The leucine zipper domain controls the orientation of AP-1 in the NFAT•AP-1•DNA complex

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Background: Heterologous transcription factors bound to adjacent sites in eukaryotic promoters often exhibit cooperative behavior. In most instances, the molecular basis for this cooperativity is poorly understood. Our efforts have been directed toward elucidation of the mechanism of cooperativity between NFAT and AP-1, two proteins that coordinately direct expression of the T-cell growth factor interleukin-2 (IL-2).

Results: We have previously shown that NFAT1 orients the two subunits of AP-1, c-Jun and c-Fos, on DNA through direct protein–protein interactions. In the present study, we have constructed cJun–cFos chimeric proteins and determined their orientation using a novel affinity-cleavage technology based on chemical ligation. We find that, in the presence of NFAT, the chimeric heterodimer binds in such a way as to preserve the orientation of the AP-1 leucine zipper, but not that of the basic region.

Conclusions: Protein–protein interactions between NFAT and the leucine zipper of AP-1 enable the two proteins to bind DNA cooperatively and coordinately regulate the IL-2 promoter. The chemical ligation technology presented here provides a powerful strategy for affinity cleavage studies, including those using recombinant proteins.

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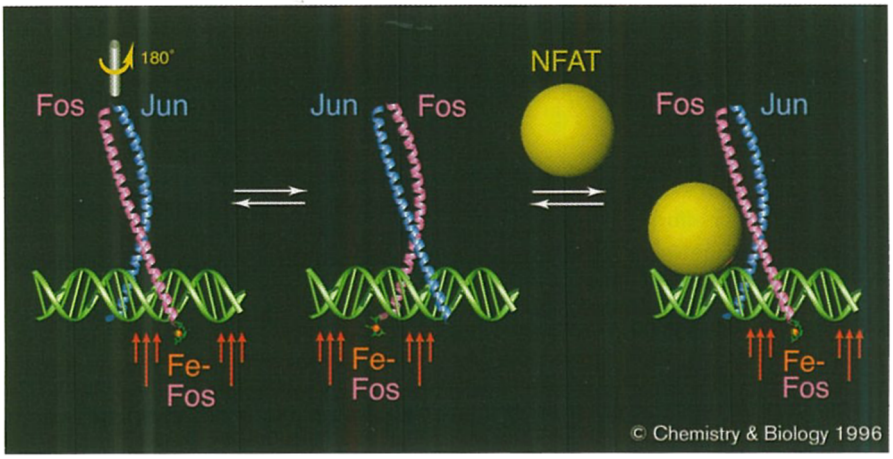
Introduction

A hallmark of eukaryotic gene expression is the ability of multiple promoter-bound proteins to activate gene transcription synergistically [1]. Although the molecular basis for this phenomenon is poorly understood, direct protein– protein contacts among components of the transcriptional machinery appear to mediate this synergistic activation [2–5]. In many cases, cooperativity is believed to result from direct protein–protein contacts among heterologous transcription factors bound to adjacent sites on the promoter [6–11]. Such cooperativity has been conclusively demonstrated in the case of the ARRE2 element of the interleukin-2 (IL-2) promoter, which is bound cooperatively by two proteins [12–14], the nuclear factor of activated T cells (NFAT) [15,16] and activator protein-1 (AP-1) [17,18]. ARRE2, an antigen receptor response element within the IL-2 promoter [19], controls activation of IL-2 gene expression in response to antigen binding to the T cell receptor [20]. The IL-2 protein thus produced delivers a growth signal to the T cell that secretes it.

NFAT comprises a family of at least four distinct proteins that share a domain (~300 amino acids long) that is responsible for DNA binding and cooperation with AP-1 [21–25]. This domain is related in structure to the Rel homology region, a DNA-binding motif found in c-Rel, NF-κB, and other members of the Rel family of transcription factors [26]. NFAT proteins also possess a common amino-terminal proline/serine-rich domain that controls their translocation from the cytoplasm to the nucleus in response to Ca^{2+} mobilization [27–29]. This translocation is regulated by a ubiquitous serine/threonine-specific phosphatase, calcineurin. The action of calcineurin is blocked by the clinically important immunosuppressive agents FK506 and cyclosporin A (CsA) [30]. There is, therefore, significant interest in NFAT as a potential target for the development of drugs that may show increased cell-type specificity and thus be less toxic than FK506 and CsA.

Although NFAT is present in the cytoplasm of resting T cells [27], AP-1 is only induced upon activation of the Ras/MAP kinase and protein kinase C signal transduction pathways [31]. AP-1 consists of two subunits, c-Fos and c-Jun, which heterodimerize and bind DNA through a homologous domain (~70 amino acids long) known as a basic-leucine zipper (bZip) [32–35]. The known isoforms of c-Jun and c-Fos form all possible heterodimer combinations [36], and most if not all of these have been shown to cooperate with NFAT [16]. X-ray crystallographic studies have revealed that the AP-1 bZip domain consists of continuous α-helices that pair to form a Y-shaped crevice where DNA binding occurs (Fig. 1) [37,38]. Residues of the basic region make direct contacts to DNA, while residues of the leucine zipper establish dimerization contacts.

Figure 1



Interaction of the AP-1 bZip domain with DNA. The two subunits of AP-1, c-Fos and c-Jun, are shown in pink and blue, respectively. c-Fos and c-Jun each contain two functional segments: an amino-terminal basic region that contacts DNA, and a carboxy-terminal leucine zipper responsible for dimerization through formation of a coiled coil.

Chemical ligation results in the attachment of the Fe–EDTA (Fe, orange sphere; EDTA, green licorice) reporter element to the amino-terminal end of the basic region, as depicted for c-Fos. AP-1 binds DNA as a mixture of orientational isomers related by interchange of c-Fos and c-Jun on DNA (left and middle structures). The orientation of c-Fos•c-Jun is locked in the cooperative complex formed between AP-1 and NFAT (depicted as a yellow sphere) on DNA. Affinity cleavage patterns are depicted by red arrows, with length roughly proportional to intensity. AP-1 alone produces a tripartite cleavage pattern, a composite of the cleavages produced by the two orientational isomers (see below, Fig. 6, lane 2), whereas the single AP-1 orientational isomer present in the cooperative NFAT•AP-1•DNA complex produces a bipartite cleavage pattern (see below, Fig. 6, lane 4).

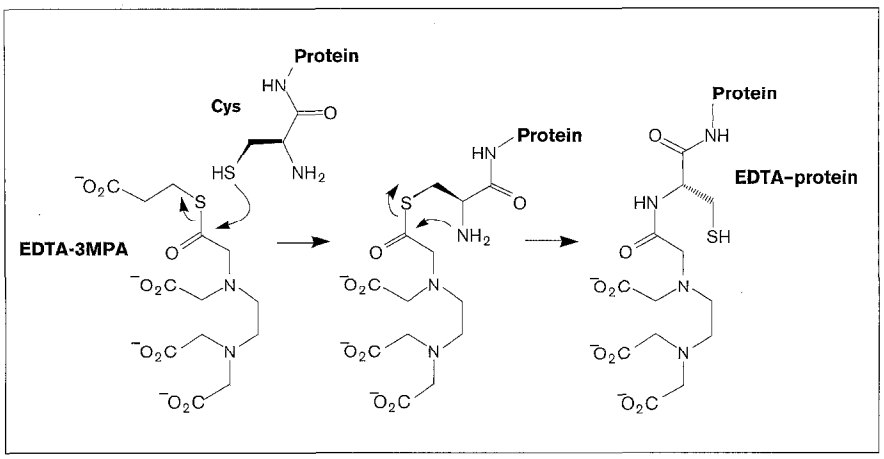
ARRE2 contains two subsites, a polypurine stretch that binds NFAT separated by two base pairs from a non-consensus AP-1 binding site [12,14,39,40]. When AP-1 binds DNA in the absence of NFAT, it does so as a mixture of orientational isomers (the positions of c-Jun and c-Fos are interchangeable). When NFAT is present, however, only a single AP-1 orientation is seen (Fig. 1; [14]). Chemical crosslinking studies indicate that NFAT and AP-1 are in close proximity when bound to ARRE2, suggesting that contacts between the two proteins are responsible for cooperativity and orientational locking [14]. Consistent with this notion, a single arginine to alanine point mutation in the c-Jun leucine zipper virtually abrogates cooperativity with NFAT, but does not affect DNA binding by AP-1 alone [41]. The involvement of the basic region in cooperativity and orientational locking could not be addressed in these mutational studies, however, because sequence alterations in this region of bZip proteins disrupt their interactions with DNA [42]. Here, we have examined directly the relative contributions of the basic region and the spacer/leucine zipper to cooperativity through the construction of c-Jun/c-Fos chimeric proteins. Our experimental approach involves equipping the basic regions of c-Fos, c-Jun, and the c-Fos•c-Jun chimeric proteins with an affinity cleavage moiety, which reports the orientation of the basic region with respect to DNA.

