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c-JUN, JUN B, and JUN D differ in their binding affinities to AP-1 and CRE consensus sequences: effect of FOS proteins

Rolf-Peter Ryseck & Rodrigo Bravo

Bristol-Myers Squibb Pharmaceutical Research Institute, Department of Molecular Biology, PO Box 4000, Princeton, New Jersey 08543-4000, USA

We have compared the binding properties of c-JUN, JUN B, and JUN D in the absence or in the presence of c-FOS, FOS B, and FRA-1 to different AP-1 and CRE-containing oligonucleotides. The results demonstrate that for a given AP-1-containing oligonucleotide the binding affinities of the different JUN proteins are always c-JUN > JUN D > JUN B. The three JUN proteins have the capacity to bind to a CRE consensus sequence with very high affinity. We have found that c-JUN, JUN B, and JUN D bind with different affinities to different oligonucleotides containing an identical AP-1 or CRE binding site, implying that the adjacent sequences influence the stability of the JUN/DNA complexes. Interestingly, an AP-1-containing oligonucleotide which binds the JUN proteins with high affinity can be converted to a CRE-containing oligonucleotide which will also bind the different JUNs very efficiently. The heterodimers formed between the different JUN and FOS proteins have an enhanced binding activity compared to the JUN:JUN homodimers. In all cases the half-lives of the JUN:FOS/DNA complexes are longer than those of the JUN:JUN/DNA complexes. The most stable complexes were obtained in the presence of FOS B, followed by FRA-1 and c-FOS.

Introduction

The transcription factor AP-1 was originally discovered in human cells as an activity that binds selectively to enhancer elements in the *cis* control region of the SV40 virus, of the human metallothionein IIA gene, and of several other vertebrate genes (Lee *et al.*, 1987a; Angel *et al.*, 1987). AP-1 can be activated by phorbol ester tumor promoters such as 12-*O*-tetradecanoylphorbol-*β*-acetate (TPA) through the protein kinase C (PKC) pathway (Angel *et al.*, 1987; Lee *et al.*, 1987b). In the meantime, it has become clear that AP-1 is not a single transcription factor, but a complex composed of different members of the *fos* and *jun* families. The first cellular gene identified to encode a transactivator similar to AP-1 was c-*jun* (Bohmann *et al.*, 1987; Angel *et al.*, 1988), the cellular homolog of the viral oncogene v-*jun* (Maki *et al.*, 1987). Two other members of this family, *jun B* (Ryder *et al.*, 1988) and *jun D* (Hirai *et al.*, 1989; Ryder *et al.*, 1989) have also been identified.

Shortly before c-*jun* was demonstrated to code for an AP-1 activity, it was found that c-FOS protein was a component of a protein complex which specifically could bind to the negative regulatory elements of adipocyte-specific genes (Distel *et al.*, 1987). Subsequent studies revealed that the exact binding site for the complex was the AP-1 sequence (Franza *et al.*, 1988; Rauscher *et al.*, 1988a) and that p39, a cellular protein normally associated with c-FOS, was also present in the complex (Franza *et al.*, 1988). Several pieces of evidence clearly demonstrated that p39 corresponds to c-JUN (Chiu *et al.*, 1988; Rauscher *et al.*, 1988a), therefore establishing that c-FOS and c-JUN form a complex *in vivo* that recognizes the AP-1 target sequence.

As in the case of c-*jun* sequences sharing a significant similarity with c-*fos* have also been described, these being *fra-1* in rat (Cohen & Curran, 1988), *fos B* in mouse (Zerial *et al.*, 1989), and *fra*-2 in human and chicken (Matsui *et al.*, 1990; Nishina *et al.*, 1990).

The initial observation that c-JUN can associate with c-FOS was very soon extended to the other members of these families. It was shown that not only c-JUN, but also JUN B and JUN D can interact with c-FOS (Nakabeppu *et al.*, 1988). On the other hand it was demonstrated that similar to c-FOS, FRA-1 is able to form a complex with c-JUN (Cohen *et al.*, 1989) and that FOS B can interact with c-JUN and JUN B (Zerial *et al.*, 1989).

Although all these complexes have the potential to bind to an AP-1 consensus sequence (TGACTCA), independent groups have obtained contradictory results regarding the binding capacity of JUN homodimers to their target sequence. In most cases, *in vitro* translated proteins using reticulocyte lysate were used for the analysis of binding to an AP-1-containing sequence as determined by gel retardation assay. In studies concerning the c-JUN homodimer, either significant binding (Nakabeppu *et al.*, 1988), weak binding (Rauscher *et al.*, 1988b; Hirai & Yaniv, 1989), or none at all (Halazonetis *et al.*, 1988; Kouzarides & Ziff, 1988; 1989; Zerial *et al.*, 1989) was obtained. Similar conflicting observations have been obtained for other JUN proteins. For example, significant binding, although five times less than that of c-JUN (Nakabeppu *et al.*, 1988), or no binding (Zerial *et al.*, 1989) were reported for JUN B. In general, in all the studies, it was found that the binding of a JUN homodimer to an AP-1 consensus sequence was weaker than that of a JUN:FOS heterodimer. A corresponding situation was found for the binding of JUN proteins to a related binding motif, that is the CRE consensus sequence (TGACGTCA), no binding or weak binding (Nakabeppu *et al.*, 1988) or strong interaction (Nakabeppu *et al.*, 1989; Hirai & Yaniv, 1989) have been reported.

These discrepancies may be due to the use of different procedures for obtaining the protein and/or test system. For example, binding to an AP-1 site was found by using purified c-JUN protein from cell extracts, DNAase I protection and transcriptional activation assays (Bohmann *et al.*, 1987). However, in this case a

Correspondence: R. Bravo

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contamination with JUN:FOS complexes cannot be excluded. Other groups have used bacterially expressed fusion proteins (Angel *et al.*, 1988; Hirai *et al.*, 1989; Abate *et al.*, 1990) for DNAase I protection and gel retardation assays.

Because all these studies have used conditions difficult to compare such as different reticulocyte lysates, oligonucleotides, amounts of protein and DNA, labelling procedures, etc., we decided to further characterize the binding properties of c-JUN, JUN B, and JUN D alone or in complex with c-FOS, FOS B, and FRA-1 to a number of different oligonucleotides containing the AP-1 or CRE consensus sequences using gel retardation assays.

