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Asymmetrical recognition of the palindromic AP1 binding site (TRE) by Fos protein complexes

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Fos and Jun proteins form a tight complex which binds specifically to the AP1 recognition sequence, a palindromic DNA element also referred to as the TPA responsive element (TRE). To elucidate the mechanism of Fos-Jun interaction with the TRE we have performed UV cross-linking studies using oligonucleotides where thymines were replaced with bromouracil. Our results indicate that both Fos and Jun directly contact the TRE but that the interaction of Fos and Jun with thymines in structurally equivalent positions in the two half sites of the TRE is different. In addition, we have carried out a comprehensive mutagenesis study of the TRE by introducing all possible point mutations plus thymine → uracil substitutions into the palindromic TRE core sequences and the adjacent nucleotides on both sides. The results of this analysis clearly show that the palindromic TRE is asymmetrical with respect to binding of Fos-Jun. We also show that a Fos protein complex with a homodimeric DNA binding site binds considerably less efficiently to TRE mutants with a perfect dyad symmetry compared with the binding to the wild-type TRE. This demonstrates that the asymmetrical recognition of the TRE is not due to the heterodimeric nature of the Fos/Jun complex but directly related to an asymmetry in the TRE sequence. The methyl groups of all four thymine residues within the TRE seem to be functionally crucial since thymine → uracil substitutions strongly reduce or abolish binding to Fos/Jun. The relevance of structurally equivalent methyl groups in the TRE core sequence is different, lending further support to the conclusion that the TRE is asymmetrical.

Key words: AP1/Fos protein complex/Jun/TPA-responsive element

Introduction

An intriguing recent development is the merging of two fields of research, the regulation of eukaryotic transcription and the function of proto-oncogene products. In this context one observation deserves particular attention, namely the discovery that Fos and Jun proteins are constituents of the transcription factor AP1 (Bohmann *et al.*, 1987; Bos *et al.*, 1988; Angel *et al.*, 1988; Rauscher *et al.*, 1988a,b; Chiu *et al.*, 1988; Lucibello *et al.*, 1988; Sassone-Corsi *et al.*, 1988a,b; for a review see Curran and Franza, 1988). The AP1 protein complex had previously been detected by virtue of its inducibility by the tumor promoter TPA and its binding to the SV40 early promoter (Angel *et al.*, 1987; Lee *et al.*, 1987). AP1 interacts with a specific palindromic DNA sequence, known as the AP1 binding site or TPA responsive element (TRE). The Fos-Jun heterodimer binds to the TRE with high affinity, while the individual constituents of the AP1 complex are unable to do so: Jun binds with at least 30-fold lower affinity and binding of Fos is undetectable (Nakabeppu *et al.*, 1988; Halazonetis *et al.*, 1988; Kouzarides and Ziff, 1988; Rauscher *et al.*, 1988c; Zerial *et al.*, 1989). While this property of Fos can at least in part be explained by its inability to form homodimers (Halazonetis *et al.*, 1988), this does not apply to Jun, as Jun can form homodimers. Since on the other hand Jun homodimers bind very inefficiently to the TRE (Halazonetis *et al.*, 1988), the cooperative binding of Fos and Jun suggests a certain degree of asymmetry in the bipartite DNA binding site of the Fos-Jun complex.

Fos and Jun interact via specific structures present in both proteins, referred to as the leucine zipper (Landschulz *et al.*, 1988; Kouzarides and Ziff, 1988; Sassone-Corsi *et al.*, 1988b; Schuermann *et al.*, 1989; Turner and Tjian, 1989; Gentz *et al.*, 1989; Ransone *et al.*, 1989). A hallmark of this structure is the presence of a heptad repeat of leucines which are believed to align laterally with the leucines in a zipper of another protein and to establish complex formation by hydrophobic bonding (Landschulz *et al.*, 1988, 1989; O'Shea *et al.*, 1989). Adjacent to the leucine zippers in Fos and Jun, there are strongly basic regions which have been identified as the DNA binding sites (Neuberg *et al.*, 1989a; Turner and Tjian, 1989; Gentz *et al.*, 1989). Site-directed mutagenesis experiments showed that the regions in Fos and Jun required for interaction with the TRE are highly conserved between the two proteins. Taken together with the palindromic nature of the TRE this observation has led to the hypothesis that similar amino acids in Fos and Jun interact with similar DNA bases, i.e. Fos and Jun interact each with one half site of the TRE.

To prove the hypothesis that both Fos and Jun interact directly with the TRE and to analyze whether the Fos-Jun complex favors a specific orientation when binding to the TRE we have performed UV cross-linking experiments. The results strongly suggest that both Fos and Jun make direct contacts with the TRE and that there is a favored orientation of the complex on the TRE. To analyze the mechanism of Fos-Jun-TRE complex formation in further detail we have performed a comprehensive structure-function analysis of the TRE by introducing all possible single base changes and testing these mutated elements for Fos-Jun binding. In addition we have substituted thymine residues with uracil or 5-bromouracil to analyze the importance of the 5-methyl groups contributed by thymine residues within the TRE. Finally, we have analyzed the binding to wild-type and mutant TREs of a Fos protein complex containing a

homodimeric DNA binding site. Our results lead to the conclusion that the TRE is asymmetrical with respect to binding of the Fos–Jun complex.

