The product of a *fos*-related gene, *fra*-1, binds cooperatively to the AP-1 site with Jun: transcription factor AP-1 is comprised of multiple protein complexes

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***fra*-1 encodes a serum-inducible protein (Fra-1) that is antigenically related to Fos. We have characterized Fra-1 expression in serum-stimulated cells using antibodies raised against several regions of this protein. Fra-1, expressed transiently in COS cells or in serum-stimulated rat fibroblasts, undergoes extensive post-translational modification, primarily by phosphorylation of serine residues. It is present in both the nucleus and the cytoplasm and participates in a protein complex with Jun. Using proteins synthesized in reticulocyte lysates, we have shown that Fra-1, like Fos, binds to the AP-1 recognition element cooperatively with Jun. A truncated Fra-1 protein that contains the leucine zipper region but not an adjacent basic amino acid domain, complexes with Jun in vitro but fails to bind AP-1 oligonucleotides. These results demonstrate that Fra-1 contributes to the DNA-binding activity ascribed to transcription factor AP-1.**

[*Key Words:* Gene regulation; *fra*-1, *jun*, AP-1]

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The c-*fos* gene is the cellular homolog of the oncogene (v-*fos*) carried by two murine osteogenic sarcoma viruses (FBJ-MSV and FBR-MSV) (Curran and Teich 1982; Curran et al. 1982; Curran and Verma 1984) and one chicken transforming virus (NK24) (Fujiwara et al. 1987; Nishizawa et al. 1987). It encodes a nuclear phosphoprotein (Fos) (Curran et al. 1984) that forms noncovalent complexes with several other cellular proteins (Curran et al. 1984; Franza et al. 1987a), including the product of another proto-oncogene, c-*jun* (Rauscher et al. 1988c). The basal level of c-*fos* mRNA and protein expression in most cell types is quite low, but it can be induced rapidly and transiently by a variety of agents, including those resulting in mitogenesis, differentiation, and neuronal cell depolarization (for review, see Curran 1988). Furthermore, c-*fos* expression can be induced in the presence of protein synthesis inhibitors (Greenberg and Ziff 1984; Muller et al. 1984). These features have led to the classification of c-*fos* as a cellular immediate-early response gene (Curran and Morgan 1987).

The Fos protein complex is associated with chromatin in isolated nuclei and binds with high affinity to DNA cellulose in vitro (Sambucetti and Curran 1986), suggesting that it might function in some aspect of gene regulation. This possibility was strengthened considerably by the demonstration of a sequence-specific interaction between DNA and the Fos complex using regulatory sequences (referred to as FSE2) from the 5′- flanking region of an adipocyte-specific gene, aP2 (Distel et al. 1987). These studies initiated the search for the nucleotide sequence motif recognized by the Fos complex. Several approaches, involving mutagenesis, competition studies, and DNA-affinity assays, identified the binding site as the consensus recognition sequence of the HeLa cell activator protein-1 (AP-1) (Franza et al. 1988; Rauscher et al. 1988b). AP-1, originally described as a DNA-binding activity specific for the enhancer elements of SV40, human metallothionein II_A ($HMTII_A$), and several TPA-inducible genes (Angel et al. 1987; Lee et al. 1987a,b), comprises several proteins including the product of c-*jun* (Jun) (Bohmann et al. 1987). The connection between Fos, Jun, and AP-1 was made clear by the identification of the Fos-associated protein p39 as Jun (Rauscher et al. 1988c).

Neither Fos nor Jun contains domains previously associated with DNA-binding proteins, such as zinc fingers or helix-turn-helix motifs. However, both proteins possess a region having periodic repeats of leucine residues every seven amino acids adjacent to highly basic domains. It has been proposed that this domain forms an α-helix with leucine residues along one face, which promotes a strong hydrophobic interaction between proteins with a similar structure (Landschulz et al. 1988). The interdigitation of the leucine residues, termed a 'leucine zipper' (Landschulz et al. 1988) would presumably stabilize homomeric or heteromeric protein

complexes and bring into juxtaposition regions of the proteins involved in DNA binding. Using proteins synthesized in vitro, we have shown that Jun exhibits low-affinity binding activity with AP-1 sites (Rauscher et al. 1988a). However, it appears that high-affinity interaction with AP-1 sites requires cooperativity between Fos and Jun, and the leucine regions of both proteins are crucial to this association and their subsequent DNA-binding activity (Rauscher et al. 1988a). There is also increasing evidence that Fos is required for the transcriptional activation of a variety of promoters (Setoyama et al. 1986; Lucibello et al. 1988; Schonthal et al. 1988). All of these findings lend weight to the hypothesis that Fos functions within the context of the cellular immediate-early response as a regulator of gene expression.

The concept of the cellular immediate-early transcriptional response to extracellular stimuli (Curran and Morgan 1987; Lau and Nathans 1987) arose by analogy to the viral genes that are expressed in the presence of protein synthesis inhibitors prior to genome replication after viral infection. Many laboratories have isolated cellular genes that, like c-*fos*, are rapidly induced even in the presence of protein synthesis inhibitors (Cochran et al. 1983; Callahan et al. 1985; Kujubu et al. 1987; Lau and Nathans 1987; Lim et al. 1987; Sukhatme et al. 1988). Included in the set of cellular immediate-early genes are both Fos-related and Jun-related genes (Ryder et al. 1987; Cohen and Curran 1988). In the case of Fos, a set of Fos-related antigens, identified using antisera directed against a synthetic peptide predicted from the sequence of Fos (amino acids 127–152), have been observed to be coinduced in many of the circumstances that result in Fos induction (Sambucetti and Curran 1986; Franza et al. 1987a). We have reported previously the cloning and in vitro expression of a cDNA encoding one of these proteins, namely *fra*-1 (Fos-related antigen-1) (Cohen and Curran 1988). In the present work, we have continued the characterization of the Fra-1 protein, which we have now identified as $p38^{fra\text{-}1}$. This protein is extensively post-translationally modified and demonstrates both nuclear and cytoplasmic localization when transiently expressed in COS-1 cells. Furthermore, in in vitro association and DNA-binding assays, the Fra-1 protein binds cooperatively with Jun to AP-1 sites in a similar fashion to that observed for Fos. These findings suggest that transcription factor AP-1 may comprise a collection of related inducible protein complexes that interact with similar sequence motifs.

Results

Specificity of antisera directed against Fra-1

Primary and secondary bleeds from rabbits inoculated with various Fra-1 polypeptides were tested for their ability to immunoprecipitate Fra-1 synthesized in vitro in rabbit reticulocyte lysates. Sera from rabbits inoculated with an amino-terminal peptide derived from Fra-1 (amino acids 2–15) or with the carboxy-terminal half of the Fra-1 protein (amino acids 136–275) were able to immunoprecipitate Fra-1, whereas sera from rabbits inoculated with a peptide corresponding to amino acids 177–188 could not (data not shown). Preimmune serum from these rabbits did not precipitate any Fra-1 protein (data not shown). The anti-amino-terminal peptide antisera (referred to as Fra-1 1.2 Ab) was not able to precipitate in vitro synthesized Fos or Jun, and was therefore considered to be specific for Fra-1, although the anti-carboxy-terminal antisera (referred to as Fra-1 3.2 Ab) did cross-react weakly with in vitro synthesized Fos (data not shown). These antisera were used in immunoprecipitation analyses of native and denatured cell lysates from rat 208F fibroblasts.

