataoka et al., 1996), and thus contribute to the versatility in dimer combinations, target site specificity and possibly affinity of binding to the DNA.

The few residues, which according to the crystal structures of the GCN4-AP-1 (Ellenberger et al., 1992), GCN4-CRE (Konig & Richmond, 1993) and Jun/Fos-AP-1 (Glover & Harrison, 1995) complexes make base specific contacts to the DNA can not entirely confer specificity of DNA binding. These residues are highly conserved among different members of the bZIP family, which have distinct preferences for the CRE versus the AP-1 site. In addition, the spacial distribution of many of these residues is also highly conserved. In fact, it has been proposed that not only DNA specific contacts but also flexibility at the protein-DNA interface is an important contributor to selective recognition of the DNA target site by different bZIP proteins. It was shown that a mutation in the GCN4 fork region between the leucine zipper and basic domains is important in half site spacing preferences (AP-1 versus CRE site) of the GCN4 dimer and perhaps other bZIP dimers (Kim et al., 1993a and Johnson, 1993). In addition, two nonconserved amino acids at positions -14 and -16 with respect to the first
leucine residue of the leucine zipper domain as well as the highly conserved asparagine residue at position -17 have been shown to be important in the distinct binding specificities of different bZIP transcription factors (Suckow et al., 1993 and Kim et al., 1993). Therefore, it is thought that residues within a short region spanning the fork region (Figure 1.2) and basic domains of bZIP proteins, which are not necessarily involved in base specific or phosphate contacts with the DNA, contribute to site specific recognition by different bZIP dimers through generation of conformational variations in the DNA contact interface (Kim et al., 1993; Metallo & Schepartz, 1994; Kim & Struhl, 1995). This may in turn effect the adaptability and orientation of the basic domain and its contact with the DNA, conferring specificity of binding.

Circular dichroism and energy transfer studies demonstrate that the Fos-Jun-AP-1 interaction results in structural transitions in both the Jun/Fos leucine zipper and basic domains. Fos and Jun leucine zipper domains adopt an α-helical conformation upon dimerization (O'Shea et al., 1989; Patel et al., 1990) to form the α-helical coiled coil interface from two protein monomers. Upon DNA binding, the Jun/Fos basic domains also undergo a structural transition from a flexible largely unstructured form to a highly α-helical structure (Patel et al., 1990). In addition, subunit exchange between Fos and Jun was shown to be rapid in the absence of DNA but decreased substantially in its presence, suggesting that binding to the AP-1 site causes a change in conformation of the heterodimer, which prevents dissociation of the dimer (Patel et al., 1994).
Circular dichroism studies indicate that the DNA also undergoes a change in conformation upon binding to Jun homodimers and Jun/Fos heterodimers. Although the spectral changes induced by the two dimers are different, in either case they indicate a change from B to A or an intermediate between the A and B conformations (John et al., 1996). In addition, DNA bending studies, mainly using the method of phasing analysis have demonstrated that the DNA undergoes a structural transition (DNA bending) upon binding of the Jun/Fos heterodimer to the AP-1 site (Kerppola et al., 1991a; Kerppola & Curran 1991b; Kerppola, 1996; Kerppola, 1997, Kerppola & Curran, 1997). However, there is some controversy in the literature in this regard (Sitlani & Crothers, 1996 and Sitlani & Crothers, 1998). In fact, several members of the bZIP family of DNA binding proteins have been shown to bend the DNA upon binding to their consensus recognition site (Paolella et al., 1994; Kerppola & Curran, 1993). The magnitude and direction of the induced bend, however, differs depending on the bZIP dimer in question (Kerppola & Curran, 1993).

1.4 DNA bending

DNA structural polymorphism had been shown to be a multidimensional phenomenon. Intrinsic, sequence dependent curvature of DNA as well as extrinsic DNA curvature, including protein induced DNA bending, are thought to be involved in many biological regulatory processes such as transcription, DNA packaging, replication and site-specific recombination. The earliest example of sequence directed curvature of DNA to be identified was that of (A)_n
tracts, a series of helically phased A residues with \( n \geq 5 \), which were shown using a variety of methods to play a role in generating curvature (reviewed in Harrington, 1993). Subsequent to this finding, a number of sequence elements have been identified which give rise to strong local directionality or distortions in DNA helix conformation (reviewed in Hagerman, 1992). These are examples of static sequence directed DNA curvature. It has been shown that the dynamic character of DNA structure, including thermal motions in the DNA helix cause variations in the roll and tilt angles between adjacent base pairs resulting in DNA flexibility which is directional (Hagerman, 1992). In addition to thermal fluctuations of the DNA, other dynamic effects such as the influence of counterions as well as sequence context must therefore be taken into account when considering DNA curvature.

DNA bending has been shown to be a common characteristic of many DNA binding proteins. However, the DNA double helix is not flexible over distances of up to a few hundred base pairs (Wang & Giaever, 1988). In light of the unfavorable and costly energetics of DNA bending therefore, two important points must be considered. First is the question of the biological purpose of DNA bending. In the case of histone proteins, which non-specifically bind to and bend the DNA, and are thereby responsible for DNA packaging into nucleosome structures (Richmond et al., 1984), DNA bending evidently serves an architectural/structural function. For sequence specific DNA binding proteins, bending at the consensus binding site is thought to be involved in the regulation of gene expression. For instance, DNA bending in this case may serve to
stabilize a tertiary nucleoprotein complex involving another protein with the DNA, it may function to facilitate the binding of other transcription factors by making DNA sequences upstream or downstream of the bend available for binding, or it may facilitate the interaction between two distantly bound transcription factors by DNA looping. A more in depth understanding of the biological functions of protein induced DNA bending, however, remains to be elucidated.

The second is the question of the mechanism by which DNA bending occurs. It was suggested in the case of histone proteins, that DNA phosphate neutralization caused by the positively charged amino acid side chains of histones could result in DNA bending into nucleosome structures (Mirzabekov & Rich, 1979). In fact it has been shown through methylphosphonate (neutral) replacement of anionic DNA phosphates and by studies using ammonium cations tethered to one face of the DNA helix, that asymmetric phosphate neutralization of the DNA backbone leads to DNA bending or "collapse" towards the neutralized DNA face (Strauss and Maher, 1994; Strauss & Maher, 1996). Electrostatic interactions as well as phosphate neutralization therefore appear to be an important driving force in protein induced DNA bending. Other mechanisms by which DNA binding proteins bend the DNA include partial or complete protein side chain intercalation into the DNA helix (reviewed in Werner et al., 1996), usually in the minor groove. An example of such an interaction is that of the TATA-box binding protein bound to the TATA element. In this case the hydrophobic side chain of an isoleucine residue partially
intercalates into the minor groove, disrupts base stacking, resulting in DNA bending away from the protein (Kim et al., 1993b and Kim et al., 1993c). As well, protein mediated DNA loop formation is another mechanism by which DNA binding proteins bend the DNA (Finzi & Gelles, 1995; Bazett-Jones et al., 1994). DNA looping results from protein binding to two distal sites on the same face of the DNA helix.

1.5 DNA bending by bZIP dimers

Basic region leucine zipper transcription factors represent a family of eukaryotic sequence specific DNA binding proteins whose members have been shown to bend the DNA at their site of binding. The direction and degree of the induced bend depends on the members and dimerization partners under investigation (Kerppola & Curran, 1993). Phasing analysis experiments demonstrate that Jun homodimers bend the DNA toward the major groove whereas Jun/Fos heterodimers bend the DNA in the opposite direction, toward the minor groove (Kerppola & Curran, 1991b; Kerppola, 1996; Kerppola, 1997; Kerppola & Curran, 1997). The bZIP domain of the Fos/Jun heterodimer displays a DNA bend in the same orientation as the full length protein, but of smaller magnitude, suggesting that regions outside of the bZIP domain contribute to DNA bending (Kerppola & Curran, 1991b and Kerppola & Curran, 1997). In fact, DNA bending studies using chimeric proteins have shown that the activation domains of Fos and Jun induce DNA bending independently from the bZIP domains (Kerppola & Curran, 1997).
While studies using the methods of circular permutation (Kerppola & Curran, 1991b), phasing analysis (Kerppola, 1997; Kerppola & Curran, 1997) and direct visualization by atomic force and electron microscopy (Griffith et al., 1994 and Becker et al., 1995) indicate that the Jun/Fos heterodimer bends the DNA at the AP-1 site, studies using other methods have reached contradictory conclusions. The X-ray crystal structure of the bZIP domains of the Fos/Jun heterodimer indicates little (at most 40°) to no bending of the AP-1 site (Glover & Harrison, 1995). In addition, phase sensitive detection as well as ligase catalyzed cyclization experiments indicate no bending by the Fos/Jun heterodimer at the AP-1 site (Sitlani & Crothers, 1996; Sitlani & Crothers, 1998). Nevertheless, reexamination of the methods used for detection of DNA bending by Fos/Jun has revealed that the lack of DNA bend detection may be explained by the limitations within some of the methods, which may give rise to anomalous results. With respect to the crystal structure, it is argued that crystal packing forces (DiGabriele & Steitz, 1993), the presence of multivalent cations which shield the electrostatic mechanism of bending by Jun/Fos (Rajaram & Kerppola, 1997 and Kerppola & Curran, 1997) and agents used to promote crystallization (Sprouse et al., 1995 and Dlakic et al., 1996) may contribute to changing the conformation of the DNA in the crystal. In addition, the crystal structure of A tracts, a series of five to six adenines, which are used as standard reference bends in several electrophoretic methods of DNA bend determination, indicate no bending of the DNA (Dickerson et al., 1994 and Goodsell et al., 1994) or in some cases bending in the opposite direction (DiGabriele et al., 1989 and DiGabriele &
Steitz, 1993), suggesting that crystal packing forces may alter DNA conformation in crystals.

Gel electrophoretic methods for DNA bend detection such as circular permutation, phasing analysis and phase sensitive detection experiments are based on the observation that curved DNA displays retarded mobility through native high percentage acrylamide gels relative to uncurved DNA of the same size. Figure 1.4 illustrates three commonly used gel retardation methods for DNA bend detection: phasing analysis, phase sensitive detection, and circular permutation analysis. In phasing analysis and phase sensitive detection, the unknown bend under investigation is helically phased (placed at different distances) from an intrinsic bend of known orientation and degree, usually the A tract ($A_{\text{tract}}$). In phasing analysis, the unknown bend is placed immediately adjacent to the intrinsic reference bend (the spacing between the two bends is small). Therefore, when the two bends are in phase, the mobility of the resulting DNA molecule is thought to be similar to the mobility of a molecule which has a bend equal to the sum of the two closely spaced bends. When the two bends are out of phase, the bends are thought to cancel each other out such that the mobility of the resulting molecule is the same as that of a molecule containing one bend the magnitude of which is equal to the difference between the two closely spaced bends. In phase sensitive detection, however, the two bends are not as closely spaced as they are in phasing analysis. The resulting DNA molecule contains two separate bends the effect of which on mobility through the gel is not as well understood. In fact it has been shown that large spacing
a) Phasing Analysis

Protein induced bend

Intrinsic bend

Protein induced bend

Intrinsic bend

in phase
slower mobility

out of phase
faster mobility

b) Phase Sensitive Detection

Protein induced bend

Intrinsic bend

Protein induced bend

Intrinsic bend

Fastest mobility

Slowest mobility

c) Circular permutation

Figure 1.4  Pictorial depiction of  a) phasing analysis  where the protein induced and intrinsic reference bends are placed immediately adjacent to one another such that the two bends display an additive effect when in phase, and cancel each other out when out of phase;  b) phase sensitive detection  where the spacer length between the the two bends is long resulting in the formation of a “C” shaped DNA molecule when the two bends are in phase, and an “S” shaped molecule when they are out of phase;  c) circular permutation analysis  where the position of the DNA bend is varied from the center to the end of a DNA molecule.  When the bend is located at the center, the curved DNA molecule displays slower mobility than if the bend is located at the end of the molecule.
between the two bends in combination with short DNA segments flanking the bends results in a reduction in the mobility difference of helically phased bends (Kerppola, 1997). In addition, reexamination of ligase catalyzed cyclization experiments using the Fos and Jun bZIP domains has led to the finding that the proteins promote the intermolecular and intramolecular cyclization of the DNA through other mechanisms besides DNA bending and thereby complicate analysis of cyclization results (Kerppola, 1996). Of the methods discussed, phasing analysis as well as circular permutation experiments have been used extensively to examine DNA bending by a variety of bZIP proteins.