In the affinity cleavage technique [43] used here, a metal–ligand complex such as Fe–EDTA is attached to a defined position on the probe molecule of interest. In the presence of an oxygen source such as hydrogen peroxide and a reducing agent, the attached Fe–EDTA catalyzes the production of a highly reactive, freely diffusable species, probably •OH [44], that attacks nearby positions in a macromolecule, causing chain cleavage. This technique has been used extensively to probe the orientation of DNA-binding proteins on their recognition sites [45–50]. Affinity cleaving has also been used to probe protein–RNA [51], protein–protein [52–54], protein–small molecule [55,56], DNA–DNA [57,58] and DNA–small molecule interactions [43]. The site-specific attachment of the Fe–EDTA moiety to the probe molecule is crucial for the successful use of this technique. In our previous studies of AP-1 orientation, the EDTA ligand was attached to c-Jun and c-Fos during solid phase synthesis of the peptides [14]. This method allows precise control over the location of the EDTA substituent [48], but unfortunately is applicable only to small proteins; indeed, even proteins the size of the c-Fos and c-Jun bZip domains (68–70 amino acids) represent a formidable synthetic challenge. EDTA derivatives that can be attached to cysteine residues have also been used productively [59–61] but cannot be selectively targeted when more than a single reactive amino acid is present.

Initially, our objective was to develop chemistry for conjugating EDTA site-specifically to proteins, regardless of their size and amino acid composition. Kent and coworkers [62,63] have recently described a method for chemical ligation of peptides that produces a native amide linkage between them. This method relies on thioester exchange with an amino-terminal cysteine residue, followed by intramolecular rearrangement to form an amide bond (Fig. 2). This acyl transfer reaction exhibits impressive selectivity for amino-terminal cysteine residues, because they are the only residues that can undergo the required rearrangement to the amide.

Figure 2

Conjugation of EDTA onto the amino terminus of a protein through chemical ligation. The amino-terminal cysteine residue of a protein attacks the thioester carbonyl of EDTA-3MPA, resulting in trans-thioesterification to form a labile EDTA–protein thioester, which then undergoes intramolecular rearrangement to produce a new amide bond.



Selectivity for acyl transfer to cysteine over lysine residues is also high, particularly at neutral pH when the lysine ε-amino group exists as a quaternary ammonium salt. We reasoned, therefore, that is should be possible to produce reagents for use in affinity cleavage studies with NFAT, by reacting a thioester of EDTA with AP-1 peptides containing an amino-terminal cysteine residue (Fig. 2). Here we report the production of such reagents and their use to probe NFAT–AP-1 interactions.

Results

Synthesis of EDTA thioester (EDTA-3MPA)

The chemical ligation procedure described by Kent and coworkers [62,63] involves the coupling of two peptide fragments, one containing a carboxy-terminal thioester and the other containing an amino-terminal cysteine residue. We reasoned that the fragment containing the thioester component could be replaced by a monothioester of the chelating ligand EDTA. Coupling of a peptide containing an amino-terminal cysteine residue with the EDTA thioester should give the peptide an affinity-cleaving function. As a first step towards this goal, we synthesized an EDTA thioester suitable for use in affinity cleaving studies.

We chose 3-mercaptopropionic acid (3MPA), a water-soluble thiol that has a relatively innocuous odor, for investigation in these studies. The 3MPA monothioester of EDTA (EDTA-3MPA) was prepared by reacting stoichiometric amounts of EDTA dianhydride and 3MPA, followed by selective anhydride hydrolysis. The EDTA-3MPA was separated from contaminating EDTA and EDTA-$(3MPA)_2$ bis-thioester by semipreparative reversed-phase high performance liquid chromatography (HPLC) and lyophilized to yield the pure trifluoracetic acid salt of EDTA-3MPA as a white powder. We have stored this highly water-soluble compound as a 250 mM aqueous stock solution for several months at –70 °C with no detectable decomposition.

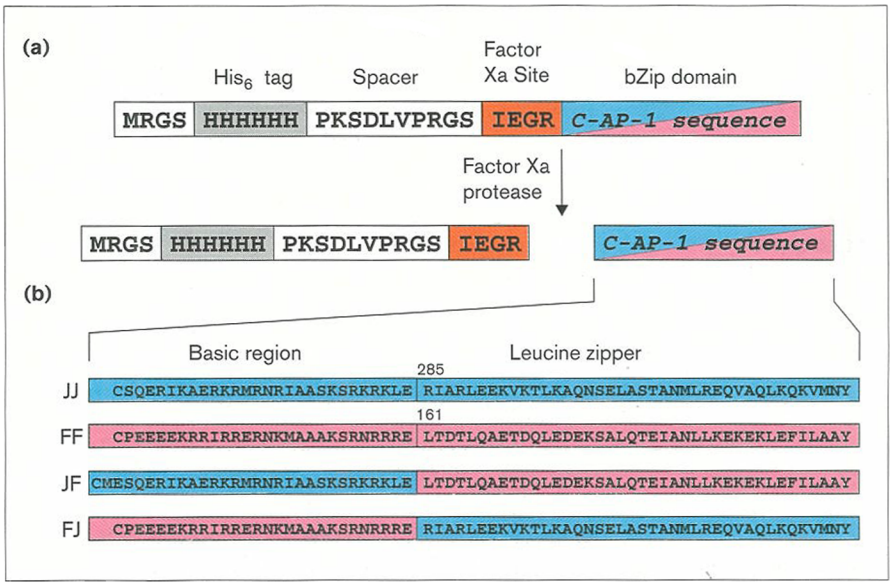
Model reactions of EDTA-3MPA with a polypeptide

To test our chemical ligation approach, we used a 10-amino-acid peptide derived from fibronectin (FB10, H_2N-CQDSETRTFY-CO_2H). Reaction of EDTA-3MPA (25 mM) with FB10 (0.4 mM) in phosphate buffer (pH 7) resulted in clean conversion of FB10 to a material with a slightly longer HPLC retention time (data not shown). To determine whether the product had an attached EDTA group and a free thiol, the product was treated with the thiol-specific reagent 4-vinylpyridine and subjected to mass spectroscopy and amino acid analyses [64]. The molecular weight of the product was consistent with the expected molecular weight of the S-ethylpyridyl modified EDTA-FB10 (1627.9 observed, 1628.7 calculated); amino acid analysis revealed the presence of the expected amino acids, including S-ethylpyridyl cysteine, in the correct proportions.

Synthesis of AP-1 affinity cleaving reagents

Escherichia coli cells do not carry out efficient post-translational removal of the initiator methionine when it is followed by cysteine [65]. We therefore decided to use selective *in vitro* proteolysis to generate native and chimeric c-Fos and c-Jun bZip proteins bearing amino-terminal cysteines. We generated expression constructs encoding a cysteine at the amino-terminal end of the bZip domain, preceded by a recognition site for the protease Factor Xa [66] and a His_6 tag [67] (Fig. 3a). Following induction, *E. coli* cells containing these constructs were lysed in a buffer containing guanidinium hydrochloride (6M) as denaturant. Lysis in the presence of methyl methanethiolsulfonate (1.5 mM), which reacts with cysteine residues to form the mixed CH_3S-disulfide, prevents the condensation of adventitious formaldehyde with the amino-terminal cysteine during handling [68] (D.A.E. and G.L.V., unpublished), and thus increases the yields of EDTA-conjugated protein. The cell lysis and purification can also be carried out under non-denaturing conditions

Figure 3



Native and chimeric AP-1 bZip proteins expressed in *E. coli*. **(a)** Schematic representation of the fusion protein construct used to generate AP-1 bZip domains having a free amino-terminal cysteine through proteolytic cleavage using Factor Xa. **(b)** Native and chimeric AP-1 bZip proteins used in this work. Color scheme is as in Figure 1, with c-Fos in pink and c-Jun in blue. Non-italicized lettering inside boxes denotes amino acid sequence in single-letter code, with the amino terminus on the left.

in the presence of thiols, with only slightly reduced yields of EDTA-conjugated protein due to formaldehyde condensation. After lysis, the denatured, S-protected recombinant proteins were purified using Ni^{2+}–nitrilotriacetic acid resin (Ni–NTA, QIAGEN). The proteins were then cleaved with factor Xa to obtain the desired bZip domains containing an amino-terminal cysteine residue, protected as the mixed CH_3S-disulfide (Fig. 3a). Two of these (JJ and FF, Fig. 3b) contain the wild-type bZip domains of c-Jun and c-Fos, respectively, whereas JF and FJ are chimeric proteins containing the amino-terminal DNA-binding (basic region) segment of c-Jun or c-Fos attached to the carboxy-terminal dimerization (leucine zipper) of the other.