Identification of the Fra-1 protein in fibroblasts

Figure 1A shows immunoprecipitation analysis of a time course of serum stimulation in rat 208F fibroblasts. Following stimulation by 20% FCS for the times indicated and pulse labeling for 15 min with [^{35}S]methionine, native cell lysates were prepared and immunoprecipitated with the Fra-1 3.2 Ab. A protein of ~38,000 daltons was observed that was not present in uninduced fibroblasts (lane 1) and was not precipitated by control sera (data not shown) but was detectable after 30 min of serum stimulation (lane 2). In this analysis, the amount of newly synthesized Fra-1 remained virtually constant from 1–4 hr after stimulation (lanes 3–6) and decreased dramatically thereafter, although a protein of 38,000 daltons was still detectable 8 and 16 hr after stimulation (lanes 7 and 8, respectively). Interestingly, the Fra-1 3.2 Ab did not appear to immunoprecipitate Fos synthesized in serum-stimulated fibroblasts (Fig. 1A), although it had been observed to cross-react slightly with in vitro-synthesized Fos (data not shown).

The Fos-binding protein p39/Jun migrates with approximately the same mobility on SDS–polyacrylamide gels as the Fra-1 protein, and therefore it was not possible to determine, from the result shown in Fig. 1A, whether the protein band at 38,000 daltons contained only Fra-1 or Fra-1 plus Jun. If Jun was present, this would imply that Fra-1, like Fos, associates with Jun in serum-stimulated fibroblasts. To address this question, the immunoprecipitation analysis was repeated using both native and denatured cell lysates. Following stimulation by 20% FCS for 45 min and pulse labeling for 30 min with [^{35}S]methionine, lysates were prepared and immunoprecipitated with anti-Fos antibodies (M Ab; against amino acids 127–152, which recognize Fos and Fos-related antigens), the Fra-1 1.2 Ab or the Fra-1 3.2 Ab. As expected, the anti-Fos antibody immunoprecipitated a set of proteins from both the native and the denatured lysates, which included Fos and Fos-related antigens at 46, 38, and 35 kD (Fig. 1B). The anti-Fos precipitate from the native lysate also contained Jun, which comigrated with the Fos-related antigen at 38 kD. Both of the anti-Fra-1 antisera precipitated the 38-kD protein under native and denaturing conditions, and a small difference in the intensity of the 38-kD protein band could be discerned between the native and the denatured ly-

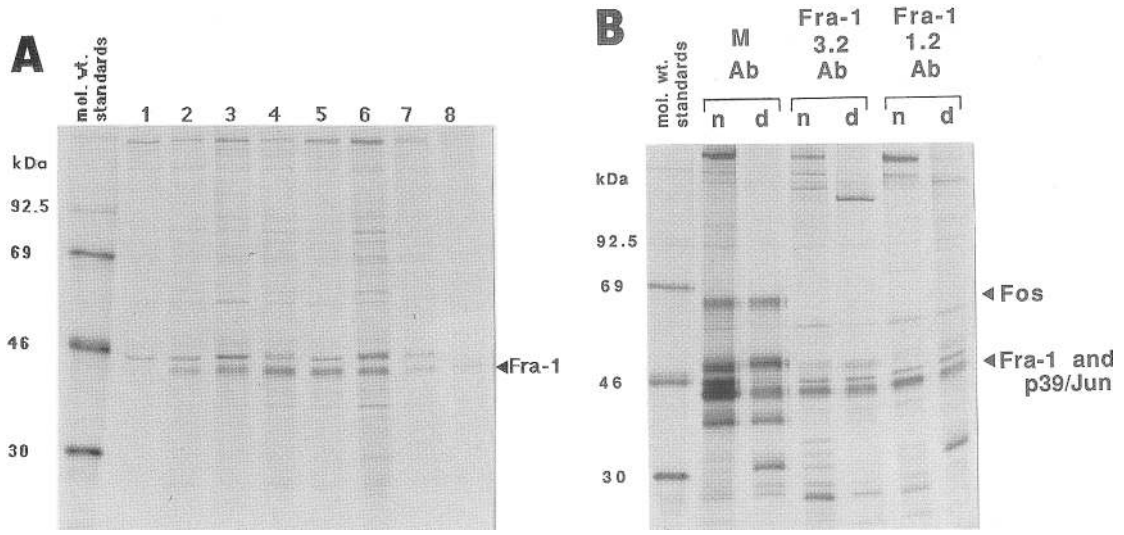

Figure 1. (*A*) Time course of induction of Fra-1 in serum-stimulated fibroblasts. 208F fibroblasts that had been serum-deprived for 24 hr were stimulated by the addition of 20% FCS for 0 min (lane *1*), 30 min (lane *2*), 60 min (lane *3*), 90 min (lane *4*), 2 hr (lane *5*), 4 hr (lane *6*), 8 hr (lane *7*), or 16 hr (lane *8*) and were then pulse-labeled with [^{35}S] methionine (300 μCi/35-mm dish) for 15 min. After the labeling period, the cells were lysed in RIPA buffer; the lysates were clarified and the supernatants were incubated with the Fra-1 3.2 Ab. Immunoprecipitates were analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography. (*B*) Immunoprecipitation of Fra-1 from native and denatured cell lysates. 208F fibroblasts that had been serum deprived for 24 hr were stimulated by the addition of 20% FCS for 45 min and then pulse-labeled with [^{35}S] methionine (1200 μCi/60-mm dish) for 30 min. Two dishes of cells were processed for each antibody used; one dish in each set was lysed after the stimulation procedure in RIPA buffer for the nondenatured lysate (n), and the other was lysed in a buffer containing 0.5% SDS and then boiled for the denatured lysate (d). After clarification of the lysates, the supernatants were incubated with anti-Fos antibodies (directed against amino acids 127–152 of Fos), the Fra-1 1.2 Ab (directed against amino acids 2–15 of Fra-1), or the Fra-1 3.2 Ab (directed against the Fra-COOH protein). Immunoprecipitates were subsequently analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography.

sate immunoprecipitates using both antisera (Fig. 1B). This result suggests that if Fra-1 associated with Jun in vivo, then possibly only a small proportion of Fra-1 was involved at any time in this complex or the anti-Fra-1 antisera precipitated this complex very inefficiently. A number of other protein species were observed in the anti-Fra-1 antibody immunoprecipitates whose significance has not yet been determined (Fig. 1B).

