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The basic region of Fos mediates specific DNA binding

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The DNA-binding domains of the members of the Fos and Jun families of proteins consist of a basic region followed by a dimerizing segment with heptad repeats of leucine. Fos-Jun heterodimers and Jun alone, but not Fos alone, bind to the symmetrical sequences TGACTCA (AP-1 site) or TGACGTCA (cAMP response element or CRE). We set out to test the hypothesis that in the Fos-Jun heterodimer the basic region of Fos confers specific DNA-binding properties equivalent to the contribution of the basic region of Jun. Fos-Jun chimeric proteins were prepared consisting of the basic region of one protein joined to the leucine repeat of the other. Heterodimers with mixed Fos and Jun leucine repeat segments showed high affinity binding to the AP-1 site or CRE whether they contained two basic regions from Jun, two basic regions from Fos, or one from each source. Heterodimers with two Fos basic regions showed somewhat greater affinity for the CRE and AP-1 site than the heterodimer with two Jun basic regions. The DNA sequence specificity and the purine and phosphate DNA contact sites for each heterodimer were similar. We conclude that in the Fos-Jun heterodimer the basic region of Fos contributes specific DNA-binding properties equivalent to those of Jun. Our results support a model in which the Fos and Jun basic regions of the Fos-Jun heterodimer each interact with symmetrical DNA half sites.

Key words: Jun/transcription factors

Introduction

Members of the Fos and Jun families of proteins, which appear to play an important role in the regulation of cell growth (Levy *et al.*, 1978; Curran and Teich, 1982; Maki *et al.*, 1987), form heterodimers that bind to specific sequences in DNA (Halazonetis *et al.*, 1988; Kouzarides and Ziff, 1988; Nakabeppu *et al.*, 1988; Rauscher *et al.*, 1988a; Gentz *et al.*, 1989; Turner and Tjian, 1989). At least some of these proteins are present in the purified mammalian transcription factor complex AP-1 (Angel *et al.*, 1987; Lee *et al.*, 1987; Rauscher *et al.*, 1988b) and are found associated with each other in cellular extracts (Rauscher *et al.*, 1988b,c). Jun proteins by themselves can bind as homodimers to AP-1 sites in DNA (consensus core sequence TGACTCA, also known as a TPA response element or TRE) and to cAMP response elements or CRE (consensus core sequence TGACGTCA, also known as ATF sites); Fos by itself does not bind to either site. However, Jun-Fos heterodimers bind to AP-1 sites or the CRE with much greater affinity than do Jun homodimers (Bohmann *et al.*, 1987; Bos *et al.*, 1988; Halazonetis *et al.*, 1988; Kouzarides and Ziff, 1988; Nakabeppu *et al.*, 1988; Rauscher *et al.*, 1988a; Sassone-Corsi *et al.*, 1988). This could be due to the formation of more stable Jun-Fos dimers compared with Jun-Jun dimers (Halazonetis *et al.*, 1988; Nakabeppu *et al.*, 1988; Rauscher *et al.*, 1988a; Gentz *et al.*, 1989), and/or the affinity of the Fos component of the dimer for the DNA site.

The physical basis of heterodimer formation between Fos and Jun proteins (or the formation of Jun homodimers) was suggested by the presence in each member of the Fos and Jun families of a region of heptad repeats of leucine residues ('leucine zipper') postulated to form amphipathic helices that mediate dimerization (Landschulz *et al.*, 1988). Studies with a model peptide and of mutated proteins with heptad repeats of leucine have supported this hypothesis and have further shown that the helices have a parallel orientation (Kouzarides and Ziff, 1988; Gentz *et al.*, 1989; Landschulz *et al.*, 1989; O'Shea *et al.*, 1989; Ransone *et al.*, 1989; Schuermann *et al.*, 1989; Turner and Tjian, 1989). Substitutions that change one or two of the repetitive leucine residues can abolish dimer formation and also result in loss of DNA-binding activity (Kouzarides and Ziff, 1988; Gentz *et al.*, 1989; Ransone *et al.*, 1989; Schuermann *et al.*, 1989; Turner and Tjian, 1989), presumably because dimerization is essential for specific binding to the symmetrical AP-1 site or CRE. In addition to the region of heptad leucine repeats, a second region of Fos and Jun (and structurally related proteins) has been shown to be essential for specific DNA binding, namely, the highly basic region of each protein adjacent to the leucine repeats (Hope and Struhl, 1986, 1987; Kouzarides and Ziff, 1988; Gentz *et al.*, 1989; Landschulz *et al.*, 1989; Neuberg *et al.*, 1989; Turner and Tjian, 1989). These two regions together are sufficient for dimerization and binding to DNA. The sequence of the basic region, as well as that of the leucine repeats, is highly conserved within the Jun family and within the Fos family, and between Jun and Fos there is also substantial conservation of the spatial arrangement of positively charged residues in the basic region (Kouzarides and Ziff, 1988; Turner and Tjian, 1989). Based on these various experimental results, the current model for the interaction of the Fos-Jun heterodimer with DNA suggests that dimerization of Fos and Jun via their leucine repeat helices positions their respective basic regions such that the Fos basic region and the Jun basic region can interact with DNA on either side of the dyad axis of the recognition site.

We set out to test a specific prediction of this model, i.e. that the Fos basic region confers specific DNA-binding properties equivalent to the DNA binding properties of the Jun basic region. For this purpose we prepared Fos-Jun chimeric proteins that contained the basic region of one

protein joined to the leucine repeat of the other. These chimeric proteins were then used to form stable heterodimers that contained either two Fos basic regions, two Jun basic regions, or one of each. The DNA-binding properties of the various dimers were then tested and were found to be nearly the same, indicating that the Fos basic region is functionally equivalent to the Jun basic region. Our results also support the inference that the difference in DNA-binding properties of Fos, Jun, and Fos + Jun is related primarily to the formation or stability of the respective dimers.

Results

Construction and expression of jun−fos chimeric plasmids

In order to generate *jun−fos* chimeric genes, we first constructed plasmids which encode the C-terminal segment of JunD (Hirai *et al.*, 1989; Ryder *et al.*, 1989) corresponding to the basic region and leucine repeats (amino acids 216−341 preceded by Met), or the central segment of Fos (van Straaten *et al.*, 1983) containing its corresponding basic region and leucine repeats (amino acids 121−207 preceded by Met and followed by Leu and Asn) (Figure 1A). These regions of Fos and Jun (including JunD) have been shown to be sufficient for heterodimer formation via their leucine repeat sequences and for sequence-specific binding of heterodimer to DNA (Halazonetis *et al.*, 1988; Kouzarides and Ziff, 1988; Nakabeppu *et al.*, 1988). Chimeric plasmids encoding the basic domain of JunD and the leucine repeat of Fos or the basic domain of Fos and the leucine repeat of JunD (Figure 1B) were constructed using restriction endonuclease cleavage sites introduced by site-directed mutagenesis as described in Materials and methods. The corresponding proteins were subsequently synthesized by translation in rabbit reticulocyte lysates of the *in vitro* transcripts of the chimeric plasmids. As indicated in Figure 1B, the truncated Jun translation product is designated JJ and the truncated Fos translation product is designated FF, the first letter representing the basic domain and the second letter representing the leucine repeat. JF is the chimeric polypeptide that has the basic domain of JunD and the leucine repeat of Fos, FJ has the basic domain of Fos and the leucine repeat of JunD. When the various translation products were examined by SDS−PAGE (Figure 2), the proteins showed the expected electrophoretic mobilities, but FF and FJ polypeptides migrated as a broad band possibly due to co-migration with reticulocyte proteins. As expected, FF and FJ, but not JJ and JF, were immunoprecipitated by antiserum against the Fos basic region (Figure 2).