Initially, we conjugated the bZip peptides with EDTA-3MPA using the reaction conditions developed for FB10. This reaction sometimes produced significant amounts of doubly conjugated material. The amount of this over-modified product was unaffected by treatment with aqueous base, suggesting that the second EDTA unit was ligated to an internal lysine sidechain. By systematic variation of the reaction conditions, we found that performing the reaction in the presence of a large excess of dithiothreitol (DTT) gave close to absolute selectivity in formation of the desired amino-terminal conjugation product, and also significantly increased the rate of the reaction. Similar observations have been made by Kent and coworkers [63] using thiophenol. Because EDTA conjugation changes the charge state as well as the mass of the protein, it affects the mobility of the molecule in an SDS-PAGE gel, allowing the reaction to be easily monitored (Fig. 4).

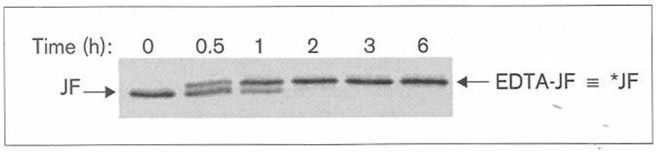
Binding and affinity cleavage

To determine the extent to which chimerism or EDTA conjugation affects the ability of the AP-1 bZip domain to bind DNA or cooperate with NFAT, we carried out electrophoretic mobility shift assays using the consARRE2 DNA site, a variant of ARRE2. ConsARRE2 contains canonical recognition sites for both NFAT and AP-1 separated by two base pairs; the two proteins exhibit strongly cooperative behavior in binding these sites [14].

DNA-binding affinity was measured using electrophoretic mobility shift assays (EMSA) under K_d-controlled conditions ([DNA] << K_d). The cooperativity in the binding of AP-1 and NFAT to DNA can clearly be seen in Figure 5 (compare lanes 3 and 8 with lane 10). At concentrations of AP-1 and NFAT that yield only partial formation of a protein–DNA complex when only one protein is added (lanes 3 and 8, respectively), the two proteins together yield essentially complete formation of a ternary NFAT•AP-1•DNA complex (lane 10). In all cases, the EDTA-conjugated proteins bind DNA with an affinity indistinguishable from their unmodified counterparts, irrespective of the presence or absence of NFAT. Thus, EDTA modification *per se* has no effect on the interactions of these proteins with DNA.

The results of affinity cleavage experiments using AP-1 bZip domains in the presence and absence of NFAT1 are shown in Figure 6. The wild-type c-Fos•c-Jun heterodimer with Fe–EDTA linked to c-Fos (*FF•JJ, lane 2) generates a tripartite cleavage pattern, resulting from attack of hydroxyl radicals in the center of the AP-1 site and on both ends outside the site; the intensity of cleavage in

Figure 4



Time-course of EDTA-3MPA conjugation to the amino-terminal cysteine of the chimeric peptide JF. The resulting peptide, *JF, has an EDTA-modified c-Jun basic region fused to the c-Fos leucine zipper.

the center is roughly double that on the ends. As discussed in detail elsewhere [14], this tripartite cleavage pattern shows that the AP-1 heterodimer binds to DNA as a pair of orientational isomers (Fig. 1). The chimeric heterodimer with Fe–EDTA attached to the c-Fos basic region (*FJ•JF, lane 3) also generates a tripartite affinity cleavage pattern, indicating that it also binds DNA as a mixture of orientational isomers. This is consistent with the fact that the basic regions of c-Jun and c-Fos form virtually identical contacts with DNA and hence are incapable of differential half-site recognition [14,37].

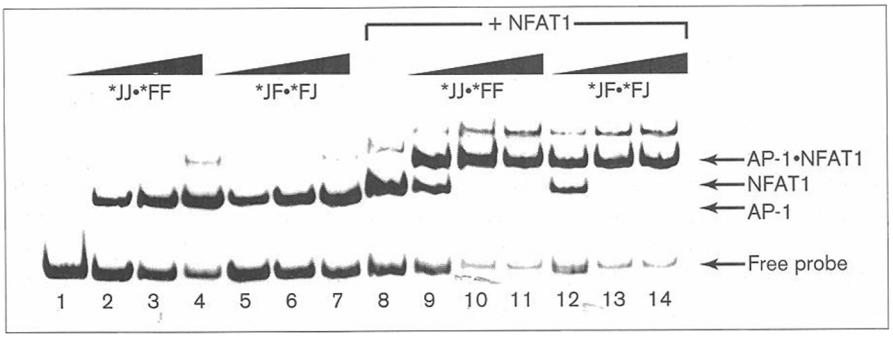
In the presence of NFAT1, *FF•JJ yields a bipartite cleavage pattern (Fig. 6, lane 4), resulting from attack of hydroxyl radicals in the center of the AP-1 site and downstream of it. When the Fe–EDTA is placed at the amino-terminal end of c-Jun only (*JJ•FF, lane 6), cleavage is observed in the center and at the upstream end of the AP-1 site. These data are fully consistent with previously reported affinity cleavage experiments that used chemically synthesized EDTA-containing peptides, demonstrating that NFAT orients AP-1 so as to position c-Jun over the upstream half-site and c-Fos over the downstream half-site [14] (Figs 1 and 7a). A chimeric heterodimer with EDTA on the c-Fos basic region (*FJ•JF, lane 5) exhibits a center/upstream bipartite cleavage pattern; placement of EDTA on the c-Jun basic domain in this chimera (*JF•FJ, lane 7) gives a center/downstream bipartite cleavage pattern. Thus, NFAT also orients the chimeric AP-1 peptides. Furthermore, since the affinity cleavage patterns change when the basic regions are swapped (compare lanes 4 and 5, and 6 and 7), we conclude that the complexes form so as to maintain the orientation of the AP-1 leucine zipper with respect to NFAT (Fig. 7).

Discussion

Here we have described a new method for the synthesis of protein-based reagents for affinity cleavage and the use of this method to probe the interactions between AP-1 and NFAT . The chemistry used in this method is a condensation between an amino-terminal cysteine residue and a thioester; the product of this reaction subsequently undergoes intramolecular rearrangement to produce a new amino-terminal amide linkage. Although this same reaction, termed chemical ligation [69], has been used by others to assemble polypeptides and proteins through segment condensation [62,63,70,71], the present example broadens the scope of chemical ligation to include the attachment of reporter groups to the amino-terminal ends of proteins. Although here we have explored only the attachment of EDTA to proteins via chemical ligation, other reporters or 'handles' such as fluorescein or biotin could presumably be attached to proteins in a similar manner, provided that they can be prepared as suitable thioesters.

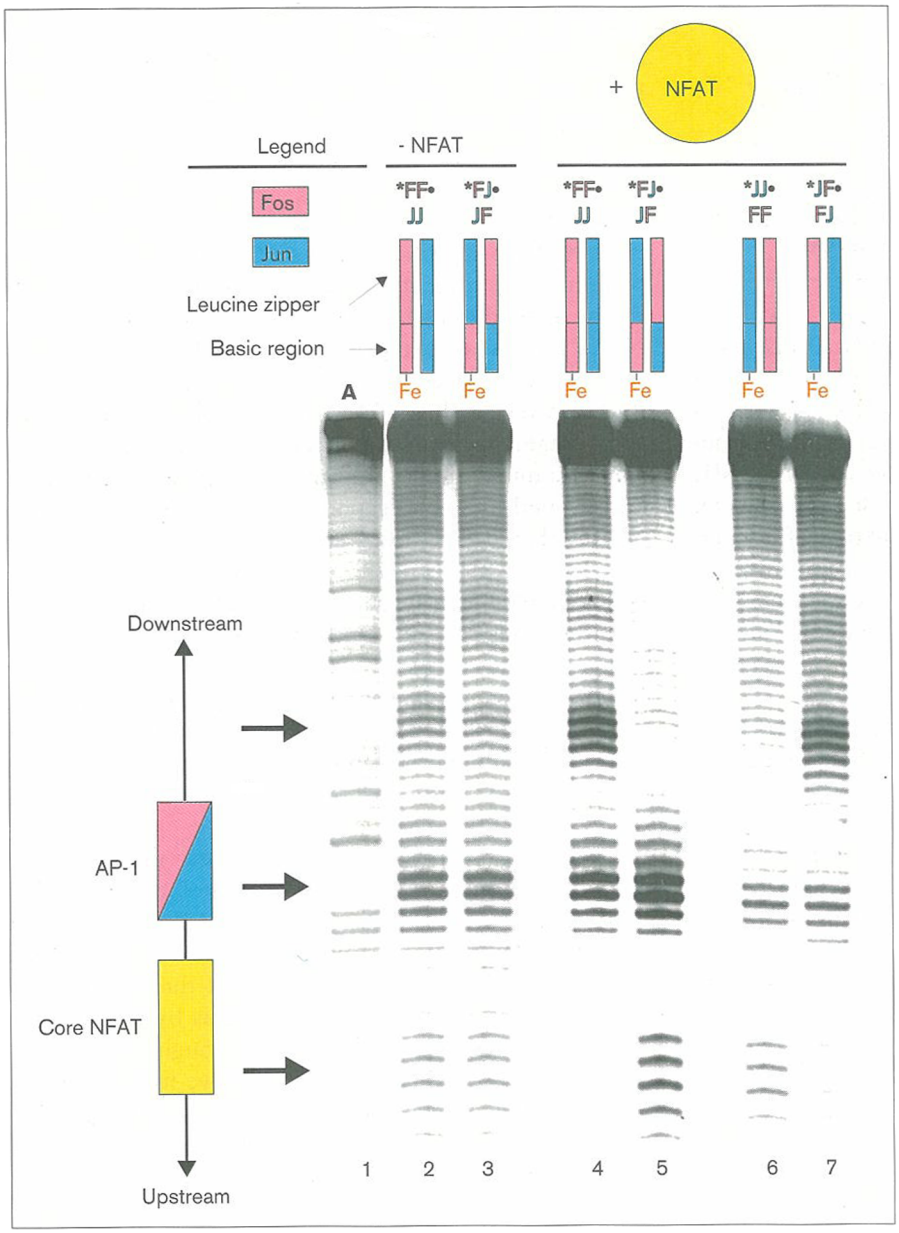
Chemical ligation has previously been used only on polypeptides produced through chemical synthesis, thus limiting the method to peptides and small proteins. Here we have demonstrated highly efficient and selective chemical ligation of proteins produced through bacterial overexpression, using *in vitro* proteolysis by Factor Xa to liberate an amino-terminal cysteine through cleavage of a fusion peptide. In this fusion peptide we included a His_6 sequence, which allowed rapid and convenient purification of the expressed protein through immobilized metal affinity chromatography. Since Factor Xa cleaves at the