Results

Binding of different JUN proteins and JUN:FOS complexes to an AP-1 containing oligonucleotide

For these studies, the different JUN (c-JUN, JUN B, and JUN D) and FOS (c-FOS, FOS B, and FRA-1) proteins were individually synthesized in an *in vitro* translation system. All the *in vitro* translated products were tested by gel electrophoresis and comparable efficients of translation were found (data not shown). The initial experiments to determine the binding capacity of the JUN proteins to an AP-1 containing oligonucleotide were done using increasing amounts of *in vitro* translated proteins. After incubation with an AP-1-containing oligonucleotide (OLIGO 1, see Table 1), the protein/DNA complex was analysed at room temperature using the gel retardation assay (Figure 1). The results show that there is a significant difference between the binding activity of the three JUN proteins. C-JUN binds very efficiently to OLIGO 1 and binding can be readily observed at the lowest concentration of protein used. In contrast, no or very weak binding was obtained with JUN B and JUN D to OLIGO 1 even at the highest concentration of protein tested, which is approximately 20-fold higher than the amount of c-JUN required to detect a significant binding. No significant differences in the binding ability of the JUN proteins was found if the reaction and gel electrophoresis were performed at 4°C (data not shown).

To compare the effect of the different FOS proteins on the binding activities of c-JUN, JUN B, and JUN D to OLIGO 1, a constant amount of each JUN protein was pre-incubated with increasing amounts of either c-FOS, FOS B, and FRA-1 before adding the AP-1-containing oligonucleotide. The results show that the binding to OLIGO 1 of c-JUN:FOS heterodimers is very similar to that of c-JUN alone (Figure 1). However, the different JUN B:FOS and JUN D:FOS complexes exhibit a dramatically increased binding compared to JUN B and JUN D alone. The effect of the three FOS proteins in the binding activity of JUN B and JUN D to OLIGO 1 is very similar. We have observed that heterodimer formation between the different *in vitro* translated JUN and FOS proteins is accompanied by an increase in the post-translational modifications of these molecules, as a consequence extra bands appear in the gel retardation assays. Competition experiments have demonstrated that the complexes formed are specific for the AP-1 containing oligonucleotides. Mutated forms like OLIGO 16 (Table 1) have been unable to compete

Table 1 List of oligonucleotides used for binding studies

CLASS	OLIGO	SEQUENCE	SOURCE
AP-1	1	TCGAAGCTA**TGAC TCA**TCCGGTCGA	ARTIFICIAL
	2	TCGAAGCTA**TGAC TCA**ACCGGTCGA	DERIVED FROM OLIGO 1
	3	TCGATACAGA**TGAC TCA**CTCCTCGA	DERIVED FROM OLIGO 12
	4	TCGATTACCA**TGAC TCA**ATTTTCGA	DERIVED FROM OLIGO 14
	5	TCGATTACCA**TGAC TCA**TTTTTCGA	DERIVED FROM OLIGO 4
	6	TCGATAGCA**TGAC TCA**TTGTTTCGA	ARTIFICIAL
	7	TCGATAGCA**TGAC TCA**GTGTTTCGA	DERIVED FROM OLIGO 6
	8	TCGAATCCA**TGAC TCA**GAGGATCGA	"FAT-SPECIFIC ELEMENT"
	9	TCGAATCCA**TGAC TCA**CAGGATCGA	DERIVED FROM OLIGO 8
	10	TCGAGTGTC**TGAC TCA**TGCTTTCGA	COLLAGENASE GENE
	11	TCGACAAAC**TGAC TCA**TAATTTCGA	RAT STROMELYSIN GENE
CRE	12	TCGATACAGA**TGACGTCA**CTCCTCGA	BOVINE PARATHYROID HORMONE
	13	TCGACAGC**TGACGTCA**GATGTCTCGA	ARTIFICIAL
	14	TCGATTACCA**TGACGTCA**ATTTTCGA	HUMAN GONADOTROPIN GENE
	15	TCGAAGCTA**TGACGTCA**TCCGGTCGA	DERIVED FROM OLIGO 1
AP-1/ CRE-LIKE	16	TCGACCGGCC**TGAC TCG**CCCTCGTCGA	KROX 24 GENE
	17	TCGAGCCAC**TGAC GCA**CACGGTCGA	N10 GENE
	18	TCGACTCCG**TGAC GCA**TGGAGTCGA	N10 GENE
	19	TCGAAACCTGC**TGAC GCA**GATGTCCTCGA	C-*FOS* GENE
	20	TCGACCAG**TGACGTAG**GAAGTCTCGA	C-*FOS* GENE
	21	TCGAGCCC**TGACGTTT**ACACTCTCGA	C-*FOS* GENE
	22	TCGAAGCTA**TGACTTCA**TCCGGTCGA	DERIVED FROM OLIGO 1
	23	TCGATTGGGG**TGACATCA**TGGGCTATCGA	C-*JUN* GENE

Only one of the strands is shown. The sequence TCGA on both ends of the oligos is derived after fill-in with the Klenow fragment of DNA polymerase I. The TCGA sequences are not part of the genomic sequences. Some of the sequences were taken from the following sources: OLIGO 8 (Kouzarides & Ziff, 1988); OLIGO 10 and OLIGO 11 (Angel *et al.*, 1987); OLIGO 12 and OLIGO 14 (Deutsch *et al.*, 1988a); OLIGO 16 (Janssen-Timmen *et al.*, 1989); OLIGO 17 and OLIGO 18 (Ryseck *et al.*, 1989); OLIGO 19, OLIGO 20, and OLIGO 21 (Treisman, 1985); OLIGO 23 (Hattori *et al.*, 1988)