Recently, a direct relationship has been shown to exist between the DNA bend angle and net charge of three amino acid residues adjacent to the N-terminus of the basic region of bZIP proteins (Kerppola & Curran 1993; Paolella et al., 1994; Paolella et al., 1997; Leonard et al., 1997; Strauss & Maher, 1997; Strauss & Maher, 1998) (Table 1.1); substitution of these amino acid residues in Jun and Fos resulted in a charge dependent proportional change in DNA bend angle, suggesting that electrostatic interactions between amino acids in this region and the negatively charged phosphodiester backbone influence DNA bending by these proteins (Leonard et al., 1997). In addition, substitution of three neutral amino acids at the N-terminus of the bZIP domain of GCN4 (proline alanine alanine) into basic amino acids (lysine lysine lysine) has been demonstrated to convert the GCN4 homodimer which does not inherently bend the DNA into a DNA bending protein (Strauss & Maher, 1997). The magnitude of bending in this case is approximately 15 degrees toward the DNA minor
Table 1.1 Charge of three amino acids adjacent to N-terminus of basic domain of several bZIP proteins correlate with DNA bending (adapted from Strauss & Maher, 1997). (min +) indicates a bend angle > 20° toward the minor groove; (min) indicates a bend angle < 20° toward the minor groove; (maj) indicates a bend angle toward the major groove. The basic amino acids are underlined.

<table>
<thead>
<tr>
<th>bZIP protein</th>
<th>amino acids adjacent to N-terminus of basic region</th>
<th>net charge of tripeptide</th>
<th>direction of DNA bend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fos</td>
<td>KRR</td>
<td>+3</td>
<td>(min +)</td>
</tr>
<tr>
<td>Fra1</td>
<td>RRR</td>
<td>+3</td>
<td>(min)</td>
</tr>
<tr>
<td>Fra2</td>
<td>KRR</td>
<td>+3</td>
<td>(min)</td>
</tr>
<tr>
<td>ATF2</td>
<td>KRR</td>
<td>+3</td>
<td>(min)</td>
</tr>
<tr>
<td>Jun</td>
<td>KAE</td>
<td>0</td>
<td>(maj)</td>
</tr>
<tr>
<td>CREB</td>
<td>KRE</td>
<td>+1</td>
<td>(maj)</td>
</tr>
<tr>
<td>ATF1</td>
<td>KRE</td>
<td>+1</td>
<td>(maj)</td>
</tr>
<tr>
<td>ZTA</td>
<td>ELE</td>
<td>-2</td>
<td>(maj)</td>
</tr>
<tr>
<td>GCN4</td>
<td>PAA</td>
<td>0</td>
<td>(maj)</td>
</tr>
</tbody>
</table>
groove. Substitution of the same three residues into negatively charged amino acids (glutamate glutamate glutamate) causes the GCN4 homodimer to bend the DNA in the opposite direction, toward the major groove by approximately 25 degrees (Strauss & Maher, 1998). The AP-1 site itself is intrinsically bent approximately 7 degrees toward the major groove (Strauss & Maher, 1997).

1.6 The ATCUN motif

The Amino Terminus Cu(II)- and Ni(II)- Binding (ATCUN) motif was first identified and characterized in albumin (Shearer et al., 1967; Peters & Blumenstock, 1967; Laussac & Sarkar, 1984). Albumin, the most abundant protein in plasma, is thought to be involved in the binding and transport of several small molecules and ions (Kragh-Hansen, 1990), including Cu(II) and Ni(II) (Giroux & Schoun, 1981). Metal binding studies of the human, bovine, and rat serum albumin showed the presence of a high affinity Cu(II) binding site located in the N-terminus of these proteins (Shearer et al., 1967; Peters & Blumenstock, 1967; Lau & Sarkar, 1971; Laussac & Sarkar, 1984). The specific ligation of Cu(II) and Ni(II) by the ATCUN motif can be detected by spectrophotometry; the visible spectra of Cu(II) ligated to ATCUN motif containing peptides displays a characteristic maximum at 525 nm (Appleton & Sarkar, 1971; Harford & Sarkar, 1997) while that of the Ni(II)-ATCUN motif complex displays a peak at 420 nm (Glennon & Sarkar, 1982).

The minimal requirement for the ligation of Cu(II) or Ni(II) by the ATCUN motif is a free amino terminus, two intervening peptide nitrogens and the
imidazole nitrogen of a histidine residue in the third position (Shearer et al., 1967; Peters & Blumenstock, 1967; Laussac & Sarkar, 1984). Comparison of protein sequences to the ATCUN motif definition has led to the finding that several proteins and peptides possess this site (Figure 1.5). Based on the requirements for metal ligation by the ATCUN motif, the molecule GlyGlyHis was designed and its metal binding characteristics studied (Lau et al., 1973); the Cu(II)-GlyGlyHis complex displayed similar visible and ESR spectral properties to that of Cu(II)-HSA (Rakhit & Sarkar, 1981). In addition, the X-ray crystal structure of Cu(II)-GlyGlyHis showed that Cu(II) is chelated by the four nitrogen ligands in a slightly distorted square planar configuration (Camerman et al., 1976) (Figure 1.6). Structural information from the N-terminus of different ATCUN motif containing proteins as well as energy minimization calculations indicate that in the absence of metal, the ATCUN motif is not a rigid structure and exists in several different conformations including a structure which can adopt a square planar conformation (Sarkar, 1976 and Gasmi et al., 1997).

Cu(II) ligated to the designed GlyGlyHis, in the presence of ascorbic acid, was demonstrated to possess anti-tumor properties in vitro and in vivo (Kimoto et al., 1983; Chiou, 1983). In fact, ATCUN motif-bound Cu(II) in the presence of redox conditions is thought to result in the generation of highly reactive hydroxyl radicals through a Fenton type mechanism as shown (Chiou, 1983):

\[
\text{ascorbate} + 2\text{Cu}^{2+} \rightarrow \text{dehydroascorbate} + 2\text{Cu}^{+} + 2\text{H}^{+} \quad (1)
\]

\[
\text{Cu}^{2+} + \text{ascorbate} + \text{O}_2 \rightarrow \text{dehydroascorbate} + \text{H}_2\text{O}_2 \quad (2)
\]
$\text{H}_2\text{O}_2 + \text{Cu}^+ \rightarrow \text{Cu}^{2+} + \text{OH}^- + \cdot\text{OH}$ \hfill (3)

These reactive radicals are thought to result in protein and DNA damage. The hydroxyl radical can travel a maximum distance of 60 Å (Mello Filho & Menghini, 1984) and based on the highly reactive nature of this radical, the ATCUN motif has been used by several laboratories to design hybrid ATCUN motif containing DNA binding proteins in order to achieve site-specific cleavage of DNA (Mack & Dervan, 1992; Nagaoka et al., 1994; Brown et al., 1995; Footer et al., 1996).
<table>
<thead>
<tr>
<th>ATCUN motif</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Serum Albumin</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>Rat Serum Albumin</td>
</tr>
<tr>
<td>Histatin 3</td>
</tr>
<tr>
<td>Neuromedin C</td>
</tr>
<tr>
<td>Neuromedin K</td>
</tr>
<tr>
<td>Human Sperm Protamine P2A</td>
</tr>
</tbody>
</table>

**Figure 1.5** Amino terminal sequences of several ATCUN motif containing proteins and peptides. The N-terminal amino acids comprising the ATCUN motif are underlined.
Figure 1.6 Crystal structure of Cu(II) ligated to the Amino Terminus Cu(II)-Ni(II) binding (ATCUN) motif (Cameron et al., 1976).
1.7 Summary

The construction of affinity cleaving proteins is of prime importance in investigating and probing the site and orientation of protein–DNA interactions. This is accomplished by the use of pre-existing metal binding motifs in hybrid proteins which also contain a DNA binding domain. The hybrid protein, which is generated has the advantage that it possesses not only the properties of the DNA binding protein of interest but it is also capable of cleaving the DNA near the protein binding site. The overall objective of this research is the design of proteins which are capable of recognizing and cleaving an unsequenced stretch of DNA at a specific site. GKH-Fos/Jun is an affinity cleaving protein containing the ATCUN motif (DNA cleavage moiety) as well as the bZIP domains of Fos and Jun protooncogenes, which was reported to bind to and cleave the DNA at the AP-1 site (Harford et al., 1996). After optimization of the DNA cleavage conditions, the objective of this work is to use AP-1 DNA cleavage patterns by GKH-Fos/Jun to determine the orientation of binding of Fos as well as other bZIP partners of Jun at the AP-1 site. In addition, this designed protein may be used as an infrequent cutter of AP-1 target sites on genomic DNA for sequence analysis as well as functional studies. Further advance in this area of research may lead to the use of such designed affinity cleaving proteins in gene and cancer therapy.
Chapter Two

Experimental Procedures
2.1 Media, enzymes and reagents

Bacterial cultures were grown in LB containing the appropriate antibiotics at a concentration of 100 μg/ml (ampicillin) and 80 μg/ml (kanamycin). Restriction endonucleases and other enzymes were supplied by Pharmacia, New England Biolabs and Boehringer-Mannheim. Radiolabeled nucleotides were supplied by Amersham. Unlabeled nucleotides were supplied by Pharmacia. Metal salts were supplied by Fisher Scientific Company.

2.2 Mutagenesis of cysteine 272(Jun), 154(cFos) and 154(GKH-Fos) into serine:

The C272-S(Jun), C154-S(cFos) and C154-S(GKH-Fos) mutations were generated using the U.S.E (Unique Site Elimination) Mutagenesis kit (Pharmacia Biotech). These mutations were carried out on expression plasmids encoding amino acids 139-211 of cFos and GKH-Fos (p2R plasmid) and 248-334 of Jun (pGEX-4-T2). Briefly, two oligonucleotide primers were used to generate multiple rounds of mutagenesis on the expression plasmids of cFos, GKH-Fos, and GST-Jun. The first primer, a target site mutagenesis primer, contained the desired mutation (the codon for serine instead of cysteine), while the second, a selection primer, changed a unique restriction site present in the original plasmid into another unique restriction site. The second primer is used in order to select mutant plasmids, which also have a high probability of containing the desired target site mutation. For a detailed discussion of this method, see Deng & Nickoloff, 1992.
2.3 Expression and purification of native and mutant (C-S) GKH-Fos/Jun and cFos/Jun heterodimers:

*E. coli* BL-21 (DE3) strain were co-transformed with the expression vector p2R containing cFos or GKH-Fos (139-211) and the kanamycin resistant gene, and the expression vector pGEX-4-T2 containing GST-Jun (248-334) and the ampicillin resistant gene. Bacteria transformed with both vectors were selected for by growing in the presence of ampicillin (100 mg/ml) and kanamycin (70 mg/ml). Cells grown in LB plus antibiotics until OD 600 reached between 0.6-1.0 were induced with IPTG (0.1 mM) for 4 h, harvested, and resuspended in 20 ml lysis buffer (25 mM KNaHPO4, 1M NaCl, 50 mM ε-amino caproic acid, 5 mM DTT, 2 mM PMSF, 0.1 mg/ml lysozyme, 0.1% Triton). The lysed bacteria were centrifuged (40 K g, 45 min) at 4°C. The supernatant, which contained a GST-Jun/(c/GKH-)Fos complex (in which the Jun moiety of the GST-Jun fusion protein was heterodimerized with the (c/GKH-)Fos polypeptide), was applied to a Glutathione Sepharose column (Pharmacia Biotech). After washing of the column with PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, 30 mM β-mercaptoethanol), the GST-Jun/(c/GKH-)Fos complex was eluted with glutathione (10 mM in 50 mM Tris-Cl pH 8.0). The GST-Jun fusion protein was then cleaved with 100 cleavage units of thrombin for 2 h at room temperature. After dialysis against loading buffer (25 mM KNaHPO4, 10% glycerol, 0.05% NP-40 and 5 mM DTT), the digested preparation was loaded onto a Bio Rex 70 cation-exchange resin (BioRad), washed with a 25 mM
KNaHPO$_4$ buffer containing 0.5 M NaCl, 10% glycerol, 0.05% NP-40 and 5 mM DTT. The (c/GKH-)Fos/Jun heterodimer was eluted with 25 mM KNaHPO$_4$ buffer containing 1 M NaCl, 10% glycerol, 0.05% NP-40 and 5 mM DTT. The purified (c/GKH-)Fos/Jun heterodimer was then dialysed against a 25 mM KNaHPO$_4$ buffer containing 10% glycerol, 0.05% NP-40 and 5 mM DTT, and its purity assessed by SDS-PAGE.