Dimer formation by chimeric proteins

To compare the dimerization properties of the Jun−Fos polypeptide pairs of ^{35}S-labeled co-translation products, each pair containing one polypeptide with the basic region of Fos, were immunoprecipitated with antiserum against the Fos basic region (Figure 3A). JJ + FF or JF + FJ resulted in immunoprecipitable JJ and JF respectively, indicating stable dimer formation. JJ + FJ yielded barely detectable immunoprecipitable JJ (seen in a longer exposure of the gel

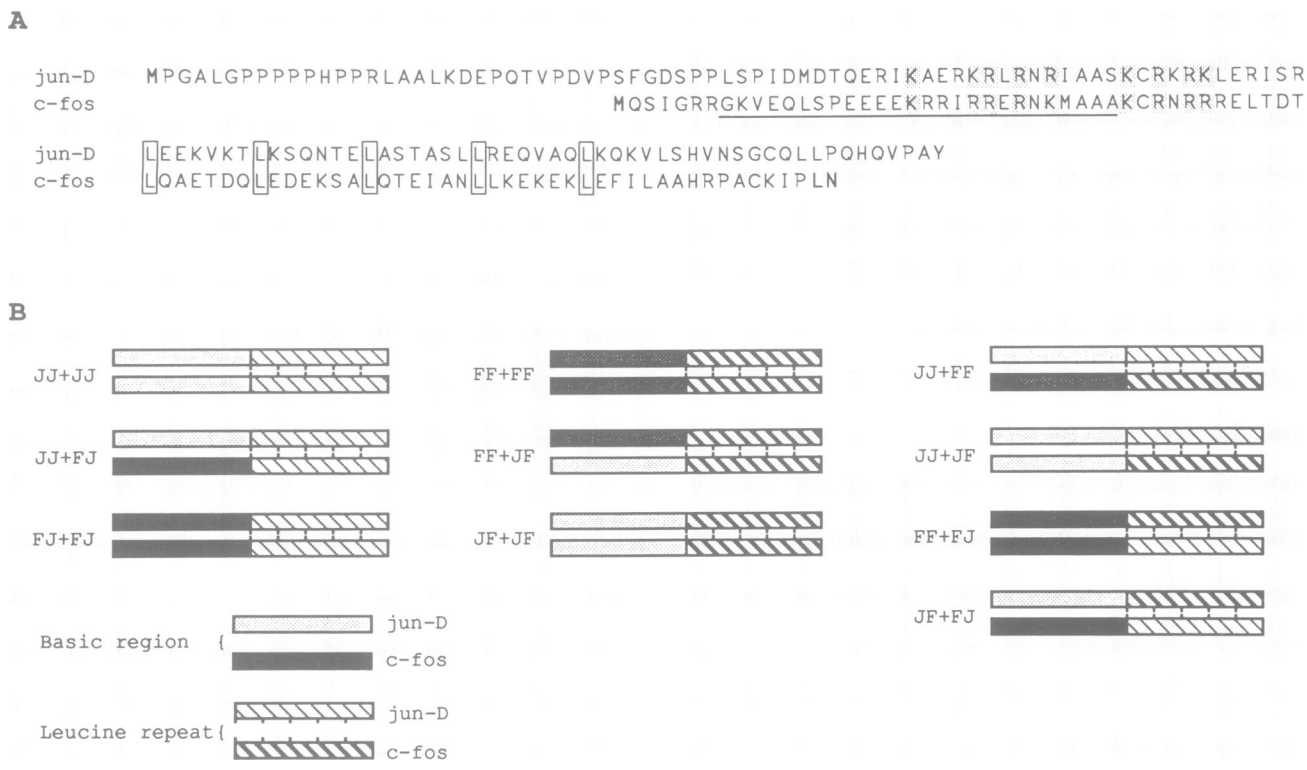

Fig. 1. The chimeric DNA-binding domains of Jun and Fos and potential dimers. (**A**) Amino acid sequences of JunD and c-Fos DNA-binding domains. The basic regions (**upper panel**) and the leucine repeat regions (**lower panel**) of the JunD and c-Fos DNA-binding domains are aligned. The conserved basic amino acids (K, R) in the basic regions are shaded, the residues making up the leucine repeats (L) are boxed and the bold characters (M, L, N) indicate the amino acids which were introduced by *in vitro* manipulation. The amino acid sequence of the synthetic peptide used to generate antiserum against the Fos basic region is underlined. (**B**) Schematic representation of the JunD (JJ), c-Fos (FF) and the chimeric (JF, FJ) DNA binding domains and potential dimers.

shown in Figure 3A), and JF + FF yielded no detectable immunoprecipitable JF. We conclude from this experiment that the dimeric proteins with one Fos and one Jun leucine repeat form more readily or are more stable during the immunoprecipitation procedure than dimers with two Jun leucine repeats, which form more readily or are more stable than dimers with two Fos leucine repeats.

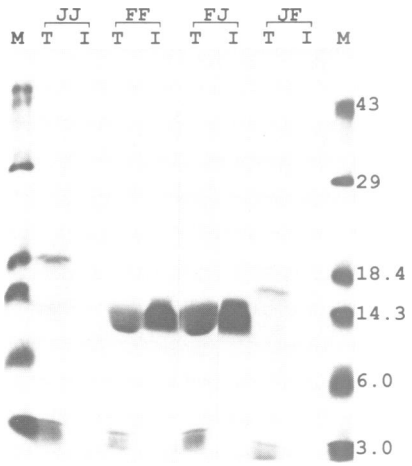

Fig. 2. Synthesis of chimeric proteins in reticulocyte lysate. One microgram of RNA prepared by *in vitro* transcription was translated in the presence of [^{35}S]methionine. Translation products (JJ, FF, FJ and JF) were precipitated by 10% trichloroacetic acid (**lanes T**) or immunoprecipitated with the antiserum against the Fos basic region (**lanes I**) and subjected to 17% SDS–PAGE. **Lanes M**, ^{14}C-labeled mol. wt standards, ovalbumin (43 kd), carbonic anhydrase (29 kd), β-lactoglobulin (18.4 kd), lysozyme (14.3 kd), bovine trypsin inhibitor (6.0 kd), and insulin (3.0 kd).

