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Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity

(gene regulation/oncogenes/leucine zipper/protein dimerization/transcription)

TSONWIN HAI*† AND TOM CURRAN‡

*Department of Medical Biochemistry and Ohio State Biotechnology Center, Ohio State University, 1060 Carmack Road, Columbus, OH 43210; and ‡Department of Molecular Oncology and Virology, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110

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ABSTRACT **The Fos/Jun and ATF/CREB families of transcription factors function in coupling extracellular signals to alterations in expression of specific target genes. Like many eukaryotic transcription factors, these proteins bind to DNA as dimers. Dimerization is mediated by a structure known as the "leucine-zipper" motif. Although Fos/Jun and ATF/CREB were previously thought to interact preferentially with different DNA regulatory elements (the AP-1/TRE and ATF/CRE sites, respectively), we find that members of these two families form selective cross-family heterodimers. The resulting heterodimers display distinguishable DNA binding specificities from each other and from their parental homodimers. These findings indicate that the Fos/Jun and ATF/CREB families of transcription factors are not as distinct as was previously thought. We suggest that they can be grouped into a superfamily of transcription factors.**

Regulation of eukaryotic gene expression is mediated by combinations of short DNA sequences that provide binding sites for sequence-specific transcription factors (ref. 1 and references therein). Two of the major classes of regulatory elements that contribute to transcriptional regulation by extracellular signals are the AP-1/TRE (2, 3) and ATF/CRE (4, 5) sequence motifs. The AP-1/TRE element (TGACTCA) was originally defined as the activator protein 1 (AP-1) binding site or the phorbol 12-*O*-tetradecanoate 13-acetate (TPA) responsive element (TRE); the ATF/CRE element (TGACGTCA) was defined as the activating transcription factor (ATF) binding site or the cAMP responsive element (CRE). Several proteins have now been shown to bind to these cis-acting elements. The AP-1/TRE site is recognized by a group of proteins, including those encoded by the c-*fos* and c-*jun* gene families (6–12). These proteins are induced by mitogenic, differentiation-inducing, and neuronal-specific stimuli (13). The ATF/CRE site is recognized by a family of proteins referred to as ATF or CRE binding proteins (CREB) (14–23). This family of proteins has been implicated in cAMP-, calcium-, and virus-induced alterations in transcription (ref. 24; for review, see refs. 25 and 26).

Previously, the Fos/Jun and ATF/CREB protein families were regarded as distinct sets of transcription factors that share the basic-region/leucine-zipper motif but have different DNA binding specificities. They bind preferentially to their respective recognition sequences: ATF/CREB proteins bind to the ATF/CRE site with a higher affinity than to the AP-1/TRE site; conversely, Fos/Jun proteins bind to the AP-1/TRE site with a higher affinity than to the ATF/CRE site (6–12, 14–23, 27–30).

Table 1 lists several of the mammalian genes that encode these two families of proteins (28–40). These genes are grouped into subfamilies according to their amino acid similarities. Within each subfamily, members share significant sequence similarity in several regions of the protein. Between subfamilies, the members are similar to each other only in their DNA-binding/leucine-zipper domains. Immunoprecipitation and DNA binding assays have indicated that members of each family form intrafamily heterodimers by using the leucine zippers. Significantly, dimerization is highly selective. For example, Fos forms heterodimers with Jun-related proteins but does not form homodimers; whereas, c-Jun forms homodimers and heterodimers with all Jun- and Fos-related proteins (6–12, 27). Similarly, members of the ATF/CREB family form selective heterodimers. For example, ATF-3 forms heterodimers with ATF-2 but not with ATF-1 (34).

Table 1. Mammalian Fos/Jun and ATF/CREB families

Family	Subfamily	Gene(s)
Fos/Jun	Fos	*fos, fra-1, fra-2, fosB*
	Jun	*c-jun, junB, junD*
ATF/CREB	CREB	*CREB, ATF-1*
	CRE-BP1	*CRE-BP1, ATF-a*
	ATF-3	*ATF-3**
	ATF-4	*ATF-4**

Alternative names for *CRE-BP1* are *ATF-2* and *HB16*. ΔCREB is a spliced variant of CREB, ATF-aΔ is a spliced variant of ATF-a, and mXBP/BP-2 is a spliced variant of CRE-BP1.
*Genomic Southern blot analysis indicated that there is more than one gene in these two subfamilies (data not shown).