Figure 5



Wild-type and chimeric AP-1 proteins bind DNA and cooperate equally well with NFAT. Electrophoretic mobility shift assays (EMSA) to measure the affinities of EDTA-modified wild-type (*JJ•*FF) and chimeric (*JF•*FJ) AP-1 heterodimers for consARRE2. Virtually identical data were obtained for the corresponding unmodified bZip domains (data not shown). AP-1 dimer concentrations are: 0 (lanes 1,8), 5 (lanes 2,5,9,12), 12.5 (lanes 3,6,10,13), and 25 nM (lanes 4,7,11,14). NFAT1 concentrations are 0 (lanes 1–7) and 2 nM (lanes 8–14). The faint upper band observed at high protein concentrations arises from non-specific DNA binding, which is commonly observed at the relatively low salt concentrations used in these studies. These bands may contribute to the background levels of non-specific DNA cleavage, but do not affect the specific cleavage patterns that provide orientational information.

Figure 6



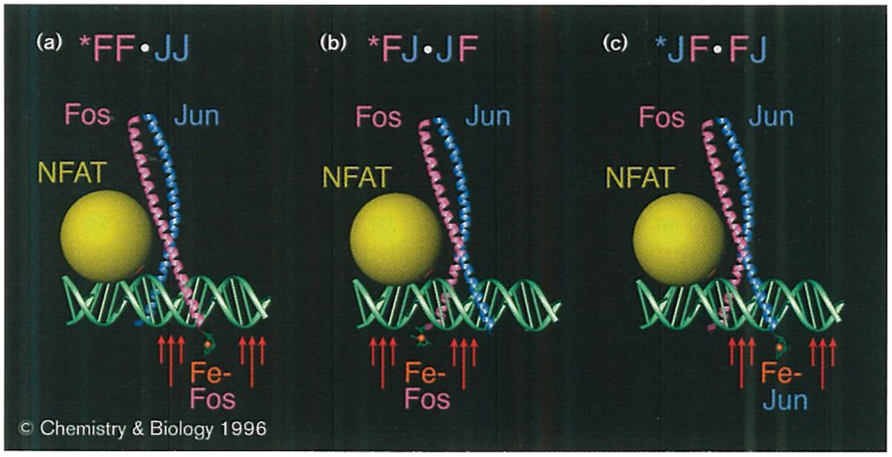
NFAT orients chimeric AP-1 peptides on DNA. A schematic representation of the DNA probe is shown with the NFAT and AP-1 subsites in yellow and blue/pink, respectively. Arrows indicate major sites of cleavage (schematized by red arrows in Figs 1 and 7). Lane 1: A-cleavage lane (A: adenine-specific Moxam-Gilbert DNA cleavage lane). Lanes 2–7: affinity cleavage experiments. Protein components are schematically shown above each lane.

carboxy-terminal end of the IEGR sequence, the entire fusion peptide could be removed from the bZip protein upon processing by the protease.

Chemical ligation has been shown [62,63] to be highly selective for an amino-terminal cysteine, even in the presence of internal cysteine and lysine residues. This selectivity derives from the irreversible rearrangement of the intermediate amino-terminal thioester linkage (Fig. 2) to the corresponding amide. Condensation of the EDTA thioester with internal cysteine residues can and presumably does take place. These internal thioesters cannot rearrange to the more stable amide, however, and are therefore removed by the large excess of exogenous thiol present in the reaction mixture. Because chemical ligation is so selective for amino-terminal cysteine residues, it can be used directly on wild-type proteins, without first mutating internal cysteine residues to another amino acid. Alternative approaches for attachment of EDTA to biosynthetically derived proteins rely on direct S-alkylation of cysteine residues [49,50,59–61], and hence require mutation of non-target cysteine residues to avoid their participation in the reaction. We should point out, however, that the AP-1 proteins used in these studies possess no internal cysteines, and therefore did not directly address the issue of EDTA-thioester formation at

Figure 7

The orientation of AP-1 in the NFAT•AP-1•DNA complex is determined by the leucine zipper. In the model, the arrangement shown in **(a)** corresponds to lane 4 of Figure 6, **(b)** corresponds to lane 5 of Figure 6, and **(c)** corresponds to lane 7 of Figure 6.



such sites. The selectivity of chemical ligation reactions using the EDTA thioester would not be expected to differ substantially from that observed in the several studies that used peptide thioesters [62,63,70]. (Native c-Fos and c-Jun do contain a cysteine residue in their bZip domains at positions 154 and 278, respectively. Both of these had been mutated to encode serine residues in the constructs from which our overexpression vectors were derived. The Cys to Ser mutations were originally introduced in these constructs to facilitate crystallographic analysis [37]. These mutations have been shown to have little effect on the DNA-binding affinity and specificity of AP-1, but render it resistant to oxidative inactivation [72].

The EDTA-thioester could, in principle, also react with the ε-amino group of internal lysine residues, but at the pH used in chemical ligation, these groups are present almost exclusively in the unreactive ammonium ion form. Only under conditions in which conjugation of the EDTA-thioester to the amine-terminal cysteine is slow (and in the absence of DTT) have we observed reaction at internal lysines. Indeed, the AP-1 peptides represent a stringent test of selectivity, because they are unusually rich in lysine residues. Under the chemical ligation conditions described here, we found that the desired coupling reaction goes essentially to completion, without producing even minor amounts of material having EDTA linked to an internal lysine. Given the high degree of selectivity observed in our and related systems [62,63,70], it is reasonable to expect that the method of reporter attachment described here will be fully applicable to much larger recombinant proteins.

The use of chemical ligation for proteins that possess redox-active disulfides is complicated by the fact that they may undergo reduction by the free thiols that are present in the ligation reaction mixture. So long as the protein can be reoxidized or perhaps even refolded following chemical ligation, disulfide reduction is not a problem. For those few cases in which the protein would undergo irreversible inactivation upon exposure to reducing agents, an alternative but related strategy could be envisaged: amino-terminal serine residues undergo selective oxidation upon treatment with periodate, and the glyoxamide thus formed is known to condense with acyl hydrazides [73], such as EDTA monohydrazide.

Chemical ligation can only modify the amino-terminus of the protein, restricting the method to targets that interact near the amino-terminus of the EDTA-modified protein. Although this is a limitation of the technique, bZip proteins are one class of DNA-binding proteins that can be usefully studied in this way. There are over 60 members of the bZip family of transcription factors [38] and they are extremely diverse in their DNA sequence specificity, dimerization behavior and biological function. The molecular basis for the unique DNA-binding properties of most bZip proteins is poorly understood, for three main reasons. First, co-crystal structures are available for only two family members, GCN4 [74,75] and AP-1 [37], and these proteins recognize virtually identical DNA sequences using highly conserved residues. Second, the naturally occurring binding sites for any given bZip protein typically vary around a consensus, and it is not known how the proteins tolerate this variation. And third, many bZip heterodimers are formed from subunits that recognize different half sites, and it is not known to what extent the heterodimers are oriented on DNA. Affinity cleavage provides a powerful means of addressing many of these issues. For example, comparison of the affinity cleavage pattern of any bZip protein with that of AP-1 (Fig. 6) [14] or GCN4 [46] directly defines the orientation, half-site spacing and alignment of the basic region with respect to its cognate half-site.