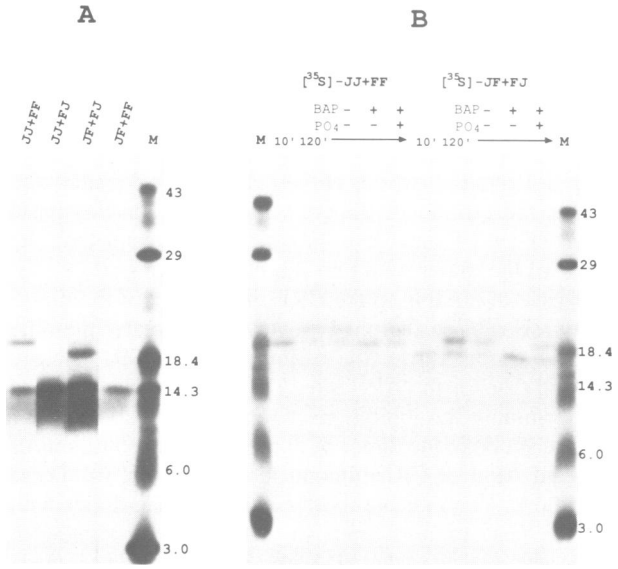

Fig. 3. Dimer formation and phosphorylation of Fos–Jun translation products. (**A**) ^{35}S-labeled co-translation products were immunoprecipitated with antiserum against the Fos basic region and analyzed by 17% SDS–PAGE. **Lane M**, mol. wt standards as described in Figure 1. (**B**) ^{35}S-labeled JJ or JF translation product was mixed with an equal volume of unlabeled FF or FJ translation product and incubated at room temperature for 10 or 120 min and precipitated with antiserum against the Fos basic region. The immune precipitate from the 120 min incubation was also incubated with or without bacterial alkaline phosphatase (BAP) [in one case with 1 mM potassium phosphate (PO_4)] at 60°C for 1 h before SDS–PAGE. Lanes M, mol. wt standards as described before.

The above experiments revealed another potentially interesting finding, namely, that the JJ or JF component of the heterodimers changes to a form with lower electrophoretic mobility during continued incubation of the dimer in the reticulocyte lysate. As shown in Figure 3B, alkaline phosphatase treatment of the immunoprecipitated heterodimer resulted in a reversal of the shift in mobility. We infer from these results that the Jun component of the dimers becomes phosphorylated, presumably by a protein kinase in the reticulocyte extract. The possible physiological significance of this observation remains to be explored.

DNA binding by chimeric proteins

To determine whether the chimeric proteins bind the consensus AP-1 site, the various translation products shown in Figure 1B were incubated with an end-labeled oligonucleotide containing the sequence ATGACTCAT and assayed for binding by retardation of the oligomer during gel electrophoresis (Figure 4A). Binding of JJ + JJ, FF + FF, FJ + FJ, and JF + JF was barely detectable or undetectable in the exposure shown in the figure, although with longer exposure slight binding was evident in the autoradiogram with JJ + JJ and FJ + FJ, but not with FF + FF and JF + JF (data not shown). In contrast to these results, JJ + FF, JJ + JF, FF + FJ, and JF + FJ were all highly active and roughly comparable to the activities of JunD + Fos (i.e. full length proteins), JunD + FF, JunD + JF, FJ + Fos, and JJ + Fos (Figure 4A). Although the assays shown in Figure 4 were done with co-translation products, the same results were obtained for the highly active pairs with mixtures of separate translation products (data not shown), indicating that functional dimers readily form on mixing the appropriate individual polypeptides. We also tested the highly active chimeric proteins for binding to the cAMP response element (CRE), ATGACGTCAT, previously shown to be a Jun- or Jun/Fos-binding site

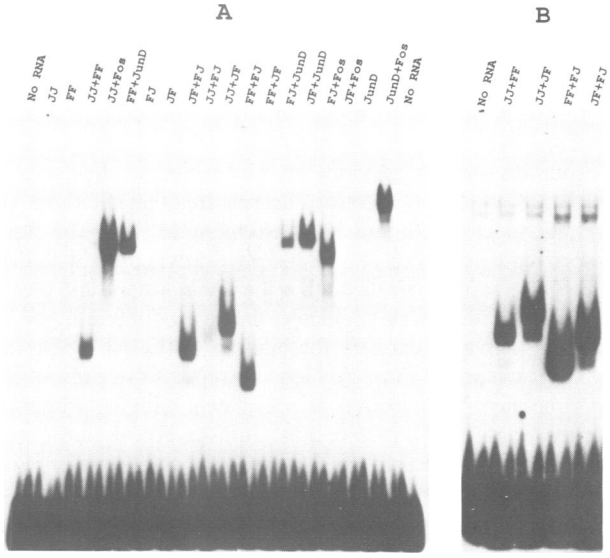

Fig. 4. Gel retardation assay for the binding of various co-translation products to the AP-1 site, ATGACTCAT (**A**) and CRE, ATGACGTCAT (**B**). Unlabeled translation products were incubated with ^{32}P-labeled fragment (1 nM of AP-1 site or 0.2 nM of CRE fragment), followed by electrophoresis and autoradiography. Translation products are indicated directly above the lanes, and 'No RNA' indicates the translation product prepared without added RNA transcript.