To clarify the question of Fra-1–Jun association in vivo, these same immunoprecipitates were subjected to high-resolution two-dimensional gel electrophoresis (Fig. 2). For reference, the anti-Fos immunoprecipitate of the native cell lysate is shown (Fig. 2A), and the positions of Fos, p39/Jun, actin (Ac), and the Fos-related antigens at 46, 38 (Fra-1), and 35 kD are indicated. This analysis revealed the presence of p39/Jun in anti-Fra-1 immunoprecipitates from native but not denatured lysates (cf. Fig. 2, B and C). This suggests that Fra-1 and Jun associate in vivo in serum-stimulated fibroblasts. To avoid complication of future analyses by the presence of Jun in Fra-1 immunoprecipitates, all subsequent work was carried out using denatured cell lysates.

The Fra-1 protein is modified post-translationally

Pulse-chase studies were performed with serum-stimulated fibroblasts and were analyzed by immunoprecipitation using the Fra-1 3.2 Ab. As shown in Figure 3, the Fra-1 protein was modified such that it shifted, even in a 15-min pulse-labeling period, from an apparent molecular weight of 35,000 to 38,000 (i.e., migrating slightly faster than p39/Jun). Thereafter, modification continues at a slower rate and Fra-1 increases in apparent molecular weight to ~40,000 (i.e., migrating slightly slower than p39/Jun). This modification process was complete within 2 hr, and thereafter, faster migrating forms of the protein began to appear, similar to those observed at the later time points of the serum-stimulation profile. These protein bands may represent cleavage or breakdown products of Fra-1 or, alternatively, they may represent loss of protein modification.

In this pulse-chase analysis, a protein species of unknown origin was observed after 2, 4, and 8 hr of the chase (arrowed in Fig. 3). This may represent a highly modified form of Fra-1 or perhaps a covalent protein complex that includes Fra-1.

The modification of Fra-1 was observed more clearly with the protein synthesized in COS-1 cells, following transient transfection with plasmids expressing Fra-1 from the human cytomegalovirus (CMV) immediate-early promoter (pCMV-*fra*-1). Figure 4 shows that in these cells, Fra-1 is modified such that it increases from an apparent molecular weight of 35,000 to ~40,000 via a series of discrete intermediates (lanes 1 and 2). This

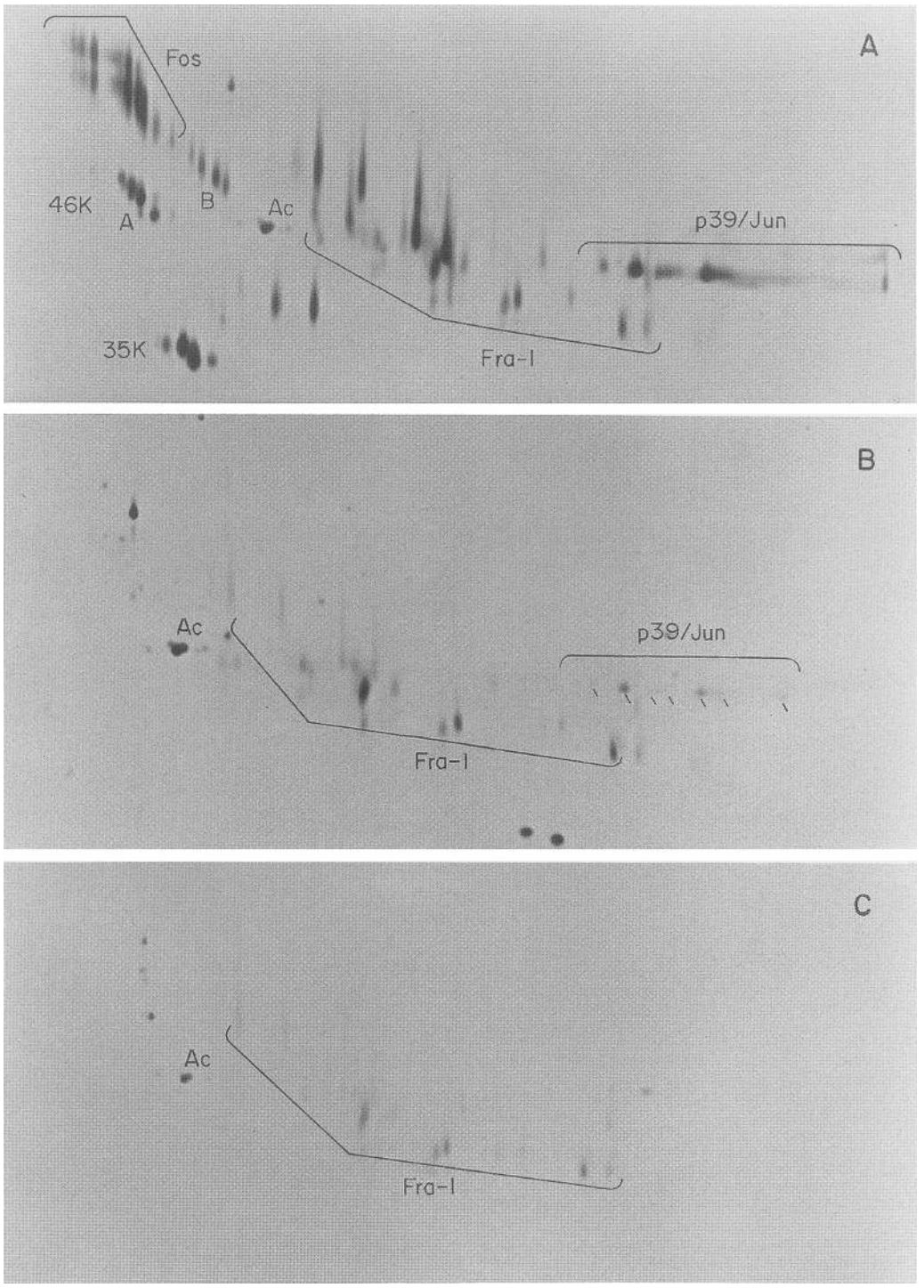

Figure 2. High-resolution two-dimensional gel electrophoresis analysis of immunoprecipitates from serum-stimulated fibroblasts. Aliquots of the samples analyzed by one-dimensional SDS–polyacrylamide gels in Fig. 1 were also analyzed on two-dimensional gels. In each case, electrophoresis was from top to bottom and isoelectric focusing (IEF) was from left (acidic) to right (basic). (*A*) The nondenatured anti-Fos antibody immunoprecipitate; (*B*) the nondenatured Fra-1 1.2 Ab immunoprecipitate; (*C*) the denatured Fra-1 1.2 Ab immunoprecipitate. The positions of the Fos, Fra-1, and p39/Jun proteins, as well as other Fos-related antigens (46K A and B, 35 K; Franza et al. 1987a) and actin (Ac), are indicated where they are present. Staphylococcus A pellets were lyphilized to dryness and resuspended in IEF sample buffer without SDS (Garrels 1983). Isoelectric focusing was broad (pH range 3.5–10.0), and the acrylamide concentration in the second gel was 10%.