Evidence has been presented elsewhere that AP-1 cooperates, either negatively or positively with a number of regulatory proteins other than NFAT, including the

transcription factors IP-1, ABP, FIP, glucocorticoid and thyroid hormone receptors, p52/54, MyoD and Elf-1 [11,38]. In most of these cases it is not known whether the mechanism of cooperativity involves direct protein–protein contacts, however. Assuming that such contacts would discriminate between c-Jun and c-Fos, as NFAT does, the cooperating protein should orient AP-1. Affinity cleavage studies can thus provide information on the mechanism of cooperative gene regulation by any protein that synergizes with AP-1. Furthermore, there are many other bZip proteins that cooperate with heterologous transcription factors. For example, the bZip protein c-Maf may interact with NFAT to control IL-4 production by T helper 2 cells [76], members of the C/EBP bZip subfamily interact synergistically with certain forms of NF-κB [10,77], and members of the ATF sub-family of bZip transcription factors have been shown to interact with various proteins, including E1a, HPV pX, CREB, NF-κB, and the retinoblastoma gene product [38,78,79].

Other major classes of transcription factors for which the present chemical ligation strategy might be applicable include basic helix-loop-helix proteins such as Myc, Max, MyoD and E47 [80–82], zinc-finger proteins such as Zif 268 and Gli [83,84], nuclear hormone receptors such as the glucocorticoid hormone and estrogen receptors [85,86], homeodomain and POU-homeodomain proteins such as engrailed, Mata/α and Oct-1 [87–89], and helix-turn-helix proteins such as the λ, 434 and Cro repressors [90–92]. Many of the proteins listed above contain zinc-bound cysteine residues that cannot be mutated without loss of function and that are reactive toward electrophiles; the present method could permit these proteins to be analyzed by affinity cleaving.

The cooperative interaction between NFAT and AP-1 is essential for activation of the IL-2 gene. Such cooperative interactions between proteins bound to adjacent sites on DNA are believed to exist in a number of other systems [6–11], and indeed may be important in the regulation of many, if not most, eukaryotic genes. Of these, the NFAT•AP-1•DNA system is probably the most extensively characterized, yet it remains poorly characterized at the molecular level. Although high-resolution structural information on the NFAT•AP-1•DNA complex is not yet available, biochemical experiments are beginning to identify regions of the AP-1 bZip that participate in cooperative protein–protein interactions with NFAT. Remarkably, mutation of a conserved arginine residue (Arg285) to alanine in the c-Jun leucine zipper results in an almost complete loss of cooperativity with NFAT, but does not affect the binding of AP-1 to DNA [41]. These experiments were unable to address the importance of the basic region, because most changes in this region disrupt AP-1•DNA interactions [42]. The experiments presented here circumvent this problem. In our chimeric constructs, we conceptually bisected the c-Jun bZip motif into two regions, a carboxy-terminal region beginning at the critical Arg285 residue, and an amino-terminal region containing all residues preceding Arg285 (Fig. 3b). These segments were exchanged with the corresponding segments from c-Fos, to generate chimeric proteins having the basic region of c-Jun fused to the leucine zipper of c-Fos and *vice versa*. The EDTA-modified chimeric AP-1 heterodimers were both oriented by NFAT, but gave an inverted affinity cleavage pattern relative to wild-type, thus indicating that the complex preserves the positioning of the AP-1 leucine zipper with respect to NFAT. We thus conclude that the leucine zipper is solely responsible for determining the strength and specificity of the protein–protein interactions between NFAT and AP-1.

Significance

Affinity cleavage is a powerful method for determining the positioning of a DNA-binding protein over its site. This method has, however, been limited by the need to use synthetic peptides and proteins, or by the requirement that the protein has a single cysteine residue. The present method, based on chemical ligation, should permit the generation of affinity-cleaving reagents from a broader range of proteins, including large native proteins produced biosynthetically, by selectively targeting the amino-terminal cysteine residue. Here we have used this method to study the structural basis for cooperativity between two transcription factors, NFAT and AP-1, on a DNA-binding site derived from the IL-2 promoter. Our earlier studies had shown that AP-1 alone binds DNA as a mixture of two orientational isomers, but that it adopts a single orientation in the cooperative complex formed with NFAT and DNA. Since both orientational specificity and cooperativity result from direct contacts between NFAT and AP-1, we used orientation to discover which region of AP-1 is responsible for cooperative interactions between the two proteins. From the affinity cleavage patterns of chimeric c-Fos/c-Jun heterodimers, it is clear that the leucine zipper region is solely responsible for orientation by NFAT. Thus we conclude that cooperativity in the NFAT•AP-1 interface results from direct contacts between NFAT and the leucine zipper region of AP-1. Using the approach described here, it should be possible to identify the residues of AP-1 that are responsible for cooperation with NFAT. Such information may aid the search for novel immunosuppressive agents that target the IL-2 gene directly. More generally, bZip proteins comprise an important superfamily of transcription factors; these proteins are central to many processes in development, tissue-specific gene expression and oncogenesis. The affinity cleaving approach reported here provides a powerful tool for examining the DNA-binding behavior of these factors, both alone and in cooperation with other regulatory proteins.

Materials and methods

EDTA-3-mercaptopropionate thioester (EDTA-3MPA)

Neat 3-mercaptopropionic acid (1.0 ml, 11.7 mmol) was added with stirring to a suspension of EDTA dianhydride (3.0 grams, 11.7 mmol) in dimethyl formamide (DMF) (15 ml). The reaction was allowed to continue for 14 h, after which the flask was heated to 60 °C and the DMF was removed under reduced pressure. 1 N NaOH (11.7 ml) was added to the resulting yellow foam and the reaction mixture was stirred for 12 h. The slurry was then centrifuged, and the supernatant was decanted from the precipitate (which contains mostly bis-thioester). Methanol (33 ml) was added to the yellow solution and the mixture was allowed to stand at 4 °C for several hours in order to precipitate EDTA. The solution was re-centrifuged, and the supernatant was decanted and then lyophilized to give a yellow powder (0.89 g). A small sample of this material (55 mg) was dissolved in water (0.5 ml), neat trifluoroacetic acid (TFA, 20 μl) was added to bring the pH to 2, and the solution was purified using reversed phase high performance liquid chromatography (HPLC) (Waters RCM 8 × 10 C18 cartridge; 2 ml min^{-1}; A = 98 % water, 2 % acetonitrile, 0.06 % TFA; B = 10 % water, 90 % acetonitrile, 0.055 % TFA; 0–5 min 100 % A, 5–20 min linear gradient to 85 % A, 20–25 min linear gradient to 0 % A; EDTA-3MPA elutes at 12.7 min). The collected fractions were pooled and lyophilized to give 26.5 mg of EDTA-3MPA as a white crystalline powder: UV/vis (water) λ_{max} 236 nm; ^{1}H NMR (D_2O) δ 4.00 (s, 4H), 3.74 (s, 2H), 3.55 (s, 2H), 3.36 (t, 2H, J = 5.6 Hz), 3.07 (t, 2H, J = 5.6 Hz), 3.00 (t, 2H, J = 6.8 Hz), 2.56 (t, 2H, J = 6.8 Hz) ppm; ^{13}C NMR (D_2O) δ 204.7, 179.0, 176.9, 172.5, 65.5, 58.4, 57.7, 56.1, 52.2, 36.3, 26.2 ppm; HRMS (negative ion FAB) for $C_{13}H_{20}N_2O_9S$ found, 379.0816; calc'd, (M–H) 379.0811.

Reaction of FB10 with EDTA-3MPA

A solution containing 0.4 mM FB10 (H_2N-CQDSETRTFY-CO_2H, Sigma), 25 mM EDTA-3MPA, 10 mM DTT, and 200 mM sodium phosphate at pH 7 was allowed to incubate at room temperature. At various time points aliquots were removed, quenched with TFA, and analyzed by reversed phase HPLC (Beckman C18 ODS ultrasphere column; 1.5 ml min^{-1}; same solvents as above; 0–5 min 85 % A, 5–20 min linear gradient to 70 % A, 20–25 min linear gradient to 0 % A; FB10 elutes at 10.4 min; FB10-EDTA elutes at 11.5 min). Both the modified and unmodified FB10 peptides were collected, lyophilized, and modified with 4-vinylpyridine as described previously. After repurification by HPLC the peptides were analyzed by amino acid analysis and mass spectroscopy (MW VP-FB10 found, 1355.9; calc'd, 1354.5; EDTA-VP-FB10: found, 1627.9; calc'd, 1628.7).