C-S mutant and native cFos, GKH-Fos and Jun were also individually purified. *E. coli* BL-21 (DE3) strain were transformed with the expression vector p2R containing cFos or GKH-Fos (139-211) and the kanamycin resistant gene, and the bacteria were grown, induced and lysed as described above. The supernatant containing the (c/GKH-)Fos peptide was loaded onto a Bio Rex 70 cation-exchange resin (BioRad), washed with a 25 mM KNaHPO$_4$ buffer containing 0.5 M NaCl, 10% glycerol, 0.05% NP-40 and 5 mM DTT, and eluted with 25 mM KNaHPO$_4$ buffer containing 0.75-1 M NaCl, 10% glycerol, 0.05% NP-40 and 5 mM DTT.

For expression and purification of Jun, *E. coli* BL-21 (DE3) strain were transformed with the expression vector pGEX-4-T2 containing GST-Jun (248-334) and the ampicillin resistant gene, the bacteria were grown, induced and lysed. The pellet containing the insoluble GST-Jun (present in inclusion bodies) was denatured in the presence of an 8M urea buffer containing 25 mM NaKHPO$_4$ and 5 mM DTT, and renatured by dialysis against 25 mM KNaHPO$_4$ buffer containing 10% glycerol, 0.05% NP-40 and 5 mM DTT. The GST-Jun fusion protein was then cleaved with 50 cleavage units of thrombin for 2 h at room
temperature. The digested preparation was loaded onto a Bio Rex 70 cation-exchange resin (BioRad), washed with a 25 mM KNaHPO$_4$ buffer containing 0.5 M NaCl, 10% glycerol, 0.05% NP-40 and 5 mM DTT. Jun was eluted with 25 mM KNaHPO$_4$ buffer containing 1 M NaCl, 10% glycerol, 0.05% NP-40 and 5 mM DTT. The purified Jun was then dialysed against a 25 mM KNaHPO$_4$ buffer containing 10% glycerol, 0.05% NP-40 and 5 mM DTT.

### 2.4 Mobility shift assays (MSAs):

In order to examine the specific DNA binding affinity between the native and C-S mutant (c/GKH-)Fos/Jun heterodimers under oxidizing conditions, the mobility shift assay was conducted under a variety of binding reaction conditions. In particular, the DTT, H$_2$O$_2$ and ascorbate concentrations were varied in the binding reaction. The protein-DNA binding reactions were carried out using 20 and 36 bp DNA fragments containing the AP-1 consensus site (5'-TGACTCA-3') (The 36 bp oligonucleotide contained two copies of this site). The $^{32}$P end-labeled double stranded oligonucleotides were obtained by annealing the complimentary oligonucleotides after their 5' ends had been phosphorylated in the presence of [$\gamma^{32}$P]-ATP and T4 polynucleotide kinase. The DNA fragments had the following sequences:

- 20 bp: (5')-GATCCCTATGACTCACGCC- (3')
  (3')-GGATACTGACTGCCGGGCTGC- (5')

- 36 bp: (5')-GATCCCGTACTCAAGCGCCATCGTGACTCAAGCGCGCA- (3')
  (3')-GGCACTGACTCGCGGTAGCAGCTGACTCGCGGTTCGA- (5')
(c/GKH)-Fos/Jun heterodimer (15-400 ng) was pre-incubated with labeled oligonucleotide for 30 min at room temperature in 25 ml of binding buffer (10 mM Tris-Cl (or 25 mM KNaHPO4), 50 mM NaCl, 25 mM KCl, 0.5 mg/ml BSA, 0.05% NP-40, 10% glycerol, 0-5 mM DTT; pH 7.5) containing ascorbate (0-12 mM), H$_2$O$_2$ (0-12 mM) and 1.0 µg of non-specific DNA (poly(dI-dC)). This mixture was then loaded onto a low ionic strength 5.4% polyacrylamide gel and electrophoresed for 2 h at 150 V at room temperature. A 0.5 X TBE running buffer was used. Following electrophoresis, the gel was dried and exposed to X-ray film (16 h, -70 °C).

2.5 Protein concentration determination:

Protein concentration determination was performed using the BioRad Bradford protein assay with detection at 595 nm. A number of standards were prepared using BSA (100-1000 µg/ml) and the concentrations of the proteins were determined from the generated standard curve.

2.6 $K_d$ determination studies:

The dissociation constants for C-S mutant and native (c/GKH-)Fos/Jun heterodimers bound to a 49 bp DNA fragment containing a single AP-1 site with the sequence

49t  (5') -CGCTCACCCGATAAAAATACGTATGACTCATATCGTTACGTG-G (3')
49b  (3') -GCCGATGGGCTATTTTTTATGCATAGTGACGTAAATGCAATGCATGCACC (5')
were determined using two independent methods relying on the electrophoretic mobility shift assay. In the first method, the heterodimer concentration was kept constant (10-100 nM depending on the experiment) and titrated with an increasing amount of DNA containing the AP-1 site. The samples were run on a mobility shift assay gel and the gel was dried and exposed to X-ray film over night. The bands corresponding to free and bound DNA were excised, counted by scintillation counting using a Beckman LS6000IC β-counter and the CPM values were converted into concentrations using the specific activity of the DNA. Scatchard analysis was then conducted to determine the dissociation constant of each heterodimer. The slope of the line corresponding to a plot of the ratio of bound to free DNA concentrations as a function of bound DNA concentration is equal to the negative reciprocal of the apparent $K_d$ value.

In parallel, the $K_d$ values were also determined by titrating a fixed amount of labeled DNA containing the AP-1 site (approximately 10 pM) with increasing concentrations of heterodimer (1 nM - 1 μM). The binding reactions were analyzed by native gel electrophoresis, the fraction of bound DNA was determined as described above by scintillation counting. The fraction of bound DNA was then plotted as a function of heterodimer concentration and the $K_d$ value estimated as the concentration of heterodimer at which 50% of the DNA is bound.
2.7 Competition electrophoretic mobility shift analysis:

The relative nonspecific DNA binding affinities of C-S mutant or native (c/GKH-)Fos/Jun heterodimers were determined and compared using a band shift competition assay. Preformed $^{32}$P-labeled AP-1 DNA-heterodimer complexes in the absence of poly(dI.dC) were incubated with increasing concentrations of unlabeled DR-2 (nonspecific) DNA with the sequence,

\[(5') - ATGCGAATTCTGACCTTTTGAACCTGGATCCATGC - (3')\]
\[(3') - TACGCTTAAGACTGGAAAATGGACCTAGGTACG - (5')\]

The concentration of labeled AP-1 used was 25 nM while the concentration of competitor DNA was varied from 0 - 350 μM (0 - 0.01 M in units of molar base pairs). The total protein concentration used was 0.25 μM (cFos/Jun C-S mutant), 0.5 μM (cFos/Jun native), 1 μM (GKH-Fos/Jun C-S mutant) and 2 μM (GKH-Fos/Jun native). The complexes were incubated for 30 min in binding buffer (20 mM Tris-Cl, 50 mM NaCl, 5 mM MgCl$_2$, 10% glycerol, 0.05% NP-40, 0.5 mg /ml BSA and 1 mM DTT; pH 7.5) in a total volume of 40 μl and resolved by electrophoretic mobility shift assays. The disappearance of the specific AP-1-heterodimer complex (percent inhibition) was then monitored as a function of the concentration of competing nonspecific DNA added and the relative binding affinities of the heterodimers for competitor was calculated (Fried, 1989):

The addition of competitor DNA (C) to the protein-DNA complex (DP) results in the equilibrium equation

\[
\frac{K}{[DP + C]} = \frac{[D + CP]}{(4)}
\]
where \( K \), the equilibrium constant of the equation, is equal to \( K_{eq}^{sp}/K_{eq}^{comp} \) (the ratio of the equilibrium constants for binding to specific DNA (\( K_{eq}^{sp} \)) and nonspecific competitor DNA (\( K_{eq}^{comp} \))). At the 50% inhibition point, where \([DP] = [D]\), the ratio of specific to nonspecific binding affinities will equal \([C]/[CP]\):

\[
K_{eq}^{sp}/K_{eq}^{comp} = [C]/[CP]
\] (5)

\([CP]\) is calculated as the total protein concentration minus the amount bound to specific DNA at the 50% inhibition point while \([C]\) can be determined from the equation \([C] = [C]_o - m[CP]\) assuming the competitor DNA is in excess. \([C]_o\) is the total concentration of competitor DNA added at the 50% inhibition point, \(m\) is the number of base pairs thought to be bound by protein (here \(m\) is set at 7 to equal the number of base pairs occupied by the protein at the AF-1 site).

### 2.8 DNA bending experiments:

Phasing analysis was conducted in order to examine and compare the DNA bending properties of the C-S mutant and native (c/GKH-)Fos/Jun heterodimers. Probes for phasing analysis were synthesized as single stranded oligonucleotides by the Hospital for Sick Children's Biotechnology Centre and had the following sequences:

**A tracts**

3a: (5')-CGCTCACCAGTATAAAAAATACATGACTCAATTACGTACGTACGTGTGG- (3')

3b: (5')-CCACACGTAACGTAATGAGATCTATGTATTTTTATACGGGTTACGCG- (3')

5a: (5')-CGCTCACCAGTATAAAAAATACGTATGACTCAATTACGTACGTGTGG- (3')

5b: (5')-CCACGTAACGTAATGAGATCTATGTATTTTTATACGGGTTACGCG- (3')

7a: (5')-CGCTCACCAGTATAAAAAATACGTACGTATGACTCAATTACGTACGTATGG- (3')
Binding reactions were performed by incubation of approximately 20-200 ng of the appropriate heterodimers (C-S mutant or native cFos/Jun or GKH-Fos/Jun (in a 3:1 molar ratio of cFos/GKH-Fos: Jun)) with approximately 20,000 CPM of the labeled duplex DNA phasing probes for 30 min at room temperature in 40 ml of reaction buffer (5% glycerol, 10 mM Tris-Cl, 50 mM NaCl, 25 mM KCl, 0.05% NP40, 0.5 mg/ml BSA and 1 mM DTT; pH 7.5 and 1.0 μg of non-specific DNA, poly(dI-dC)). The protein bound and free AP-1 phasing probes were resolved by loading the mixtures onto a low ionic strength 9% nondenaturing polyacrylamide gel (30 : 1 acrylamide : bisacrylamide) and electrophoresed for 16 h at 150V at 4°C. A 0.5 X TBE running buffer was used. Following electrophoresis, the gel was dried and exposed to X-ray film (16 h, -70°C).

