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Difference in transcriptional regulatory function between c-Fos and Fra-2

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ABSTRACT
Fra-2, one of the Fos-related antigens, is promptly expressed after the growth stimulation of fibroblasts, but its induction peak is later than that of c-Fos. In this report, we examined biochemical properties of Fra-2 and compared them with those of two other Fos family proteins, c-Fos and Fra-1. Like c-Fos and Fra-1, Fra-2 formed stable heterodimers with c-Jun, JunB or JunD in vitro and all these complexes had specific DNA-binding activity to AP-1-binding sites (AP-1 sites) or related sequences. When transiently introduced into a mouse embryonic carcinoma cell line, F9, with reporter genes containing the AP-1 site from the collagenase gene, fra-2 plus c-jun suppressed the transactivation by c-jun alone. This property of Fra-2 is in clear contrast to that of c-Fos, which stimulates the transcriptional activity of c-Jun by forming a stable heterodimer. Analysis of chimeric proteins between c-Fos and Fra-2 indicated that this difference is mainly attributable to their C terminal-half regions. Interestingly, this suppressive effect of Fra-2 was not observed in the combination with JunD: fra-2 plus junD, like c-fos plus junD, had higher transcriptional activity than junD alone. Fra-1 showed essentially the same transcriptional regulatory properties as Fra-2. These differential properties greatly expand the potential range of regulatory functions of the Fos family proteins.

INTRODUCTION
A growing body of evidence indicates that protooncogenes c-fos and c-jun play important roles in cellular growth and differentiation. The protein products of c-fos and c-jun (c-Fos and c-Jun, respectively) function cooperatively as transcriptional regulators in a heterodimeric complex (1-5). c-Jun, unlike c-Fos, also forms homodimers, although these are less stable than Fos/Jun heterodimers. The dimeric complexes interact with activator protein-1 (AP-1)-binding sites (TGACTCA) to regulate transcription (6-10). Dimerization of Fos and Jun is mediated by the leucine zipper structure (11). Dimer formation results in the juxtaposition of conserved basic regions of each protein that form a bipartite DNA binding domain (12, 13).

To date, several related genes of these protooncogenes have been reported; fra-1 (14, 15), fosB (16) and fra-2 (17-19) as fos-related genes and junB (20) and junD (21,22) as jun-related genes. Among the same family, the gene products share common biological functions; however, they are also expected to have distinct functions as was shown by comparative studies between c-Jun and JunB (23, 24).

Fra-2 was initially detected as a serum-inducible Fos-related antigen (17). The induction peak of this immediate-early gene product is later than that of c-Fos. Unlike c-Fos, Fra-2 is synthesized at a low level even in growth-arrested chicken embryo fibroblasts (CEF) or logarithmically growing CEF (17, 19). We have molecularly cloned the gene from chicken genomic library. Overexpression of fra-2 gene, like c-fos gene (25, 26), caused cellular transformation of CEF (17). Although Fra-2 was shown to form a complex with c-Jun in vivo, its biochemical properties are poorly known.

In this study, we firstly cloned fra-2 cDNA and demonstrated its biochemical properties in vitro, such as dimer-forming activity with Jun family proteins and specific DNA-binding activity to AP-1 sites. We next examined the transcriptional regulatory function of Fra-2 using transient transfection assay in a mouse embryonic carcinoma cell line, F9. The results will be compared with those for two other Fos family proteins, c-Fos and Fra-1.

MATERIALS AND METHODS
Library screening
We constructed a chicken cDNA library in λgt10 using 5 μg of poly(A) RNA isolated from CEF that were serum-starved and then treated with 10% calf serum for 60 min in the presence of 10 μg/ml cycloheximide. The cDNAs were synthesized with a cDNA cloning kit (BRL) using random primer and a synthetic

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oligonucleotide (5'-CTGGCATTGTCCTCCTCCTCCTCCTC-GA-3') that is located in the 3'-noncoding region of the fra-2 gene. Recombinants (6 x 10^6) were screened by hybridization with the fragments from exon 1 and exon 4 of fra-2 genomic DNA (17). From 31 positive clones, several long inserts were subcloned into pUC118 plasmid. From two λ clones (λ004 and λ030), the entire coding sequence of fra-2 cDNA was inserted into pSP64 in the sense orientation to generate pSP-Fra-2. By nucleotide sequence analysis using the diodeoxyxynucleotide chain termination method, the entire coding sequence was shown to be identical to the sequence that had been predicted from the fra-2 genomic DNA analysis (17) and the deduced amino acid sequence has a strong homology (87%) to human Fra-2 protein (18).