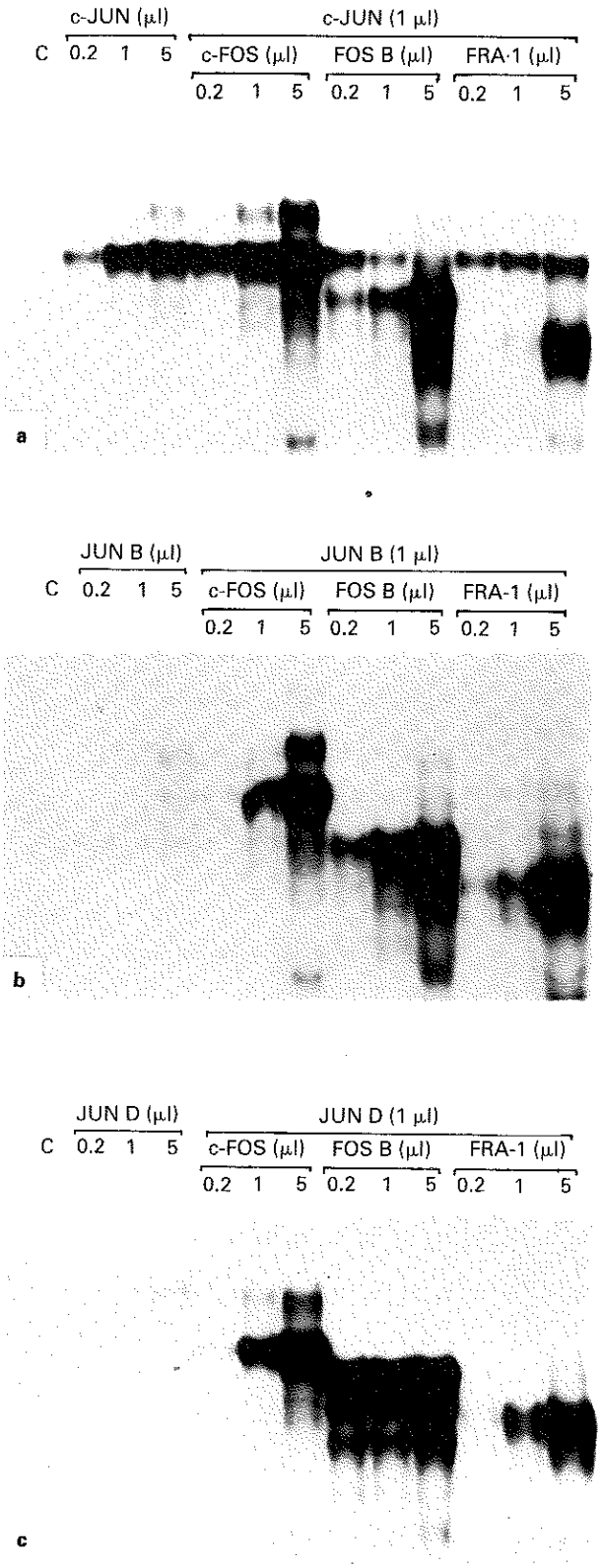

Figure 1 Binding of different JUN:FOS protein complexes to an AP-1 containing oligonucleotide. Gel retardation analyses using increasing amounts of *in vitro* translated c-JUN (**a**), JUN B (**b**), or JUN D (**c**) alone or a constant amount (1 μl) of each JUN protein in combination with increasing amounts of *in vitro* translated FOS proteins and 0.5 pmol/ml of [^{35}P]-labelled AP-1 OLIGO 1 (see Table 1). The amount of JUN and FOS proteins used in each case were comparable. In lane C (control) 6 μl of reticulocyte lysate incubated with the labelled AP-1 OLIGO 1. In all cases the lysate volume was adjusted to 6 μl. Free oligonucleotide is not shown

efficiently in contrast to AP-1 consensus oligonucleotides (data not shown).

These results confirm that the three JUN proteins efficiently interact with at least three of the members of the FOS family and show that the FOS proteins favor the binding of the JUN proteins to a given AP-1 consensus sequence.

Binding of JUN proteins and JUN:FOS complexes to different oligonucleotides containing AP-1 and CRE consensus sequences

The results presented above clearly demonstrate that *in vitro* translated c-JUN protein efficiently binds to an AP-1 consensus sequence as determined by gel retardation assays. These observations are in conflict with some previous reports including ours (Zerial *et al.*, 1989). One possible explanation for the different results could be the source of reticulocyte lysates, for instance, due to differences in their ability to post-translationally modify the proteins or in their efficiency of expression. However, comparable results were obtained using various *in vitro* translation kits. In general, the binding of the JUN proteins alone or in the presence of FOS proteins to a given AP-1 consensus sequence was always very reproducible (data not shown). Other significant differences that could explain the conflicting results were the oligonucleotide sequences used or the way of performing the labelling and the gel retardation assays. To examine the first possibility, we performed gel retardation assays using a number of oligonucleotides containing the same AP-1 consensus sequence but with different flanking regions, some corresponding to natural sequences, others slightly modified (see Table 1). To complete this study, some CRE-containing oligonucleotides as well as AP-1-like and CRE-like sequences were used.

To compare the binding affinities of the protein complexes, the concentration of the different oligonucleotides was determined by measuring the optical density at 260 nm. Then, they were end-labelled with an excess of α-[^{32}P]dCTP to obtain similar specific activities. The incorporation and the amount of oligonucleotide was further determined using a 15% denaturing polyacrylamide gel (data not shown).

As shown in Figure 2a, the binding of c-JUN varies greatly with different oligonucleotides containing an identical AP-1 consensus sequence (OLIGO 1 to OLIGO 11, see Table 1), suggesting that the AP-1 flanking regions play an important role in the c-JUN/DNA interaction. For example, the change of one base pair in the 3′ flanking region (compare for instance OLIGO 1 and OLIGO 2, a change of T to A adjacent to the AP-1 consensus) dramatically alters the binding properties. Whereas in some cases c-JUN binds strongly to an AP-1-containing oligonucleotide, in others, the binding is at least an order of magnitude lower. It is important to note that strong binding is found in sequences like ATGACTCAPy, whereas the binding is dramatically reduced in the case of an ATGACTCAPu motif. However, with the extended motif CTGACTCAT (OLIGO 10 and OLIGO 11), in one case a high affinity is observed, whereas in the other the binding is rather poor, pointing to the importance of other nucleotides for efficient binding. These observations explain the contradictory results of different groups. A sequence