Since members of each family can form selective intrafamily heterodimers by using the leucine-zipper structure, it was reasonable to ask if these two families of proteins could form interfamily heterodimers. Furthermore, would such heterodimers have altered DNA binding specificities? Therefore, we investigated the possibilities of cross-family heterodimerization and the effects of such dimerization on DNA binding specificities. The approach involved translation of *in vitro*-transcribed mRNA in reticulocyte lysate and examination of the resulting polypeptides by two assays: coimmunoprecipitation to detect heterodimer formation and gel-shift analysis to determine DNA binding specificities. Herein we report studies on three members of the Fos/Jun family (c-Fos, Fra-1, and c-Jun) and four members of the ATF/CREB family (ATF-1, ATF-2, ATF-3, and ATF-4).

MATERIALS AND METHODS

DNA and Proteins. The following oligonucleotides were synthesized and used for gel-shift experiments: CRE site, TCGATTGGCTGACGTCAGAGAGAG; AP-1 site, TCGACGTGACTCAGCGCGCATCGTGACTCAGCGCGC; and Enk-2 site, TCGAAGGGCCTGCGTCAGCTGCAGCCCGCCGG. The corresponding binding sites are underlined.

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†To whom reprint requests should be addressed.

Although the AP-1 probe contained two binding sites, it showed no difference from the probe containing a single site when assayed with Fos and Jun (F. J. Rauscher III and T.C., unpublished results). ATFs, Jun, Fos, and Fra-1 were synthesized in rabbit reticulocyte lysates programmed with the *in vitro*-transcribed mRNA in the presence of [^{35}S]methionine or unlabeled methionine according to the protocol recommended by the manufacturer (Promega). The Jun antibody was raised against C-terminal amino acids 224–334 of c-Jun. The Fos antibody was made against rat c-Fos amino acids 1–131 (41). The Fra-1 antibody was raised against rat Fra-1 amino acids 136–275 (7).

Immunoprecipitation. Reticulocyte lysate (2 μl) containing ATF proteins was mixed with 2 μl of reticulocyte lysate containing Jun or Fos and incubated at 37°C for 30 min before immunoprecipitation by 2 μl of the appropriate antibody. Immunoprecipitation was carried out in phosphate-buffered saline containing 0.1% Nonidet P-40, 100 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, aprotinin (25 μg/ml), and leupeptin (2 μg/ml) as described (42, 43). The precipitates were analyzed on SDS/12% polyacrylamide gels.

DNA Binding. Reticulocyte lysate (2 μl) containing ATFs, 2 μl of lysate containing Fos or Jun, or 1 μl of ATFs mixed with 1 μl of Fos or Jun was incubated at 37°C for 30 min and assayed for gel-shift activity using the oligonucleotides described above under the conditions described (34). The assays were all performed under conditions of DNA excess. The same batches of proteins and labeled oligonucleotides were used in the reactions presented in any given figure panel.

RESULTS

Selective Cross-Family Dimerization. The coimmunoprecipitation results are summarized in Table 2 and shown in Fig. 1. The Fos and Jun antibodies used in this study do not recognize any of the ATF proteins. Therefore, the appearance of ATF proteins in immunoprecipitates is indicative of dimerization. As shown in Fig. 1, ATF-2 and ATF-3 selectively formed dimers with Jun but not with Fos or Fra-1. ATF-4, however, dimerized with all three proteins: Jun, Fos, and Fra-1. Although, it appeared to associate with Fra-1 with higher affinity than with Jun or Fos, this preference was variable in different experiments. ATF-1, in contrast, did not form dimers with any of the Fos/Jun proteins analyzed here (data not shown). Thus, members of the ATF/CREB family of transcription factors form selective heterodimers with members of the Fos/Jun family.