Protein overexpression and purification

NFAT1 was overexpressed and purified as described previously [14]. DNA encoding the chimeras was generated using megaprimer PCR [93], ligated into plasmid pLM1 [94] and transformed into *E. coli* BL-21 (DE3) pLysS (Novagen). After growth to mid-log phase, the cells were induced by the addition of isopropyl-β-D-galactopyranoside to 1 mM for 2–4 h. Following centrifugation, the cell pellet was resuspended in 20 ml of buffer Gn (6 M guanidinium hydrochloride, 100 mM sodium phosphate, 10 mM Tris-hydrochloride, 1.5 mM methyl methanethiolsulfonate (Aldrich), pH 8) and stirred vigorously for 1 h at room temperature. The resulting mixture was centrifuged at 15 000 rpm in a Sorvall SA-600 rotor for 20 min. The supernatant was removed and mixed with 1 ml of Ni^{2+}-NTA resin (Qiagen) which had previously been washed with buffer Gn. This slurry was stirred for 45 min and then loaded into a 1.5-cm diameter column and the liquid allowed to flow through. The resin was washed extensively, first with 2 × 7 ml buffer Gn, then with 6 × 7 ml buffer U (8 M urea, 100 mM sodium phosphate, 10 mM Tris-hydrochloride, pH 6.5), and finally with 4 × 7 ml buffer PiBS (50 mM PIPES, 100 mM NaCl, pH 7.6). The proteins were eluted with 3 × 5 ml buffer ABS (50 mM acetic acid, 100 mM NaCl, pH 2.7), concentrated to < 1 ml by centrifugal ultrafiltration (Ultrafree-15, 5000 MWCO, Millipore), and brought up to 3 ml with buffer PiBS to which was added 2 mM $CaCl_2$. The protein was proteolyzed with 20 μg of Factor Xa (New England Biolabs) for 0.5–4 h. The reaction was stopped by adding dansyl-EGR-chloromethyl ketone (Calbiochem) to 9 μM, pepstatin A to 1.3 μg ml^{-1}, and leupeptin to 11 μg ml^{-1}. Next, the solution was gently stirred with 1 ml of fresh Ni^{2+}-NTA resin for 45 min to bind any uncleaved protein as well as the cleaved amino-terminal fusion peptide. Finally, the resin was rinsed with 3 × 3 ml buffer PiBS and concentrated by centrifugal ultrafiltration. The concentrated stock was stored at −70 °C in 100-μl aliquots.

For proteins that are irreversibly inactivated upon denaturation, we recommend carrying out the purification in the presence of 10 mM β-mercaptoethanol, using the procedure recommended by Qiagen. In such cases, the protein should not be treated with methyl methanethiolsulfonate.

Protein conjugation

Conjugations were carried out in buffer PiBS with DTT added to a final concentration of 300 mM, pH 7–7.5. Neutralized 3PA-EDTA was added to a 100-μl aliquot of protein to a final concentration of 25 mM, and the reaction was allowed to incubate at room temperature. The reaction was followed by removing aliquots and analyzing them by mass spectroscopy and/or SDS gel electrophoresis on a 15 % polyacrylamide gel (Protogel, National Diagnostics). Under these conditions, the reaction is complete in less than 12 h. The protein was then concentrated against PiBS by centrifugal ultrafiltration (Centricon-3, Amicon), and quantified by BCA assay (Pierce). Peptides stored at −70 °C are stable indefinitely. Mass spectral data: MW EDTA-JJ found, 8317; calc'd 8318; MW EDTA-FF found, 8379; calc'd 8378; MW EDTA-JF found, 8512; calc'd 8513; MW EDTA-FJ found, 8442; calc'd 8444.

Binding assays and affinity cleavage

Stock solutions of peptides containing 25 μM JJ or FJ and 75 μM FF or JF (with either or both peptide EDTA-modified) were prepared and stored at −70 °C. Peptide dilutions were made by diluting these stocks into doubly distilled water, either with or without an equimolar amount of ferric ammonium sulfate (FAS). Final heterodimer concentration was 50 nM in affinity cleavage experiments, or as noted in EMSA. NFAT1 was added to a final concentration of 2 nM (EMSA) or 40 nM (affinity cleavage). A DNA restriction fragment containing the consensus ARRE2 site was radiolabeled at the 3′ end using Klenow DNA polymerase and [α^{33}P]dNTP. The binding buffer contained a final concentration of 14 mM HEPES pH 7.5, 60 mM KCl, 0.1 % Nonidet P-40, 1 mM DTT, 5 μg ml^{-1} poly dI•dC, and 5 % glycerol (binding reactions only) in a total volume of 20 μl. The reactions were allowed to equilibrate for 30 min at room temperature, after which binding reactions were loaded directly on a 6 % native polyacrylamide gel (Protogel, National Diagnostics) run at 150 V in 0.5 × TBE buffer. Affinity cleavage was initiated by adding 1 μl of 250 mM sodium ascorbate and 1 μl of 20 mM H_2O_2. Cleavage was allowed to continue for several min and then quenched with 2 μl of 3 M sodium acetate with 0.5 mg ml^{-1} tRNA followed by 60 μl cold absolute ethanol. The reactions were incubated on ice for 30 min and centrifuged at 14 000 rpm, 4 °C for 30 min. The supernatant was removed and discarded, and the pellet was resuspended in loading dye (95 % formamide, 12.5 mM EDTA, 25 mM thiourea, 1 mg ml^{-1} bromophenol blue, and 1 mg ml^{-1} xylene cyanol), denatured at 90 °C, and run on a 15 % denaturing gel (Sequagel, National Diagnostics). Gels were dried and imaged by phosphorimaging or autoradiography.

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References

1. Tjian, R. & Maniatis, T. (1994). Transcriptional activation: a complex puzzle with few easy pieces. *Cell* **77**, 5–8.