2.9 Analysis of DNA bend parameters:

The mobilities (μ) of the protein bound and free phasing probes were measured as the distance in millimeters from the center of the electrophoresis well to the center of the band. The mobilities were normalized to the average mobility of the appropriate set of phasing probes (protein bound and free
probes) to give relative mobilities ($\mu_{rel}$). Relative mobilities were then plotted as a function of the distance in base pairs between the center of the intrinsic bend (the A tract) and the center of the AP-1 site. The best fit of the relative mobilities to the cosine function

$$\mu_{rel} = \left(A_{PH} \right)/2 \cos \left[2\pi (S - S_T)/P_{PH} \right] + 1$$

(6)

(taken from Strauss & Maher, 1997) was then determined. $\mu_{rel}$ is the relative mobility of the phasing probe; $A_{PH}$, the amplitude of the phasing function is related to the magnitude of the bend; $S$ is the spacer length in base pairs between the centers of the two bends; $S_T$ is the spacer length at which the two bends are predicted to cancel each other out giving rise to the probe with the fastest mobility; $P_{PH}$ is the helical periodicity of DNA sequences between the two bends (assumed to be 10.5 base pairs per turn of the DNA helix). The direction of DNA bending was determined by correlating the spacer lengths at which the minima and maxima of the phasing function are reached with what is known of the magnitude and orientation of the reference bend. The A tract (the reference bend) bends the DNA $20^\circ$ toward the minor groove. The minima are obtained when the protein induced and intrinsic bends are in phase (or cooperate), resulting in the slowest probe mobility while the maxima correspond to the spacer lengths at which the two bends cancel each other out resulting in the fastest probe mobility.
2.10 Generation of DNA cleavage substrates:

Two DNA substrates of different sizes containing the consensus AP-1 target site but with different flanking sequences were used for cleavage reactions, and were both generated by PCR. The first DNA fragment, 100 bp in length, was amplified from the plasmid AP-1 pRSETA. This plasmid was constructed by digestion of the pRSETA vector with the restriction enzymes HindIII and XhoI, followed by band purification of the digested vector, and subsequent ligation with a 100 bp AP-1 site containing DNA fragment with complimentary restriction sites (Figure 2.1 a). The second 126 bp DNA fragment was amplified from the AP-1 PSE420 plasmid, which was similarly constructed by digestion of the PSE420 vector with the KpnI and PstI restriction enzymes followed by purification of the pre-cut vector, and ligation with a 40 bp AP-1 site containing DNA fragment with complimentary restriction sites (Figure 2.1 b). Single strand 5'-end labeled duplex 100 and 126 bp DNA fragments were generated using PCR amplification by 5'-end labeling one of the PCR reaction primers prior to the PCR reaction.

PCR reactions contained 0.02 ng/μl template DNA (AP-1 PSE420 or AP-1 pRSETA), 0.25 ng/μl of the appropriate primers, 100 μM each of dATP, dCTP, dGTP and dTTP, 1 unit vent DNA polymerase (Pharmacia Biotech) in a total volume of 100 μl buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂). The DNA fragments were initially denatured (96°C, 5 min), and amplified by 29 cycles of incubation (96°C, 60 s; 57°C, 30 s; 72°C, 90 s) followed by final extension
Figure 2.1  a) Construction of AP-1 pRSETA plasmid: pRSETA vector was digested with HindIII and XhoI restriction enzymes, purified, and ligated in the presence of 100 bp AP-1 DNA with complimentary ends, resulting in the generation of the plasmid AP-1 pRSETA; b) Construction of AP-1 PSE420 plasmid: the PSE420 vector was digested with KpnI and PstI restriction enzymes, the digested plasmid purified, and ligated in the presence of a 40 bp AP-1 site containing DNA with complimentary ends, generating AP-1 PSE420.
After PCR amplification, the single strand 5'-end labeled duplex DNA substrates were band purified by polyacrylamide gel electrophoresis. The sequences of the 100 and 126 bp DNA fragments (with the AP-1 site presented in bold) are:

126 bp fragment:

(5') - GCGCTTTAATTTGGCGATGCTAGCTATAGTTCTAGAGGTACCGATTCGCAGGCG
(3') - CGCGAAATTAAAACGCTACGATCGATATCAAGATCTCCATGCTAAAGG

ATGACTCATGGCGACCTCGTCGCTGCAGCTGGCGCCATCGATACGCGTACGTCGCGACCG
TACTGAGTACCGCTGGAGCAGCGACGTCGACCGCGGTAGCTATGCGCATGCAGCGCTGGC
CGGACATGTAC (3')
GCCTGTACATG (5')

100 bp fragment:

(5') - AAGCTTCCGGATCGCGCATCCCCGGATCGCGCATCGCGCATCCCCGGATCGCGCATCGTGAC
(3') - AGGCCTAGCGCTAGGGCTAGCGCTAGGGCTAGCGCTAGCACTG

TCAGCGATCGGATCGCGCATCGGATCGCGCATCGCGCATCGG
AGTCGCTAGCCTAGCGCTAGCGCTAGCGCTAGCGCTAGCG

2.11 DNA cleavage reactions:

The GKH-Fos/Jun heterodimer and equimolar Cu(II) were first allowed to equilibrate for 45 min at room temperature. The following reagents were added prior to the addition of DNA: 5 ml of 5 X reaction buffer (50 mM Tris (pH 7.5), (0-25 mM) DTT, 125 mM KCl, 250 mM NaCl, 0.5% NP-40, 50% glycerol). The reaction mixture also contained 1.0 μg of non-specific DNA (poly(dI-dC)), 0.25 mM H₂O₂, 2.5 mM ascorbate and approximately 20,000 CPM of ³²P-end labeled DNA substrate. Water was added to a final volume of 25 ml. The cleavage
reaction was allowed to proceed for various times (1-16 h) at 37°C. The reaction was stopped by the addition of SDS to a final concentration of 0.4% (wt/v). The DNA was then extracted by phenol-chloroform and mixed with 4 μl of gel loading dye. Approximately 10,000 CPM of DNA substrate was loaded onto a denaturing 15% (1:20 crosslinked) urea polyacrylamide gel. The gel was pre-run for 1 h at 1700V, followed by electrophoresis for 3 h, dried and exposed to X-ray film overnight at room temperature.

2.12 DNaseI footprinting:

The DNaseI footprinting method used is a modified version of a method described by Leblanc and Moss (Kneale, 1994). (c/GKH)-Fos/Jun heterodimers and single strand 5'-32P-end labeled duplex 49 bp DNA fragment,

49t (5') -CGCTCACCGATAAAAATACGTATGACTCATTTACGTACGTGG- (3')
49b (3') -GCGAGTAGTCATTTTTTTATGCATAGTGAATGCAATGCACC- (5')

were allowed to equilibrate for 30 min in a binding buffer containing 5% glycerol, 10 mM Tris-Cl pH 7.5, 1 mM DTT, 0.2 mg/ml BSA, 0.1 mg/ml poly(dI-dC), 60 mM KCl, 20 μM EDTA, 0.5 mM MgCl₂ and 0.5 mM CaCl₂ in a total volume of 100 μl. Thereafter, 10 units of the DNaseI enzyme was added to each reaction tube.

After exactly 2.5 min of digestion, the reactions were stopped by the addition of 100 μl stop solution containing 1% SDS (wt/v), 200 mM NaCl, and 20 mM EDTA. One volume of phenol: chloroform (200 μl) was then added to extract the reaction products. After brief centrifugation, the aqueous phase was
removed. To the aqueous phase, 50 µl of 3M NaOAc, 1 µl of 10 mg/ml tRNA and 1 ml of 100% ethanol was added and the DNA precipitated by incubation at -70°C for 20 min, and centrifuged at 14,000 rpm for 20 min. The supernatant was then removed and the radioactive DNA pellet was washed by the addition of 500 µl 80% ethanol (-20°C), centrifuged for 5 min and the supernatant again removed. The DNA pellet was dried in a speedVac for 10 min and resuspended in a loading buffer containing 7 M urea, 0.1 X TBE, 0.05% xylene cyanol and 0.05% bromophenol blue. The DNA was denatured by heating the samples at 90°C for 3 min. Approximately 15,000 CPM of DNA substrate was then loaded onto a denaturing 15% (1:20 crosslinked) urea polyacrylamide gel. The gel was pre-run for 1 h at 1700 V, followed by electrophoresis for 3 h, dried and exposed to X-ray film overnight at -70°C.
Chapter Three

Results and Discussion
The designed GKH-Fos/Jun heterodimer was previously reported to site-specifically cleave the DNA 35 bp 5' of the AP-1 site in the presence of Cu(II), H$_2$O$_2$ and ascorbate (Harford et al., 1996). In an effort to increase the efficiency of DNA cleavage by this heterodimer, which requires a 16-20 hour incubation time before detection of substantial cleavage, the conserved cysteine residues (C272 in Jun, C154 in cFos and GKH-Fos) present in the DNA binding domain of these proteins were mutated to serine residues. These cysteine residues are thought to be sensitive to the oxidizing nature of the cleavage reaction, and therefore weaken the heterodimer-AP-1 DNA interaction. Indeed, a stable Jun/Fos-AP-1 complex was attained for X-ray crystallography only when these cysteine residues were mutated into serines (Glover & Harrison, 1995).

3.1 Expression and purification of native and C-S mutant GKH-Fos/Jun and cFos/Jun heterodimers:

Previously, expression plasmids for the DNA binding and dimerization domains of GKH-Fos and Jun were constructed and the proteins were individually expressed in *E. coli* BL21 (DE3) strain and purified (Harford et al., 1996). GKH-Fos (139-211) was expressed in a p2R expression vector and purified using BioRex 70 cation exchange column chromatography while Jun (248-334) was expressed as a GST fusion protein in pGEX-4T-2 vector and subsequently purified through a Glutathione Sepharose column and BioRex 70 cation exchange column after cleavage with thrombin. Although the level of expression of both GST-Jun and GKH-Fos was high, approximately 30% of total *E. coli* protein
content, GST-Jun was highly insoluble (approximately 95% of GST-Jun was present in inclusion bodies), while GKH-Fos was present equally in the soluble and insoluble fractions. GST-Jun was solubilized in the presence of 8M urea and renatured. However, the renatured protein no longer bound to the Glutathione Sepharose column with high affinity, presumably due to improper refolding.

In an attempt to increase the solubility of GST-Jun during expression in *E. coli*, the expression plasmid of GST-Jun was co-transformed with that of GKH-Fos (or cFos). GST-Jun and (c/GKH-)Fos were then co-expressed and co-purified. Co-expression with (c/GKH-) Fos did increase the solubility of GST-Jun. Figure 3.1 displays an SDS-PAGE analysis of the expression and purification of the C-S mutant GKH-Fos/Jun heterodimer. The native and C-S mutant GKH-Fos/Jun as well as the cFos/Jun heterodimers were purified in the same manner. A band with the apparent molecular weight of 10 kD corresponds to purified C-S mutant GKH-Fos (9kD) and C-S mutant Jun (11 kD) which can not be distinguished in this case by SDS-PAGE. A band of molecular weight 37 kD corresponds to the GST-Jun fusion protein, while a band of molecular weight 26 kD corresponds to the GST moiety alone. The purity of the (c/GKH-)Fos/Jun heterodimer was estimated to be at least 95%. This analysis demonstrates that the purification procedure was adequate for subsequent experiments.
Figure 3.1 SDS-PAGE analysis of the purification of the C-S mutant GKH-Fos/Jun heterodimer. a: molecular weight markers, b,e: eluant from glutathione affinity resin, c: soluble cell extract, d: flow through of glutathione affinity resin, f: product of thrombin cleavage of GST-Jun and GKH-Fos, g: flow through of BioRex cation exchange column, h,i: eluants from wash with 25 mM phosphate buffer containing 0.5 M NaCl, 0.05% NP-40, 10% glycerol and 1 mM DTT j-o: purified C-S mutant GKH-Fos and Jun monomers eluted by successive washes (1-6) with 1 M NaCl, 25 mM phosphate buffer containing 0.05% NP-40, 10% glycerol and 1 mM DTT.
3.2 Effect of the mutation of a conserved cysteine residue into serine on the DNA binding activity of the GKH-Fos/Jun heterodimer under various oxidizing conditions.

Gel-MSA results presented here demonstrate that the mutant C-S GKH-Fos/Jun heterodimer binds to the AP-1 site with higher affinity compared to the native heterodimer under oxidizing conditions. Figure 3.2 displays a gel-MSA analysis of the mutated C-S GKH-Fos/Jun heterodimer (with a 1:3 molar ratio of Jun:GKH-Fos in order to ensure quantitative heterodimerization) binding to a 36 bp DNA containing 2 AP-1 sites in the presence of 0.25 mM ascorbate, 0.25 mM H$_2$O$_2$ and various (0-4 mM) DTT concentrations. The need for the presence of reducing agents in the binding reaction appears to be abolished for the mutant C-S GKH-Fos/Jun heterodimer; the mutant C-S GKH-Fos/Jun heterodimer binds to the AP-1 DNA equally well under 4, 2 and even 0 mM DTT concentrations. The oxidation of the conserved cysteine residues in Jun and Fos has previously been shown to occur rapidly when the concentration of reducing agents in the buffer (be it chemical reducing agents such as DTT or nuclear redox factor (REF-1)) fall below 0.2 mM (Xanthoudakis & Curran, 1994). The fact that the C-S mutant GKH-Fos/Jun dimer retains DNA binding activity in the absence of reducing agents demonstrates that the DNA binding activity of the mutant dimer is no longer sensitive to oxidation.