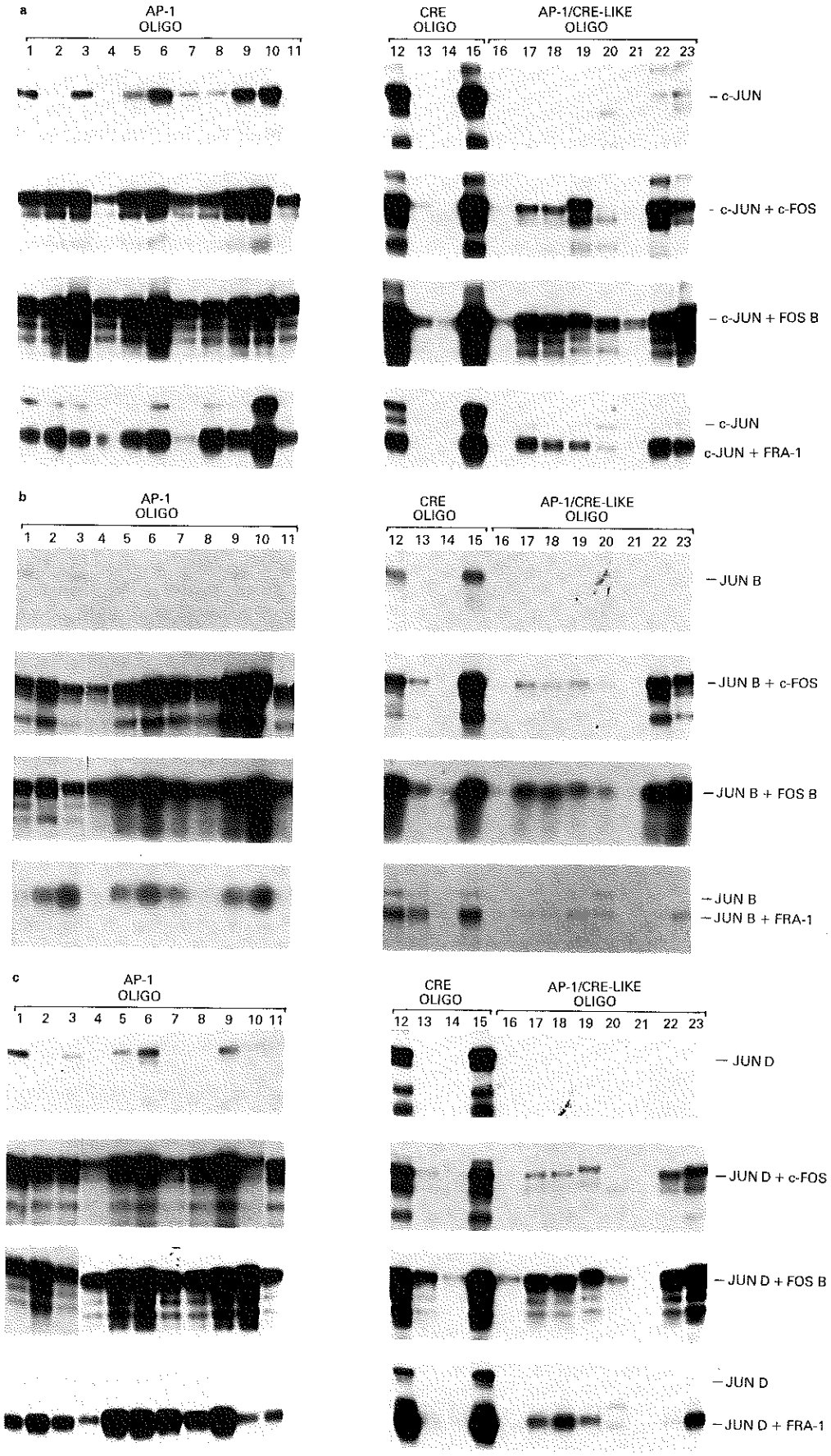

Figure 2 Binding of JUN proteins and JUN:FOS complexes to different AP-1 and CRE containing oligonucleotides. For each of the following assays, 0.5 pmol ml^{-1} of labelled oligonucleotide was used. Binding of **(a)** c-JUN, **(b)** JUN B, and **(c)** JUN D alone or in combination with the different members of the FOS family. A comparable amount of *in vitro* translated JUN or FOS proteins was used in each case. Oligonucleotide numbers correspond to those in Table 1

corresponding to OLIGO 7 was used by Halazonetis *et al.* (1988) and to OLIGO 8 by Kourizarides and Ziff (1988), both obtained a very weak or no binding of c-JUN. On the other hand, a longer version of OLIGO 1 was used by Nakabeppu *et al.* (1988) obtaining a good binding for c-JUN.

FOS proteins substantially increase the binding of c-JUN to all the AP-1-containing oligonucleotides tested. However, clear differences in affinity can still be found using different oligonucleotides, confirming and extending recent observations (Risse *et al.*, 1989). Interestingly, our results show that the FOS proteins, i.e., c-FOS, FOS B, and FRA-1, differentially affect the binding activity of c-JUN, FOS B being the most efficient, followed by FRA-1 and c-FOS.

More dramatic differences have been found using different CRE-containing oligonucleotides. Two of them bind strongly to c-JUN alone (OLIGO 12 and OLIGO 15), whereas two others (OLIGO 13 and OLIGO 14) show no detectable binding. It is important to note that the CRE-containing OLIGO 12 and OLIGO 15 are derived from the AP-1-containing OLIGO 3 and OLIGO 1, respectively, which also bind c-JUN efficiently. On the other hand, CRE-containing OLIGO 14 is derived from the AP-1-containing oligonucleotide OLIGO 4, which binds poorly to c-JUN. These results imply that if the binding to a given AP-1-containing oligonucleotide is strong, then its corresponding CRE will present a similar or even stronger binding and *vice versa*. In contrast to the AP-1-containing oligonucleotides, only a slight increase in the binding of c-JUN was observed in the presence of FOS proteins for all the CRE-containing oligonucleotides tested.

Oligonucleotides containing an AP-1-like or CRE-like consensus sequence (OLIGO 16 to OLIGO 23) bind weakly, if at all, to c-JUN. Change of the CRE consensus TGACGTCA to TGACTTCA (OLIGO 15 versus OLIGO 22) reduces the binding of c-JUN homodimer at least fifty times. The presence of FOS proteins restores the binding of the c-JUN, however, to different levels, for all oligonucleotides of the AP-1/CRE-like class. The c-JUN:FOS B complex binds to an AP-1-like sequence best, and in many cases, as efficiently as to an authentic AP-1 consensus core sequence.

To further support the finding that c-JUN can bind to a CRE-containing oligonucleotide with high affinity, competition experiments between the AP-1 OLIGO 3 and its CRE counterpart OLIGO 12 (see Table 1) were performed. The results demonstrate that the CRE OLIGO 12 effectively competes with the AP-1 OLIGO 3 for c-JUN binding (not shown). In fact, the CRE sequence is a better competitor than AP-1 itself, in agreement with the above observation that CRE OLIGO 12 binds c-JUN stronger than AP-1 OLIGO 3.

In contrast to c-JUN, JUN B has a low affinity for all the AP-1 oligonucleotides used, but in combination with FOS proteins, the binding affinity is significantly increased, to a level similar to that of c-JUN (Figure 2b). Surprisingly, JUN B efficiently binds to the CRE-containing OLIGO 12 and OLIGO 15, although weaker than c-JUN. The presence of the FOS proteins increases the binding of JUN B to the CRE OLIGO 12 and OLIGO 15 to a level similar to that of c-JUN. With the CRE OLIGO 13 and OLIGO 14, the affinity in the presence or absence of FOS proteins is as weak as that obtained with c-JUN (Figure 2b). JUN B only binds to the AP-1-like or CRE-like sequences in the presence of c-FOS, FOS B, or FRA-1. However, the binding is in general weaker than that observed for the c-JUN:FOS complexes. As for c-JUN, JUN B in the presence of FOS B presents the strongest binding with any given oligonucleotide.

Similarly to c-JUN, JUN D can bind alone to some of the AP-1-containing oligonucleotides. However, the binding of JUN D for these sequences is weaker than that observed for c-JUN but much stronger than for JUN B (Figure 2c). Again, as observed for c-JUN and JUN B, an enhancement in JUN D binding is obtained in the presence of all three FOS proteins. Like c-JUN and JUN B, JUN D can also bind to the CRE-containing OLIGO 12 and OLIGO 15 with high affinity. None of the AP-1-like or CRE-like sequences tested were recognized by JUN D alone, but a significant binding was observed in many cases in the presence of the FOS proteins, the best being usually heterodimers with FOS B. A summary of these results is shown in Table 2.