Results

UV crosslinking of Fos and Jun to the TRE

To analyze whether both or only one of the two proteins in the Fos–Jun complex bind directly to the TRE and whether there is a preferential orientation of the complex we took advantage of the presence of thymine residues in the TRE. It had been shown previously that if the interacting protein is in close proximity to a thymine residue the cross-linking by UV of protein and DNA can be considerably enhanced by replacing the thymine with 5-bromouracil (BU) (Ogata and Gilbert, 1977). Therefore, if the cross-linking is greater with a BU-containing probe than with a probe containing thymine in the same position the cross-linked protein must be in close contact with that DNA base. We chose to replace the thymines at position 1 in the upper or lower strand with BU (oligonucleotides 1/BU upper and 1/BU lower, respectively) and analyzed the cross-linking of Fos and Jun to this probe in comparison to the normal TRE oligonucleotide (not containing BU). For this experiment, the ^{32}P-labeled oligonucleotide was incubated with the protein complex, exposed to UV and separated on an SDS–polyacrylamide gel. Proteins with cross-linked DNA were then made visible by autoradiography. Figure 1 shows the results of this experiment. While cross-linking was rather inefficient with the TRE oligonucleotide (on longer exposures cross-linking of Fos and Jun can be seen) there was strong cross-linking with oligonucleotides 1/BU upper and 1/BU lower. By comparing the mobility of the crosslinked proteins with that of Fos (JR220) and c-Jun (leftmost lanes) it becomes obvious that both proteins were cross-linked to the BU-containing oligonucleotides, Fos preferentially to 1/BU-lower and Jun preferentially to 1/BU-upper. A very similar pattern of cross-linked proteins was found in three independent experiments. There is a third band above the cross-linked c-Jun protein whose intensity is stronger when BU is present in the upper strand and which is not seen in the absence of a Fos–Jun complex suggesting that this band is probably not unspecific. It may thus represent a Fos–Jun–TRE complex or could be due to binding of another protein to this complex. The rightmost lanes in Figure 1 show controls where the individual proteins were used for cross-linking to an oligonucleotide containing BU in position 1 in both strands (BU-1). As expected no protein–DNA complexes could be detected. Likewise, no cross-linking was observed when reticulocyte lysate alone was used (data not shown). The specificity of the UV cross-linking is evident for two reasons. First, substitution of thymines outside the TRE with BU did not enhance cross-linking efficiency relative to that seen with the TRE oligonucleotide (data not shown). Second, the reticulocyte lysate contains a great number of proteins, but cross-linking is only seen with Fos and Jun.

Our data clearly show that both Fos and Jun are in close contact with the thymine residues at position 1 in both strands. In addition, the preferential contacts between Fos and thymine (1) in the lower strand and Jun and thymine (1) in the upper strand strongly suggests that the Fos–Jun complex favors a specific orientation when binding to the TRE and thus points to some degree of asymmetry in the binding site.

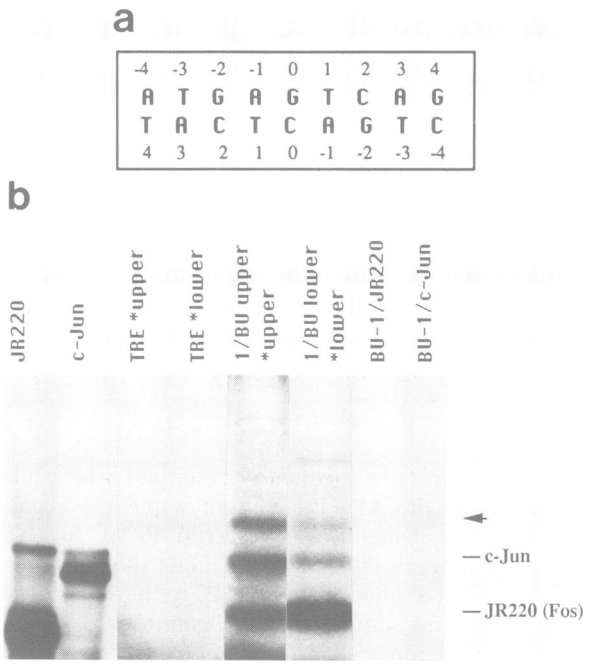


Fig. 1. (**a**) Sequence of the TRE plus one adjacent base pair on either side. (**b**) UV cross-linking of Fos–Jun complex to the TRE. A complex of *in vitro* translated Fos (JR220) and Jun protein was incubated with the ^{32}P-labeled TRE oligonucleotide, UV-irradiated and analyzed on a 10% SDS–polyacrylamide gel. Either wild-type TRE was used or oligonucleotide in which the thymine residue at the indicated positions were substituted with 5-bromouracil (BU). *indicates which of the two complementary strands was labeled. BU-1 contains BU at position 1 in both the upper and lower strand. In the rightmost lanes cross-linking was carried out with the individual proteins. For reference the [^{35}S]methionine labeled *in vitro* translated proteins are shown in the two leftmost lanes.

Mutagenesis of the TRE

To study the interaction of the Fos–Jun complex with the TRE in greater detail we performed a detailed structure–function analysis. Oligonucleotides containing all possible single base changes within the palindromic TRE sequence plus the adjacent nucleotide on either side were analyzed for Fos–Jun binding in band shift assays. If the TRE were partly asymmetrical, as suggested by the UV cross-linking results shown in Figure 1, one would expect to obtain different results with oligonucleotide probes containing equivalent mutations in both half sites of the TRE.