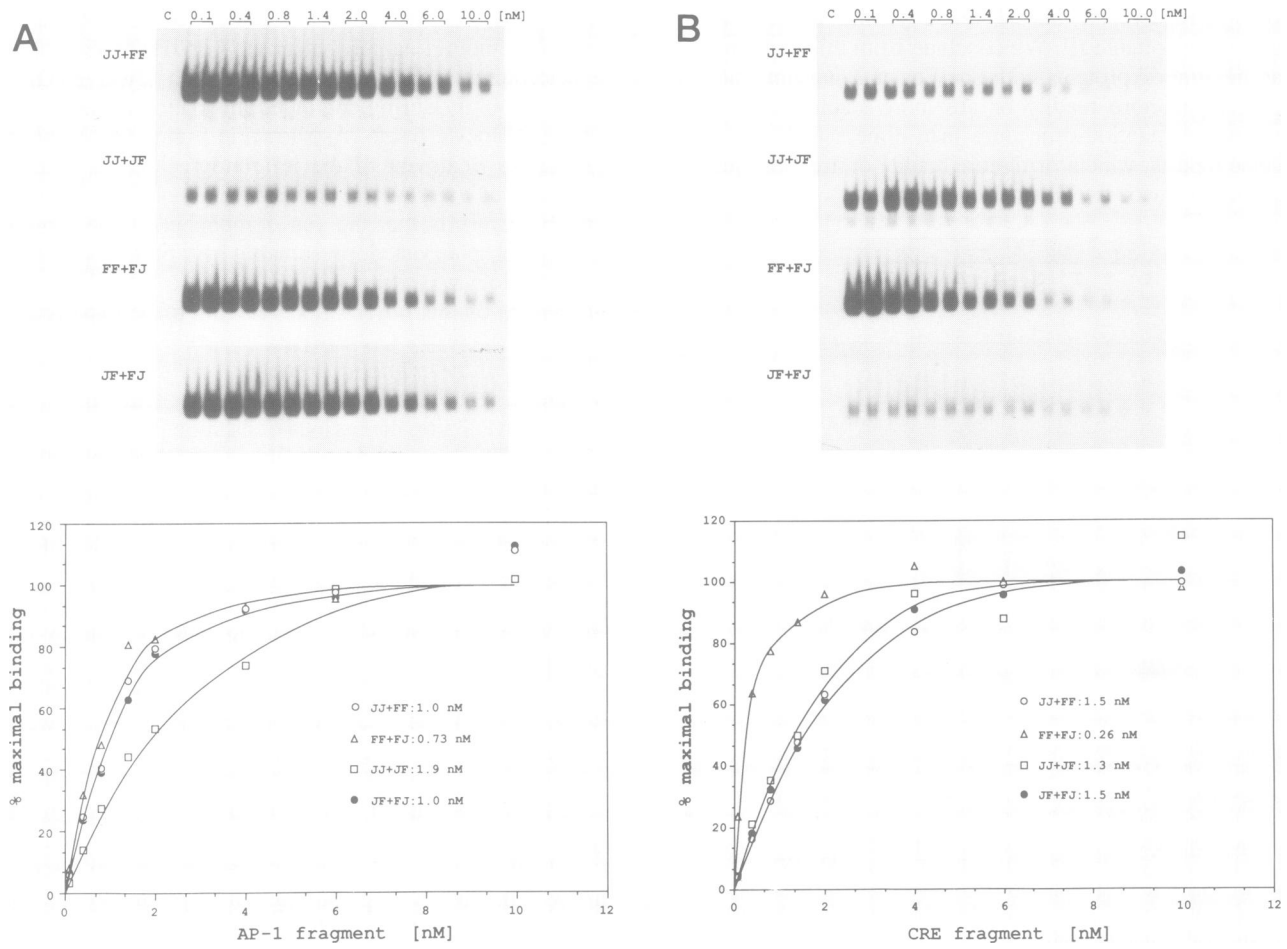

Fig. 5. Affinity of various dimers for the AP-1 site (**A**) and CRE (**B**), estimated by the gel retardation assay. Various concentrations of unlabeled fragments and 0.1 nM of ^{32}P-labeled fragment were incubated with the indicated co-translation products at room temperature for 20 min, followed by electrophoresis and autoradiography (upper panel). Each reaction was analyzed in duplicate. Total concentration of DNA fragment is shown above each lane; **lanes C** indicate incubation of 0.1 nM fragment with translation 'product' prepared in the absence of added RNA transcript. Gel areas corresponding to the retarded fragments were excised and counted. In the lower panel the percentage of maximal binding is shown as a function of fragment concentration. The concentration of fragment required for 50% of maximal binding is given in the figure.

(Nakabeppu *et al.*, 1988; Rauscher *et al.*, 1988a). The results were similar to those obtained with the AP-1 site (Figure 4B).

From this series of experiments we conclude (i) that strong binding to an oligonucleotide site requires that the dimeric binding species contains one polypeptide with the Jun dimerizing leucine repeat and the other polypeptide with the Fos leucine repeat, (ii) that either the homodimeric Jun basic regions or the Fos basic regions or the heterodimeric Jun/Fos basic regions are adequate for binding to the AP-1 site or the CRE when the Jun−Fos leucine repeats are present in the same dimer. The results are consistent with the proposal that the relative affinities of Fos, Jun, or Fos + Jun for a DNA site are related to the formation of stable dimers via their leucine repeat regions.

Affinities of chimeric proteins for the AP-1 site and the CRE

Next, we estimated the affinities of the four highly active dimers for the AP-1 site and CRE by determining the amount of bound oligonucleotide as a function of oligonucleotide concentration (Figure 5). For each dimer the same preparation of translation products was used in the AP-1 site and CRE experiments. For the AP-1 site the affinity of a dimer with two Fos basic regions (FF + FJ) is approximately the same as the affinity of dimers with mixed Jun/Fos basic regions (JJ + FF and JF + FJ), which is greater than the affinity of the dimer with two Jun basic regions (JJ + JF). In the case of binding to the CRE the affinity of the dimer with two Fos basic regions is several fold greater than the others. For this dimer the affinity for the CRE clearly exceeded that for the AP-1 site. This result was confirmed by determining the inhibition of binding of the [^{32}P]AP-1 oligonucleotide to the dimer by increasing concentrations of unlabeled AP-1 or CRE oligonucleotide (Figure 6). These experiments also indicate more clearly that the dimer with two Jun basic regions binds to the CRE with somewhat greater affinity than to the AP-1 site (Figure 6). We conclude that the dimeric Fos basic region confers somewhat greater affinity for the AP-1 site and the CRE compared to dimeric Jun basic regions.

Specificity for DNA-binding sites

To further compare the binding of dimers with Jun−Jun, Fos−Fos, or Jun−Fos basic regions, we used oligonucleotides with base substitutions in the AP-1 site or CRE and examined the ability of these variant oligonucleotides to bind to chimeric proteins (Table I) or to compete with the AP-1

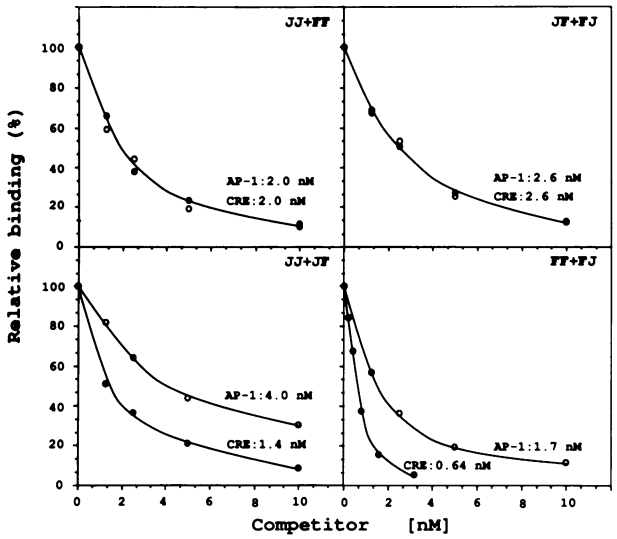

Fig. 6. Comparison of affinities of various dimers for the AP-1 site and CRE. Unlabeled AP-1 (open circles) or CRE (closed circles) 'competitor' fragments were incubated with the indicated co-translation products (JJ + FF, JF + FJ, JJ + JF and FF + FJ) at room temperature for 15 min, and then ^{32}P-labeled AP-1 fragment (0.1 nM) was added and equilibrated for 20 min at room temperature prior to electrophoresis. Gel areas corresponding to the retarded fragments were excised and counted. The relative binding is shown as a function of the concentration of unlabeled fragment. The concentration of unlabeled fragment required for 50% reduction in binding of the labeled fragment is given in each graph.