To determine if JUN B could affect the binding of c-JUN to the oligonucleotide we first mixed different proportions of both proteins separately obtained by *in vitro* translation. However, no effect was visible, possibly due to the fact that no heterodimers are formed under these conditions (data not shown). Therefore, various amounts of c-*jun* and *jun* B RNA were cotranslated and the resulting complexes were used for the gel retardation assay. Under these conditions the formation of heterodimers is possible (K. Kovary and R. Bravo, unpublished). As shown in Figure 3, lane 1 c-JUN at certain concentrations presents a strong binding to the AP-1 containing oligonucleotide in the absence of JUN B. In contrast, if a similar amount of c-JUN is used but in the presence of a five fold excess of JUN B a weak binding is observed (Figure 3, lane 7) suggesting that JUN B inhibits the binding of c-JUN by the formation of c-JUN/JUN B heterodimers.

The effect of oligonucleotide concentration in the binding of JUN proteins to an AP-1 or CRE sequence

To further analyse the differences in the binding of the JUN proteins to AP-1 or CRE-containing oligonucleotides we performed gel retardation assays using different concentrations of oligonucleotides labelled to a similar specific activity. Figure 4 shows that c-JUN efficiently binds to the AP-1 OLIGO 1 and the CRE OLIGO 12 at a concentration of 1 pmol ml^{-1} and that a weak

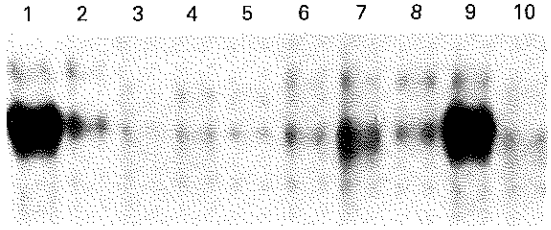

Figure 3 JUN B prevents c-JUN from binding to an AP-1 sequence. Various amounts of c-*jun* and *jun* B mRNA were *in vitro* translated either alone or together and the protein products were incubated with [^{32}P]-labelled AP-1 OLIGO 1. Relative amounts of protein used for the assay as determined by immunoprecipitation (c-JUN:JUN B) Lane 1, 10:0; lane 2, 1:0; lane 3, 0:10; lane 4, 0:5; lane 5, 0:1; lane 6, 1:1; lane 7, 5:10; lane 8, 1:1; lane 9, 10:1; lane 10, 1:10

Table 2 Summary of the relative binding of the different JUNs and JUN:FOS complexes to various AP-1- and CRE-containing oligonucleotides

	AP-1											*CRE*				*AP-1/CRE-LIKE*							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
c-JUN	3	2	3	1	3	3	1	1	3	4	1	4	1	1	4	1	1	1	1	2	1	2	2
c-JUN + c-FOS	4	4	4	3	4	4	3	3	4	4	3	5	1	1	5	1	3	3	4	2	1	4	3
c-JUN + FOS B	4	4	5	4	4	5	4	4	4	4	4	5	3	2	5	2	4	4	4	4	2	4	5
c-JUN + FRA-1	4	4	4	3	4	4	2	4	4	4	3	4	1	1	4	1	3	3	3	2	1	4	3
JUN B	1	1	1	1	1	1	1	1	1	1	1	2	1	1	2	1	1	1	1	1	1	1	1
JUN B + c-FOS	3	4	3	3	4	4	4	4	5	5	3	3	2	1	4	1	2	2	2	2	1	4	3
JUN B + FOS B	4	4	3	3	4	4	4	4	4	5	4	4	3	1	4	1	3	3	3	3	1	4	4
JUN B + FRA-1	2	3	3	1	3	3	3	1	3	3	1	3	3	1	3	1	1	2	2	2	1	2	2
JUN D	3	1	2	1	2	3	2	1	3	2	1	4	1	1	4	1	1	1	1	1	1	1	1
JUN D + c-FOS	4	4	4	2	4	4	3	4	4	3	4	4	2	1	4	1	2	2	2	1	1	3	3
JUN D + FOS B	4	4	4	4	5	5	4	4	5	5	4	5	3	2	5	3	4	4	4	3	1	4	5
JUN D + FRA-1	3	4	3	3	4	4	4	4	4	3	3	5	2	1	5	1	3	3	3	2	1	2	3

The gel retarded bands were quantitated by densitometer scanning. For easier comparison, the highest value obtained (OLIGO 15, c-JUN + FOS B, Figure 2a) was taken as 100% and the relative binding of the various oligonucleotides was grouped in 5 categories as follows:
1: no binding observed
2: $<1\%$
3: $1\%-<10\%$
4: $10\%-<50\%$
5: $50\%-100\%$

binding can be observed at a concentration of 0.1 pmol ml^{-1}. In contrast, c-JUN shows no detectable binding to AP-1 OLIGO 2 and OLIGO 4 at the concentration of 1 pmol ml^{-1}. With the latter oligonucleotides, significant binding of c-JUN is observed at 10 pmol ml^{-1}. However, this interaction is still weaker than the binding to OLIGO 1 and OLIGO 12 at a 10 times lower concentration, specially in the case of OLIGO 4. As shown in Figure 4 JUN B has the capacity of binding alone to an AP-1 consensus sequence but with an affinity approximately 10 times lower than that of c-JUN. JUN B shows a significant binding with OLIGO 1 and OLIGO 12 at 10 pmol ml^{-1} whereas, with OLIGO 2 and OLIGO 4, only a very weak binding is observed. JUN D shows significant binding to OLIGO 1 and OLIGO 12 at a concentration of 1 pmol ml^{-1}, however, this binding is weaker than c-JUN. Only low affinity binding can be detected with OLIGO 2 and OLIGO 4 at the highest concentration used. These results confirm that different oligonucleotides containing an identical AP-1 consensus sequence vary in their binding properties and demonstrate that the three JUN proteins alone can bind to AP-1 or CRE consensus sequences although with very different affinities. The order of binding affinity for different AP-1- or CRE-containing sequences is c-JUN > JUN D > JUN B.