The results of this study are displayed in Figure 2 and an evaluation of the data is shown in Figure 3. As expected, while under the conditions used no protein–DNA complexes were detectable with Fos or Jun alone, strong cooperative binding was observed with the Fos–Jun complex. Analysis of the mutant TRE oligonucleotides yielded four major results. (i) The left half site of the TRE is less sensitive to base substitutions than the right half site (compare position −1 to 1 and position −2 to 2). (ii) There is hardly any symmetry with respect to the effect of equivalent mutations in both half sites (compare −3/A to 3/T or −2/T to 2/A or −1/C to 1/G). (iii) The guanine residue at position 0 is less sensitive to substitution than the other bases, although A and especially T decrease binding efficiency. (iv) The

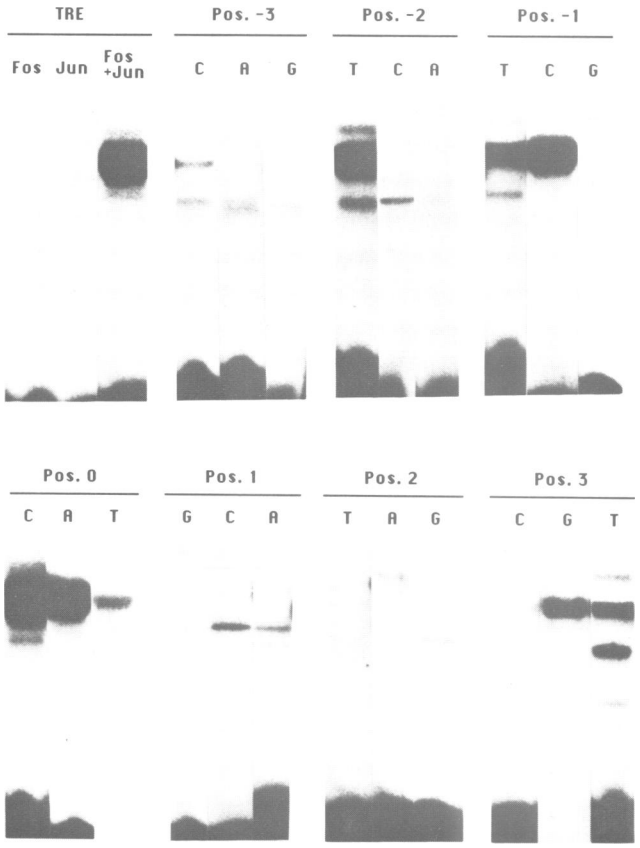


Fig. 2. Gel retardation assay illustrating the effect of TRE point mutations on Fos−Jun binding affinity. Wild-type TRE or mutant double-stranded oligonucleotides end-labeled with ^{32}P were incubated with a complex of *in vitro* translated Fos (JM) and Jun proteins and separated by non-denaturing PAGE. For the TRE the lack of binding of the individual proteins is also shown. The indicated positions of each point mutation refer to the upper strand (compare Figure 1a).

Name	Sequence	Binding of Fos/Jun
TRE	A T G A G T C A G	+++
-3/C	A **C** G A G T C A G	±
-3/A	A **A** G A G T C A G	−
-3/G	A **G** G A G T C A G	−
-2/T	A T **T** A G T C A G	+++
-2/C	A T **C** A G T C A G	−
-2/A	A T **A** A G T C A G	−
-1/T	A T G **A** G T C A G	++
-1/C	A T G **C** G T C A G	+++
-1/G	A T G **G** G T C A G	−
0/C	A T G A **C** T C A G	+++
0/A	A T G A **A** T C A G	++
0/T	A T G A **T** T C A G	+
1/G	A T G A G **G** C A G	−
1/C	A T G A G **C** C A G	−
1/A	A T G A G **A** C A G	−
2/T	A T G A G T **T** A G	−
2/A	A T G A G T **A** A G	−
2/G	A T G A G T **G** A G	−
3/C	A T G A G T C **C** G	±
3/G	A T G A G T C **G** G	+
3/T	A T G A C T C **T** G	+
-4/T	**T** T G A G T C A G	++++
-4/G	**G** T G A G T C A G	++
-4/C	**C** T G A G T C A G	++
4/C	A T G A G T C A **C**	+++
4/T	A T G A G T C A **T**	+
4/A	A T G A G T C A **A**	+++

Binding was estimated by scanning of autoradiogrammes.
++++: >200% of binding TRE; +++: 75-100%; ++: 25-75%; +: 5-25%; ±: <5%; -: 0%.

Fig. 3. Evaluation of band shift data from Figure 1a. Names of the oligonucleotides indicate the position (compare Figure 1a, upper strand), followed by the substituted base. Mutated positions are marked by a dot.

nucleotides at positions −4 and 4 also play a role in determining binding affinity; their importance, however, seems to be much lower than the core nucleotides between −3 and 3. These results suggest that the Fos−Jun binding site may extend beyond the palindromic structure and that there is some degree of functional asymmetry in the structurally symmetrical core of the TRE.

To substantiate the results shown in Figures 2 and 3 and to be able to estimte the binding affinity of Fos−Jun for the mutated TRE sequences we performed competition experiments using the TRE oligonucleotide, the corresponding mutated TRE (i.e. the oligonucleotide used as the probe) and an oligonucleotide (rd) of random sequence but of the same size as competitors in band shift assays (Figure 4, data not shown). While no competition was seen with rd, the two other competitors (TRE or the corresponding mutated TRE) competed efficiently with the probe. No effect on complex formation was seen when single-stranded TRE oligonucleotides were used as competitors (data not shown). These results show that the binding of Fos−Jun to the mutated TREs represents specific DNA binding. A quantitation of the competition experiments is shown in Figure 4, where the relative binding is plotted against the molar ratio of competitor: probe. Determination of the molar excess of the mutated oligonucleotide competitor required to achieve 50% competition (i.e. half maximal binding) allowed the different oligonucleotides to be ranked with respect to their affinity for the Fos−Jun complex (Table I). The results are in good agreement with the binding data (Figures 2, 3). The TRE oligonucleotide is the best competitor in all cases; oligonucleotides 0/C, −1/C and −2/T require 3-, 6- and 7-fold higher concentrations for 50% competition (+++ with respect to binding; see Figure 3); with oligonucleotides −1/T and 0/A 10- and 22-fold higher concentrations, respectively, were needed to achieve 50% competition (++ in Figure 3). These results indicate that any base change in the palindromic TRE core reduces the affinity for the Fos−Jun complex but there is a wide spectrum with respect to the observed effects.