Cross-Family Dimerization Alters DNA Binding Specificity. We next examined the DNA binding activities of the cross-family heterodimers by gel-shift analyses using oligonucleotides containing three related regulatory elements: ATF/CRE (TGACGTCA), AP-1 (TGACTCA) from metallothionein II$_A$, and Enk-2 (TGCGTCA) from the proenkephalin regulatory region. The results are summarized in Table 3 and shown in Figs. 2 and 3. As shown in Fig. 2*A*, both Jun and ATF-2 associated with the CRE as homodimers (lanes 4 and 5), although the relative affinity of ATF-2 for the CRE was greater than that of Jun. Incubation of ATF-2 with Jun prior to the addition of the probe resulted in the formation of a DNA–protein complex with an intermediate mobility on the

Table 2. Selective heterodimer formation between ATF and Fos/Jun proteins

	Heterodimer formation			
	ATF-1	ATF-2	ATF-3	ATF-4
Jun	−	+	+	+
Fos	−	−	−	+
Fra-1	−	−	−	+

+, Heterodimer formed; −, no heterodimer formed.

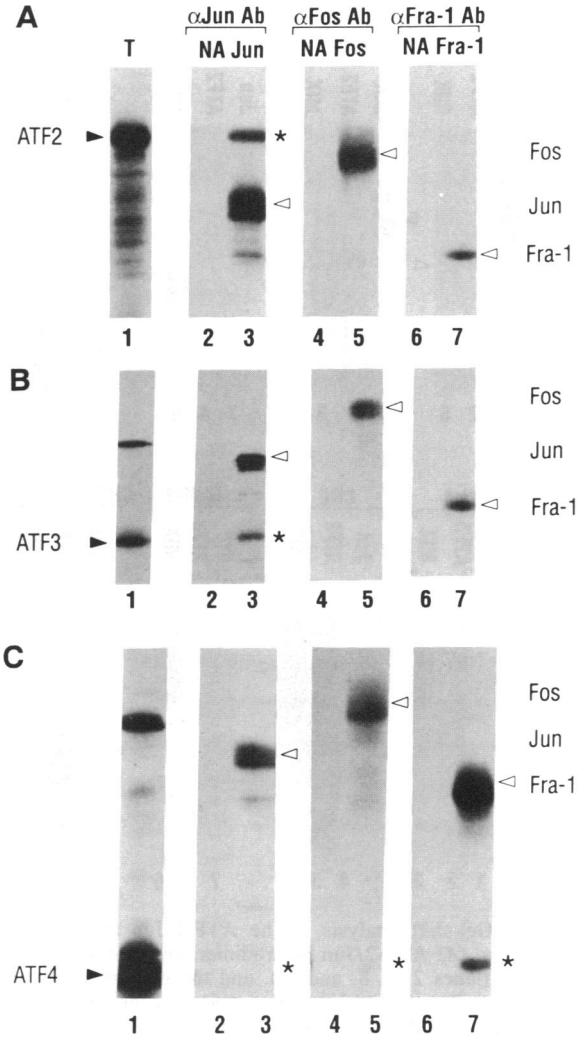

FIG. 1. Coimmunoprecipitation of ATF proteins with Jun, Fos, and Fra-1. (*A*) Analysis of ATF-2. ATF-2 protein was mixed with Jun (lane 3), Fos (lane 5), or Fra-1 (lane 7) before immunoprecipitation. Control experiments (lanes 2, 4, and 6) were carried out in parallel by immunoprecipitating the ATF-2 protein with the appropriate antibody without preincubating with the corresponding Jun/Fos proteins. Lane 1 shows the total ATF-2 protein from the translation reaction (T). (*B*) Analysis of ATF-3. Same as *A* except that ATF-3 was used in this experiment. Lane 1 shows the total ATF-3. (*C*) Analysis of ATF-4. Same as *A* except ATF-4 was used. Solid arrowheads, ATF-2, ATF-3, or ATF-4; open arrowheads, Fos, Jun, and Fra-1; asterisks, coimmunoprecipitated ATF-2, ATF-3, or ATF-4; Ab, antibody; NA, not added.