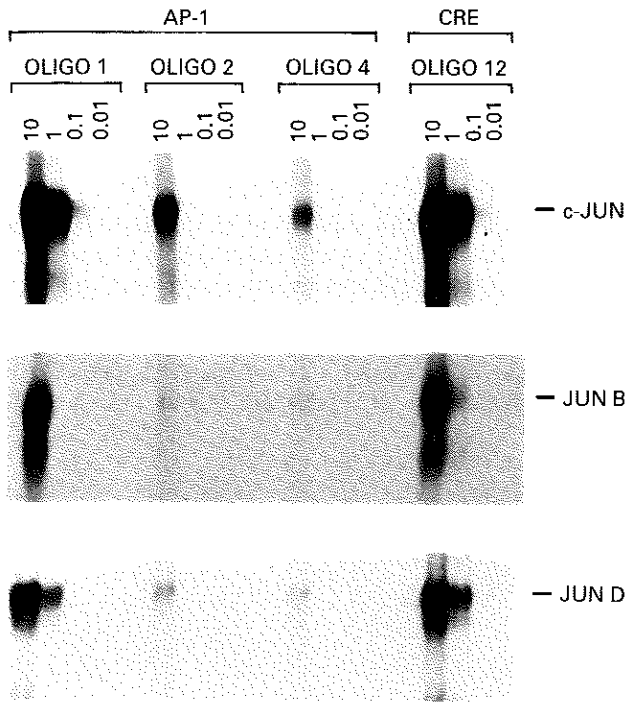

Figure 4 Dependence upon the oligonucleotide concentration of the binding of JUN proteins to an AP-1 or CRE sequence. For each assay, 5 μl of *in vitro* translated c-JUN, JUN B, or JUN D was used. The AP-1-containing oligonucleotides OLIGO 1, OLIGO 2, and OLIGO 4 as well as the CRE-containing oligonucleotide OLIGO 12 were labelled to high specific activity and used at the indicated amounts (pmol ml^{-1})

Dissociation rates of different DNA/protein complexes

The results described above suggest that the affinity of the various JUN and JUN:FOS complexes for an AP-1 or CRE consensus sequence differ significantly. As the affinity for the binding site is a function of both the rate of association and the rate of dissociation, we compared these functions for the various JUN:JUN and JUN:FOS complexes by using oligonucleotides with significantly different binding affinities. Because the association between the proteins and DNA was extremely rapid and therefore difficult to measure, only the half-lives of the protein/DNA complexes for the different JUN homodimers or JUN:FOS heterodimers using several oligonucleotides were compared (Figure 5).

For the different assays, the proteins were first incubated with the labelled AP-1-containing oligonucleotide to form the protein/DNA complex, then a 1000-fold excess of unlabelled oligonucleotide was added and the mixture incubated for various lengths of time. Samples were taken at different periods of time and immediately analysed by gel electrophoresis. As the unlabelled oligonucleotide is in vast excess, it will replace the [^{32}P]-labelled oligonucleotide that dissociates during the incubation time. Therefore, the decrease in the intensity

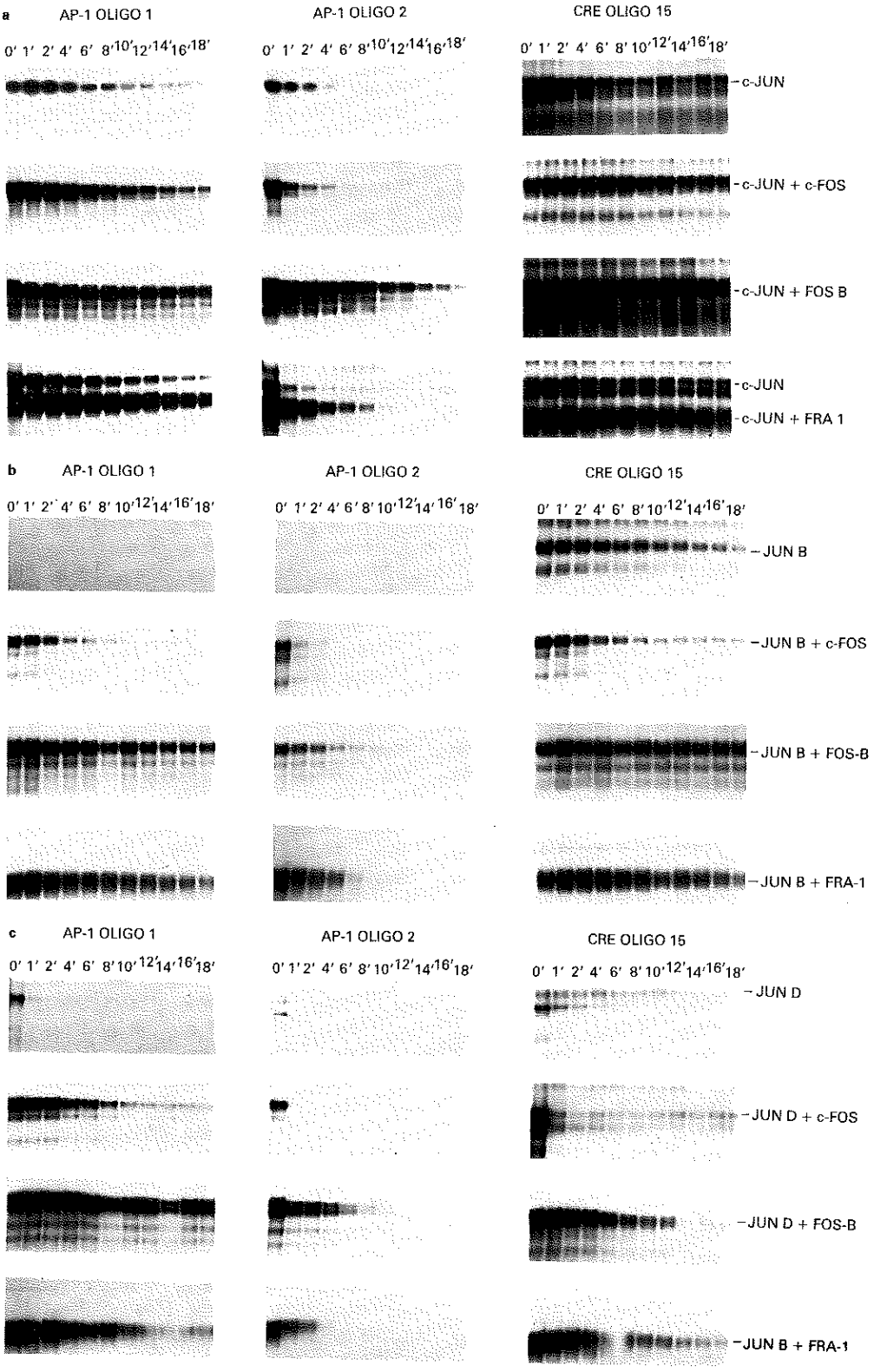

Figure 5 Dissociation rate of the different DNA/protein complexes. *In vitro* translated c-JUN, JUN B, or JUN D alone or in combination with c-FOS, FOS B, or FRA-1 were incubated with [^{32}P]-labelled AP-1 OLIGO 1 or OLIGO 2, or with CRE OLIGO 15 at a concentration of 0.5 pmol ml^{-1} for 20 min at room temperature. After loading the first aliquot on a gel (time point 0'), a 1000-fold excess of unlabelled OLIGO 1 was added and further incubated at room temperature for the indicated times. The DNA protein complexes were resolved in a 7% polyacrylamide gel as described in Materials and methods. Similar exposure times are shown with the exception of c-JUN:OLIGO 2 which was exposed three times longer and JUN B:OLIGO 1 and JUN B:OLIGO 2 which were exposed 10 times longer

of the gel shift band that occurs with time reflects the dissociation rate of the protein/DNA complex.