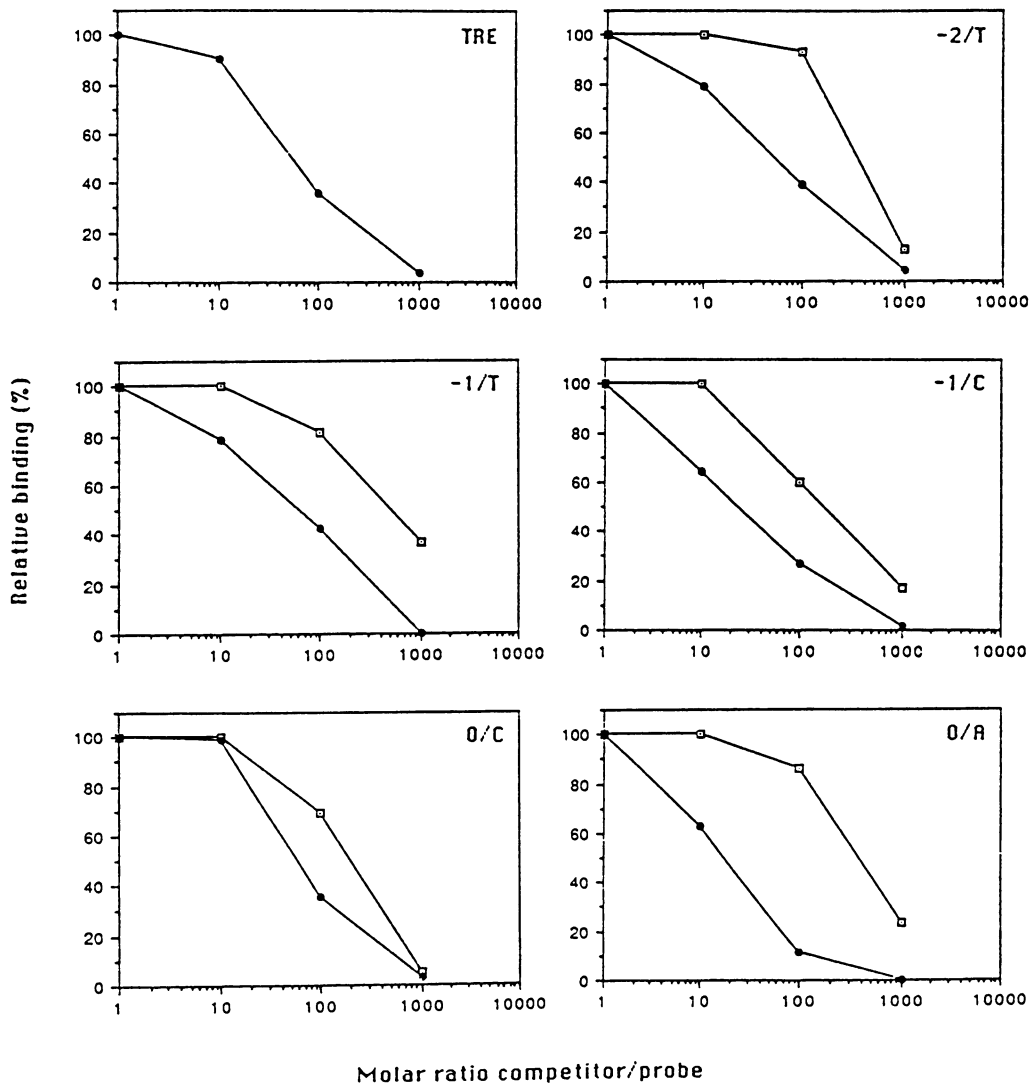


Fig. 4. Quantitation of competition experiments by density scanning of band shift autoradiographs. Relative binding is plotted against the molar ratio of competitor: probe. Binding was normalized to 100 for molar ratio = 1. The probes used in the different assays are indicated in the upper right corner of each plot. Results are shown for competition by the respective unlabeled oligonucleotide (i.e. the same DNA as used as the probe; open squares) and by the TRE oligonucleotide (filled circles).