Table I. Effects of base substitution in the AP-1 site or CRE on binding of different dimers

Binding site	Relative binding activity				
	Jun + Fos	JJ + FF	JJ + JF	FF + FJ	JF + FJ
AP-1(wt)	100	100	100	100	100
AP-1(3T)	16	26	16	11	24
AP-1(4T)	2.1	3.0	<2	<2	<2
AP-1(6A)	12	22	4.3	7.4	16
Ap-1(7A)	21	47	16	14	35
CRE(wt)	100 (48)	100 (83)	100 (190)	100 (220)	100 (89)
CRE(6A)	19	23	18	25	19
CRE(6T)	3.4	3.8	2.4	<2	2.6
CRE(6C)	14	13	11	5.3	9.1
CRE(1C,10G)[a]	ND	7.1	7.7	2.8	7.3

Each end-labeled fragment (0.2 nM) was incubated with various dimers for 20 min at room temperature prior to electrophoresis and gel areas corresponding to the retarded fragments were excised and counted. Binding activity relative to the AP-1(wt) or CRE(wt) fragment is shown. The binding activity of the CRE(wt) fragment relative to the AP-1(wt) fragment is shown in parenthesis.
[a]Separate experiment; ND, not done.

site for binding (Figure 7). An overall comparison of the results indicates that with one exception, each base pair substitution has similar effects on the relative binding by the various dimers tested. The exception is the T6 → A substitution which showed a greater effect on binding by JJ + JF than by the other dimers tested in both the direct binding assay (Table I) and the competition assay (Figure 7). Thus there is little difference in binding site specificity between the Fos and Jun basic regions. Some base pair substitutions resulted in a marked decrease in binding whereas others had a lesser effect (summarized in Figure 7).

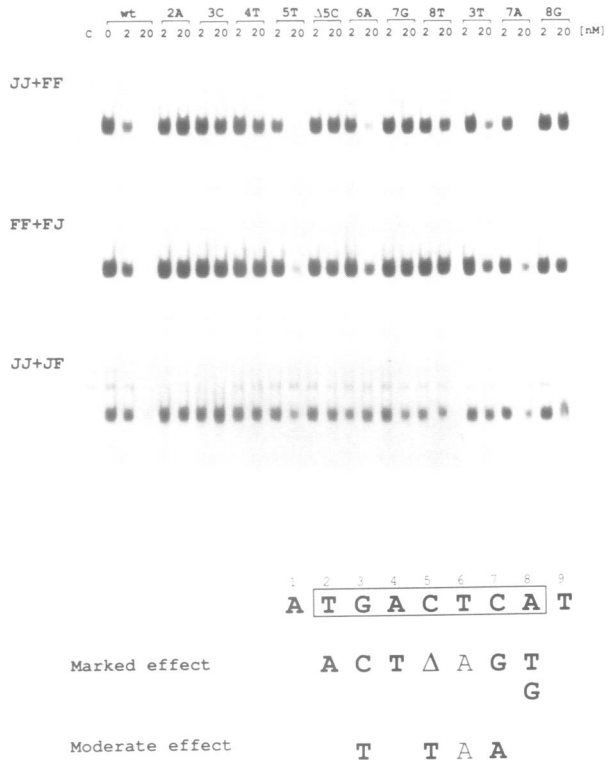

Fig. 7. Effects of base substitution in the AP-1 site on binding of different dimers. Co-translation products (JJ + FF, FF + FJ and JJ + JF) were incubated with (2 or 20 nM) or without (0 nM) unlabeled fragments, which have one base substitution in the AP-1 binding site, at room temperature for 15 min, and ^{32}P-labeled AP-1 fragment (0.1 nM) was added and the mixture was equilibrated for 20 min at room temperature prior to electrophoresis. Autoradiograms are shown in the upper panel with the substituted base indicated directly above the lanes. The effects of base substitution are summarized in the lower panel and described in the text.

Particularly notable are the approximately comparable effects of symmetrical changes at positions 2 and 8, and 3 and 7, but not at positions 4 and 6 (for the dimers with mixed J/F basic regions). In the case of the CRE, although few altered oligonucleotides were tested (Table I), the results also indicate that binding by each of the dimers is affected to similar extents by the base-substituted oligomers tested. Change in G6 to any other base led to a decrease in binding, most marked with the T6 substitution. Also of note is the importance of positions 1 and 10, which flank the core CRE sequence; flanking nucleotides also affect the *in vivo* response of the CRE to cAMP, presumably mediated by the CRE-binding protein CREB (Deutsch *et al.*, 1988). This effect explains our previously reported finding that the CRE (in that case with 1C, 10G flanking nucleotides) had only ~10% of the binding activity of the AP-1 site when tested with Jun alone or Jun plus Fos (Nakabeppu *et al.*, 1988).

Nucleotide contacts of Jun–Fos proteins

To compare the physical interactions of the various dimers with the AP-1 site or CRE we determined the effect of purine methylation on binding as described in Materials and methods. As seen in Figure 8, the methylation interference patterns for each DNA strand were essentially identical for each of the dimeric proteins tested: Jun + Fos, JJ + FF, JJ + JF, FF + FJ and JF + FJ. The inferred base contacts are shown for both the AP-1 site and the CRE (Figure 8).