Figure 3.3 displays a comparison of the DNA binding activity of the native and C-S mutant GKH-Fos/Jun heterodimer under varying H$_2$O$_2$ and ascorbate concentrations (0.2-13 mM). While at 12 mM ascorbate and 12 mM H$_2$O$_2$ buffer
Figure 3.2 Gel-MSA analysis of C-S mutant and native GKH-Fos/Jun heterodimer DNA binding activity under varying DTT concentrations. The ability of the C-S mutant and native GKH-Fos/Jun heterodimers (3:1 molar ratio GKH-Fos:Jun) to bind to a 36 bp DNA fragment containing two AP-1 sites was tested under increasing DTT concentrations. Lanes a-c correspond to C-S mutant GKH-Fos/Jun-AP-1 interaction, a: 0 mM DTT, b: 2 mM DTT, c: 4 mM DTT. Lanes d-f correspond to native GKH-Fos/Jun-AP-1 interaction, d: 0 mM DTT, e: 2 mM DTT, f: 4 mM DTT. All binding reactions included approximately 150 ng GKH-Fos/Jun heterodimer, < 100 pM DNA, 1 µg poly(dI.dC), 0.25 mM H2O2 and 0.25 mM ascorbate.
Figure 3.3 Gel-MSA comparison of native and C-S mutant GKH-Fos/Jun heterodimers under varying oxidizing conditions. The ability of the C-S mutant and native GKH-Fos/Jun heterodimers to bind to a 20 bp DNA fragment (<10 pM) containing one copy of the AP-1 consensus site were compared under varying H$_2$O$_2$ and ascorbate concentrations. In all reactions, the GKH-Fos:Jun molar ratio was 1:1. a: DNA alone, b: 50 ng native GKH-Fos/Jun, c: 50 ng C-S mutant GKH-Fos/Jun (lanes b and c correspond to binding reactions carried out under 0.25 mM ascorbate and 0.25 mM H$_2$O$_2$ concentrations), d: 100 ng native GKH-Fos/Jun, e: 100 ng C-S mutant GKH-Fos/Jun (lanes d and e correspond to binding reactions carried out under 2.5 mM ascorbate and 2.5 mM H$_2$O$_2$ concentrations, f: 100 ng native GKH-Fos/Jun, g: 100 ng C-S mutant GKH-Fos/Jun (lanes f and g correspond to binding reactions carried out under 12 mM ascorbate and 12 mM H$_2$O$_2$ concentrations. All binding reactions also included 1 mM DTT and 1 µg poly (dI.dC).
concentrations the DNA binding capability of the native GKH-Fos/Jun heterodimer is lost, the mutant C-S GKH-Fos/Jun heterodimer retains its capability to bind to the AP-1 site. Collectively these results indicate that the mutant C-S GKH-Fos/Jun heterodimer is not sensitive to oxidizing conditions or the absence of reducing agents and appears to retain its AP-1 binding affinity under these conditions. Thus, we achieved one of our primary goals to produce a Fos/Jun dimer which is capable of binding stably to the AP-1 site in the absence of DTT, which may interfere with the free radical mediated mechanism of cleavage. We were also able to reduce the amount of glycerol present in the binding buffer from 10% to 5% (v/v) without compromising the stability and DNA binding activity of the designed protein. Stable binding of the designed protein to the DNA under the cleavage reaction conditions is a pre-requisite for more efficient DNA cleavage by the GKH-Fos/Jun heterodimer.

3.3 DNA cleavage:

Site-specific DNA cleavage by the GKH-Fos/Jun heterodimer was originally reported to occur approximately 35 bp 5' of the AP-1 site on each DNA strand on a 100 bp AP-1 site containing DNA substrate. However, the 100 bp DNA substrate was synthetically generated and contained impurities and degradation fragments. In order to overcome the problem of degradation and generate pure DNA substrates for cleavage experiments, PCR amplification was used to generate 100 bp and 126 bp AP-1 site containing DNA substrates from plasmids AP-1 pRSETA and AP-1 PSE420, respectively. The 100 bp and 126 bp
AP-1 DNA fragments (containing different sequences flanking the AP-1 site) were used in cleavage reactions with the C-S mutant GKH-Fos/Jun heterodimer in the presence of Cu(II), ascorbate and H₂O₂. The cleavage reaction time was varied from 4 h to overnight incubations. The site of specific cleavage appeared to be 25 – 38 bp 5' of the AP-1 site on each DNA strand in the 100 bp and 126 bp DNA fragments. Figure 3.4 illustrates the sites of cleavage on both DNA cleavage substrates used.

However, results from cleavage experiments were not consistently reproducible; depending on the protein preparation, DNA cleavage did not always occur. In addition, using certain protein preparations, DNA cleavage occurred not only in the cleavage reaction mixtures containing Cu(II)-GKH-Fos, but also in the control reactions containing cFos (which lacks the ATCUN motif). In order to ensure the presence of the GKH cleavage moiety of the GKH-Fos protein sample, and the absence of the motif from the cFos sample, N-terminal sequence analysis was conducted on these proteins. Results from N-terminal sequencing confirmed the presence of the ATCUN motif in the GKH-Fos preparation and its absence from the cFos sample.

As shown in Figures 3.5 – 3.8, incubation of the 100 bp and 126 bp AP-1 site containing DNA substrates with DNaseI alone, 40 μM Cu(II) or in some cases ascorbate and H₂O₂ alone resulted in the generation of a similar pattern of DNA cleavage products as those generated by the cleavage reaction. The finding that treatment of the AP-1 DNA substrates with DNaseI alone generates a similar pattern of DNA cleavage as that induced by Cu(II) GKH-Fos/Jun, suggests that
Figure 3.4  Sequence of DNA cleavage substrates used in cutting reactions showing sites of cleavage. The arrows indicate positions of observed site-specific DNA cleavage by the C-S mutant and native GKH-Fos/Jun heterodimers. The site of cleavage seems to occur from 25-38 bp 5' of the AP-1 site on each DNA strand on both the 126 bp and 100 bp DNA cleavage substrates.
Figure 3.5  Denaturing polyacrylamide gel analysis of 100 bp top strand end-labeled AP-1 DNA cleavage under various conditions. Approximately 0.01 pmoles of DNA was used in each reaction. M: DNA marker, arrows indicate the size (in number of bases) of the corresponding fragments. a: DNA incubated overnight at 37°C with 1 μM Cu(II) in the presence of 0.25 mM ascorbate, 0.25 mM H₂O₂, b: DNA alone incubated overnight at 37°C in the presence of 0.25 mM ascorbate and 0.25 mM H₂O₂, lanes c-e correspond to DNA incubated at 37°C with 50 nM C-S mutant Cu(II)-GKH-Fos/Jun, 0.25 mM ascorbate and 0.25 mM H₂O₂, c: 4 h incubation, d: 6 h incubation, e: overnight incubation, f: DNA alone digested for 10 s with 1 unit of DNaseI, g: DNA-C-S mutant GKH-Fos/Jun complex digested for 10 s with 1 unit of DNaseI, h: DNA-cFos/Jun complex digested for 10 s with 1 unit of DNaseI, i: DNA incubated with 50 nM Cu(II)-GKH-Fos/Jun native, 0.25 mM ascorbate and 0.25 mM H₂O₂ overnight at 37°C. The main DNA fragments generated (as indicated by arrows) are 14, 16, 18 and 25 bases long.
Figure 3.6 Denaturing polyacrylamide gel analysis of 100 bp bottom strand end-labeled AP-1 DNA cleavage under various conditions. Approximately 0.01 pmoles of DNA was used in each cleavage reaction. M: DNA marker, arrows indicate the size (in number of bases) of the corresponding fragments. a: DNA incubated overnight at 37°C with 1 μM Cu(II) in the presence of 0.25 mM ascorbate and 0.25 mM H₂O₂, lanes b-d correspond to DNA incubated at 37°C with 50 nM C-S mutant Cu(II) GKH-Fos/Jun, 0.25 mM ascorbate and 0.25 mM H₂O₂ (b: 4 h incubation, c: 6 h incubation, d: overnight incubation), e: DNA alone incubated overnight at 37°C, f: DNA incubated overnight at 37°C in the presence of 0.25 mM ascorbate and 0.25 mM H₂O₂, g: DNA alone digested for 10 s with 1 unit of DNaseI. The main DNA fragment generated (as indicated by arrows) is 15 bases long.
Figure 3.7 Denaturing polyacrylamide gel analysis of 126 bp top strand end-labeled AP-1 DNA cleavage under various conditions. Approximately 0.01 pmoles of DNA was used in each reaction. M: DNA marker, arrows indicate the size (in number of bases) of the corresponding fragments. a: DNA alone digested for 10 s with 1 unit of DNaseI, b: DNA-C-S mutant GKH-Fos/Jun complex digested for 10 s with 1 unit of DNaseI, c: DNA alone incubated overnight at 37°C, d: DNA incubated overnight at 37°C in the presence of 0.25 mM ascorbate and 0.25 mM H₂O₂, e: DNA incubated overnight at 37°C with 1 μM Cu(II) in the presence of 0.25 mM ascorbate, 0.25 mM H₂O₂, f: DNA incubated overnight at 37°C with 50 nM C-S mutant cFos/Jun, g: DNA incubated at 37°C with 50 nM C-S mutant Cu(II)-GKH-Fos/Jun, 0.25 mM ascorbate and 0.25 mM H₂O₂. The main DNA fragments generated (as indicated by arrows) are 17 and 25 bases long.
Figure 3.8 Denaturing polyacrylamide gel analysis of 126 bp bottom strand end-labeled AP-1 DNA cleavage under various conditions. Approximately 0.01 pmols of DNA was used in each reaction. M: DNA marker, arrows indicate the size (in number of bases) of the corresponding fragments. a: DNA alone incubated overnight at 37°C, b: DNA incubated overnight at 37°C in the presence of 0.25 mM ascorbate and 0.25 mM H₂O₂, c: DNA incubated overnight at 37°C with 1 μM Cu(II) in the presence of 0.25 mM ascorbate and 0.25 mM H₂O₂, d: DNA incubated overnight at 37°C with 50 nM C-S mutant cFos/Jun, 0.25 mM ascorbate and 0.25 mM H₂O₂, lanes e and f: DNA incubated overnight at 37°C with 50 nM C-S mutant Cu(II)-GKH-Fos/Jun, 0.25 mM ascorbate and 0.25 mM H₂O₂, g: DNA alone incubated overnight at 37°C, h: DNA incubated overnight at 37°C with 50 nM C-S mutant Cu(II)-GKH-Fos/Jun, 0.25 mM ascorbate and 0.25 mM H₂O₂, i: DNA alone digested for 10 s with 1 unit of DNaseI, j: DNA-C-S mutant GKH-Fos/Jun complex digested for 10 s with 1 unit of DNaseI. The main DNA fragment generated (as indicated by arrows) is 22 bases long.
perhaps contamination of the cleavage reaction mixture with endoI, a bacterial endonuclease present in the BL-21 (DE3) E. coli strain, and not "specific" cleavage by the designed protein, was responsible for generating the observed DNA cleavage. In fact, in support of this possibility, endoI has been found to be extremely difficult to remove from bacterial DNA preparations, even after alkaline lysis treatment (Pulleyblank, 1998).