**Plasmids**

pSP-c-Fos(rat), pGEM-c-Jun(rat) (10), and pSP-Fra-1(rat) (15) were kindly provided by Dr. T. Curran. pGEM-junB(mouse) and pGEM-junD(mouse) were generous gifts from Dr. D. Nathans (20, 22). Plasmids containing chimeric genes between c-fos and fra-2 were constructed as follows. The c-fos gene was first mutagenized to create a BamHI site in the coding region (nucleotide position 556-561 according to the numbering described in 27), which resulted in no substitution of the encoded amino acids. For this purpose, the EcoRI-Xhol fragment of c-fos (rat) cDNA was blunt-ended, ligated to XbaI linkers, and cloned into the XbaI site of pUC119 to generate pSP-Fra-1. The BamHI fragment of pUC-c-fosM1, which contains the N-terminal portion of the c-fos coding region and the BamHI-XbaI fragment of pSP-Fra-2, which contains the C-terminal portion of the fra-2 coding region were isolated. These fragments were cloned into the XbaI site of pSP64 in the sense orientation to generate pSP-FosF2. The Sall-BamHI fragment of pSP-Fra-2 and BamHI-XbaI fragment of pUC-c-fosM1 were ligated to BamHI-XbaI fragment of pSP64 to generate the reciprocal construct, pSP-F2Fos.

For the construction of expression plasmids, EcoRI-Xhol fragment of c-fos (rat) cDNA was blunt-ended, ligated to XbaI linkers, and cloned into the XbaI site of the RSV promoter expression vector pRSV-2 (29), kindly gifted by Dr. K. Ozato, to generate pRSV-c-Fos. EcoRI-EcoRI fragments of pGEM-c-Jun, pGEM-junB and pGEM-475-junD, as well as EcoRI-XbaI fragment of pSP-Fra-1 were isolated. Each fragment was blunt-ended, ligated to Sall linkers and cloned into the Sall site of pRSV-2. These expression plasmids were designated as pRSV-c-Jun, pRSV-JunB, pRSV-JunD, and pRSV-Fra-1, respectively. The Sall-Sall fragment of pSP-Fra-2, the XbaI-XbaI fragment of pSP-FosF2 and the Sall-XbaI fragment of pSP-F2Fos were also cloned into pRSV-2 in the sense orientation to generate pRSV-c-Fra-2, pRSV-FosF2 and pRSV-F2Fos, respectively.

To construct the reporter chloramphenicol acetyl transferase (CAT) plasmid used in the transfection experiments, we first constructed a starting plasmid designated proCAT. The Hind-7-Hind-7 fragment of CAT gene (CAT GenBloc (Pharmacia)), the Hpal-BamHI fragment of pSV0CAT (30) (containing SV40 poly(A) signal) and the BamHI-Hind-7 fragment of p55Cl (31) (containing β-interferon promoter, a kind gift from Dr. T. Taniguchi) were isolated. These fragments were ligated to the PvuII-Hind-7 fragment of pUC19 to generate proCAT. The reporter CAT plasmid, poolTRECAT was constructed by inserting the synthetic oligonucleotides of col TRE (see the section entitled gel shift analysis) into the BamHI site of proCAT which is located just upstream of the β-interferon promoter sequence.

**In vitro transcription and translation**

Plasmid DNAs carrying various cDNAs were linearized by digestion with appropriate restriction enzymes and were transcribed in vitro using SP6 RNA polymerase for 1.5 hr at 40°C or T7 RNA polymerase for 1.5 hr at 37°C. Standard reaction mixtures for in vitro translation contained 7-12 μl of micrococcal nuclease-treated rabbit reticulocyte lysate (Promega Biotech), 0.1 μg of transcribed RNA, 1 μl of 1 mM amino acids minus methionine and 10 μCi of 35S-methionine. Unlabeled proteins were synthesized in the absence of 35S-methionine in lysates supplemented with 1 mM methionine. The mixtures were incubated for 60 min at 30°C. Labelled protein products were analyzed by SDS-polyacrylamide gel electrophoresis and their radioactivities were measured by using an RI image analyzer (AMBIS).