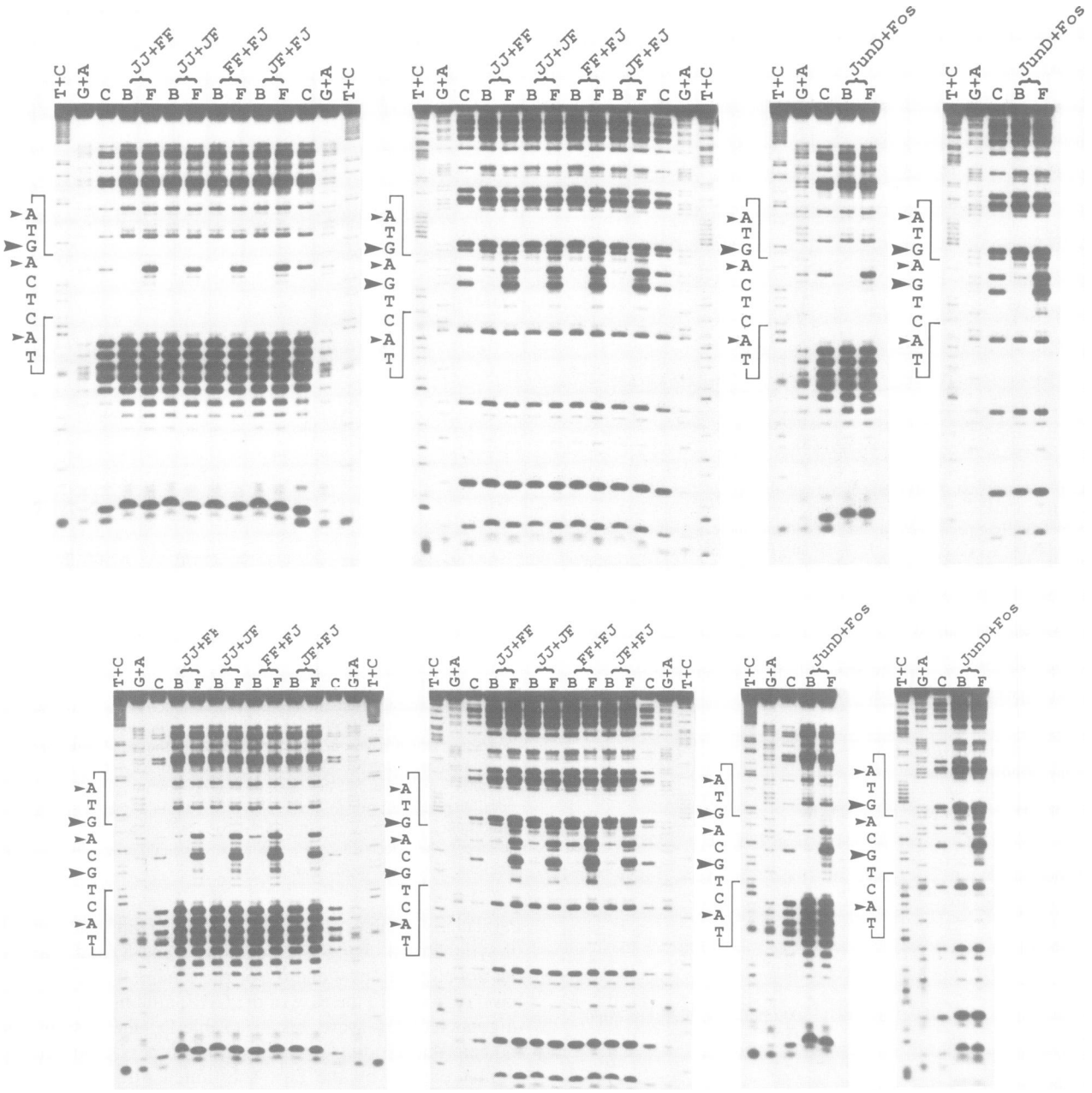

Fig. 8. Methylation interference. AP-1 (**upper panel**) or CRE (**lower panel**) fragments, end-labeled with ^{32}P on either strand, were treated with dimethyl sulfate, incubated with the indicated dimers, and separated by electrophoresis into a protein-bound fraction (**lanes B**) and unbound fraction (**lanes F**). Each fraction was cleaved at methylated purine residues and analyzed by electrophoresis in 12% polyacrylamide−7 M urea gel. Controls (**lanes C**) consisted of fragments that were not incubated with dimer. On the side of each panel are purine and pyrimidine sequence tracks of each strand and the deduced sequence of the binding site. Inferred purine contacts are indicated by arrowheads, with larger arrowheads indicating the primary contacts.

Each of the guanine bases in the core sequence of the AP-1 site or CRE is contacted, as are the adenine bases, though the latter signals are weaker. There is also evidence of interference with binding by methylation of the flanking adenine residues in each case. A parallel set of experiments to detect phosphate contacts by the various dimers indicated that ethylation of phosphate groups throughout the core sequences of the AP-1 site or CRE and in the flanking nucleotide on either side, interfered with binding by each of the dimers (Figure 9). Again, there was no detectable difference between dimers with Jun−Jun, Fos−Fos, or Fos−Jun basic regions, including full length Fos−Jun dimers. We conclude that there are extensive, symmetrical contacts between the dimers and the DNA bases and phosphate groups in the AP-1 site and CRE, and that the contacts made by dimers with the different basic regions are very similar. Further, full length Fos−Jun dimers did not show evidence of DNA interactions additional to those seen with the truncated dimers.

Discussion

The main conclusion of this report is that the basic region of Fos contributes specific DNA-binding properties to Fos−Jun heterodimers. Dimers with either two Fos basic regions, two Jun basic regions or one of each could