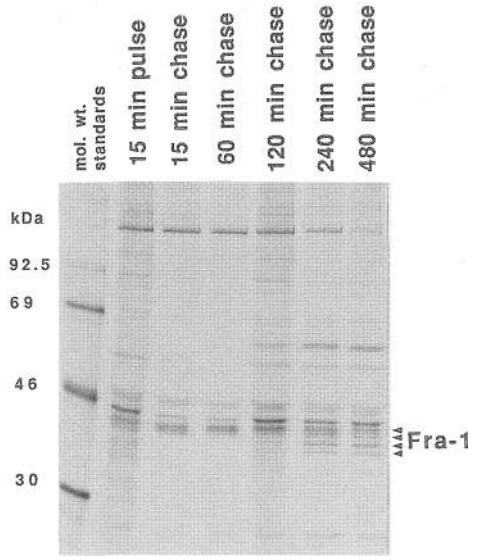

Figure 3. Pulse-chase analysis of the Fra-1 protein. 208F fibroblasts were serum-deprived for 24 hr and stimulated by the addition of 20% FCS for 60 min, followed by pulse labeling with [^{35}S]methionine (375 μCi/35-mm-dish) for 15 min. At that time, the medium was removed, and unlabeled medium containing 20% FCS was added. The incubation was continued for the times indicated, and the cells were then lysed under denaturing conditions. The clarified lysates were immunoprecipitated by the Fra-1 3.2 Ab and analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography.

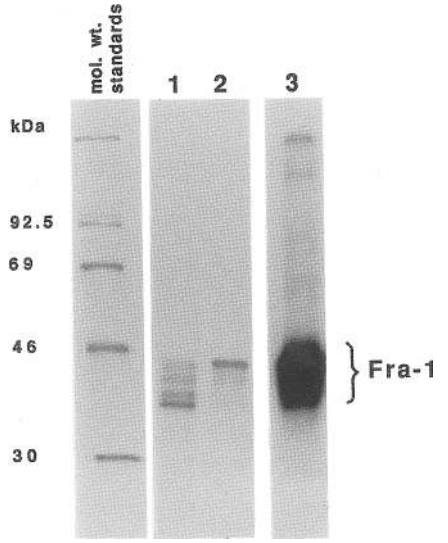

Figure 4. Post-translational modification of Fra-1 in COS cells. COS-1 cells that had been transiently transfected with pCMV-*fra*-1 for 48 hr were used for pulse-chase analysis and phosphate-labeling experiments. In the pulse-chase studies, the cells were labeled for 15 min with 200 μCi of [^{35}S]methionine (lane *1*), followed by a 60-min chase with unlabeled medium (lane *2*). Clarified extracts were immunoprecipitated with anti-Fos antibody. In the phosphate-labeling experiments, the cells were labeled with 4 mCi of [^{32}P]orthophosphate/35-mm dish for 30 min at 37°C. The clarified supernatant was immunoprecipitated with M antibody (lane *3*).

modification process was complete within 60 min. As indicated in Figure 4 (lane 3), a significant contribution to the modification of Fra-1 is phosphorylation. It was determined by phosphoamino acid analysis that most of the phosphorylation occurs at serine residues (data not shown). The differences in the rates of post-translational modification in COS cells and fibroblasts may be related to the effects of serum stimulation. In the case of Fos, we have noticed that cell-surface stimulation activates the enzymes involved in post-translational modification (Lee et al. 1989). A similar situation may be occurring with Fra-1. The several distinct intermediates detected in COS cells are similar in apparent molecular weight to those observed at late time points in the pulse-chase experiment (Fig. 3). This suggests that Fra-1 may undergo temporally regulated dephosphorylation.

Localization of Fra-1

COS-1 cells expressing Fra-1 following transient transfections with pCMV-*fra*-1 were used for indirect immunofluorescence studies to localize this protein within the cell. Figure 5 shows the pattern of immunofluorescence observed 48 hr after transfection of COS-1 cells with either a Fos- or a Fra-1-expressing plasmid. Immunostaining of cells expressing Fra-1 was detected in both the nucleus and the cytoplasm, whereas the staining was located exclusively in the nucleus in cells expressing Fos. A similar result was obtained in indirect immunofluorescence studies of serum-stimulated fibroblasts using anti-Fos antibodies, although the intensity of staining was much less than that observed in transfected COS cells.

Fra-1 and Jun associate in vitro

Rat cDNA inserts containing the entire open reading frame of c-*fos*, c-*jun*, or *fra*-1 were ligated in the sense orientation into the *Eco*RI site of pSP65, and these recombinant plasmids were used as templates for the transcription of specific RNAs from the SP6 promoter. In addition, RNA was transcribed from the pFra-COOH plasmid (that contains a truncated *fra*-1 cDNA insert encoding amino acids 136–275) using purified *Escherichia coli* RNA polymerase. The structure of the cDNA inserts is shown in Figure 6A. The transcribed RNAs were translated in vitro in rabbit reticulocyte lysates, and the products were then mixed together in various combinations to allow the proteins to associate; they were then immunoprecipitated with antisera that recognized one (but not the other) protein in the mixture. In this way, the second protein would only be precipitated by the noncognate antisera by virtue of an association with the first protein. In Figure 6B, it can be seen that the anti-Fos antibody, which recognizes Fos and Fra-1 but not Jun, precipitated Jun when it was mixed with either Fos or Fra-1 (lanes 1 and 2, respectively). This indicates that

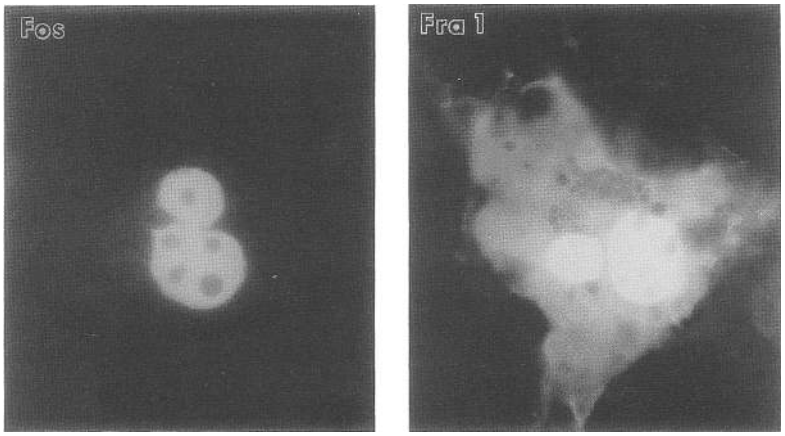

Figure 5. Immunofluorescence analysis of Fra-1 and Fos expressed in COS cells. COS-1 cells were transiently transfected by pCMV-*fos* (Fos) or pCMV-*fra*1 (Fra-1) for 48 hr and then processed for indirect immunofluorescence analysis using the anti-Fos antibody and rhodamine-conjugated goat anti-rabbit IgG. Immunofluorescence was not detected in untransfected controls (not shown).