Table 3. Summary of the relative DNA binding specificities of various ATF/Fos/Jun heterodimers

Heterodimer	Binding specificity(ies)
ATF-2/Jun	CRE > ENK-2 > AP-1
ATF-3/Jun	CRE, AP-1, ENK-2
ATF-4/Fra-1	CRE > AP-1
ATF-4/Fos	CRE
ATF-4/Jun	CRE

Relative affinity of CRE, AP-1, and Enk-2 sites for a given dimer is indicated in order by ">." DNA concentrations in all reaction mixtures were equivalent, and the protein concentrations were estimated to be about the same. Based on this estimation, we assigned the affinities according to the relative intensities of the complexes obtained in gel-shift assays.

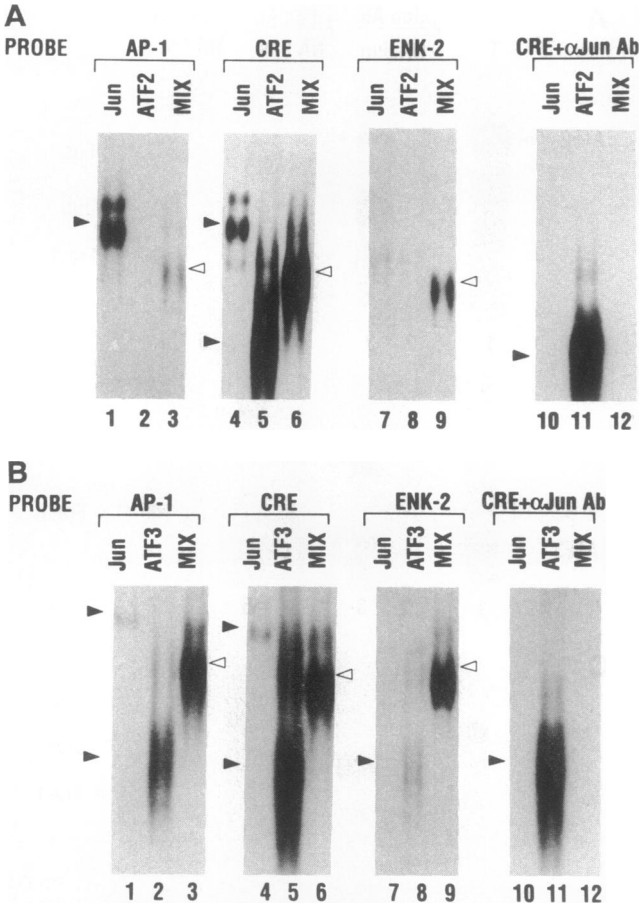

FIG. 2. Gel-shift analysis of the ATF-2/Jun and ATF-3/Jun heterodimers. (*A*) ATF-2/Jun heterodimer. Jun (lanes 1, 4, 7, and 10), ATF-2 (lanes 2, 5, 8, and 11), and the mixture of these two proteins (lanes 3, 6, 9, and 12) were assayed for their ability to bind AP-1 (lanes 1–3), CRE (lanes 4–6 and 10–12), and Enk-2 (lanes 7–9) sites. Anti-Jun antibody (1 μl) was added to the reaction mixtures after binding was completed (lanes 10–12). The mixtures were incubated at 4°C for 1 hr before loading onto the gel. Solid arrowheads, homodimeric complexes; open arrowheads, heterodimeric complexes. (*B*) ATF-3/Jun heterodimer. Same as *A* except ATF-3 was used instead of ATF-2.

gel (lane 6). This indicates that the ATF-2/Jun heterodimer binds to the CRE probe. The presence of Jun in this complex was confirmed by the specific loss of this band after incubation with an anti-Jun antibody (lane 12). A super-shifted band was detected close to the origin of the gel (not shown in Fig. 2). An unrelated antibody, the anti-Fos antibody, did not alter the mobility of this complex (data not shown). The apparent binding affinity of the ATF-2/Jun heterodimer to the CRE probe was much greater than that to the AP-1 or Enk-2 probe. In addition, this heterodimer displayed a binding activity that was distinguishable from its parental homodimers by the following characteristics: (*i*) unlike the Jun homodimer, the heterodimer did not bind well to the AP-1 site and (*ii*) the heterodimer associated with the Enk-2 probe better than did either homodimer.