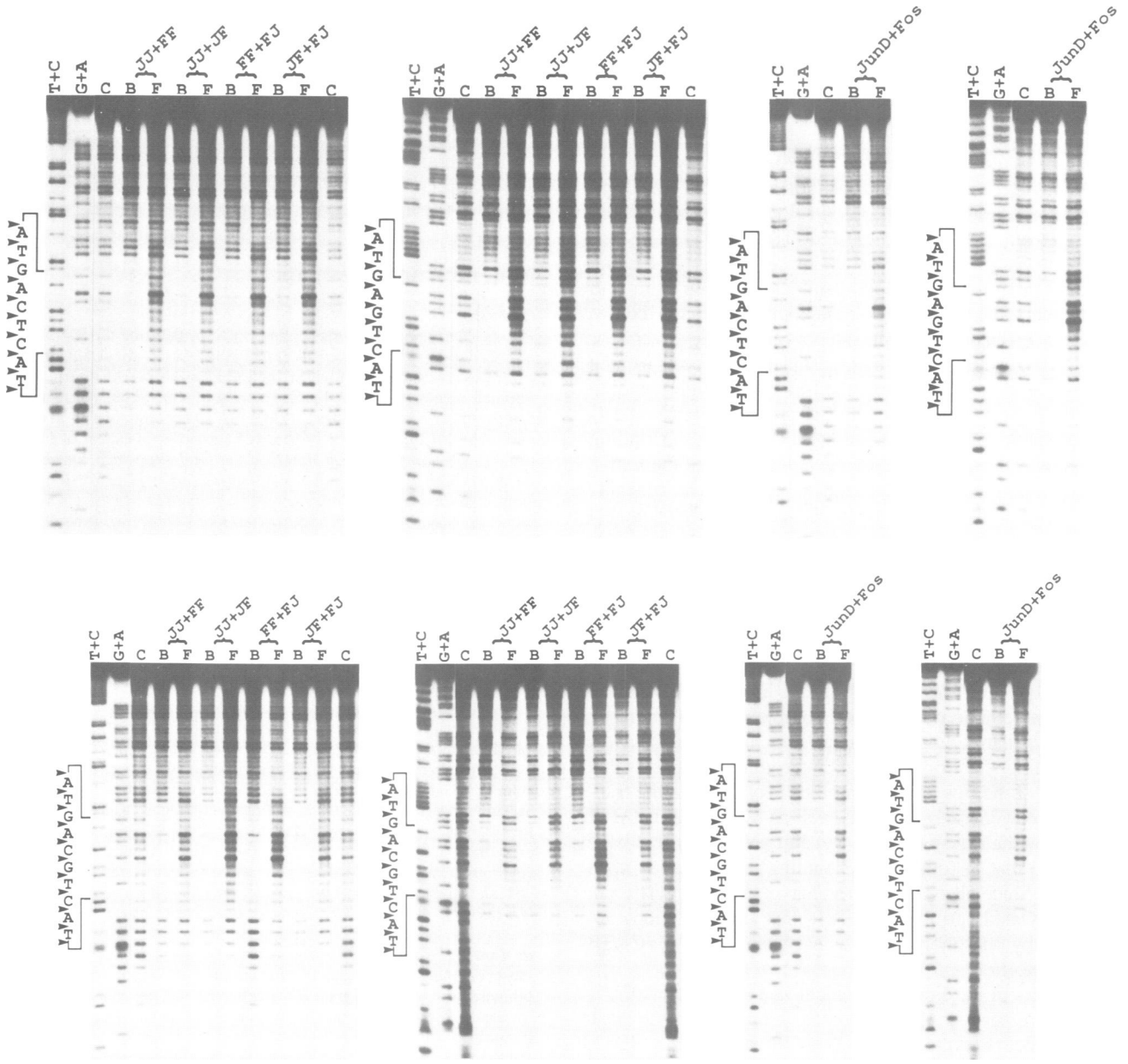

Fig. 9. Ethylation interference. AP-1 (**upper panel**) or CRE (**lower panel**) fragments, end-labeled with ^{32}P on either strand, were treated with ethyl nitrosourea, incubated with the indicated dimers, and separated by electrophoresis into a protein-bound fraction (**lanes B**) and unbound fraction (**lanes F**). Each fraction was cleaved at ethylated phosphate residues and analyzed as described in Figure 8. Controls (**lanes C**) consisted of fragments that were not incubated with dimer. To the left of each panel are purine and pyrimidine sequence tracks of each strand and the deduced sequence of the binding site. Inferred phosphate contacts are marked by arrowheads.

specifically bind to an AP-1 site or CRE. Judging by the results of the DNA methylation and ethylation interference experiments and the effects of DNA base substitutions, all dimers, regardless of the source of the basic regions, bound to the DNA in a similar way. However, there was some difference in affinities estimated by the gel retardation assay; in particular, dimers with two Fos basic regions had a 3- to 4-fold greater affinity for the CRE than those with two Jun basic regions. Our findings support a current model (Gentz *et al.*, 1989; Landschulz *et al.*, 1989; Neuberg *et al.*, 1989; O'Shea *et al.*, 1989; Turner and Tjian, 1989) in which the basic regions of the Fos–Jun heterodimer (joined via their parallel leucine repeat regions) bind in an equivalent way to each symmetrical half of the DNA binding site.

The ethylation and methylation interference experiments give an indication of the extent of the interaction of Fos–Jun heterodimers with a DNA-binding site and define some of the nucleotide contacts. Since full length Fos–Jun heterodimers showed the same interference footprints as truncated heterodimers, it appears that those parts of the proteins that are outside the basic and leucine repeat segment do not contribute to significant interactions with the binding site. The methylation interference results suggest that there are symmetrical major groove contacts between a dimer and each guanine of the core sequence of the AP-1 site or CRE, including the central guanine of the AP-1 site. In addition, methylation of each adenine in the core sequence, and the adenine of the flanking nucleotide on either side, had a lesser effect on protein binding. The importance of the flanking nucleotides is evident in the comparison of efficiency of

binding of dimers to CREs with A−T versus C−G flanking nucleotides. Thus there are extensive protein−DNA interactions in the core AP-1 site and CRE and the flanking nucleotide on each end.

It is clear from previous reports that the greater DNA-binding activity of mixtures of Fos and Jun compared to Jun alone or Fos alone is correlated with the formation of stable Fos−Jun heterodimers (Halazonetis *et al.*, 1988; Nakabeppu *et al.*, 1988; Neuberg *et al.*, 1989; Rauscher *et al.*, 1988a; Gentz *et al.*, 1989; Turner and Tjian, 1989). In our experiments also we find a correlation between DNA-binding activities of Fos and Jun polypeptides and their ability to form dimers that are stable to immunoprecipitation. Mixtures with only Fos leucine repeats did not form stable dimers nor bind to DNA, mixtures with only Jun leucine repeats formed a small amount of stable dimer and had slight binding activity, and mixtures with both Fos and Jun leucine repeats formed stable dimers and were highly active in the binding assay. Since these properties were independent of the source of the basic regions, the differences in formation of stable dimers and ability to bind to the DNA site are attributable to the leucine repeat regions of each interacting pair. Our experiments thus support the conclusion that the formation of stable dimers is a critical determinant of the ability of any given pair of Jun- or Fos-related proteins to bind to an AP-1 site or CRE. Still unanswered are questions regarding what determines 'complementarity' between two leucine repeat sequences and whether stable Jun homodimers are present in cellular transcription complexes.

The fact that certain CRE sequences are high affinity *in vitro* binding sites for Fos−Jun heterodimers suggests that members of the Fos and Jun families may regulate the transcription of genes via CREs as well as by binding to AP-1 sites. How the activity of Fos and Jun proteins is regulated relative to that of (other) CRE-binding proteins is an open question. Since all of these proteins are found in different phosphorylated forms, it is likely that their activities are regulated by phosphorylation, as has been shown for CREB (Yamamoto *et al.*, 1988).

Members of the Fos family identified so far (Fos, Fra1, FosB) have very similar amino acid sequences in the basic and leucine repeat segments (van Straaten *et al.*, 1983; Cohen and Curran, 1988; Zerial *et al.*, 1989), and members of the Jun family identified so far (c-Jun, JunB, and JunD) have very similar sequences in their basic and leucine repeat segments (Bohmann *et al.*, 1987; Maki *et al.*, 1987; Angel *et al.*, 1988; Ryder *et al.*, 1988; Hirai *et al.*, 1989; Ryder *et al.*, 1989). As expected, both FosB and Fra1, like Fos, interact with Jun proteins and enhance DNA binding to an AP-1 site (Rauscher *et al.*, 1988a; Cohen *et al.*, 1989; Zerial *et al.*, 1989; Nakabeppu, unpublished). The various heterodimers between Fos family members and Jun family members thus appear to recognize the same or very similar sites in DNA. If there are differences in transcriptional effects of the nine possible heterodimers they would presumably be due to other interactions specific for each heterodimer, mediated by those parts of the Jun and Fos proteins that are different within each family. It is curious that all the Fos and Jun proteins, whose 'complementary' leucine repeat sequences allow them to form stable dimers, have such similar DNA recognition domains. Based on the heterodimer model, one would anticipate finding partners for Fos or Jun (and similar proteins) with 'complementary' leucine repeat sequences but different DNA binding domains, thus allowing recognition of an array of non-symmetrical DNA sequences by various combinations of monomers (Landschulz *et al.*, 1989). Both Fra1 and FosB, as well as JunD, were initially identified by use of antiserum to the basic region or by isolation of cDNAs that hybridized with probes that encoded the basic region. Other screening methods may turn up more distantly related cryptic DNA-binding proteins that interact with Fos or Jun via 'leucine zippers'.