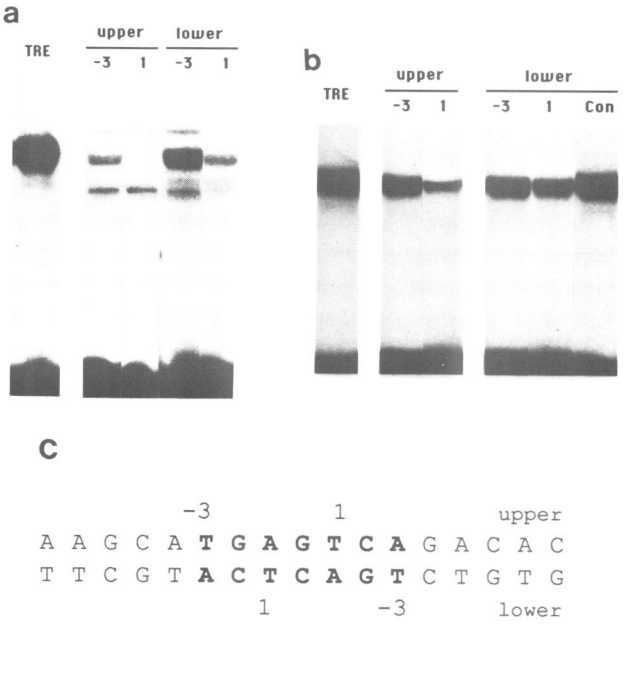
Relevance of methyl groups in the TRE

A convenient way to analyze whether specific functional groups in a DNA recognition sequences are involved in establishing base–amino acid contacts is the use of oligonucleotides lacking such groups or with substitutions in specific positions. Since the TRE core contains four thymine residues we sought to analyse the role of their methyl groups in Fos–Jun binding. This was done by substituting individual thymines with uracil. Since thymine → uracil substitutions may give rise to slight conformational alterations we also used oligonucleotides containing BU instead of thymine. BU introduces similar conformational alterations to uracil, but the bromine can functionally replace the methyl group (Ogata and Gilbert, 1977). Therefore, if an oligonucleotide binds efficiently to both the TRE and a BU-substituted oligonucleotide, but with less affinity to the uracil substituted probe, it is very likely that the methyl group of the corresponding thymine plays a crucial role in establishing contacts with the protein complex. The results of these experiments are shown in Figure 5. Substitution of any of the four thymines with uracil resulted in a significant

Table I. Evaluation of competition experiments

Oligonucleotide	Competition by TRE[a]	Competition by mutated oligonucleotide (probe)[a]	Ratio [competition by mutated oligo (probe): competition by TRE]
A. Substitutions with T, C or A (see Figure 5)			
−2/T	6.0	43	7
−1/T	7.5	73	19
−1/C	3.0	18	6
0/A	2.1	48	22
0/C	7.2	21	3
B. Substitutions with U (see Figure 7)			
−3/U upper	0.86	120	140
−3/U lower	2.4	120	50
1/U lower	1.1	>300	>250

[a]Values indicate the nanomolar concentration of competitor giving 50% inhibition (corresponding to 50% binding in Figures 5 and 7). The concentration of the probe was in all assays 0.12 nM.



Oligonucleotide	Binding of Fos/Jun
TRE	+++
-3/U upper	+
1/U upper	-
-3/U lower	++
1/U lower	+
-3/BU upper	+++
1/BU upper	+
-3/BU lower	+++
1/BU lower	++

Fig. 5. (**a**) Gel retardation assay to analyze the relevance of thymine methyl groups. Each of the thymines present in the TRE was individually replaced with uracil. The effect of these point mutations on Fos–Jun binding was analyzed in a band shift assay with wildtype TRE as the reference. (**b**) Same analysis as in (**a**) except that 5-bromouracil was used to replace thymine residues. The control (con) shows the binding of the Fos–Jun complex to an oligonucleotide containing a BU outside the TRE. (**c**) Evaluation of data shown in (**a**) and (**b**) by scanning of autoradiograph (compare Figure 2b). U: uracil, BU. 5-bromouracil. The positions of each substituted thymine are indicated in the sequence at the top. The core sequence is shown in bold letters.

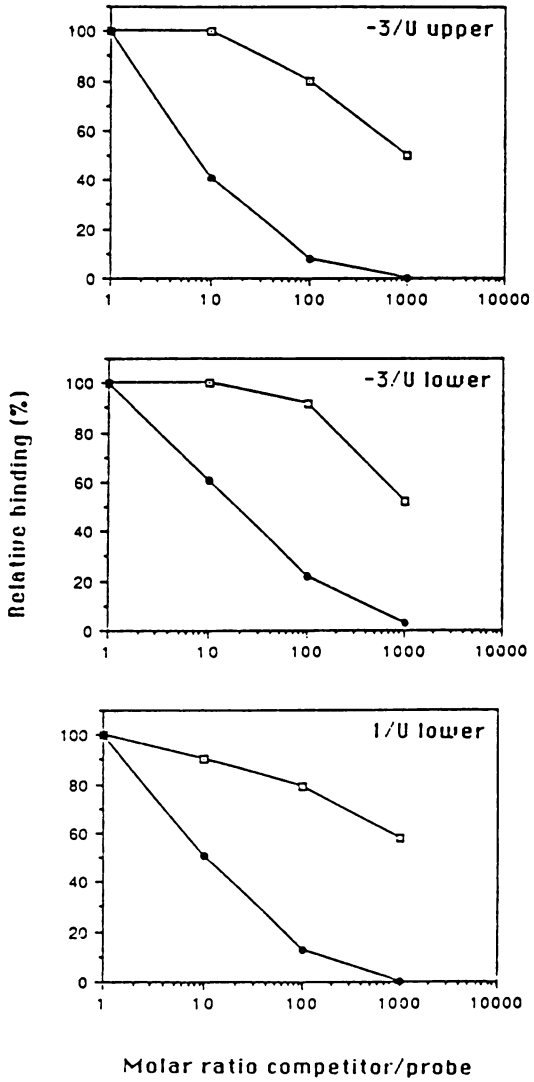


Fig. 6. Quantitation of competition experiments shown in Figure 5a. For details see legend to Figure 4.

decrease in binding, especially in positions −3 and 1 in the upper strand and position 1 in the lower strand, where the T → U mutation almost abolished binding activity. In contrast, the interaction with the TRE was affected to a much lesser degree by substitution with BU in all four cases. In agreement with these observations are the competition experiments shown in Figure 6 and Table Ib. −3/U lower, which binds less efficiently than the TRE (++ in Figure 5c) but better than the other uracil substituted oligonucleotides requires a 50-fold higher concentration relative to the TRE oligonucleotide for 50% competition. With −3/U upper and 1/U lower (+ in Figure 5c) a 140- and >250-fold excess of competitor relative to the TRE is needed. These results suggest that the methyl groups of all four thymine residues participate in Fos–Jun binding. Interestingly, equivalent substitutions in both strands gave quantitatively different results (compare −3 upper with −3 lower and 1 upper with 1 lower, in Figures 5, 6 and Table Ib) providing further evidence for the functional asymmetry of the TRE.