In contrast to the ATF-2/Jun heterodimer, the ATF-3/Jun heterodimer interacted with all three probes with similar affinities (Fig. 2*B*). This heterodimer bound to the Enk-2 site efficiently, although neither parental homodimer associated with the site appreciably. Thus, ATF-3 and Jun interact cooperatively with the Enk-2 element. The presence of Jun in the heterodimeric complex (on the CRE site) was confirmed by an antibody inhibition experiment (lane 12).

Fig. 3 shows the DNA binding specificities of ATF-4 heterodimers formed with Jun, Fos, and Fra-1. The ATF-4/Jun heterodimer associated weakly with the CRE site but did not bind to the AP-1 or Enk-2 probe appreciably (Fig. 3*A*). The ATF-4/Fos heterodimer, however, interacted cooperatively with the CRE site: the affinity of this heterodimer to the CRE site was significantly greater than that of either homodimer (Fig. 3*B*). This dimer did not bind to the AP-1 or Enk-2 site. The ATF-4/Fra-1 heterodimer formed complexes with both the CRE and the AP-1 probes but did not bind to the Enk-2 probe (Fig. 3*C*). The interaction of this heterodimer with the AP-1 site is weak (indicated by an open arrow in lane 3). The presence of Fra-1 in this heterodimeric complex was confirmed by a super shift in its mobility in the presence of Fra-1 antibody (lane 12). Like the ATF-4/Fos dimer, ATF-4/Fra-1 associated with the CRE site cooperatively. The presence of Fra-1 in this heterodimeric complex was confirmed by an antibody inhibition experiment (lane 15). The complexes detected on the gel with the Enk-2 probe were nonspecific, as they were not affected by any of the antibodies used (data not shown). Finally, ATF/CREB and Fos/Jun pairs that failed to form heterodimers in the coimmunoprecipitation assay (Fig. 1 and Table 2) also did not form detectable heterodimeric complexes on the DNA (data not shown).

DISCUSSION

Four major conclusions can be drawn from this study. (*i*) Members of the ATF/CREB family form selective cross-family heterodimers with members of the Fos/Jun family (Table 2). (*ii*) The heterodimers have different binding activities than their parental homodimers. (*iii*) Different heterodimers have distinguishable binding specificities (Table 3). (*iv*) The cross-family heterodimers can bind to either the ATF/CRE or the AP-1 site, depending on the dimer composition, although in general they exhibit a preference for the CRE site.

Similar results of cross-family heterodimer formation between ATF-2 and c-Jun have been reported by several laboratories (28–30). One implication of these observations is that the distinction between the ATF/CREB and the Fos/Jun families of transcription factors is not as clear as was previously thought. These two families of proteins were distinguished originally by their consensus recognition sequences. DNA binding studies using either purified proteins from nuclear extracts or proteins synthesized from cloned genes *in vitro* indicated preferential DNA binding to their respective recognition sequences. This distinction is now blurred by the observation that ATF/CREB and Fos/Jun proteins form heterodimers with various binding specificities and affinities depending on the dimer composition. We suggest that the ATF/CREB and Fos/Jun proteins can be grouped into a transcription factor superfamily.

Can the specificity of dimerization described here be explained by the corresponding leucine-zipper sequences? Although detailed structural features of leucine zippers are not available, studies of the Fos and Jun proteins suggested that two types of interactions are important for dimer specificity: (*i*) hydrophobic interactions between the conserved 4,3 repeats typical of the coiled-coil helices (the 4,3 repeat is made up of amino acids at positions **a** and **d** along one face of the α-helix; see refs. 44–46 for the nomenclature) and (*ii*) electrostatic interactions between the residues located in between the conserved hydrophobic amino acids (44–46). The dimerization specificities displayed by ATF-3 and ATF-4 may, in part, be explained by their 4,3 repeat sequences. ATF-4, which behaves like Jun in terms of dimerization specificity, is very similar to Jun in its 4,3 repeat region. In contrast, ATF-3 has features of both the Fos and Jun families. In the Fos family, the alternate nonleucine apolar residues have been characteristically replaced by two lysines: one