Alternatively, comparison of the similar DNA cleavage pattern induced by Cu(II) alone in the presence of ascorbate and H₂O₂, to that induced by the cleavage reaction, suggests that "specific" cleavage may have been due to the susceptibility of the DNA fragment to cleavage by excess Cu(II) (which may have become free in solution over the course of the cleavage reaction incubation time) in the reaction mixture. A third possibility is that DNA bending induced by the GKH-Fos/Jun dimer may be responsible for altering DNA structure such that sequences approximately 35 bp 5' of the AP-1 site become readily susceptible to cleavage. In support of the possibility that the originally observed DNA cleavage by Cu(II)-GKH-Fos/Jun was due to experimental artifacts, is the fact that glycerol, which was present in the cleavage reaction mixture is a hydroxyl radical quencher. It has been reported that glycerol concentrations as low as 0.5% (v/v) significantly inhibit cleavage of DNA by the hydroxyl radical (Dixon et al., 1991). The Cu(II)-GKH-Fos/Jun cleavage reaction mixtures contained 5-10% (v/v) glycerol. The presence of such high glycerol concentrations would therefore prevent the hydroxyl radical mediated cleavage of DNA.
3.4 DNaseI footprinting of the cFos/Jun and GKH-Fos/Jun heterodimers:

In order to examine and compare the specificity of binding of the cFos/Jun and GKH-Fos/Jun heterodimers and to ensure that the addition of the GKH motif N-Terminus of the Fos bZIP domain has not altered the DNA binding specificity of the GKH-Fos/Jun heterodimer, DNaseI footprinting experiments were conducted. In addition, DNaseI footprinting is a method used to detect DNA looping (Hochschild, 1991). One model by which to explain the observed distance between the site of cleavage by GKH-Fos/Jun and the AP-1 site was that a DNA looping phenomenon induced by the Cu(II)-GKH motif may bring distant DNA sites into close proximity to the AP-1 site, presumably the site of GKH-Fos/Jun binding. DNaseI footprinting experiments were therefore conducted to examine the proposed DNA looping model.

DNaseI footprinting is an assay used to locate specific protein-DNA contacts. The basis of the method is that the DNA region (the phosphodiester back bone) that is bound by the protein is protected from digestion by DNaseI while the rest of the DNA is cleaved. DNaseI is an endonuclease, which interacts with a 6 bp DNA contact region within the minor groove and bends the DNA towards the major groove (Suck, 1994; Weston et al., 1992). The enzyme then cleaves the DNA phosphodiester backbone through a mechanism involving an activated water molecule (Stryer, 1988). If the DNA flanking the site of protein binding is looped, DNaseI footprinting of the complex results in the appearance of an enhanced and diminished pattern of cleavages (Hochschild, 1991). This
cleavage pattern reflects the distortion of the phosphate backbone, i.e. the narrowing and widening of the minor groove on the inside and outside of the loop, respectively, which results in a change in the susceptibility of DNA to cleavage by DNaseI.

The specific interaction of both the bZIP domains of the Fos/Jun heterodimer (Abate et al., 1990) as well as the full length dimer (Allegretto et al., 1990) with the AP-1 site has been previously examined by DNaseI footprinting. In both cases a clear area of protection was observed, which corresponds to the AP-1 site. The X-ray crystal structure of the Fos/Jun bZIP domains bound to the AP-1 site indicates the presence of electrostatic interactions between the basic region side chains and the DNA phosphodiester backbone and hydrogen bonding and van der Waals contacts between the conserved amino acid side chains of Jun and Fos and the bases of the AP-1 site (Glover & Harrison, 1995). Figure 3.9 illustrates a DNaseI protection assay of the interaction between a 49 bp AP-1 site containing DNA fragment and the C-S mutant as well as native cFos/Jun and GKH-Fos/Jun heterodimers. Results indicate that the addition of the GKH motif N-Terminus of the Fos bZIP domain has not altered the DNA binding specificity of the Fos/Jun heterodimer; all four heterodimers bind to a region of approximately 7 bp, as indicated by a bracket, which corresponds to the AP-1 site. However, the higher concentration of protein required for the GKH-Fos/Jun dimer to exhibit complete protection of the AP-1 site reflects a decreased stability and perhaps decreased specificity of the GKH-Fos/Jun-AP-1 interaction relative to that of the cFos/Jun-AP-1 complex. In addition, DNaseI footprinting
Figure 3.9 DNaseI footprinting titration experiment with C-S mutant and native cFos/Jun and GKH-Fos/Jun proteins bound to a 49 bp DNA fragment containing the AP-1 site. Lane M represents a 2 base DNA marker, lanes a, h and l: DNaseI digestion of approximately 0.01 pmoles of free AP-1 oligo-nucleotide; lanes b-d, e-g and i-n represent DNaseI digestion of approximately 0.01 pmoles of AP-1 oligonucleotide, which had been pre-incubated with increasing concentrations of heterodimer as indicated: lanes b, c and d: 10 nM, 50 nM, and 250 nM, respectively, of C-S mutant cFos/Jun, lanes e, f and g: 10 nM, 50 nM, and 250 nM, respectively, of native cFos/Jun, lanes i, j and k: 10 nM, 50 nM, and 250 nM, respectively, of C-S mutant GKH-Fos/Jun, lanes m, n and o: 10 nM, 50 nM, and 250 nM, respectively, of native GKH-Fos/Jun. The bases corresponding to the AP-1 site are indicated by a bracket.
results do not indicate the presence of DNA looping induced by the GHK-Fos/Jun heterodimer; the cleavage patterns resulting from DNaseI footprinting of the cFos/Jun and GKH-Fos/Jun-DNA complexes appear to be similar. The DNaseI footprint pattern of the Cu(II) bound GKH-Fos/Jun heterodimer also appears to be similar to that of the cFos/Jun heterodimer and therefore does not indicate DNA looping.

3.5 Comparison of the specific DNA binding affinity of the native and C-S mutant cFos/Jun and GKH-Fos/Jun heterodimers under reducing conditions.

We have used electrophoretic mobility shift titration experiments in order to determine and compare the DNA binding constants of the C-S mutant and native (c/GKH-)Fos/Jun heterodimers under reducing conditions (2 mM DTT). The obtained apparent $K_d$ values correspond to the specific binding affinity of the heterodimers for AP-1 site containing DNA in the presence of a large excess of nonspecific competitor DNA (poly(dI.dC)). The apparent $K_d$ values were determined by two different gel retardation based methods. According to the first method used, the apparent $K_d$ values were obtained by conducting a DNA titration experiment: a fixed amount of protein (from 5 - 100 nM depending on the experiment) was titrated with increasing concentrations of $^{32}$P-labeled AP-1 site containing DNA. Figures 3.10 depicts an example of such an experiment conducted with the C-S mutant and native cFos/Jun heterodimers. The free and protein bound DNA were resolved by electrophoresis, the bands excised,
Figure 3.10  DNA titration experiment of the C-S mutant and native cFos/Jun heterodimers. Approximately 50 ng of cFos/Jun heterodimer (in a 3:1 molar ratio of cFos:Jun) was used in each binding reaction with increasing concentrations of a 49 bp AP-1 DNA.  

a) DNA titration of C-S mutant cFos/Jun heterodimer with (lane 1: 2.8 nM, lane 2: 5.7 nM, lane 3: 8.1 nM, lane 4: 12.5 nM, lane 5: 30.1 nM, lane 6: 44.2 nM, lane 7: 53.1 nM, lane 8: 73.9 nM, lane 9: 86.2 nM) AP-1 DNA.  
b) DNA titration of native cFos/Jun heterodimer with (lane 1: 4.3 nM, lane 2: 4.1 nM, lane 3: 13.7 nM, lane 4: 21.8 nM, lane 5: 27.5 nM, lane 6: 35.9 nM, lane 7: 46.3 nM, lane 8: 50.6 nM, lane 9: 62.1 nM) AP-1 DNA.
counted by scintillation counting, and the values converted to concentrations using the specific activity of the DNA. The apparent $K_d$ values were then determined by Scatchard analysis with the slope of the line of bound versus bound/free DNA corresponding to $-1/K_d$ (Figures 3.11 and 3.12). Using the DNA titration experiments, we determined the apparent $K_d$ values as $8.4 \pm 2.2$ nM, $15.7 \pm 4.5$ nM, $56.8 \pm 9.1$ nM and $96.1 \pm 21.7$ nM for the C-S mutant cFos/Jun, native cFos/Jun, C-S mutant GKH-Fos/Jun and native GKH-Fos/Jun heterodimers, respectively. To further confirm these results, the dissociation constants were determined in parallel by titrating a fixed amount of DNA (approximately 10 pM) with increasing concentrations of heterodimer. Figure 3.13 illustrates such a protein titration experiment using the native cFos/Jun and GKH-Fos/Jun heterodimers. Under conditions in which the protein concentration is in great excess (more than 10 fold in excess) of the DNA concentration, the protein concentration at which half of the DNA is bound is a good approximation of the $K_d$ value (Carey, 1991). The apparent binding constants for the four heterodimers were estimated accordingly (Figure 3.14) as $4.2$ nM, $12.1$ nM, $26.8$ nM and $73.9$ nM for the C-S mutant cFos/Jun, native cFos/Jun, C-S mutant GKH-Fos/Jun and native GKH-Fos/Jun heterodimers, respectively.

The apparent $K_d$ values obtained by the two independent methods are within a two fold difference of one another and therefore agree well. Figure 3.15 displays a bar graph depiction (for comparative purposes) of the apparent AP-1 binding constants of the C-S mutant and native cFos/Jun and GKH-Fos/Jun
Figure 3.11  Representative $K_d$ determination experiment: scatchard analysis of C-S mutant and native cFos/Jun heterodimers binding to a 49 bp AP-1 DNA. The slope of the Scatchard plot is equal to the negative reciprocal of the dissociation constant and the x-intercept equals the total active dimer concentration ($P_T$). Scatchard plot analysis using 4 separate such titration experiments for each protein reveals an average dissociation constant ($K_d$) of $8.4 \pm 2.2$ nM for the C-S mutant cFos/Jun dimer and $15.7 \pm 4.5$ nM for the native cFos/Jun dimer.
Figure 3.12  Representative $K_d$ determination experiment: scatchard analysis of C-S mutant and native GKH-Fos/Jun heterodimers binding to a 49 bp AP-1 DNA. The slope of the Scatchard plot is equal to the negative reciprocal of the dissociation constant and the x-intercept equals the total active dimer concentration ($P_T$). Scatchard plot analysis using 4 separate such titration experiments for each protein reveals an average dissociation constant ($K_d$) of $56.8 \pm 9.1$ nM for the C-S mutant GKH-Fos/Jun dimer and $96.1 \pm 21.7$ nM for the native GKH-Fos/Jun dimer.
Figure 3.13  Kd determination assay of native cFos/Jun and GKH-Fos/Jun heterodimers by protein titration. a) Titration of radiolabeled 49 bp AP-1 probe (<10 pM) with increasing amounts of native cFos/Jun heterodimer. The amount of cFos/Jun heterodimer ranged from 0.6 to 1000 nM. Lane 1: 0.6 nM, 2: 1.3 nM, lane 3: 6.3 nM, lane 4: 13 nM, lane 5: 19 nM, lane 6: 25 nM, lane 7: 63 nM, lane 8: 250 nM, lane 9: 1000 nM. b) Titration of AP-1 probe with increasing amounts of native GKH-Fos/Jun heterodimer. The amount of GKH-Fos/Jun heterodimer ranged from 1 to 3000 nM. Lane 1: 1 nM, 2: 6 nM, lane 3: 13 nM, lane 4: 25 nM, lane 5: 75 nM, lane 6: 125 nM, lane 7: 625 nM, lane 8: 1250 nM, lane 9: 3000 nM.
Figure 3.14  Semilogarithmic plot of fraction of AP-1 bound as a function of heterodimer concentration. The Kd values are estimated as the heterodimer concentration at which half of the DNA is bound. The DNA binding constants shown above correspond to the average of 4 separate experiments. The error bars represent one standard deviation of the mean.
Figure 3.15  Bar graph depiction of heterodimer $K_d$ values. The AP-1 binding constants shown in black bars correspond to those obtained from Scatchard analysis. The apparent $K_d$ values are $8.4 \pm 2.2$ nM (C-S mutant cFos/Jun), $56.8 \pm 9.1$ nM (C-S mutant GKH-Fos/Jun), $15.7 \pm 4.5$ nM (native cFos/Jun) and $96.1 \pm 21.7$ nM (native GKH-Fos/Jun). The AP-1 binding constants shown in empty bars correspond to the values obtained from protein titration experiments. The apparent $K_d$ values are $4.2$ nM (C-S mutant cFos/Jun), $26.8$ nM (C-S mutant GKH-Fos/Jun), $12.1$ nM (native cFos/Jun) and $73.9$ nM (native GKH-Fos/Jun).
heterodimers. The cysteine to serine mutation results in an approximately two fold increase in the DNA binding affinity of the cFos/Jun heterodimer. According to the crystal structure of the Fos/Jun-AP-1 complex (Glover & Harrison, 1995), the cysteine-serine residue is not involved in making DNA specific contacts. The substitution of an oxygen atom in serine for the sulfur in cysteine was thought not likely to induce any major changes in the local structure of the basic domain. However, OH is a better proton donor than SH, and this could have an affect on the stability of the α helical basic domain. Any change in helix stability may in turn affect DNA binding affinity. The slight decrease in $K_d$ may also reflect a modest increase in stability of the C-S mutant Fos/Jun heterodimer due to a decrease in its susceptibility to oxidation.