![Figure 1](image_url)  
**Figure 1.** Time course of heterodimer formation between Fos family proteins and c-Jun. (A). Fos family proteins and c-Jun were separately synthesized in vitro in the presence or absence of 35S-methionine, respectively. Labeled Fos family proteins and unlabeled c-Jun were combined at similar molecular concentrations and incubated at 37°C. At the time indicated, proteins were immunoprecipitated by anti-Jun antiserum and analyzed by electrophoresis on 10% SDS-polyacrylamide gel. On the right, molecular weights calculated by utilizing conjugated molecular weight markers are shown. (B) Radioactivities in the bands of Fos family protein shown in (A) were quantitated by using an RI image analyzer. The percentages of Fos family proteins that were recovered in the immunoprecipitates as heterodimers were plotted. Open circles, triangles, and squares indicate c-Fos/c-Jun, Fra-1/c-Jun and Fra-2/c-Jun, respectively.
**Heterodimer formation**

For *in vitro* association, reticulocyte lysates containing Fos family proteins or Jun family proteins were combined at equimolar concentrations and incubated at 37°C. At various times, aliquots were removed and diluted to 100 μl with ice-cold RIPA buffer, and proteins were immunoprecipitated with rabbit polyclonal antiserum such as anti Jun antiserum (32), anti Fra-2 pep 2 antiserum (17) and anti Fos pep 1 antiserum (33). The immunoprecipitation procedure was essentially the same as described (26).

**Gel shift analysis**

Unlabeled translation products were combined and incubated at 37°C for 30 min, and then 5 μl of binding buffer [10 mM Tris-HCl (pH 8.0), 50 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol (DTT), 5% glycerol, 5% sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF)] and 1 μg of poly(dI-dC) (Pharmacia) were added. After 15 min at room temperature, 32P-labeled oligonucleotide probe (0.5 ng) was added and incubation was continued for 15 min. DNA-protein complexes were resolved on a 5% polyacrylamide gel in 1×TBE [50 mM Tris-borate (pH 8.3), 1 mM EDTA] by electrophoresis at 200 V for 2 hr. The sequences of the oligonucleotides used for the probe DNA were as follows. (AP-1-binding sites or related sequences are shown in bold letters.)

**FSE2**

5'-TCGACTATTAAAACATGACCTCAGAGGAAAAC-3'
3'-GATAATTTTGACTAGTCTCTTTTAGCT-5'

**collagenase TRE**

5'-GATCTATAAGCATGAGTCAGACACTCTGG-3'
3'-ATATTTCTGACTGCTCTGGAGACCTAG-5'

c-Jun TRE
5'-GATCCGACCCGGGGGTGACATCATGGGCTA-3'
3'-GTCGGCGCCTACTTAGTACCCGATCTAG-5'

**somatostatin CRE**

5'-GATCTCCCTGCTGAGTCAGAGGAGAGAGAGAG-3'
3'-AGGAAACCCACTGCAGTCCTCTCTCTCTCT-5'

**Cell culture and transfection assays**

F9 teratocarcinoma cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and were transfected by the modified calcium phosphate coprecipitation method (34). Six hours before transfection, 3×10⁵ cells were plated in 60 mm dishes with 2.5 ml of medium. The cells were cotransfected with 4 μg of pcoITRECAT as the reporter plasmids and various amounts of expression plasmid(s) carrying the c-fos, c-jun or a related gene. DNA was brought to 12 μg by addition of the pUC119 plasmid. Sixteen to twenty hours after transfection, cells were washed twice with DMEM and incubated in DMEM containing 10% FBS for 1 day before harvest. Cell lysates were prepared as described previously (30) and protein concentrations were determined by

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**Figure 2.** Heterodimer formation by Fos family proteins with JunB or JunD. Proteins were separately synthesized, mixed and incubated for 30 min at 37°C. Fos and Fra-1 were immunoprecipitated by anti-Fos antiserum, and Fra-2 was immunoprecipitated by anti-Fra-2 antiserum and analyzed by SDS-polyacrylamide gel electrophoresis. The left, molecular weight markers are shown. The positions of immunoprecipitated Fos family proteins are indicated on the right. The arrows on the left indicate the positions of coimmunoprecipitated JunB and JunD proteins.

**Figure 3.** AP-1-binding activity of heterodimers. Each protein was synthesized *in vitro* and mixtures were incubated for 30 min at 37°C. DNA-binding activity was determined by gel shift analysis using 0.5 ng of 32P-labeled oligonucleotides containing the AP-1 site of FSE2 and collagenase TRE (A) or the AP-1-like sequence from c-jun (c-jun TRE) or somatostatin (somatostatin CRE) genes (B) (Materials and Methods). In samples with competitor (+), unlabeled oligonucleotide (50 ng) was added. Arrowhead indicate the position of the specific bands formed by the protein-DNA complexes.
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Figure 4. Transactivation of pcolTRECAT reporter plasmid by various combinations of Fos family proteins and Jun family proteins that were transiently expressed in F9 cells. F9 cells were cotransfected with a total of 10 μg of DNA containing pcolTRECAT (4 μg), various amounts of the expression plasmids of c-Jun(A), JunB (B) or JunD (C) and pUC119 as a carrier DNA, without (open circles) or with the expression plasmids of Fos (open triangles), Fra-1 (open squares), or Fra-2 (closed circles) (2 μg). Some of the transfection were carried out in duplicate and the results of two independent experiments are indicated as the average CAT activity.