Asymmetrical recognition of the TRE by a homodimeric Fos protein complex

The asymmetrical recognition of the TRE could be due to either the orientation of the heterodimeric Fos–Jun protein complex or to an asymmetry in the TRE itself. If the former hypothesis were correct binding of a homodimeric protein complex should bind with increased affinity to a perfectly symmetrical sequence, i.e. a TRE with corresponding base substitutions in the residues adjacent to the palindromic core sequence. To address this question we analyzed the binding of a Fos protein complex containing a homodimeric DNA binding site. We have previously shown that a Fos protein with a Jun leucine zipper (termed ψ-Fos) is able to form homodimers with Fos (Neuberg *et al.*, 1989b). As shown

Table II. Binding of Fos–Jun compared to binding of ψ-Fos–Fos to wild-type and mutated TREs

Oligonucleotide		Binding of	
Name	Sequence	Fos–Jun[a]	ψ-Fos–Fos[a]
TRE	ATGAGTCAG	100	100
−4/T	TTGAGTCAG	>250	>350
−4/G	GTGAGTCAG	130	150
−4/C	CTGAGTCAG	83	27
4/C	ATGAGTCAC	120	149
4/T	ATGAGTCAG	24	2
5/A	ATGAGTCAA	87	25

[a]Binding assays were performed as in Figures 2 and 3 and quantitated by density scanning of autoradiographs. Results were normalized to 100% for binding to the TRE.

in Table II, the binding properties of Fos–ψ-Fos differ substantially from those of Fos–Jun for several of the tested mutant TREs, especially those which due to a mutation are structurally perfectly symmetrical (−4/C and 4/T). This finding clearly shows that the asymmetrical recognition of the TRE is due to the structure of the TRE itself rather than to the bound protein complex. Binding was also analyzed in competition experiments which gave very similar results (data not shown).

Discussion

It had previously been shown that Fos can be cross-linked by UV to DNA when bound in a protein complex to the TRE in the promoter region of the aP2 gene (Distel *et al.*, 1987). However, from this study it was not clear whether Fos interacts with specific bases within the TRE itself or whether it contacts adjacent nucleotides, e.g. by forming salt bridges or hydrogen bonds with the phosphate backbone. Both mechanisms would be compatible with the observed cooperative binding of Fos and Jun to the TRE. To address this question we have performed UV cross-linking experiments using oligonucleotides containing BU within TRE, which should increase the efficiency of cross-linking if the protein to be analyzed were in close contact with that residue. Our results clearly show that cross-linking of both Fos and Jun to the TRE is greatly enhanced if the oligonucleotide contains BU at position 1 in either the lower or upper strand. We therefore conclude that both proteins establish contacts to the TRE itself. Since the TRE has a palindromic structure (TGAGTCA) and on the other hand the DNA binding sites of Fos and Jun are highly homologous, it is likely that each of the two proteins recognizes one half site of the TRE. This way similar nucleotide–amino acid interactions would occur between Fos and the TRE and between Jun and the TRE. However, the UV cross-linking experiments strongly suggest that the binding site is functionally not perfectly symmetrical since Fos seems to favor thymine 1 in the lower strand and Jun thymine in the upper strand. There thus seems to be a preferred orientation for the binding of the Fos–Jun complex to the TRE.

To analyze this interaction in further detail we performed a detailed structure–function analysis of the TRE by analyzing the binding of Fos–Jun to oligonucleotides containing single point mutations in the TRE. The results of this study (see Figures 2 and 3; Table I) were unexpected since equivalent mutations in both half sites of the TRE in several cases gave opposite results. Thus replacing guanine in position −2 with thymine or adenine in position −1 with cytosine in the left half had only a slight effect on binding, while the corresponding mutation cytosine → adenine in position 2 or thymine → guanine in position 1 practically destroyed binding. These results suggest that there is some degree of asymmetry in the TRE, a conclusion that is in agreement with the UV cross-linking experiments (see above). This conclusion is further substantiated by the experiment where specific methyl groups were eliminated from the TRE by substituting uracil for thymine (see Figures 5, 6 and Table I). All four thymine methyl groups appear to play a crucial role in binding, and again equivalent mutations in both half sites had different effects. Finally, the nucleotides adjacent to the TRE core (positions −4 and 4) are relevant to some extent since substitutions in this position can affect binding. It is thus possible that the nucleotides adjacent to the palindromic core of the TRE determine which protein binds preferentially to which half site.

The fact that a structurally symmetrical binding site is functionally (i.e. with respect to its binding properties) asymmetrical is not unique to the TRE. A similar situation has for instance been found with the glucocorticoid response element (for a review see Beato, 1989) and the binding site for the yeast transcription factor GCN4 (Hope and Struhl, 1987). In the former case, a homodimeric protein complex binds to an imperfectly symmetrical DNA sequence and mutations in the right half site are far more detrimental for binding than base substitutions in the left half site (Beato, 1989). The GCN4 protein binds as a homodimer to a sequence that is very similar to the TRE. It has been shown that the central base pair (C–G) is important for binding, suggesting that the two half sites overlap (Hill *et al.*, 1986; Hope and Struhl, 1987), a situation that is similar to the binding of Fos–Jun to the TRE (see Figures 2 and 3).