The results (Figure 5) show that the half-lives of the different JUN:JUN/DNA or JUN:FOS/DNA complexes vary depending on the AP-1-containing oligonucleotide used. All the complexes formed with AP-1 OLIGO 2 have a significantly shorter half-life than those with AP-1 OLIGO 1. The CRE OLIGO 15 forms

the most stable complexes with the c-JUN and JUN-B homodimers and with the FOS heterodimers.

The half-life of the c-JUN/OLIGO 1 complex is approximately 6 to 8 min in contrast to that for the c-JUN/OLIGO 2 complex which is approximately 1 min, demonstrating that different AP-1-containing oligonucleotides can have at least a 5-fold difference in their dissociation rate when complexed with c-JUN. These results also suggest that the base pairs flanking the AP-1 binding sequence can play an important role in stabilizing the protein/DNA complex. Interestingly, the complex formed between c-JUN and the CRE OLIGO 15 is much more stable than that with AP-1 OLIGO 1, presenting a half-life of approximately 14 to 18 min, supporting the observations that c-JUN efficiently binds to a CRE consensus sequence. The presence of c-FOS slightly prolongs the half-life of the complex with AP-1 OLIGO 1 and OLIGO 2. However, the presence of FOS B dramatically affects the half-lives of these complexes prolonging them for both OLIGO 1 and OLIGO 2 at least 5-fold. FRA-1 has an intermediate effect in the stabilization of these complexes. As illustrated in Figure 5a, the differences in the half-lives of the complexes with OLIGO 1 and OLIGO 2 are observed even in the presence of the FOS proteins. The effect of FOS proteins in the stability of the CRE OLIGO 15 complexes is less pronounced than that observed with the AP-1 containing oligonucleotides, possibly due to the fact that the c-JUN/OLIGO 15 complex itself is more stable than those with OLIGO 1 and OLIGO 2. In contrast to published results (Rauscher *et al.*, 1988b), the minimal effect of c-FOS we have observed is possibly due to a different AP-1 oligonucleotide used in that study.

As shown in Figure 5b and 5c the half-lives of the complexes between AP-1, OLIGO 1 and OLIGO 2 with JUN B and JUN D are significantly shorter than those with c-JUN. The complex between JUN D and OLIGO 1 has a half-life of less than 30 sec in contrast to that of c-JUN which is approximately 6 to 8 min. Like c-JUN the complex JUN D/OLIGO 2 has an even shorter half-life, which in the case of JUN D is difficult to estimate, but probably is only of a few seconds (Figure 5c). Due to the very weak binding of JUN B to both AP-1-containing oligonucleotides it was not possible to estimate the half-lives of the complexes. Interestingly, the complex between JUN B and the CRE OLIGO 15 is very stable with a half-life of approximately 8 min. In contrast, the complexes between JUN D with either the CRE OLIGO 15 or AP-1 OLIGO 1 have a half-life of less than 1 min. It is important to note that the complex JUN B/OLIGO 15 is as stable as that between the heterodimer c-JUN:c-FOS and AP-1 OLIGO 1. These results confirm the above observations that the JUN proteins can interact with a CRE binding site and suggest that this motif may be the primary binding site for JUN B.

The presence of FOS proteins increases the stability of the complexes between JUN B and JUN D with OLIGO 1 and OLIGO 2 following the same order as that observed with c-JUN, in which FOS B has the strongest positive effect. In the case of JUN B and JUN D the effect of c-FOS on the half-lives of their complexes with both AP-1-containing oligonucleotides is more evident than that observed with c-JUN, possibly due to the initial difference in stability of the complexes between the JUN proteins. The results illustrate that the FOS proteins similarly affect the stability of the complexes formed with AP-1 OLIGO 1 and OLIGO 2. In every case the OLIGO 2 complexes were the least stable. The fact that all JUN:JUN homodimers and JUN:FOS heterodimers form more stable complexes with OLIGO 1 than with OLIGO 2 further confirms that the flanking regions play an important role in these interactions. The effect of FOS proteins on the stability of the JUN B and JUN D complexes with CRE OLIGO 15 is less dramatic than that observed with the AP-1 containing oligonucleotides. c-FOS has no significant effect on the stability of these complexes and FOS B and FRA-1 have a weak effect.

Discussion

One of the major questions asked of gene families is what are the common and what are the specific tasks the single members have. The *jun* and *fos* genes differ in many ways from each other, but also possess common features. At the expression level, c-*jun* (Lamph *et al.*, 1988; Quantin & Breathnach, 1988; Ryder & Nathans, 1988; Ryseck *et al.*, 1988) and *jun* B (Ryder *et al.*, 1988) are barely present in quiescent fibroblasts but their expression dramatically increases following serum stimulation, whereas *jun D* is expressed at significant levels in quiescent cells and slightly increases after induction of cell proliferation (Hirai *et al.*, 1989). In the case of the members of the *fos* family, c-*fos*, *fos B*, and *fra-1* are expressed at very low levels in quiescent cells and the three of them are significantly induced after serum addition (Greenberg & Ziff, 1984; Kruiger *et al.*, 1984; Müller *et al.*, 1984; Zerial *et al.*, 1989), however the induction of *fra-1* is considerably slower (Cohen & Curran, 1988). Although the products of these six genes coexist after serum stimulation of quiescent fibroblasts, in general, they are differentially expressed during development and in adult tissues (Dony & Gruss, 1987; Hirai *et al.*, 1989; Wilkinson *et al.*, 1989), suggesting that the various members of the *jun* and *fos* families have distinctive functions. These have been difficult to envisage considering the previous evidence that all the proteins encoded by the *jun* family have a similar affinity for an AP-1 binding site and that they interact with all proteins of the *fos* family in a similar fashion.

Our results however, show that there are clear differences in the binding affinities of the various JUN homodimers and JUN:FOS heterodimers to an AP-1 site supporting the notion that these proteins may have similar but distinctive cellular functions. We have demonstrated that the affinity of c-JUN is at least ten times higher than that of JUN B and JUN D for a given AP-1 binding site and that although the presence of FOS proteins increases the binding of the JUN molecules, they do so differentially, FOS B being the most effective followed by FRA-1 and c-FOS. It is important to note that JUN B displays very weak binding to all the AP-1 containing oligonucleotides tested including several derived from natural sequences, suggesting that JUN B possibly requires the presence of FOS proteins or other molecules for its normal cellular function. In our studies we have also found that the binding to the AP-1 site depends not only on the JUN or JUN:FOS complexes involved, but also on the sequence flanking the AP-1 (TGACTCA) motif. For instance, the c-JUN

homodimer can bind to some of the oligonucleotides used in our studies with 5 to 10 times higher affinity than to other sequences, which differ in only one base pair in the immediate AP-1 flanking region. These differences in binding persist even in the presence of the FOS proteins implying that flanking regions stabilize the protein/DNA complex either by direct contact with the protein or conversely, that certain sequences decrease the binding of the proteins by steric hindrance. Further studies involving X-ray analysis of complexes between JUN:JUN or JUN:FOS dimers and DNA will be necessary for solving this matter.