using a Bio-Rad protein assay kit. CAT activities were assayed by standard methods (30) using cell extracts containing equal amounts of protein. The radioactivities were quantitated by counting 14C spots on the TLC plate with an RI image analyzer (AMBIS).

RESULTS
Fra-2 forms stable complexes with c-Jun, JunB and JunD
To analyze the complex-forming activity of Fra-2 with c-Jun, Fra-2 and c-Jun were synthesized in rabbit reticulocyte lysates in the presence or absence of 35S-methionine, respectively. They were mixed at similar molecular concentrations, incubated at 37°C for the time indicated (Fig. 1A) and immunoprecipitated with anti-Jun antiserum. Coimmunoprecipitated Fra-2 protein was detected after gel electrophoresis. Quantitation of the radioactivity (Fig. 1B) clearly indicated that Fra-2 protein forms a stable complex with Jun with similar kinetics to that of c-Fos or Fra-1, and most of the Fra-2 protein forms the complex within 10 min.
To examine whether Fra-2 can form complexes with other Jun family proteins, labeled Fra-2 was mixed with labeled JunB or JunD, incubated for 30 min and immunoprecipitated with anti-Fra-2 antiserum. As shown in Fig. 2, JunB and JunD were coimmunoprecipitated with Fra-2, indicating that Fra-2 forms a stable complex with JunB or JunD, as was reported for c-Fos (35). Fra-1 also formed complexes with these Jun family proteins (Fig. 2).

AP-1-binding activity of Fra-2/Jun family proteins
To analyze whether Fra-2/Jun complex has specific DNA-binding activity, both proteins were separately synthesized in vitro, mixed and analyzed by gel shift assay using several 32P-labelled DNA probes that contain a single AP-1-binding site. As representative AP-1-binding sites, the FSE2 region of the aP2 gene and an oligonucleotide from the human collagenase gene that contains TPA-responsive element (TRE) were tested. Using these two oligonucleotides as probes, specific band shifts were detected on incubation with Fra-2 plus c-Jun as well as c-Fos plus c-Jun and Fra-1 plus c-Jun (Fig. 3A). The shifted bands almost disappeared

Figure 5. Inhibitory effect of Fra-2 or Fra-1 on the transcriptional stimulation by c-Fos plus c-Jun. Expression plasmids of c-Fos and c-Jun (2 μg each) were cotransfected with various amounts (0–3 μg) of Fra-2 (open triangles) or Fra-1 (open circles) expression plasmids on F9 cells. The transactivation was quantitated in the terms of the CAT activity from pcolTRECAT.
they were not competed out by upon addition of unlabeled probes as competitors. Furthermore they were not competed out by the mutated FSE2 probe (32) that has two nucleotides conversion in the AP-1 site (data not shown). Control experiments indicated that c-Jun alone does not form a specific band under these assay conditions (Fig. 3A and our unpublished results).

We also tested other DNA probes containing an AP-1 like sequence (one base insertion) that is located in the 5' flanking region of the chicken c-jun gene and a representative cAMP-responsive element (CRE) sequence in the rat somatostatin gene. All of these three complexes were shown to bind to these probes with reduced affinity compared with the binding to the representative AP-1 sites (Fig. 3B). Nucleotides containing these AP-1 like sequences competed the shifted bands of Fig. 3A less effectively, when added to the reaction mixtures instead of homologous competitors (data not shown). Fra-2/JunB and Fra-2/JunD also have an activity to bind these representative AP-1 sites or these two related sequences (data not shown). Even using more sensitive conditions of gel shift assay, we never observed any specific binding by Fra-2 alone, or Fos plus Fra-2, or Fra-1 plus Fra-2 (data not shown).