Fra-1 and Jun associated in vitro, as had been observed for Fos and Jun (Rauscher et al. 1988a). When Fos and Fra-1 lysates were mixed and immunoprecipitated with Fos-specific antisera, no Fra-1 protein was precipitated (lane 3), showing that these two proteins did not associate under these conditions. The truncated Fra-1 protein (Fra-COOH), which contains virtually all of the Fra-1 leucine zipper region except for the first leucine, retained its ability to associate with Jun (lane 4) but was still unable to associate with Fos (lane 5). It is not possible from this result to quantify the relative degree of association of Fra-COOH with Jun as compared with Fra-1 with Jun, because the two immunoprecipitates were performed with different antisera. In lane 2, the anti-Fos (M) antibody was used and, hence, only the Jun that was associated with Fra-1 was precipitated. In lane 4, the Jun-specific antibody was used and, therefore, all of the Jun present (i.e., both that bound to the Fra-COOH and free Jun) was precipitated.

Fra-1–Jun complexes formed in vitro bind to AP-1 sites

In previous DNA-binding assays using the FSE2 oligonucleotide, it had been shown that anti-Fos peptide anti-

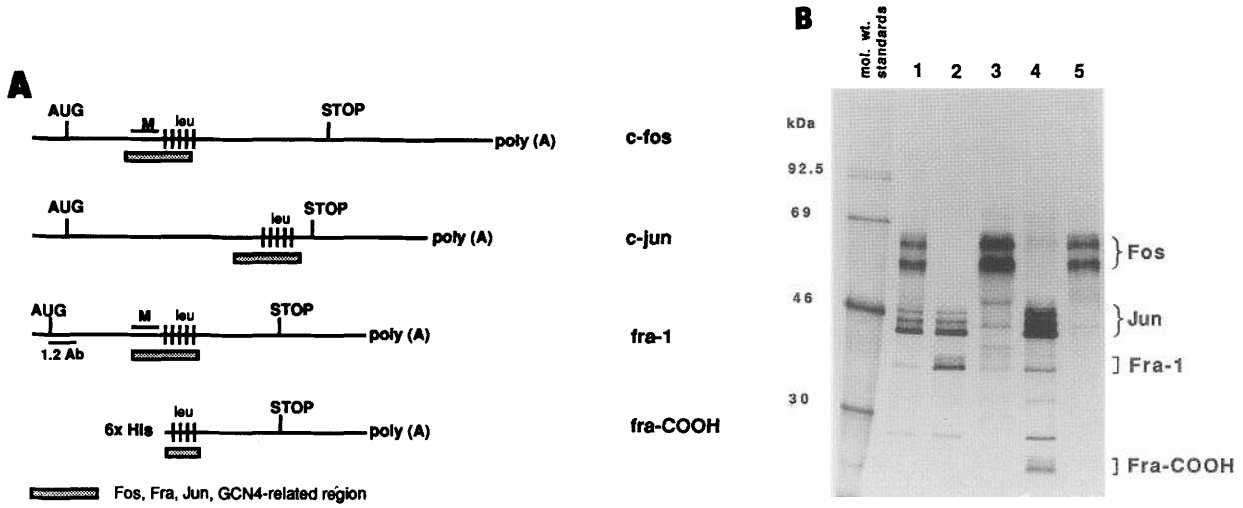

Figure 6. (*A*) Structure of cDNA clones used for in vitro expression. The structure of the c-*fos*(rat) cDNA is from Curran et al. (1987), and M indicates the position of the 127- to 152-amino-acid region used to generate the anti-Fos antibody that also recognizes the Fos-related antigens. The structure of the c-*jun*(rat) cDNA is based on a comparison of the c-*jun*(human) (Bohmann et al. 1987) and the c-*jun*(mouse) (Ryseck et al. 1988) sequences. The structure of *fra*-1(rat) is from Cohen and Curran (1988), and 1.2 Ab indicates the position of the 2–15 amino acid region used to generate the Fra-1 1.2 Ab. The structure of *fra*-COOH is described in Materials and methods. The positions of the proposed leucine zipper (leu) (Lanschulz et al. 1988) and the region of sequence similarity between Fos, Fra-1, Jun, and GCN4 (Vogt et al. 1987; Cohen and Curran 1988) are shown. (*B*) Association of Fra-1 and Jun in vitro. *fos*, *jun*, *fra*-1, and *fra*-COOH RNAs were translated in vitro; the translates were mixed together in various combinations and allowed to associate at 37°C for 30 min. The mixtures were then immunoprecipitated with an antibody that recognizes only one of the proteins in the mixture. (Lane *1*) Fos and Jun precipitated with the anti-Fos antibody; (lane *2*) Fra-1 and Jun precipitated with the anti-Fos antibody; (lane *3*) Fos and Fra-1 precipitated with the Fos-1 antibody (kind gift of D. Slamon); (lane *4*) Jun and Fra-COOH precipitated with Jun-specific antibody (kind gift of D. Bohmann and R. Tjian); (lane *5*) Fos and Fra-COOH precipitated with the anti-Fos antibody.

bodies could disrupt the gel-shift activity present in adipocyte nuclear extracts (Distel et al. 1987). In view of the fact that this antibody recognizes both Fos and its related antigens, it is conceivable that proteins such as Fra-1, as well as Fos, were contributing to the gel-shift activity that was observed. Therefore, it was decided to test whether the in vitro association of Fra-1 and Jun was significant in terms of AP-1 binding, as has been observed for the Fos–Jun complex (Rauscher et al. 1988a).

Unlabeled Fra-1, Fos, Jun, and Fra-COOH proteins were synthesized in vitro in rabbit reticulocyte lysates, and these proteins were either mixed together in various combination or used alone in gel retardation assays employing a ^{32}P-labeled oligonucleotide containing the AP-1 site from the HMTII$_A$ gene. Nonspecific AP-1 activity contributed by the reticulocyte lysate was abolished by preincubation with the poly(dI-dC) nonspecific competitor prior to addition of the oligonucleotide. The results of this analysis are shown in Figure 7. No binding to oligonucleotides containing AP-1 sites was observed with in vitro synthesized Fos, Fra-1, or Fra-COOH on their own, although Jun alone displayed a low, but detectable, level of binding activity that was clearly visible on longer exposures of the gel. Jun has been shown to bind to AP-1 sites with a low apparent affinity compared with the Fos–Jun complex (Rauscher et al. 1988a). However, a dramatic increase in AP-1 binding activity was observed when the Fos–Jun and Fra-1–Jun complexes were allowed to form. In both cases, the binding activity of the complex could be abolished by preincubation with anti-Fos antibodies before addition of the oligonucleotide. Interestingly, the formation of the complex between Fra-COOH and Jun did not result in the generation of AP-1 binding activity; indeed, the basal level of Jun binding was abolished. As expected, the protein mixtures that did not associate in vitro (i.e., Fos plus Fra-1 and Fos plus Fra-COOH) also did not display AP-1 binding activity. Similar patterns of results were obtained with other oligonucleotides containing AP-1 sites, including the FSE2 and gibbon ape leukemia virus (GALV) oligonucleotides (data not shown).