The existence of related molecules such as the *jun* and *fos* families with the common biological function but with different activities could provide the cell with a mechanism for fine regulation. For example, one can postulate that under physiological conditions where c-JUN is expressed at low levels, it will bind to only those promoters containing a high affinity AP-1 site, but when c-JUN is expressed at high levels it will bind to the majority of its target genes. However, to efficiently bind to the very low affinity AP-1 sites, c-JUN would require the presence of one of the FOS proteins. As these differentially affect the binding of c-JUN, the type of low affinity sites recognized will depend on the FOS protein involved, adding another level of selection to the putative target genes. A similar situation could be postulated for the other JUN proteins. Considering that the various *jun* and *fos* genes are differentially expressed during development it is not difficult to envisage that several combinations of these proteins will naturally occur, and therefore the expression of different sets of genes will be controlled by these proteins in different cell types. Further *in vivo* and *in vitro* studies using reporter genes driven by different AP-1 containing sequences are necessary for the better characterization of the transacting activities of the different members of the *jun* and *fos* families. Similarly, the addition or the depletion of single JUN and/or FOS proteins in *in vitro* and *in vivo* systems will be essential for the understanding of their cellular function.

The observation that the binding activity of JUN B is much weaker than that of c-JUN is important considering the reports of Chiu *et al.* (1989) and Schütte *et al.* (1989). For example, Chiu *et al.* (1989) describe a weak activation by JUN B of a CAT construct driven by the AP-1 sequence of the collagenase gene (OLIGO 10 in our studies). In contrast, with c-JUN they found a strong activation. According to our results these observations can be explained by the enormous difference in binding activities between c-JUN and JUN B to the AP-1 sequence used. In a similar manner, the finding of Chiu *et al.* (1989) that overexpression of JUN B leads to an inhibition of CAT activation by c-JUN is in agreement with our results (Figure 3) showing that the presence of JUN B inhibits the binding of c-JUN to an AP-1 consensus sequence. The observation that JUN B activates the expression of a reporter plasmid containing three copies of an AP-1 sequence by Chiu *et al.* (1989) could be due to an increase in the chances of binding of JUN B. Indeed, we have observed binding of JUN B to an oligonucleotide containing three consensus AP-1 sequences (not shown).

Our results are also in agreement with the observations of Schütte *et al.* (1989). For example, their finding that *jun B* + c-Ha *ras* have less efficient transformation activity than c-*jun* + c-Ha *ras*, can be due to the weaker binding of JUN B to AP-1 sequences. Their observation that *jun B* inhibited the transforming potential of c-*jun* can be explained by the possible formation of JUN B:c-JUN heterodimers which would have lower affinity for AP-1 binding sites than the c-JUN homodimers. The observation that the transformation potential of c-*jun* and *jun B* in the presence of c-Ha *ras* is significantly increased by cotransfection with c-*fos*, agrees with our results showing that the binding of c-JUN and specially of JUN B to an AP-1 sequence is dramatically increased in the presence of c-FOS, being at least 10-fold stronger than that of JUN B homodimers. It is not known which amino acids in JUN B account for the weaker binding to an AP-1 consensus sequence, but it is possible that not only those amino acids laying in the conserved basic part of the helix-turn-helix region (the DNA-binding domain), but also more N-terminal residues influence the stability of the JUN B:JUN B/DNA or JUN B:FOS/DNA complexes. This effect of the N-terminal sequence has been recently reported for c-JUN (Cohen & Curran, 1990).

Interestingly, the JUN:JUN and JUN:FOS complexes can bind to some CRE elements very strongly, but as for AP-1, the binding is influenced by the flanking base pairs leading to another possible mechanism of fine gene regulation in the cell by these families of proteins. These findings add a new level of complexity to that of gene regulation mediated by AP-1 or CRE sequences, specially considering the increasing number of different ATF/CREB proteins (Hai *et al.*, 1989) and the recent observations that c-JUN can form heterodimers with some (Benbrook & Jones, 1990; Macgregor *et al.*, 1990) but not all of the CREB proteins (Dwarki *et al.*, 1990). It would not be surprising if the various CREB proteins differ in their binding affinities to different CREs and that their binding is strongly influenced by the neighboring sequences as suggested by the observations of Deutsch *et al.* (1988b) and Hai *et al.* (1989). A similar situation can be envisaged for other DNA binding proteins like octamer binding proteins or certain zinc finger subfamilies.

Materials and methods

In vitro *transcription and translation*

In vitro transcription of the different *jun* and *fos* mouse cDNAs subcloned in pBluescript(KS)+, pGemini 1 or pTZ18R was carried out as previously described (Zerial *et al.*, 1989). *In vitro* translation was performed using the reticulocyte lysates of NEN, Amersham, BRL (results of Figure 1), and Promega (all other results shown).

Gel retardation assay

The annealing of the two strands of a corresponding double-stranded oligonucleotide was performed in 10 mM Tris-HCl, pH 8.0, and 10 mM $MgCl_2$, starting at 70°C and cooling down slowly to room temperature. The correct sequence of the oligonucleotides was confirmed after recloning and sequencing with the T7 DNA polymerase kit from Pharmacia.

One pmol of the oligonucleotides was end-labelled with [^{32}P]dCTP by filling in the TCGA overlapping ends using the Klenow fragment of DNA polymerase I. Six pmols of the radioactive deoxynucleotide were used to assure complete labelling of the double-strand oligonucleotide. Labelling of the

oligonucleotide was controlled by gel electrophoresis in a 15% denaturing polyacrylamide gel. *In vitro* translated proteins (3 μl) were added to a buffer (14 μl) containing 10 mM Hepes, pH 8.0, 0.1 μM EDTA, 2 mM DTT, 50 mM KCl, 5 mM $MgCl_2$, 5 mM spermidine, 17% glycerol, 5 $\mu g\,ml^{-1}$ poly dI:dC and incubated for 10 min on ice. If not otherwise indicated, 0.01 pmol of the labelled oligonucleotide was then added and the mixture was incubated for 10 to 15 min at room temperature. The DNA-protein complexes were resolved on a 7% polyacrylamide gel (39:1 acrylamide:bisacrylamide) in 20 mM Tris-boric acid, pH 8.3, 0.25 mM EDTA run at room temperature for 16 h at 100 V. Quantitation of the retarded oligonucleotides was performed by densitometer scanning of autoradiograms.

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