2. Sauer, F., Hansen, S.K. & Tjian, R. (1996). DNA template and activator–coactivator requirements for transcriptional synergism by *Drosophila* bicoid. *Science* **270**, 1825–1828.
3. Sauer, F., Hansen, S.K. & Tjian, R. (1995). Multiple TAFIIs directing synergistic activation of transcription. *Science* **270**, 1783–1788.
4. Xu, H.E., Kodadek, T. & Johnston, S.A. (1995). A single GAL4 dimer can maximally activate transcription under physiological conditions. *Proc. Natl. Acad. Sci. USA* **92**, 7677–7680.
5. Miner, J.N. & Yamamoto, K.R. (1991). Regulatory crosstalk at composite response elements. *Trends Biochem. Sci.* **16**, 423–426.
6. Diamond, M.I., Miner, J.N., Yoshinaga, S.K. & Yamamoto, K.R. (1990). Transcription factor interactions: selectors of positive or negative regulation from a single DNA element. *Science* **249**, 1266–1272.
7. Wasylyk, B., Wasylyk, C., Flores, P., Begue, A., Leprince, D. & Stehelin, D. (1990). The *c-ets* proto-oncogenes encode transcription factors that cooperate with c-Fos and c-Jun for transcriptional activation. *Nature* **346**, 191–193.
8. Du, W., Thanos, D. & Maniatis, T. (1993). Mechanisms of transcriptional synergism between distinct virus-inducible enhancer elements. *Cell* **74**, 887–898.
9. Hill, C.S. & Treisman, R. (1995). Differential activation of c-Fos promoter elements by serum, lysophosphatidic acid, G proteins and polypeptide growth factors. *EMBO J.* **14**, 5037–5047.
10. Stein, B., Cogswell, P. & Baldwin, A.S., Jr. (1993). Functional and physical associations between NF-κB and C/EBP family members: a Rel domain–bZIP interaction. *Mol. Cell. Biol.* **13**, 3964–3974.
11. Wang, C.-Y., Bassuk, A.G., Boise, L.H., Thompson, C.B., Bravo, R. & Leiden, J.M. (1994). Activation of the granulocyte-macrophage colony-stimulating factor promoter in T cells requires cooperative binding of Elf-1 and AP-1 transcription factors. *Mol. Cell. Biol.* **14**, 1153–1159.
12. Jain, J., McCaffrey, P.G., Valge-Archer, V.E. & Rao, A. (1992). Nuclear factor of activated T cells contains Fos and Jun. *Nature* **356**, 801–804.
13. Jain, J., Miner, Z. & Rao, A. (1993). Analysis of the preexisting and nuclear forms of nuclear factor of activated T cells. *J. Immunol.* **151**, 837–848.
14. Chen, L., *et al.*, & Verdine, G.L. (1995). Only one of the two DNA-bound orientations of AP-1 found in solution cooperates with NFATp. *Curr. Biol.* **5**, 882–889.
15. Shaw, J.-P., Utz, P.J., Durand, D.B., Toole, J.J., Emmel, E.A. & Crabtree, G.R. (1988). Identification of a putative regulator of early T cell activation genes. *Science* **241**, 202–205.
16. Rao, A. (1994). NFATp: a transcription factor required for the coordinate induction of several cytokine genes. *Immunol. Today* **15**, 274–281.
17. Lee, W., Haslinger, A., Karin, M. & Tjian, R. (1987). Activation of transcription by two factors that bind promoter and enhancer sequences of the human metallothionein gene and SV40. *Nature* **325**, 368–372.
18. Curran, T. & Franza, J. (1988). Fos and Jun: the AP-1 connection. *Cell* **55**, 395–397.
19. Durand, D.B., Shaw, J.-P., Bush, M.R., Replogle, R.E., Belagaje, R. & Crabtree, G.R. (1988). Characterization of antigen receptor response elements within the interleukin-2 enhancer. *Mol. Cell. Biol.* **8**, 1715–1724.
20. Crabtree, G.R. & Clipstone, N.A. (1994). Signal transmission between the plasma membrane and the nucleus of T lymphocytes. *Annu. Rev. Biochem.* **63**, 1045–1083.
21. McCaffrey, P.G., *et al.*, & Hogan, P.G. (1993). Isolation of the cyclosporin-sensitive T cell transcription factor NFATp. *Science* **262**, 750–754.
22. Northrop, J.P., *et al.*, & Crabtree, G.R. (1994). NFAT components define a family of transcription factors targeted in T cell activation. *Nature* **369**, 497–502.
23. Ho, S.N., Thomas, D.J., Timmerman, L.A., Li, X., Francke, U. & Crabtree, G.R. (1995). NFATc3, a lymphoid-specific NFATc family member that is calcium-regulated and exhibits distinct DNA binding specificity. *J. Biol. Chem.* **270**, 19898–19907.
24. Masuda, E.S., *et al.*, & Arai, N. (1995). NFATx, a novel member of the nuclear factor of activated T cells family that is expressed predominantly in the thymus. *Mol. Cell. Biol.* **15**, 2697–2706.
25. Hoey, T., Sun, Y.-L., Williamson, K. & Xu, X. (1995). Isolation of two new members of the NF-AT gene family and functional characterization of the NF-AT proteins. *Immunity* **2**, 461–472.
26. Wolfe, S.A., *et al.*, & Verdine, G.L. An unusual Rel-like architecture in the DNA-binding domain of NFATc. *Nature*, in press.
27. Flanagan, W.M., Corthésy, B., Bram, R.J. & Crabtree, G.R. (1991). Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. *Nature* **352**, 803–807.
28. Shibasaki, F., Price, E.R., Milan, D. & McKeon, F. (1996). Role of kinases and the phosphatase calcineurin in the nuclear shuttling of transcription factor NF-AT4. *Nature* **382**, 370–373.
29. Luo, C., *et al.*, & Rao, A. (1996). Interaction of calcineurin with a domain of the transcription factor NFAT1 that controls nuclear import. *Proc. Natl. Acad. Sci. USA* **93**, 8907–8912.
30. Liu, J., Farmer, J.D., Jr., Lane, W.S., Friedman, J., Weissman, I. & Schreiber, S.L. (1991). Calcineurin is a common target of cyclophilin–cyclosporin A and FKBP–FK506 complexes. *Cell* **66**, 1–9.
31. Karin, M. (1995). The regulation of AP-1 activity by mitogen-activated protein kinases. *J. Biol. Chem.* **270**, 16483–16486.
32. Landschulz, W.H., Johnson, P.F. & McKnight, S.L. (1988). The leucine zipper: a hypothetical structure common to a new class of DNA-binding proteins. *Science* **240**, 1759–1764.
33. Sassone-Corsi, P., Ransone, L.J., Lamph, W.W. & Verma, I.M. (1988). Direct interaction between fos and jun nuclear oncoproteins: role of the 'leucine zipper' domain. *Nature* **336**, 692–695.
34. Kouzarides, T. & Ziff, E. (1988). The role of the leucine zipper in the Fos–Jun interaction. *Nature* **336**, 646–651.
35. Nakabeppu, Y. & Nathans, D. (1989). The basic region of Fos mediates specific DNA binding. *EMBO J.* **8**, 3833–3841.
36. Angel, P. & Karin, M. (1991). The role of Jun, Fos and the AP-1 complex in cell proliferation and transformation. *Biochim. Biophys. Acta* **1072**, 129–157.
37. Glover, J.N.M. & Harrison, S.C. (1994). The crystal structure of the heterodimeric bZip transcription factor c-Fos:c-Jun bound to DNA. *Nature* **373**, 257–261.
38. Hurst, H.C. (1994). Transcription factors 1: bZip proteins. *Protein Profile* **1**, 123–168.
39. Boise, L.H., *et al.*, & Thompson, C.B. (1993). The NFAT-1 DNA binding complex in activated T cells contains Fra-1 and JunB. *Mol. Cell. Biol.* **13**, 1911–1919.
40. McCaffrey, P.G., Jain, J., Jamieson, C., Sen, R. & Rao, A. (1992). A T cell nuclear factor resembling NF-AT binds to an NF-κB site and to the conserved lymphokine promoter sequence 'Cytokine-1'. *J. Biol. Chem.* **267**, 1864–1871.
41. Peterson, B.R., Sun, L.J. & Verdine, G.L. (1996). A critical arginine residue mediates cooperativity in the contact interface between NFAT and AP-1. *Proc. Natl. Acad. Sci. USA* **93**, 13671–13676.
42. Suckow, M., Von Wilcken-Bergmann, B. & Müller-Hill, B. (1993). The DNA binding specificity of the basic region of the yeast transcriptional activator GCN4 can be changed by substitution of a single amino acid. *Nucl. Acids Res.* **21**, 2081–2086.
43. Dervan, P.B. (1986). Design of sequence-specific DNA-binding molecules. *Science* **232**, 464–471.
44. Pogozelski, W.K., McNeese, T.J. & Tullius, T.D. (1995). What species is responsible for strand scission in the reaction of $[Fe^{II}–EDTA]_2$ and H_2O_2 with DNA? *J. Am. Chem. Soc.* **117**, 6428–6433.
45. Sluka, J.P., Horvath, S.J., Glasgow, A.C., Simon, M.I. & Dervan, P.B. (1990). Importance of minor-groove contacts for recognition of DNA by the binding domain of Hin recombinase. *Biochemistry* **29**, 6551–6561.
46. Oakley, M.G. & Dervan, P.B. (1990). Structural motif of the GCN4 DNA binding domain characterized by affinity cleaving. *Science* **248**, 847–850.
47. Oakley, M.G., Mrksich, M. & Dervan, P.B. (1992). Evidence that a minor groove-binding peptide and a major groove-binding protein can simultaneously occupy the same site. *Biochemistry* **31**, 10969–10975.
48. Mack, D.P., Sluka, J.P., Shin, J.A., Griffin, J.H., Simon, M.I. & Dervan, P.B. (1990). Orientation of the putative recognition helix in the DNA-binding domain of Hin recombinase complexed with the *Hix* site. *Biochemistry* **29**, 6561–6567.
49. Ebright, Y.W., Chen, Y., Pendergrast, P.S. & Ebright, R.H. (1992). Incorporation of an EDTA–metal complex at a rationally selected site within a protein: application to EDTA–iron DNA affinity cleaving with catabolite gene activator protein (CAP) and Cro. *Biochemistry* **31**, 10664–10670.
50. Dumoulin, P., Ebright, R.H., Knegtel, R., Kaptein, R., Granger-Schnarr, M. & Schnarr, M. (1996). Structure of the LexA repressor–DNA complex probed by affinity cleavage and affinity photo-cross-linking. *Biochemistry* **35**, 4279–4286.
51. Heilek, G.M., Marusak, R., Meares, C.F. & Noller, H.F. (1995). Directed hydroxyl radical probing of 16S rRNA using Fe(II) tethered to ribosomal protein S4. *Proc. Natl. Acad. Sci. USA* **92**, 1113–1116.
52. Ghaim, J.B., Greiner, D.P., Meares, C.F. & Gennis, R.B. (1995). Proximity mapping the surface of a membrane protein using an artificial protease: demonstration that the quinone-binding domain of subunit I is near the amino-terminal region of subunit II of cytochrome *bd*. *Biochemistry* **34**, 11311–11315.