It is interesting to note that the Jun protein interacts preferentially with thymine 1 in the right half site while Fos favors the equivalent thymine in the other half site. Since on the other hand mutations of the A–T base pair in position −1 and of the other base pairs in that half site have less pronounced effects on binding than mutations in position 1, one could speculate that the free energy of the binding of Fos–Jun to the TRE is determined predominantly by the interaction between Jun and DNA and that Fos has a stabilizing function. However, further experiments are required to analyze the validity of this hypothesis.

To distinguish whether the asymmetrical recognition of the TRE by Fos–Jun is related to the heterodimeric nature of the protein complex or a feature of the TRE DNA sequence we have analyzed the binding of a ψ-Fos–Fos complex which contains a homodimeric DNA binding site. This complex was found to bind much less efficiently to mutated TREs where the bases adjacent to the palindromic core were changed in such a way as to generate a sequence of perfect dyad symmetry over nine residues (Table II). This observation clearly indicates that the observed asymmetrical recognition is directly related to asymmetrical features of the TRE. It thus appears that the two half sites of the TRE are not identical. This is, on the one hand, due to the presence of different bases 5′ and 3′ to the TRE core, but

may on the other hand also indicate that the two half sites overlap as in the case of GCN4 (see above).

Another potentially interesting feature of our analysis is the fact that it is now possible to derive a complete consensus sequence for maximum binding of Fos–Jun to the TRE. One should note, however, that this consensus sequence: 5′-A/T T G/T A/C G/C T C A G/C/A (upper strand) is only valid for single base substitutions since multiple alterations have not been analyzed and would probably destroy binding. Using the set of mutant oligonucleotides described here it will now be interesting to test the possibility that complexes consisting of different members of the Fos and Jun families (Cohen *et al.*, 1989; Nakabeppu *et al.*, 1988; Zerial *et al.*, 1989; Hirai *et al.*, 1989) may show differential binding properties. These mutant oligonucleotides will also be useful to identify amino acid–nucleotide interactions in the Fos–Jun–TRE complex to get closer to understanding the mechanism of Fos–Jun–TRE complex formation and eventually the control of AP1-dependent gene expression.

Materials and methods

Plasmids

All genes used for *in vitro* transcription were cloned with the expression vector pTZ18 (Pharmacia). The mouse c-*jun* cDNA and JR220 have been described elsewhere (Ryseck *et al.*, 1988; Schuermann *et al.*, 1989). JM is a hybrid v-*fos*–mouse c-*fos* corresponding to a mouse cDNA containing the 5 FBJ-MSV-specific point mutations. JR220 encodes a truncated v-Fos protein of 220 amino acids; the JM protein is 380 amino acids long. The ψ-Fos construct has been described in Neuberg *et al.* (1989b).

In vitro transcription – translation and in vitro reconstitution of Fos – Jun complexes

One microgram of linearized plasmid was incubated with 0.5 mM nucleoside triphosphates (ATP, CTP, GTP, UTP), 1 U of RNasin (Genofit), 0.4 OD_{260} U'CAP'-nucleotide (Boehringer) and 5 U of T7 RNA polymerase for 30 min at 37°C in a total volume of 50 μl transcription buffer (40 mM Tris pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 10 μM dithiothreitol, 5 μg bovine serum albumin). Another 5 U of enzyme were added, and the reaction mixture was incubated for another 30 min. *In vitro* transcribed RNA was mixed with 60 μl of reticulocyte lysate (Amersham). The reaction mixture was adjusted to 150 μl with H_2O and incubated at 30°C for 1 h. Complex formation was carried out by incubating *in vitro* translated c-Jun and Fos in 50 mM potassium acetate, 0.5 mM 2-mercaptoethanol for 1 h at 30°C.

Oligonucleotides

All oligonucleotides were synthesized using an Applied Biosystems model 380A DNA synthesizer by the β-cyanoethyl-phosphate-amidite method and were purified by gel electrophoresis after removal of trityl groups. Purified complementary strands were mixed in equal amounts in 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 0.1 M NaCl, heated to 90°C and allowed to cool to room temperature overnight to permit the formation of a duplex DNA. DNAs (20 ng) were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase and the labeled DNAs were purified by passing through NACS columns (BRL).

Gel retardation assay

Binding reactions were performed by pre-incubating 5 μl Fos (JM; see above) and 5 μl Jun *in vitro* translation mixtures with 1 μg of poly(dI-dC) in a buffer containing 10 mM HEPES, pH 7.9, 60 mM KCl, 4% Ficoll, 1 mM EDTA and 1 mM dithiothreitol (DTT) for 60 min at room temperature (Barberis *et al.*, 1987). Two femtomoles of double-stranded oligonucleotide ^{32}P-labeled by polynucleotide kinase were added and incubation was continued for 30 min at room temperature. The reaction mixtures were separated on 4% polyacrylamide gels at room temperature at 10 V/cm.

TRE oligonucleotide (corresponding to the sequence of the collagenase TRE; Angel *et al.*, 1987):

5′AAGCATGAGTCAGACAC (upper strand);

rd oligonucleotide:

5′GCGACTAACATCGATCG (upper strand).

UV cross-linking

For UV cross-linking experiments (Ogata and Gilbert, 1977; Chodosh *et al.*, 1986; Kumar and Chambon, 1988) complex formation between Fos–Jun and the ^{32}P-labeled oligonucleotide was performed essentially as described in 'gel retardation assay' with the only difference being that a 10-fold higher concentration of radioactive oligonucleotide was used. An Eppendorf tube containing the reaction mixture was placed on ice and irradiated at a distance of 5–6 cm for 5.5 min by using an inverted UV-transilluminator at 303 nm. The irradiated proteins were dentatured by boiling for 5 min in SDS sample buffer and separated on a 10% SDS–polyacrylamide gel. The yield of cross-linked TRE was in the range of 1%.