**Effect of Fra-2 on transcription from a promoter with an AP-1-binding site**

The in vitro analysis described above failed to reveal any differences between Fra-2 and other Fos family proteins in their ability either to form a heterodimer with Jun proteins or to bind AP-1 sites as the heterodimer. Since Fos plus Jun transactivate the transcription from a promoter with an AP-1 site much more effectively than Jun alone does, we next examined the effect of Fra-2 on the transcription using transient expression experiments in F9 embryonic teratocarcinoma cells. As a reporter plasmid, we constructed pcolTRECAT, which has an AP-1 site from the collagenase gene just upstream of the TATA box from the β-interferon gene and was fused to the CAT gene. The reporter plasmid (4 μg) was cotransfected with different amounts of Jun expression vectors with or without Fra-2, c-Fos, or Fra-1 expression vectors (2 μg). Even in the absence of c-Fos, c-Jun alone activated the transcription of the reporter CAT gene (Fig. 4A). When combined with Fos expression plasmid, Jun expression plasmid elevated CAT activity, in good agreement with previous reports (2, 9). On the contrary, Fra-2 plus Jun and Fra-1 plus Jun reduced the CAT activity to a lower level compared with Jun alone. The inhibitory effect of Fra-2 and Fra-1 on gene activation by Jun was observed at all dosages of the transfected Jun plasmid (1–6 μg). This is the first clear difference between Fos and its related antigens in our observations. The stimulatory effect of c-Fos and inhibitory effect of Fra-2 and Fra-1 were further confirmed by a set of transfection experiments in which 0.5–A3 μg of expression plasmid of Fos family proteins was cotransfected with a constant amount (2 μg) of Jun expression plasmid (data not shown). To test whether Fra-2 or Fra-1 can inhibit the stimulatory effect of c-Jun plus c-Fos, we transected various amounts of Fra-2 with constant amounts of c-Fos (2 μg) and c-Jun (2 μg). As shown in Fig. 5, Fra-2 or Fra-1 efficiently suppressed the transactivation by c-Fos/c-Jun in a dose-dependent manner.

To demonstrate whether this inhibitory effect of Fra-2 and Fra-1 could be observed in conjunction with other Jun family proteins, similar experiments were performed by substituting JunB (Fig. 4B) or JunD (Fig. 4C) for c-Jun. When the transactivation by c-Jun, JunB or JunD homodimers was compared before analyzing the effects of Fos family proteins, Jun was shown to be the most potent transactivator (compare Fig. 4A and B, C). JunB and JunD showed only a weak activity to enhance the transcription from the reporter CAT gene.

When expression plasmids of Fos family proteins were added with JunB expression plasmid, again a clear difference was detected between Fos and its related antigens. Cotransfection with c-Fos enhanced the transactivation of JunB drastically, as described previously (24), while CAT production by Fra-2 plus JunB or Fra-1 plus JunB was kept at as low a level as with JunB alone (Fig. 4B).

We next cotransfected expression plasmids of these three Fos family proteins with JunD expression plasmid. c-Fos enhanced the transactivation by JunD drastically, as was the case with c-Jun or JunB. Interestingly, both Fra-1 and Fra-2 also stimulated the activity, though their stimulatory effects were reproducibly lower than that by c-Fos (Fig. 4C, and unpublished results). These results indicate that both Fra-2 and Fra-1 can modulate transactivation by Jun family proteins either stimulatively or suppressively, depending upon the partner in the heterodimer.

**Analysis of chimeric proteins between c-Fos and Fra-2**

There was clear difference between c-Fos and Fra-2 in their effects on the transactivation by c-Jun. Expression plasmids of c-Fos, Fra-2 and their chimeras (2 μg) were transfected with c-Jun expression plasmid (2 μg) together with the reporter plasmid pColTRECAT (4 μg). Each transfection was carried out in duplicate and the results of two independent transfection experiments are indicated on the right as average relative activity normalized with respect to CAT activity with c-Jun alone, taken as 100. Open triangles indicate the junction of chimeric proteins. BD and LZ indicate basic domain and leucine zipper structure, respectively. Open boxes and stippled boxes indicate the regions derived from c-Fos and Fra-2, respectively.

Figure 6. Structures of the chimeric proteins between c-Fos and Fra-2 and their effects on the transactivation by c-Jun. Expression plasmids of c-Fos, Fra-2 and their chimeras (2 μg) were transfected with c-Jun expression plasmid (2 μg) together with the reporter plasmid pColTRECAT (4 μg). Each transfection was carried out in duplicate and the results of two independent transfection experiments are indicated on the right as average relative activity normalized with respect to CAT activity with c-Jun alone, taken as 100. Open triangles indicate the junction of chimeric proteins. BD and LZ indicate basic domain and leucine zipper structure, respectively. Open boxes and stippled boxes indicate the regions derived from c-Fos and Fra-2, respectively.
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