53. Rana, T.M. & Meares, C.F. (1991). Iron chelate mediated proteolysis: protein structure dependence. *J. Am. Chem. Soc.* **113**, 1859–1861.
54. Ermácora, M.R., Ledman, D.W. & Fox, R.O. (1996). Mapping the structure of a non-native state of staphylococcal nuclease. *Nat. Struct. Biol.* **3**, 59–66.
55. Schepartz, A. & Cuenoud, B. (1990). Site-specific cleavage of the protein calmodulin using a trifluoperazine-based affinity reagent. *J. Am. Chem. Soc.* **112**, 3247–3249.
56. Hoyer, D., Cho, H. & Schultz, P.G. (1990). A new strategy for selective protein cleavage. *J. Am. Chem. Soc.* **112**, 3249–3250.
57. Dreyer, G.B. & Dervan, P.B. (1985). Sequence-specific cleavage of single-stranded DNA: oligodeoxynucleotide–EDTA•Fe(II). *Proc. Natl. Acad. Sci. USA* **82**, 968–972.
58. Moser, H.E. & Dervan, P.B. (1987). Sequence-specific cleavage of double helical DNA by triple helix formation. *Science* **238**, 645–650.
59. Rana, T.M. & Meares, C.F. (1990). Specific cleavage of a protein by an attached iron chelate. *J. Am. Chem. Soc.* **112**, 2457–2458.
60. Haywood, M.M., Adrian, J.C., Jr. & Schepartz, A. (1995). Convenient syntheses of bifunctional metal chelates. *J. Org. Chem.* **60**, 3924–3927.
61. Ermácora, M.R., Delfino, J.M., Cuenoud, B., Schepartz, A. & Fox, R.O. (1992). Conformation-dependent cleavage of staphylococcal nuclease with a disulfide-linked iron chelate. *Proc. Natl. Acad. Sci. USA* **89**, 6383–6387.
62. Dawson, P.E., Muir, T.W., Clark-Lewis, I. & Kent, S.B.H. (1994). Synthesis of proteins by native chemical ligation. *Science* **266**, 776–779.
63. Lu, W., Qasim, M.A. & Kent, S.B.H. (1996). Comparative total synthesis of turkey ovomucoid third domain by stepwise solid phase peptide synthesis and native chemical ligation. *J. Am. Chem. Soc.* **118**, 8518–8523.
64. Friedman, M., Krull, L.H. & Cavins, J.F. (1970). The chromatographic determination of cystine and cysteine residues in proteins as S-β-(4-pyridylethyl)cysteine. *J. Biol. Chem.* **245**, 3868–3871.
65. Hirel, P.-H., Schmitter, J.-M., Dessen, P., Fayat, G. & Blanquet, S. (1989). Extent of amino-terminal methionine excision from *Escherichia coli* proteins is governed by the sidechain length of the penultimate amino acid. *Proc. Natl. Acad. Sci. USA* **86**, 8247–8251.
66. Nagai, K. & Thøgersen, H.C. (1984). Generation of β-globin by sequence-specific proteolysis of a hybrid protein produced in *Escherichia coli*. *Nature* **309**, 810–812.
67. Hochuli, E., Bannwarth, W., Döbeli, H., Gentz, R. & Stüber, D. (1988). Genetic approach to facilitate purification of recombinant proteins with a novel metal chelate absorbent. *Biotechnology* **6**, 1321–1325.
68. Shiraishi, H., Kataoka, M., Morita, Y. & Umemoto, J. (1993). Interactions of hydroxyl radicals with tris(hydroxymethyl)aminomethane and Good's buffers containing hydroxymethyl or hydroxyethyl residues produce formaldehyde. *Free Rad. Res. Commun.* **19**, 315–321.
69. Kemp, D.S. & Carey, R.I. (1993). Synthesis of a 39-peptide and a 25-peptide by thiol capture ligations: observation of a 40-fold rate acceleration of the intramolecular O,N-acyl-transfer reaction between peptide fragments bearing only cysteine protective groups. *J. Org. Chem.* **58**, 2216.
70. Canne, L.E., Bark, S.J. & Kent, S.B.H. (1996). Extending the applicability of native chemical ligation. *J. Am. Chem. Soc.* **118**, 5891–5896.
71. Lee, D.H., Granja, J.R., Martinez, J.A., Severin, K. & Ghadiri, M.R. (1996). A self-replicating peptide. *Nature* **382**, 525–528.
72. Abate, C., Patel, L., Rauscher, F.J., III, & Curran, T. (1990). Redox regulation of Fos and Jun DNA-binding activity *in vitro*. *Science* **249**, 1157–1161.
73. Geoghegan, K.F. & Stroh, J.G. (1992). Site-directed conjugation of nonpeptide groups to peptides and proteins via periodate oxidation of a 2-amino alcohol. Application to modification at N-terminal serine. *Bioconj. Chem.* **3**, 138–146.
74. Ellenberger, T.E., Brandl, C.J., Struhl, K. & Harrison, S.C. (1992). The GCN4 basic region leucine zipper binds DNA as a dimer of uninterrupted α helices: crystal structure of the protein–DNA complex. *Cell* **71**, 1223–1237.
75. König, P. & Richmond, T.J. (1993). The X-ray structure of the GCN4-bZIP bound to ATF/CREB site DNA shows the complex depends on DNA flexibility. *J. Mol. Biol.* **233**, 139–154.
76. Ho, I.C., Hodge, M.R., Rooney, J.W. & Glimcher, L.H. (1996). The proto-oncogene *c-maf* is responsible for tissue-specific expression of interleukin-4. *Cell* **85**, 973–983.
77. LeClair, K.P., Blanar, M.A. & Sharp, P.A. (1992). The p50 subunit of NF-κB associates with the NF-IL6 transcription factor. *Proc. Natl. Acad. Sci. USA* **89**, 8145–8149.
78. Gong, Q., Huang, Z. & Wicks, W.D. (1995). Interaction of retinoblastoma gene product with transcription factors ATFa and ATF2. *Arch. Biochem. Biophys.* **319**, 445–450.
79. Kaszubska, W., *et al.*, & Whelan, J. (1993). Cyclic AMP-independent ATF family members interact with NF-κB and function in the activation of the E-selectin promoter in response to cytokines. *Mol. Cell. Biol.* **13**, 7180–7190.
80. Ferré-D'Amaré, A., Prendergast, G., Ziff, E. & Burley, S. (1993). Recognition by Max of its cognate DNA through a dimeric b/HLH/Z domain. *Nature* **363**, 38–45.
81. Ma, P.C.M., Rould, M.A., Weintraub, H. & Pabo, C.O. (1994). Crystal structure of MyoD bHLH domain–DNA complex: perspectives on DNA recognition and implications for transcriptional activation. *Cell* **77**, 451–459.
82. Ellenberger, T.E., Fass, D., Arnaud, M. & Harrison, S.C. (1994). Crystal structure of transcription factor E47: E-box recognition by a basic region helix-loop-helix dimer. *Genes Dev.* **8**, 970–980.
83. Pavletich, N.P. & Pabo, C.O. (1991). Zinc finger–DNA recognition: crystal structure of a Zif268–DNA complex at 2.1 Å. *Science* **252**, 809–817.
84. Kaptein, R. (1992). Zinc-finger structures. *Curr. Opin. Struct. Biol.* **2**, 109–115.
85. Luisi, B.F., Xu, W.X., Otwinowski, Z., Freedman, L.P., Yamamoto, K.R. & Sigler, P.B. (1991). Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. *Nature* **352**, 497–505.
86. Schwabe, J.W.R., Chapman, L., Finch, J.T. & Rhodes, D. (1993). The crystal structure of the estrogen receptor DNA-binding domain bound to DNA: how receptors discriminate between their response elements. *Cell* **75**, 567–578.
87. Wolberger, C., Vershon, A.K., Liu, B., Johnson, A.D. & Pabo, C.O. (1991). Crystal structure of a Matα2 homeodomain–operator complex suggests a general model for homeodomain–DNA interactions. *Cell* **67**, 517–528.
88. Kissinger, C.R., Liu, B., Martin-Blanco, E., Kornberg, T.B. & Pabo, C.O. (1990). Crystal structure of an engrailed homeodomain–DNA complex at 2.8 Å resolution: a framework for understanding homeodomain-DNA interactions. *Cell* **63**, 579–590.
89. Klemm, J.D., Rould, M.A., Aurora, R., Herr, W. & Pabo, C.O. (1994). Crystal structure of the Oct-1 POU domain bound to an octamer site: DNA recognition with tethered DNA–binding modules. Cell **77**, 21–32.
90. Jordan, S.R. & Pabo, C.O. (1988). Structure of the lambda complex at 2.5 Å resolution: details of the repressor–operator interactions. *Science* **242**, 893–899.
91. Aggarwal, A.K., Rodgers, D.W., Drottar, M., Ptashne, M. & Harrison, S.C. (1988). Recognition of a DNA operator by the repressor of phage 434: a view at high resolution. *Science* **242**, 899–907.
92. Mondragón, A., Wolberger, C. & Harrison, S.C. (1989). Structure of phage 434 Cro protein at 2.35 Å resolution. *J. Mol. Biol.* **205**, 179–188.
93. Sarkar, G. & Sommer, S.S. (1990). The 'megaprimer' method of site-directed mutagenesis. *BioTechniques* **8**, 404–407.
94. Sodeoka, M., Larson, C.J., Chen, L., LeClair, K.P. & Verdine, G.L. (1993). A multifunctional plasmid for protein expression by ECPCR: overproduction of the p50 subunit of NF-κB. *BioMed. Chem. Lett.* **3**, 1089–1094.