As an alternative approach to studying the DNA-binding activity of the Fra-1–Jun complex, DNA-affinity precipitation assays (DNAP, Franza et al. 1987b) were performed. In this procedure, [^{35}S]methionine-labeled proteins were incubated with biotinylated oligonucleotides in the presence of the poly(dI-dC) nonspecific competitor, and DNA–protein complexes were recovered using streptavidin-agarose (BRL, Bethesda, Maryland). Following elution from this matrix, the proteins were analyzed on SDS–polyacrylamide gels. Previous studies had shown that the Jun protein alone could be recovered using the GALV AP-1 site (Rauscher et al. 1988a). In Figure 8, it can been seen that there was only very low level of recovery of Fos alone (lane 1) or Fra-1 alone (lane 2), using the double-stranded GALV AP-1 site and that

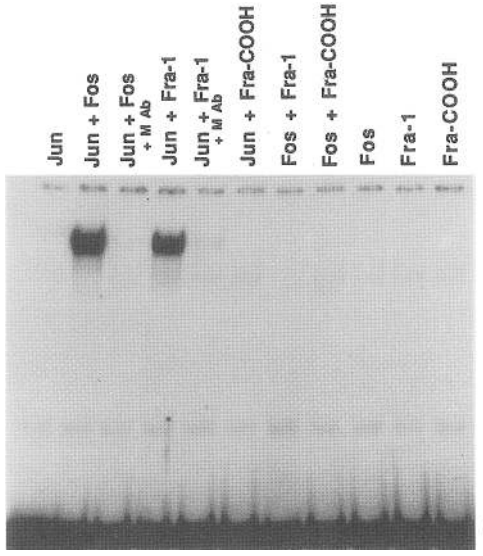

Figure 7. In vitro formed Fra-1–Jun complexes bind to oligonucleotides containing AP-1 sites. Gel retardation assays were performed using unlabeled Fos, Jun, Fra-1, and Fra-COOH proteins synthesized in vitro and the ^{32}P-labeled oligonucleotide (oligo) containing the AP-1 site from the HMTII$_A$ gene (Rauscher et al. 1988b). Where mixtures of proteins were used, the proteins were allowed to associate for 30 min at 37°C before use in this assay. Addition of antisera was done during the preincubation with the poly(dI-dC) competitor DNA.

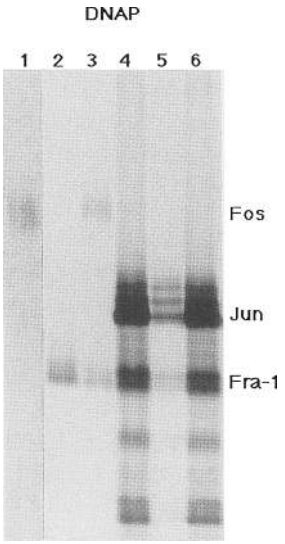

Figure 8. Fra-1–Jun complexes bind to double-stranded DNA using the DNAP assay. The biotinylated oligonucleotides used in this DNAP assay were as described elsewhere (Rauscher et al. 1988a). The binding reactions contained [^{35}S]methionine-labeled Fos (lane *1*), Fra-1 (lane *2*), Fos plus Fra-1 (lane 3), or Fra-1 plus Jun (lanes *4–6*) in a total volume of 80 μl containing 20 pmoles of biotinylated oligonucleotide, 50 mM KCl, and a 20-fold excess (by mass) of poly(dI-dC) nonspecific competitor DNA in DNAP solution B. Proteins that bound to the oligonucleotide were recovered on streptavidin–agarose and analyzed by SDS–polyacrylamide electrophoresis. The oligonucleotides used in each lane were as follows: (Lanes *1–4*) DGALV (double stranded); (lane *5*) HIV LTR enhancer (HIVEN3C1/2; Franza et al. 1987b); (lane *6*) somatostatin CRE.

mixing of these two proteins prior to incubation with the oligonucleotide did not enhance the recovery of either protein (lane 3). However, mixing of Fra-1 and Jun prior to incubation with either the GALV AP-1 site (lane 4) or the closely related cAMP responsive element (CRE) from the somatostatin gene (lane 6) resulted in a significant recovery of both Fra-1 and Jun. Using an unrelated control oligonucleotide derived from the HIV LTR enhancer (lane 5), there was a greatly reduced recovery of Jun and virtually no recovery of Fra-1.

Discussion

We have previously described the cloning and characterization of a cDNA clone encoding a Fos-related antigen, referred to as *fra*-1 (Cohen and Curran 1988). This cDNA was isolated from an expression library (constructed from serum-stimulated fibroblasts) by virtue of the antigenic similarity of its protein product to Fos in the peptide domain (amino acids 127–152) used to raise anti-Fos antibodies. Sequence analysis of *fra*-1 indicated that the protein encoded by this cDNA (Fra-1) contains several regions with significant homology to Fos, including the region that is also similar to the Jun and GCN4 proteins, and it seems likely that c-*fos* and *fra*-1 have diverged from a common ancestor (Cohen and Curran 1988). Investigation of the inducibility of *fra*-1 mRNA revealed several points of interest, namely (1) the kinetics of *fra*-1 induction in fibroblasts following serum stimulation are delayed and somewhat protracted compared with c-*fos*; (2) like c-*fos*, *fra*-1 is superinduced in the presence of protein synthesis inhibitors; and (3) the conditions that result in the expression of *fra*-1 overlap with, but are not identical to, those that result in the expression of c-*fos* (Cohen and Curran 1988).

We have now extended the analysis of Fra-1 with the assistance of antisera directed against this protein. We have been able to identify Fra-1 in serum-stimulated fibroblasts as a protein migrating at approximately the same position on SDS–polyacrylamide gels as the p39/Jun Fos-associated protein (Fig. 1). It was observed that Fra-1 was extensively modified post-translationally, both in serum-stimulated fibroblasts and in COS cells expressing this protein following transient transfection (Figs. 3 and 4). A significant element of the modification involves phosphorylation, mainly of serine residues (Fig. 4). The large increase in apparent molecular weight associated with phosphorylation of both Fos and Fra-1 may occur in the conserved carboxy-terminal region that contains several stretches of serine residues. Localization of the Fra-1 protein by indirect immunofluorescence in transiently transfected COS cells revealed that although there was a significant amount of Fra-1 present in the nucleus, this protein may also be present in the cytoplasm of the cell (Fig. 5). Thus, although there are overlaps between Fos and Fra-1 in terms of their expression and physical characteristics, there are also clear differences.