It has been demonstrated that the full length Fos/Jun heterodimer binds to the AP-1 site with a $K_d = 2 \times 10^{-10}$ M (Smeal et al., 1989). Comparison of this value with the $K_d$ value that we have obtained for the bZIP domains of the Fos/Jun heterodimer, $1.57 \times 10^{-8}$ M indicate that regions outside of the bZIP domain must be required for further stabilizing the Jun/Fos-AP-1 complex. In fact similar studies comparing the apparent DNA binding affinity of the bZIP domain (Santiago-Rivera et al., 1993) and full length (Williams et al., 1993) CREB homodimer, another member of the bZIP family transcription factors, indicate that the full length CREB dimer binds the CRE site with a 200 fold higher affinity than the bZIP domain alone. Our $K_d$ value of $1.57 \times 10^{-8}$ M of the cFos/Jun (native)--AP-1 interaction is within a three fold difference of a previously reported value of $5 \times 10^{-8}$ for the AP-1 binding constant of the bZIP domains of
the Fos/Jun heterodimer (John et al., 1996). The apparent three fold difference in these values is not surprising given the fact that sequences outside of the core 7-nucleotide AP-1 site have been shown to affect the DNA binding affinity of the Fos/Jun dimer (Kerppola & Curran, 1994).

The addition of GKH motif to the N-Terminus of the Fos bZIP domain results in a 6-7 fold lowering of DNA binding affinity of the Fos/Jun heterodimer. This drastic increase in $K_d$ value was unexpected for several reasons. The GKH tripeptide was attached to the N-terminus of Fos. Based on structural information from the Jun/Fos-AP-1 complex, the GKH motif, which lies outside of the DNA binding (basic) region of Fos, was thought not to interfere with or disrupt any specific protein-DNA contacts, which would result in a lower AP-1 binding affinity. In addition, lysine, the second residue of the ATCUN motif has a high propensity for $\alpha$ helicity (Johnson et al., 1994) and was chosen as the second residue of the ATCUN motif because the basic region of Fos is highly $\alpha$ helical upon DNA binding. Furthermore, the positively charged side chain of lysine was thought to enhance DNA binding by the designed protein due to favorable electrostatic interactions with the DNA phosphate backbone.
3.6 Comparison of the binding affinities of the native and C-S mutant GKH-Fos/Jun and cFos/Jun heterodimers for nonspecific DNA.

To examine what effect the addition of the GKH motif to Fos has on the nonspecific DNA binding affinity of the Fos/Jun heterodimer, gel shift competition experiments were conducted and the relative nonspecific DNA binding affinities of the C-S mutant and native cFos/Jun and GKH-Fos/Jun heterodimers determined. To preformed $^{32}$P-labeled AP-1-heterodimer complexes, increasing concentrations of nonspecific competing DNA ligand, DR-2 (the retinoic acid receptor response element) was added and the stability of the specific protein-DNA complex was compared to that of the nonspecific DNA-protein complex. Figure 3.16 illustrates an example of such an experiment conducted with the C-S mutant cFos/Jun and GKH-Fos/Jun heterodimers. The protein bound and free AP-1 DNA were resolved by electrophoresis, the bands excised, counted by scintillation counting, and the percent inhibition of the specific protein-DNA complex plotted as a function of increasing DR-2 concentration added. At the 50% inhibition point, the nonspecific DNA binding affinity of the heterodimers was determined (see Experimental Procedures) (Figure 3.17).

Comparison of the nonspecific DNA binding affinities of the GKH-Fos/Jun and cFos/Jun heterodimers indicates that addition of the positively charged GKH motif to the basic domain of Fos results in an approximately 4 fold increase in affinity of the Fos/Jun dimer for nonspecific DNA (DR-2). In support
Figure 3.16 Gel retardation competition experiment quantifying the relative nonspecific DNA binding affinities of a) C-S mutant cFos/Jun and b) C-S mutant GKH-Fos/Jun heterodimers. Preformed radiolabeled AP-1 (<10 pM)-heterodimer complexes were incubated with increasing amounts of unlabeled nonspecific DNA (DR-2) and the disappearance of the AP-1-heterodimer complex was monitored. The total protein concentration used was 2.5 X 10⁻⁷ M for C-S mutant cFos/Jun and 1 X 10⁻⁶ M for C-S mutant GKH-Fos/Jun. The concentration of DR-2 ranged from 0.34 - 174 μM; lane 1: 0.34 μM, lane 2: 0.68 μM, lane 3: 1.38 μM, lane 4: 2.72 μM, lane 5: 5.44 μM, lane 6: 10.9 μM, lane 7: 21.8 μM, lane 8: 43.5 μM, lane 9: 87 μM, lane 10: 174 μM DR-2.
Figure 3.17 Semilogarithmic plot of the percent inhibition of specific AP-1 heterodimer complexes as a function of added nonspecific DNA (DR-2) in units of molar base pairs. The relative competition binding affinities (K\text{comp}) of the heterodimers were calculated as described in Experimental Procedures. The competition binding affinities were determined as $6.4 \times 10^{-5}$ M (C-S mutant cFos/Jun), $6.9 \times 10^{-5}$ M (native cFos/Jun), $1.9 \times 10^{-5}$ M (C-S mutant GKH-Fos/Jun) and $1.8 \times 10^{-5}$ M (native GKH-Fos/Jun). The error bars represent one standard deviation of the mean of 4 separate experiments.
of our results, it has been shown that the presence of the conserved basic cluster in the basic domain of bZIP proteins is not responsible for specific binding to the AP-1/CRE consensus sites, but it is responsible for introducing great affinity for nonspecific DNA (Metallo et al., 1997). The increase in nonspecific DNA binding affinity by the basic cluster is thought to be due to nonspecific electrostatic interactions with the phosphodiester backbone. Our results suggest that the presence of the GKH motif adjacent to the DNA binding domain of Fos contributes to the nonspecific electrostatic element of the Fos/Jun-DNA interaction and thereby increases the affinity of the Fos/Jun dimer for nonspecific DNA.

3.7 Comparison of DNA bending by cFos/Jun and GKH-Fos/Jun heterodimers.

The next question we addressed was whether the addition of the GKH motif to Fos alters the DNA bending properties of the Jun/Fos heterodimer. Using the method of phasing analysis, the Jun and Fos bZIP domains have been shown to bend the DNA (by approximately 12°) at the AP-1 site (Kerppola & Curran, 1996; Kerppola, 1997). However, DNA bending studies of the Jun/Fos bZIP domains using ligase mediated cyclization experiments (Sitlani & Crothers, 1998) and phase sensitive detection (Sitlani & Crothers, 1996) yield the opposite result and do not show DNA bending. To examine and compare DNA bending by cFos/Jun and GKH-Fos/Jun heterodimers, electrophoretic phasing analysis was conducted. This method is based on the phase dependent mobility variation
of protein-DNA complexes in which the phasing probes' spacer distance between the center of the bend under investigation and the center of an intrinsic bend of known magnitude and direction is varied. The reference bend used is a series of 6 Adenines, called an A tract, which contains an intrinsic bend of approximately 18 - 20° toward the minor groove. When the two bends are in phase, they have an additive effect in which the mobility of the DNA probe corresponds to the mobility of a probe containing one bend which equals the sum of the two bends, resulting in slow migration through the acrylamide gel. When the two bends are out of phase, they cancel each other out, resulting in faster mobility through the gel.

Figure 3.18 displays electrophoretic phasing analysis of the C-S mutant cFos/Jun and GKH-Fos/Jun heterodimers. Identical results were obtained for phasing analysis of the native cFos/Jun and GKH-Fos/Jun heterodimers. DR-2, which lacks the AP-1 site was included in the reaction mixture as an internal control for variations in the consistency of the gel and corrections for probe mobility. For analysis of the phasing data, relative mobilities (distance in mm traveled by each protein complexed or free DNA probe normalized to the average mobility of each set of probes) were plotted as a function of spacer distance in bp between the centers of the two bends. The points were then fit to a cosine function (6) (Figure 3.19). The direction of the protein induced bend is determined by correlating the spacer distance, which gives rise to the minimum and maximum of the phasing function, to what is known of the direction of the intrinsic reference bend. For both cFos/Jun and GKH-Fos/Jun heterodimers, the
Figure 3.18 Electrophoretic mobility shift analysis of C-S mutant cFos/Jun and GKH-Fos/Jun heterodimers bound to phasing analysis probes. The heterodimers were incubated with AP-1 phasing probes containing varying spacer lengths between the centers of the AP-1 site and the intrinsic bend, and the protein bound and free probes were resolved by electrophoresis. Lane designations indicate the separation in base pairs between centers of the AP-1 site and A tract.
Figure 3.19  Relative mobilities of C-S mutant cFos/Jun and GKH-Fos/Jun complexes and free probes plotted as a function of the separation in base pairs between the centers of the AP-1 site and the A tract. Relative mobilities correspond to the average of 5 separate phasing analysis experiments conducted with each heterodimer, and the vertical bars represent one standard deviation of the mean of the 5 experiments. The points in each set of data were fit to Equation 6 (a cosine function).
first minimum of the phasing function corresponds to a spacer length of approximately 10.5 bp or one turn of the DNA helix while the second minimum corresponds to a spacer length of two turns of the DNA helix. The maximum of the phasing function is reached at a spacer length of approximately 16 bp or one and a half turns of the DNA helix. Given the fact that the A tract contains an 18-20° bend toward the minor groove, these results indicate that both the cFos/Jun and GKH-Fos/Jun heterodimers bend the DNA toward the minor groove. However, the higher amplitude of the phasing function corresponding to the GKH-Fos/Jun heterodimer compared to that of the cFos/Jun heterodimer indicates that the magnitude of the bend induced by GKH-Fos/Jun heterodimer is greater than that induced by the cFos/Jun heterodimer.

It has recently been demonstrated that the charge of three amino acid residues located N-terminus to the basic domain of bZIP proteins is critical in determining the magnitude and direction of DNA bending by different bZIP dimers (Kerppola & Curran, 1993; Paolella et al., 1994; Paolella et al., 1997; Leonard et al., 1997; Strauss & Maher, 1997; Strauss & Maher, 1998). In fact, it has been proposed that similar to the mechanism by which histone proteins have been shown to bend the DNA, asymmetric DNA phosphate neutralization by the charge of amino acids in this region of the basic domain is the mechanism by which bZIP proteins bend their target DNA (Strauss & Maher, 1997; Strauss & Maher, 1998). The three critical amino acids adjacent to the N-terminus of several bZIP proteins are underlined in Figure 1.3 and displayed in Table 1.1. A net positive charge in this region is thought to correspond to DNA bending away
from the leucine zipper domain (toward the minor groove) as in the case of Fos, while a net negative charge corresponds to a bend toward the leucine zipper region (toward the major groove) as in the case of Jun. The net DNA bending induced by bZIP heterodimers appears to be the difference between the two bends (if the bZIP monomers bend the DNA in opposite directions) or their sum (if the bZIP monomers bend the DNA in the same direction). The net DNA bend induced by the Jun/Fos heterodimer is the difference between the DNA bend induced toward the major groove by Jun and that induced by Fos toward the minor groove, resulting in an overall DNA bend toward the minor groove. In our design, the positively charged GKH motif is attached immediately adjacent to the three critical amino acids in Fos. From our results, the increase in DNA bending induced by the GKH-Fos/Jun heterodimer compared to the cFos/Jun heterodimer suggests that perhaps the charge of amino acids further N-terminal to the three critical amino acids identified previously also contribute to the electrostatic bending mechanism.