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References

Angel,P., Imagawa,M., Chiu,R., Stein,B., Imbra,R.J., Rahmsdorf,H.J., Jonat,C., Herrlich,P. and Karin,M. (1987) *Cell*, **49**, 729–739.

Angel,P., Allegretto,E.A., Okino,S.T., Hattori,K., Boyle,W.J., Hunter,T. and Karin,M. (1988) *Nature*, **332**, 166–171.

Barberis,A., Saperti-Furga,G. and Busslinger,M. (1987) *Cell*, **50**, 347–359.

Beato,M. (1989) *Cell*, **56**, 335–344.

Bohmann,D., Bos,T.J., Admon,A., Nishimura,T., Vogt,P.K. and Tjian,R. (1987) *Science*, **238**, 1386–1392.

Bos,T.J., Bohmann,D., Tsuchie,H., Tjian,R. and Vogt,P.K. (1988) *Cell*, **52**, 705–712.

Chiu,R., Boyle,W.J., Meek,J., Smeal,T., Hunter,T. and Karin,M. (1988) *Cell*, **54**, 541–552.

Chodosh,L.A., Carthew,R.W. and Sharp,P.A. (1986) *Mol. Cell. Biol.*, **6**, 4723–4733.

Cohen,D.R., Ferreira,P.C.P., Gentz,R., Franza,B.R.,Jr and Curran,T. (1989) *Genes Dev.*, **3**, 173–184.

Curran,T. and Franza,B.R.,Jr (1988) *Cell*, **55**, 395–397.

Distel,R.J., Ro,H.-S., Rosen,B.S., Groves,D.L. and Spiegelman,B.M. (1987) *Cell*, **49**, 835–844.

Gentz,R., Rauscher,F.J.,III, Abate,C. and Curran,T. (1989) *Science*, **243**, 1695–1699.

Halazonetis,T.D., Georgopoulos,K., Greenberg,M.E. and Leder,P. (1988) *Cell*, **55**, 917–924.

Hill,D.E., Hope,I.A., Macke,J.P. and Struhl,K. (1986) *Science*, **234**, 451–457.

Hirai,S.I., Ryseck,R.P., Mechta,F., Bravo,R. and Yaniv,M. (1989) *EMBO J.*, **8**, 1433–1439.

Hope,I.A. and Struhl,K. (1987) *EMBO J.*, **6**, 2781–2784.

Kouzarides,T. and Ziff,E. (1988) *Nature*, **336**, 646–651.

Kumar,V. and Chambon,P. (1988) *Cell*, **55**, 145–156.

Landschulz,W.H., Johnson,P.F. and McKnight,S.L. (1988) *Science*, **240**, 1759–1764.

Landschulz,W.H., Johnson,P.F. and McKnight,S.L. (1989) *Science*, **243**, 1681–1688.

Lee,W., Mitchell,P. and Tjian,R. (1987) *Cell*, **49**, 741–752.

Lucibello,F.C., Neuberg,M., Hunter,J.B., Jenuwein,T., Schuermann,M., Wallich,R., Stein,B., Schönthal,A., Herrlich,P. and Müller,R. (1988) *Oncogene*, **3**, 43–51.

Nakabeppu,Y., Ryder,K. and Nathans,D. (1988) *Cell*, **55**, 907–915.

Neuberg,M., Schuermann,M., Hunter,J.B. and Müller,R. (1989a) *Nature*, **338**, 589–590.

Neuberg,M., Adamkiewicz,J., Hunter,J.B. and Müller,R. (1989b) *Nature*, **341**, 243–245.

Ogata,R. and Gilbert,W. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 4973–4976.

O'Shea,E.K., Rutkowski,R. and Kim,P.S. (1989) *Science*, **243**, 538–542.

Ransone,L.J., Visvader,J., Sassone-Corsi,P. and Verma,I.M. (1989) *Genes Dev.*, **3**, 770–781.

Rauscher,F.J.,III, Sambucetti,L.C., Curran,T., Distel,R.J. and Spiegelmann,B.M. (1988a) *Cell*, **52**, 471–480.

Rauscher,F.J.,III, Cohen,D.R., Curran,T., Bos,T.J., Vogt,P.K.,

Bohmann,D., Tjian,R. and Franza,B.R.,Jr (1988b) *Science*, **240**, 1010–1016.
Rauscher,F.J,III, Voulalas,P.J., Franza,B.R.,Jr and Curran,T. (1988c) *Genes Dev.*, **2**, 1687–1699.
Ryseck,R.-P., Hirai,S.I., Yaniv,M. and Bravo,R. (1988) *Nature*, **334**, 535–539.
Sassone-Corsi,P., Lamph,W.W., Kamps,M. and Verma,I.M. (1988a) *Cell*, **54**, 553–560.
Sassone-Corsi,P., Ransone,L.J., Lamph,W.W. and Verma,I.M. (1988b) *Nature*, **336**, 692–695.
Schuermann,M., Neuberg,M., Hunter,J.B., Jenuwein,T., Ryseck,R.-P., Bravo,R. and Müller,R. (1989) *Cell*, **56**, 507–516.
Turner,R. and Tjian,R. (1989) *Science*, **243**, 1689–1694.
Zerial,M., Toschi,L., Ryseck,R.-P., Schuermann,M., Müller,R. and Bravo,R. (1989) *EMBO J.*, **8**, 805–813.

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