The obvious question that arises from the comparison of Fos and Fra-1 is whether the differences observed between these proteins, either at the structural or at the expression level, reflect differences in function. To begin to address this question, we wished to determine whether Fra-1, either alone or as a part of a protein complex, possessed a DNA-binding activity. In a parallel study to this one, it was demonstrated that formation of the complex between Fos and its associated protein p39/Jun could be reconstituted in vitro (Rauscher et al. 1988a) and that the complex thus formed between in vitro synthesized proteins behaved in gel retardation assays in an analogous fashion to Fos protein complexes isolated from cultured cells. Therefore, as a starting point, we investigated the ability of Fra-1 to associate in vitro with either Jun or Fos, and we have demonstrated that Fra-1 and Jun associate in vitro, but Fra-1 and Fos do not (Fig. 6B). The ability to form complexes between Fra-1 and Jun is perhaps not surprising, given that Fra-1 and Fos display 77% conservation in the leucine zipper domain. If, as seems likely from recent mutagenesis studies (R.L. Gentz, F.J. Rauscher III, and T. Curran, in prep.), the leucine-containing region is responsible for Fos–Jun interactions, it is not unreasonable to expect that Fra-1 could substitute for Fos in these associations. Indeed, we would predict that the Fra-COOH protein (which contains a virtually intact leucine structure) would also associate with Jun, and this was, in fact, observed to be the case (Fig. 6B). These results also demonstrate that the first leucine is not required for protein complex formation. It is probable that the association of Fra-1 and Jun in vitro reflects the ability of these proteins to complex in vivo because anti-Fra-1 immunoprecipitates from nondenatured extracts of serum-stimulated fibroblasts also contain Jun. It is difficult to assess the extent of Fra-1-Jun binding in vivo because we do not know how efficiently anti-Fra-1 antisera precipitate this complex. As these proteins have virtually identical mobilities on SDS–polyacrylamide gels, it is not possible simply to compare the intensity of the band at 38 kD in the native and denatured lysates in Figure 1B to determine the proportion of Fra-1 that is able to associate with Jun. Clearly, protein complex formation requires more than the simple leucine repeat, as Fos and Fra-1 did not associate in the coprecipitation assay (Fig. 6B). This suggests that although the leucine repeat motif may be indicative of a protein domain involved in complex formation, other amino acids in this region may be responsible for the specificity of the interaction.

In gel-shift and DNAP assays (Figs. 7 and 8), Fra-1 performed exactly as Fos, i.e., it enhanced AP-1 site binding activity by cooperating with Jun. The specificity of the interaction of the Fos–Jun complex and the Fra–Jun complex was indistinguishable using several AP-1 sites and the CRE from the somatostatin gene. It is possible that these protein complexes have the same DNA-binding specificity but different transcriptional activities in vivo. Because Fra-1 is induced slightly later than Fos following cell-surface stimulation (Cohen and Curran 1988), this would allow a temporal regulation of genes containing AP-1 motifs. With this in mind, we are now conducting experiments to determine the relative

stabilities of Fos and Fra-1 complexes with Jun and the affinities of these complexes for various AP-1-containing sites. Furthermore, we wish to determine whether, and to what extent, Fra-1 can compete with Fos for binding to Jun.

Interestingly, the deleted Fra-1 protein (Fra-COOH), although capable of complexing with Jun, did not enhance DNA-binding activity. Indeed, in gel-shift assays, this mutant protein abolished the low-level AP-1 binding activity associated with Jun alone. This result suggested that Jun binds to DNA via a homomeric complex (probably a dimer) and that in the heteromeric Fos–Jun or Fra-1–Jun complexes, regions of both proteins contribute to DNA binding. The region responsible for this effect must lie in the amino-terminal portion of Fra-1 that was deleted in Fra-COOH. The strongest candidate region is the very basic stretch of amino acids adjacent to the leucine zipper. This region is highly conserved between Fos and Fra-1 (Cohen and Curran 1988) and exhibits similarity with the corresponding domain of Jun (Vogt et al. 1987). The formation of heteromeric Fra-1–Jun and Fos–Jun complexes does not require cotranslation, thus, Fos or Fra-1 can displace Jun from a homomeric complex. In the gel-shift assay, Fra-COOH acted as a *trans*-dominant suppressor of Jun. This suggests that Fra-COOH may be a very useful biological probe for investigating the function of AP-1 complexes, as it may suppress AP-1 DNA-binding activity when overexpressed in cells.

The results presented here demonstrate that the cellular immediate-early gene *fra*-1 encodes a protein that contributes to the DNA-binding activity described as AP-1. Indeed affinity-purified preparations of AP-1 contain at least three Fra proteins in addition to Fos, Jun, and several other unrelated proteins (Rauscher et al. 1988c). Although Fra-1 is not expressed in all circumstances in which Fos is expressed, several Fra proteins are usually present in these situations (Franza et al. 1987b; J.L. Sonnenberg et al., in prep.). We are attempting to isolate cDNA clones encoding these proteins from expression libraries using antibodies that recognize the entire family. c-*jun* is also a member of an inducible gene family, and one closely related gene has been identified (*jun*-B) (Ryder et al. 1987). The most conserved region between c-*jun* and *jun*-B corresponds to the leucine zipper, the putative DNA-binding domain, and in vitro association between Jun B and Fos has recently been demonstrated (Nakabeppu et al. 1988). Thus, transcription factor AP-1 may be comprised of multiple protein complexes with similar DNA-binding specificities. The subunits of these complexes may be independently or coordinately expressed, depending upon the cell type or the nature of the inducing stimulus. In this way, the products of a small number of genes may represent a complex component of a signal transduction cascade. These proteins are induced by many different second messenger signals and may function in regulating the expression of a large number of target genes to mediate long-term adaptive responses of the cell to environmental cues.

Materials and methods

Cell culture

Rat fibroblast 208F cells (Quade 1979) were maintained in Dulbecco–Vogt Modified Eagle's minimum essential medium (DMEM) containing 10% fetal calf serum (FCS) and antibiotics. For serum stimulation experiments, the cells were deprived of serum for 24 hr (DMEM + 0.5% FCS) and then stimulated by the addition of 20% FCS for various times as indicated. COS-1 cells (Gluzman 1981) were maintained in Iscove's modified Dulbecco's medium (IMDM) containing 10% FCS and antibiotics. For transfections, 3×10^5 to 4×10^5 COS-1 cells were seeded onto 35-mm dishes (with or without coverslips) and allowed to proliferate for 24 hr.

Transfections

COS cells were transfected as described by Cullen (1987), with some modifications. The subconfluent monolayer of COS cells was washed twice with prewarmed Ca/Mg phosphate-buffered saline (PBS) and was then transfected with a mixture of 400 μg DEAE–dextran (Pharmacia; m.w. 500,000) and 0.5–1.0 μg of DNA in 400 μl PBS/35-mm dish for 30–45 min at 37°C. At that time, 2 ml of IMDM supplemented with 125 μg/ml chloroquine and 2% Fungizone was added to each dish and incubation continued at 37°C for 2 hr. This medium was then removed and replaced with IMDM containing 10% DMSO for 2 min at room temperature; finally, this was replaced with fresh IMDM medium and the cells were incubated at 37°C for 48 hr.