Materials and methods

Plasmids

Plasmids carrying 475−*junD* hybrid cDNA, or human c-*fos* cDNA were described previously (Nakabeppu *et al.*, 1988). Mouse c-*fos* cDNA plasmid, pGEMfos3 was kindly provided by J.G.Belasco and M.E.Greenberg. Plasmids carrying the DNA-binding domain of *junD* or human c-*fos* were constructed as follows. Truncated cDNA of *junD* corresponding to amino acids 216−341 (Ryder *et al.*, 1989) were joined to the 5′ untranslated region of 475 cDNA cloned into pGEM2, at the *Nco*I site which contains the initiation codon of clone 475 (Lau and Nathans, 1987). [The 5′ untranslated segment of 475 cDNA conferred more efficient translation of the downstream sequence (Nakabeppu *et al.*, 1988)]. The *Hha*I−*Xho*II fragment of human c-*fos* cDNA was treated with T4 DNA polymerase to produce blunt ends and joined to the 5′ untranslated region of 475 cDNA at the *Nco*I site that had been filled in with the Klenow fragment of DNA polymerase I. Into the plasmid, a universal translational terminator fragment (GCTTAAT-TAATTAAGC, Pharmacia) was inserted at the *Sma*I site in the polylinker of the vector pGEM2. The resulting plasmid encodes the DNA-binding domain of human c-Fos corresponding to residues 121−207 (which are identical to those of mouse c-Fos) (van Straaten *et al.*, 1983) preceded by Met and followed by Leu and Asn.

To generate *jun*−*fos* chimeric genes, a unique *Kpn*I site (GGTACC) was introduced by *in vitro* mutagenesis into the plasmids carrying DNA encoding the binding domain of JunD or c-Fos by inserting the sequence GTAC just before the codon (CTG in *junD*, CTC in c-*fos*) for the first leucine of the heptad repeat. This resulted in conservative nucleotide changes (Thr/ACA to Thr/ACG in *junD* or Arg/CGC to Arg/CGG in c-*fos*) in the codon preceding the insert. After the mutagenized plasmids were digested with *Kpn*I and treated with T4 DNA polymerase to remove the protruding 3′ end, and digested with *Sca*I, the fragment encoding the *junD* basic domain was ligated to the fragment encoding the c-*fos* leucine repeat, or vice versa.

In vitro transcription and translation

Plasmids containing various cDNA sequences downstream of the SP6 promoter were linearized with *Hin*dIII, treated with proteinase K followed by phenol/chloroform extraction and ethanol precipitation. Templates were transcribed with SP6 RNA polymerase and the resulting transcripts were translated in micrococcal nuclease-treated rabbit reticulocyte lysates (Promega) with or without [^{35}S]methionine as described previously (Nakabeppu *et al.*, 1988). Proteins were analyzed by electrophoresis on 17% SDS−polyacrylamide gels followed by fluorography and autoradiography.

Gel retardation assay for DNA binding

All oligonucleotides containing the AP-1 binding site or related sites were synthesized and cloned into pBSKS plasmids. The cloned oligonucleotides were excised from the plasmids by *Sal*I and *Bam*HI and purified, and then they were end-labeled by the Klenow fragment of DNA polymerase I in the presence of [^{32}P]dATP and dCTP. The binding assay was performed as described previously (Nakabeppu *et al.*, 1988). To quantify binding, gel areas corresponding to the retarded bands were excised and radioactivity retained was measured in a scintillation counter.

Methylation interference assay

*Xho*I−*Sst*I or *Xba*I−*Kpn*I fragments containing the AP-1 site or CRE were purified and labeled on the 3′ end of *Xho*I or *Xba*I sites by the Klenow fragment of DNA polymerase I with [^{32}P]dATP and dCTP. Each fragment (1 pmol) was treated with dimethyl sulfate (Aldrich) according to the procedures described by Baldwin (1988). The binding reaction was carried out with 5 μl (JJ + FF, FF + FJ and JF + FJ) or 10 μl (JJ + JF and JunD + Fos) of co-translation products in 50 μl reaction volume containing ~50 fmol of the methylated DNA fragments. DNA−protein complexes and free probe were separated by polyacrylamide gel electrophoresis, as

described previously (Nakabeppu *et al.*, 1988). DNA from the entire gel was transferred electrophoretically to a DEAE membrane (Schleicher and Schuell NA45) in 0.25 × TBE (1 × TBE is 89 mM Tris, 89 mM boric acid, 2 mM EDTA) overnight at 4°C. Bound and unbound DNA were eluted from the membrane, and extracted with phenol/chloroform followed by precipitation with ethanol. To cleave the DNA fragments at methylated purine residues, the fragments were resuspended in 100 μl of 1 M piperidine, heated to 90°C for 30 min and lyophilized. The DNA fragments were resuspended in 95% formamide, 20 mM EDTA containing marker dyes and subjected to electrophoresis through 12% polyacrylamide−7 M urea gels.

Ethylation interference assay

DNA fragments containing the AP-1 site or CRE, labeled at either 3′ end, were treated with ethyl nitrosourea (Pfaltz and Bauer) by the procedures described by Sakonju and Brown (1982). Conditions for the binding reaction and the separation of the DNA−protein complexes were identical with those described for methylation interference assay. The DNA fragments were cleaved by NaOH.

Immunoprecipitation of translation products

10−20 μl of translation products were diluted to 200 μl with cold RIPA buffer (10 mM Tris−HCl pH 8.0, 150 mM NaCl, 1% NP40, 1% deoxycholate, 0.1% SDS) (Curran *et al.*, 1984) and 5 μl of anti-Fos serum (see Figure 1A) provided by Dr K.Ryder was added. After 1 h incubation on ice, 40 μl of 50% suspension of Protein A−Sepharose CL-4B (Pharmacia) in RIPA buffer was added and incubated with continuous rocking at 4°C for 1 h. The immune complexes were washed with 0.5 ml of RIPA buffer three times, and prepared for SDS−PAGE.

Assays for dimer formation

To analyze dimer formation of various proteins, suitable pairs of *in vitro* transcripts (JJ + FF, JJ + FJ, JF + FJ, JF + FF) were co-translated in the presence of [^{35}S]methionine, or ^{35}S-labeled JJ or JF translation product which was treated with RNase A (80 μg/ml) for 30 min at 30°C to stop translation, was mixed with an equal volume of non-radioactive FF or FJ translation product treated with RNase A and incubated at room temperature for the times noted in the text. Resulting dimers were immunoprecipitated with anti-Fos serum and subjected to SDS−PAGE. For the dephosphorylation experiments, immune complexes were washed with RIPA buffer and then twice with 0.5 ml of 10 mM Tris HCl pH 8.0 and resuspended in 40 μl of the Tris buffer. The washed immune complexes were incubated at 60°C for 1 h with 400 U of bacterial alkaline phosphatase (BRL) in the presence or absence of 1 mM potassium phosphate (pH 8.0) to inhibit phosphatase activity.

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