3.8 Correlation between DNA bending, DNA binding affinity and DNA binding specificity.

The addition of the positively charged GKH motif results in increased DNA bending and decreased DNA binding affinity and specificity of the Fos/Jun heterodimer. Our results suggest that the energetic cost of the increased DNA bending by GKH-Fos/Jun is paid for through a decrease in DNA binding affinity and specificity of the heterodimer. In fact, there is much evidence
highlighting the link between DNA bending and DNA binding affinity. The CAP protein, which bends its DNA target by approximately 90° (Schultz et al., 1991) has been shown to bind to its prebent target site (constrained in a minicircle) with a 200 fold increased affinity than it does to linear DNA. This suggests that prebending of the CAP binding site contributes to the free energy of binding by the CAP protein (Kahn & Crothers, 1992). Studies of the EcoRI endonuclease binding to and bending its target site have demonstrated that the energetic cost of distorting the DNA restriction site is paid for by a decrease in DNA binding affinity of the EcoRI endonuclease (Lesser et al., 1993).

Selective association of DNA binding proteins to their specific consensus target site frequently involves a structural transition in the protein, DNA or both, as in the case of the Fos/Jun-AP-1 interaction. Sequence specific DNA binding proteins are thought to initially sample nonspecific DNA sites before finding their specific target which accommodates both the structural transitions involved in the specific protein-DNA interaction and the formation of the complementary hydrogen bonding, protein-phosphate (electrostatic) as well as van der Waals interactions. The sampling of nonspecific DNA, or facilitated diffusion, is thought to occur by sliding of proteins on DNA and facilitated target location by direct intersegment transfer (in this case the protein is transferred from one nonspecific DNA site to another distal nonspecific site brought together by transient diffusion of DNA loops) (von Hippel & Berg, 1994). The DNA binding protein then diffuses along the DNA in search of its specific target sequence. In order to accomplish sequence specific binding, the protein must differentiate
between specific and nonspecific DNA sites. The affinity of the protein for specific as opposed to nonspecific DNA therefore is crucial in this regard.

Many DNA binding proteins are known to bend the DNA upon binding to their target site. However, DNA bending is an energetically unfavorable phenomenon. It is possible that the energetic cost associated with DNA bending at the target site is compensated in the context of a ternary complex in which the interaction of additional proteins with the protein-DNA complex confers additional stability. However, if one considers the energetics of the interaction between the DNA binding protein and target DNA alone, the unfavorable energy of DNA bending decreases the affinity of the protein for specific DNA. The decrease in affinity for specific DNA affects the protein's preference for specific as opposed to nonspecific DNA and therefore results in a decrease in DNA binding specificity. The above scenario corresponds to the DNA binding characteristics of a protein which bends specific but not nonspecific DNA. If the DNA binding protein bends both nonspecific as well as specific DNA, then the energy cost of DNA bending will no longer be a factor that affects the ratio of the protein's affinity for specific and nonspecific DNA. DNA bending in this case may serve to contribute to selective DNA recognition. This is suggested to be the mechanism by which the E. coli CAP protein, which bends both specific and nonspecific DNA, recognizes its target site (Erie et al., 1994).

It has previously been shown using chimeric CRE-BP1 and GCN4 proteins, both members of the bZIP family, that the presence of the N-terminal basic cluster, which has been demonstrated to be involved in DNA bending
(Paolella et al., 1997), reduces DNA binding affinity and introduces high affinity for nonspecific DNA (Metallo et al., 1997). This is in support of our findings of the apparent destabilizing effect of the addition of the basic GKH motif to the N-terminus of the basic cluster of Fos. It is, however, interesting to note that despite the presence of the naturally occurring basic N-terminal residues in Fos, the Fos/Jun heterodimer binds with high affinity to the AP-1 site. Examination of the residues further N-terminus to the basic residues in Fos reveals the presence of four negatively charged glutamate residues immediately adjacent to the basic cluster. The negative charge of the amino acids in this region may serve to some what neutralize the positive charge and its destabilizing effect.

In order for gene expression to occur, DNA consensus sequences buried within a nucleosomal structure must become available for binding by general transcription factors as well as the RNA polymerase transcription complex. Nucleosomal displacement or chromatin remodeling can then in turn facilitate the binding of other transcription factors to DNA. It has recently been shown that the Jun/Fos heterodimer is capable of disrupting the structure of a nucleosome by binding to the AP-1 site (Ng et al., 1997). In fact, the strategic location of the AP-1 site immediately down stream of the transcription start site in several promoters (Verdine et al., 1993 and Ng et al., 1997) supports the purpose of this nucleosome unraveling/remodeling characteristic of the Jun/Fos heterodimer. Perhaps by disrupting nucleosome structure at the AP-1 site, DNA sequences upstream of the AP-1 site, i.e. at the transcription start site may become available for binding by proteins of the general transcription machinery.
It is possible that DNA bending induced by the Jun/Fos heterodimer contributes to its ability to disrupt nucleosome structure.

If one considers DNA bending by bZIP proteins in the context of a highly condensed and organized chromatin structure, it is possible that differences in affinity of binding as well as the magnitude and direction of DNA bending induced by different bZIP dimer combinations, could have significant consequences and versatility in their effect on nucleosome structure. In fact, it has been suggested that small differences in DNA bending (as small as 5-10°) introduced to a constrained ternary protein-DNA complex involving not only the bZIP dimer but also other proteins of the transcription complex as well as the nucleosomes, could have significant effects on the thermodynamic stability of the complex and transcription of genes (Hagerman, 1996). Given the apparent destabilizing effect of DNA bending caused by charged residues in and around the basic regions of several bZIP proteins, it is of interest to correlate the distribution of basic residues in this region of different bZIP dimers, in a comparative manner, to their DNA binding affinity and specificity. These investigations may enhance our understanding of the significance of DNA bending in what determines the specificity of function of different members of the bZIP family of transcription factors.
Chapter Four

Summary
The designed GKH-Fos/Jun heterodimer was previously reported to cleave DNA containing the AP-1 site in the presence of Cu(II), ascorbate and H$_2$O$_2$. However, the efficiency of DNA cleavage was low. In an attempt to increase the efficiency of DNA cleavage, a conserved cysteine residue located in the basic region of both Fos and Jun, which is sensitive to oxidation was mutated into serine. DNA binding studies demonstrate that the C-S mutated GKH-Fos/Jun heterodimer binds AP-1 containing DNA in the presence of strong oxidizing conditions and in the absence of reducing agents. DNA cleavage studies conducted with the C-S mutated heterodimer yield irreproducible and inconsistent results. In addition, DNA cleavage patterns similar to those caused by the designed protein are observed by digestion of DNA with DNaseI alone or with Cu(II) alone in the presence of ascorbate and H$_2$O$_2$. These results suggest that the originally observed cleavage may have been due to endoI contamination or susceptibility of the DNA sites to degradation by Cu(II) (which may have become free in solution over the reaction time).

We conducted studies to determine the effect of the addition of the positively charged GKH motif to the N-terminus of Fos on the DNA binding properties of the Fos/Jun heterodimer. DNaseI footprinting experiments demonstrate that the GKH-Fos/Jun heterodimers retain specific binding to the AP-1 site. However, studies of $K_d$ determination show that the addition of the GKH motif to Fos decreased the specific DNA binding affinity of the Fos/Jun heterodimer for the AP-1 site approximately 6-7 fold. In addition, results from gel retardation competition experiments indicate an approximately 4 fold
increase in the affinity of the GKH modified Fos/Jun heterodimer for nonspecific DNA (DR-2). DNA bending studies were also conducted to compare the DNA bending properties of the GKH-Fos/Jun and cFos/Jun heterodimers. Results indicate that both GKH-Fos/Jun and cFos/Jun bend the DNA toward the minor groove. However, the GKH-Fos/Jun heterodimer exhibits increased DNA bending in this direction than does the unmodified cFos/Jun heterodimer. Collectively these results suggest that the energetic cost of increased DNA bending resulting from the addition of the positively charged GKH motif to N-terminus of Fos is manifested through a decrease in both specificity and affinity of binding of the modified heterodimer to the AP-1 site.
Chapter Five

Future Perspectives
5.1 The design

The positive charge of the ATCUN motif in the form of a GKH tripeptide results in a decrease in specificity as well as affinity of binding of the GKH-Fos/Jun heterodimer to the AP-1 site. In future designs it might be more favorable to use a neutral amino acid such as glycine or alanine as the second residue of the ATCUN motif. Alternatively, it is possible to use negatively charged amino acids such as glutamate as the first and second residues of the ATCUN motif. The negative charge in this region may have an effect on the positive charge of the naturally occurring basic amino acids located adjacent to the N-terminus of Fos and result in further stabilization of the protein-DNA interaction. In fact, Fos contains four glutamate residues immediately adjacent to the basic tripeptide comprising the region whose charge has been shown to be critical in determining the magnitude and direction of DNA bending induced by bZIP proteins.

5.2 Studies using designed ATCUN-Fos/Jun dimer

In order to pursue the DNA cleavage experiments, the conditions for cleavage must be further optimized. In particular, glycerol, an avid hydroxyl radical quencher, which was present at high concentrations (5% v/v) in the original cleavage reaction mixture must be removed in order to avoid interference with the hydroxyl radical mediated mechanism of DNA cleavage.

Having optimized cleavage reaction conditions, it will be possible to use the modified Fos/Jun heterodimer as a tool to study what determines the
orientation of binding of different bZIP dimers to the palindromic AP-1 site. The modified heterodimer will selectively cleave the DNA on one side of the AP-1 site (on the side of GKH-Fos) depending on its orientation of binding. It has been suggested that several factors including the nature of DNA sequences flanking the AP-1 site (Rajaram & Curran, 1997), as well as interactions between other transcription factors such as NFAT (Erlanson et al., 1996) and AP-1 proteins confer a preferred orientation of binding to bZIP heterodimers. Having a bZIP dimer, which is capable of selective DNA cleavage on one side of the AP-1 site enables us to change the flanking sequences of the AP-1 site, analyze the pattern of cleavage of the dimer and determine binding orientation by the protein. It will also be possible to analyze the effect of different dimerization partners of Fos on the orientation of binding of these different bZIP dimer combinations to the palindromic AP-1 site. Such studies will enhance our understanding of what determines functional specificity of different bZIP proteins and dimer combinations, and further our knowledge of the mechanistic flow of transcription and signaling.

In addition, comparison of protein sequences to the requirements of the ATCUN motif has led to the discovery that several proteins and peptides including a DNA binding protein, human sperm protamine P2a (McKay et al., 1986) possess this Cu(II) and Ni(II) binding structure (Figure 1.1). The presence of the ATCUN motif could have significant physiological implications with regards to the functions of these proteins. We believe that Cu(II)-ATCUN motif
attached to Fos/Jun is one model system by which to investigate the possibility of metal related, ATCUN motif mediated toxicity and damage to DNA.

5.3 **Significance of charge of amino acids N-terminus to the basic region of bZIP proteins**

Addition of the positively charged GKH tripeptide to N-terminus of Fos bZIP domain results in a decrease in AP-1 binding affinity, an increase in nonspecific DNA binding affinity, and increase in DNA bending of the Fos/Jun heterodimer. Given our results correlating increased DNA bending with a decrease in DNA binding affinity and specificity, it is of interest to study the effect of charge in the region in and adjacent to the N-terminus of different bZIP proteins on not only DNA bending, but also the stability and specificity of the bZIP dimer-DNA interaction. These investigations may enhance our understanding of the significance of DNA bending in affecting the energetics of protein-DNA interactions and perhaps the specificity of function of different members and the bZIP family of transcription factors.
Chapter Six

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