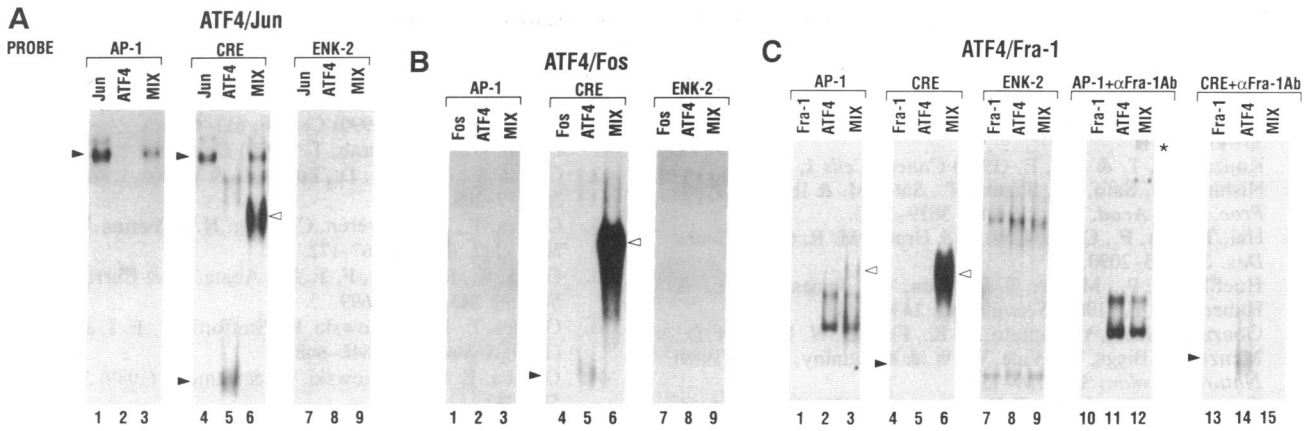

FIG. 3. Gel-shift analysis of the ATF-4 heterodimers. (*A*) ATF-4/Jun heterodimer. Jun (lanes 1, 4, and 7), ATF-4 (lanes 2, 5, and 8), and the mixture of these two proteins (lanes 3, 6, and 9) were assayed for their ability to bind AP-1 (lanes 1–3), CRE (lanes 4–6), and Enk-2 (lanes 7–9) sites. Solid arrowheads, homodimeric complexes; open arrowhead, heterodimeric complex. (*B*) ATF-4/Fos heterodimer. Same as *A* except Fos was used instead of Jun. (*C*) ATF-4/Fra-1 heterodimer. Same as *A* except Fra-1 was used instead of Jun. Anti-Fra-1 antiserum (1 μl) was added to the reaction mixture after binding was completed (lanes 10–15). Asterisk, antibody-super-shifted band.

between the second and third leucines and the other one between the fourth and fifth leucines. The inability of ATF-3 to form stable dimers with Fos-related proteins may be explained by the lysine residues between the fourth and fifth leucines. ATF-2 and Jun form stable dimers, but ATF-2 and Fos do not. This distinction may be due to the differences in the potential electrostatic interactions between these two pairs of proteins. ATF-1 fails to dimerize with any of the Fos/Jun proteins examined here, perhaps because it has four leucine-heptad repeats, whereas, Fos and Jun have five leucine repeats in the zipper region.

Accumulating evidence indicates that protein dimerization plays an important role in transcriptional regulation (for reviews, see refs. 47 and 48). As shown here, a complex array of dimers can be generated from a relatively small number of components. This diversity provides a great variety of mechanisms for transcriptional regulation. What are the physiological functions of the heterodimers reported in this study? Although we can only speculate, it seems reasonable that the dimers are involved in signal transduction processes within the nucleus. One common feature of the promoters that contain ATF/CRE, AP-1, and related binding sites is that they respond to environmental stimuli (for example, mitogens, phorbol esters, viral infection, and peptide hormones that elevate cAMP levels). It has been postulated that the ATF/CRE/AP-1 sites in the promoters mediate these responses. Consistently, members of this superfamily have been shown to increase their activities or their levels upon cell stimulation (13). Thus, this group of transcription factors may represent a nuclear component of a signal transduction pathway that functions in homeostasis. The selective dimerization and altered DNA binding specificities demonstrated in this report provide an explanation for the involvement of similar regulatory elements in diverse physiological responses that require stimulus–transcription coupling.

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