Cell labeling, lysis, and immunoprecipitation

In pulse-labeling experiments with 208F fibroblasts, subconfluent monolayers of cells in 35-mm (or 60-mm) dishes were stimulated by the addition of 20% dialyzed FCS for the times shown. The cells were then labeled for 15 min (unless stated otherwise) with 300 μCi (or 1200 μCi for 60-mm dishes) of [^{35}S] methionine. For preparation of native cell lysates, the labeled medium was removed and the cells were lysed by the addition of 1 ml RIPA buffer essentially as described (Curran et al. 1984, 1985). For denatured cell extracts, the labeled medium was removed and replaced with 200 μl of lysis buffer containing 0.5% SDS. The cells were scraped from the dish in this buffer and then boiled for 5 min. Transfected COS cells were pulse-labeled with 100–200 μCi [^{35}S]methionine for 15 min as described (Curran and Teich 1982). Pulse-chase labeling of both fibroblasts and transfected COS cells was as described (Curran et al. 1984), except that in the fibroblast experiments the extracts were always prepared under denaturing conditions. For phosphoamino acid analysis, transfected COS cells were labeled with 1–2 mCi [^{32}P]orthophosphate per dish in phosphate-free saline supplemented with phosphate-free FCS (PO_4 medium) for 60 min at 37°C. Clarification of cell extracts, immunoprecipitation, and SDS–polyacrylamide gel electrophoresis were as described previously (Curran et al. 1984, 1985).

Immunofluorescence

For immunofluorescence studies, COS cells were grown and transfected on coverslips in 35-mm dishes. After 48 hr of transfection, indirect immunofluorescence was performed using the anti-Fos antibodies against the M peptide (amino acids 127–152) (Curran et al. 1985) and rhodamine-conjugated goat anti-rabbit IgG, as described by Curran et al. (1984). Untransfected and mock-transfected cell controls, as well as preimmune sera controls, were included in each experiment and gave negative results.

Construction of recombinant cDNA plasmids

The present study made use of previously constructed pSP65 recombinant plasmids containing the c-*fos*(rat) cDNA insert (Curran et al. 1987), the *fra*-1(rat) cDNA insert (Cohen and Curran 1988), and the c-*jun*(rat) cDNA insert (Rauscher et al. 1988a). Plasmids containing the cDNA inserts in the sense orientation with respect to the SP6 promoter were used as templates for the in vitro transcription of *fos*, *fra*-1, and *jun* RNAs. For the transfection of COS cells, the 1.5-kb *Eco*RI *fra*-1 cDNA insert (or the 2.1-kb c-*fos* cDNA insert) was cloned by blunt-end ligation between the *Hin*dIII and *Bam*HI sites of the CMV vector, which was a kind gift of Bryan Cullen (Duke University Medical School). This vector contains the human CMV immediate-early promoter. The resulting recombinant plasmids were referred to as pCMV-*fra*-1 and pCMV-*fos* for simplicity.

For the generation of a truncated version of the Fra-1 protein (i.e., amino acids 136–275), the pSP65-*fra*-1 plasmid was digested with *Pvu*II, and *Bam*HI linkers were added. The ligation mixture was then digested with *Bam*HI and *Hin*dIII to generate a fragment encoding the carboxy-terminal half of Fra-1. This fragment was inserted into the expression vector pDS56-6xHis (Gentz et al. 1989) digested with *Bam*HI and *Hin*dIII to generate the recombinant plasmid pFra-COOH. Amino acid 135 of Fra-1 is the first leucine residue of the repreating leucine structure; therefore, the truncated protein Fra-COOH contains a leucine zipper domain that is virtually intact.

Expression and purification of Fra-COOH

E. coli cells containing pFra-COOH were grown at 37°C in LB medium plus ampicillin (100 μg/ml) and kanamycin (25 μg/ml). At an OD_{600} of 0.6 isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and incubation was continued for an additional 3 hr. Cells were harvested by centrifugation and lysed using 6 mM guanidinium–hydrochloride (pH 8.0). The cell debris was removed by centrifugation, and the supernatant was directly applied to a Ni^{2+} chelate column (Hochuli et al. 1987). The washing steps and elution of the protein were as described (Gentz et al. 1989).

Generation of antisera against Fra-1

Two hydrophilic peptides predicted from the *fra*-1 cDNA sequence were produced by an automated peptide synthesizer and were used to generate antisera directed against the Fra-1 protein. The amino-terminal peptide (peptide 1) extends from amino acids 2–15 and corresponds to the sequence YRDFGEPGPSSGAG. The second peptide (peptide 2) extends from amino acids 177 to 188 and corresponds to the sequence PEEDKKDTGGTS. A tyrosine residue was added at the amino–terminal end of peptide 2, and both peptides were cross-linked to thyroglobulin using gluteraldehyde. Peptide–carrier complexes (5 mg/ml in PBS) were used to inoculate rabbits (1 mg of complex per inoculation). Preimmune serum was collected from each animal and, following the primary inoculation, booster inoculations were administered after 4 and 8 weeks; serum was removed from the animals 10 days after each of the booster injections. The resulting antisera were referred to as Fra-1 1.1 Ab and Fra-1 1.2 Ab for the two bleeds from the animal inoculated with peptide 1, an Fra-1 2.1 Ab and Fra-1 2.2 Ab for the two bleeds from the animal inoculated with peptide 2.

The Fra-COOH protein, purified on the Ni^{2+} chelate column, was also used to inoculate rabbits as described above. The resulting antisera were referred to an Fra-1 3.1 Ab and Fra-1 3.2 Ab.

In vitro synthesis of proteins

Recombinant pSP65 plasmids containing the various cDNA inserts were linearized with appropriate enzymes, and RNAs were transcribed in vitro using SP6 polymerase as described by Curran et al. (1987). The pFra-COOH plasmid was transcribed into RNA by *E. coli* RNA polymerase in the presence of capping structure 7mGpppA, as described previously (Stueber et al. 1984; Bujard et al. 1988). These RNAs were used to program protein synthesis by messenger-dependent rabbit reticulocyte lysates (Promega Biotech), essentially as described (Rauscher et al. 1988a).

In vitro association of proteins

For association experiments with in vitro-synthesized proteins, equal volumes of the reticulocyte lysates to be mixed (usually 2 μl) were combined and incubated at 37°C for 30 min. For immunoprecipitation analysis of these associations, the mixtures were diluted to 100 μl with cold RIPA buffer and appropriate antibodies were added, followed by standard incubation and washing procedures (Curran et al. 1984, 1985).

Gel retardation and DNAP assays

These assays were performed exactly as described by F.J. Rauscher et al. (1988), except that in the DNAP assay the poly(dI-dC) competitor DNA was used at a concentration of 5 μg/μl in water (not solution B), and the final reaction volume was 80 μl.

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