Fos-Associated Protein p39 Is the Product of the *jun* Proto-oncogene

FRANK J. RAUSCHER III, DONNA R. COHEN, TOM CURRAN, TIMOTHY J. BOS, PETER K. VOGT, DIRK BOHMANN, ROBERT TJIAN, B. ROBERT FRANZA, JR.*

The Fos protein complex and several Fos-related antigens (FRA) bind specifically to a sequence element referred to as the HeLa cell activator protein 1 (AP-1) binding site. A combination of structural and immunological comparisons has identified the Fos-associated protein (p39) as the protein product of the *jun* proto-oncogene (c-Jun). The p39/Jun protein is one of the major polypeptides identified in AP-1 oligonucleotide affinity chromatography extracts of cellular proteins. These preparations of AP-1 also contain Fos and several FRA's. Some of these proteins bind to the AP-1 site directly whereas others, like Fos, appear to bind indirectly via protein-protein interactions. Cell-surface stimulation results in an increase in c-*fos* and c-*jun* products. Thus, the products of two proto-oncogenes (and several related proteins), induced by extracellular stimuli, form a complex that associates with transcriptional control elements containing AP-1 sites, thereby potentially mediating the long-term responses to signals that regulate growth control and development.

THE ISOLATION AND CHARACTERIZATION OF RETROVIRAL oncogenes (v-*onc*) and their cellular progenitor genes (c-*onc*) has led to the development of specific reagents that are used to investigate the molecular basis of normal and neoplastic cell growth. A unifying theme arising from these studies is that many oncogene products appear to function in information transmission pathways (signal transduction processes) between and within cells. Several proto-oncogenes encode proteins similar or identical to growth factors and their receptors, G proteins, protein kinases, or nuclear proteins whose expression increases in response to extracellular stimuli (*1*). One of these inducible proto-oncogenes encodes the nuclear protein Fos. We have been pursuing studies on the normal cellular *fos* gene (c-*fos*) as well as the *fos* oncogene (v-*fos*) in an effort to elucidate the role of Fos in the transduction of signals within the nucleus (*2*).

The v-*fos* gene was first described as the transforming gene carried by the FBJ murine sarcoma virus (MSV) that encoded the *fos* protein (*3*). It is responsible for the induction of osteogenic sarcomas (in rodents) by FBJ-MSV (*4*). The v-*fos* product is expressed as a nuclear phosphoprotein with an apparent molecular size of 55 kD ($p55^{v\text{-}fos}$) (*3*). Its carboxyl-terminal 49 amino acids are translated from a different reading frame from c-*fos* as a result of an out-of-frame deletion (*5*, *6*). The c-*fos* protein product, which has an apparent molecular size of 62 kD, is also a nuclear phosphoprotein but it undergoes much more extensive phosphorylation modification than v-Fos (*7*, *8*). Although its expression in most cell types is low, c-*fos* can be transiently induced by several orders of magnitude as a result of mitogenic, differentiation, or neural cell depolarizing stimuli (*2*).

Both $p55^{v\text{-}fos}$ and $p62^{c\text{-}fos}$ can be a part of a nuclear protein complex (*6*, *9*). The major Fos-associated protein (FAP) in fibroblasts is a 39-kD protein (p39), which was first identified as an FBJ-MSV transformation-associated protein (*10*). Like Fos, p39 is phosphorylated primarily on serine residues (*7*, *8*). Two other FAP's have been identified. One has been detected only in PC12 cells (p40) and the other (p48) is present in very low amounts in fibroblasts and PC12 cells (*9*). The Fos-p39 complex is formed in the nucleus and much of the posttranslational modification of the two proteins appears to occur in the nucleus (*7*). In addition to detecting Fos and FAP's in immunoprecipitation assays, antibodies against Fos amino acids 127 to 152 also recognize a set of Fos-related antigens (FRA's) (*9*). These are nuclear proteins and they are induced by many of the agents and conditions known to increase Fos expression (*9*, *11*). A gene encoding one of the FRA's (*fra*-1) has been cloned and characterized (*12*). The amino acid sequence of its expression product is similar to that of Fos in a region related to the yeast transcription factor GCN4 and the *jun* oncogene (*13*). The *jun* proto-oncogene (c-*jun*) product is structurally and functionally identical to a member of the set of proteins that interact with AP-1 sites (*14*–*16*).

Fos, Fos-associated proteins, and Fos-related antigens are associated with chromatin in isolated nuclei and bind to DNA-cellulose in vitro (*17*), and the v-*fos* gene stimulates transcription from selected promoters in trans (*18*). These data have led to the proposal that Fos may be regarded as a marker for a set of inducible genes that function in coupling short-term signals, elicited by cell-surface stimulation to long-term adaptive responses by regulating expression of specific target genes (*19*). The first suggestion of a specific DNA-binding property associated with Fos immunoreactive proteins was the inhibition by antibodies to Fos (anti-Fos) of a gel-shift complex that involved a regulatory sequence (FSE2, Fos-specific element 2), derived from an adipocyte gene (*aP2*) (*20*). A combination of several procedures—DNA affinity precipitation assays (DNAP) (*21*, *22*), mutagenesis, competition, and antibody inhibition (*23*)—identified the DNA target sequence as the AP-1 binding site. Specifically, AP-1 motifs in the gibbon ape leukemia virus long terminal repeat (LTR), the human immunodeficiency virus (HIV)

F. J. Rauscher III, D. R. Cohen, and T. Curran are at the Roche Institute for Molecular Biology, Nutley, NJ 07110. T. J. Bos and P. K. Vogt are in the Department of Microbiology, University of Southern California School of Medicine, Los Angeles, CA 90033. D. Bohmann and R. Tjian are at the Howard Hughes Medical Institute, Department of Biochemistry, University of California at Berkeley, Berkeley, CA 94720. B. R. Franza, Jr., is at the Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724.

*To whom correspondence should be addressed.

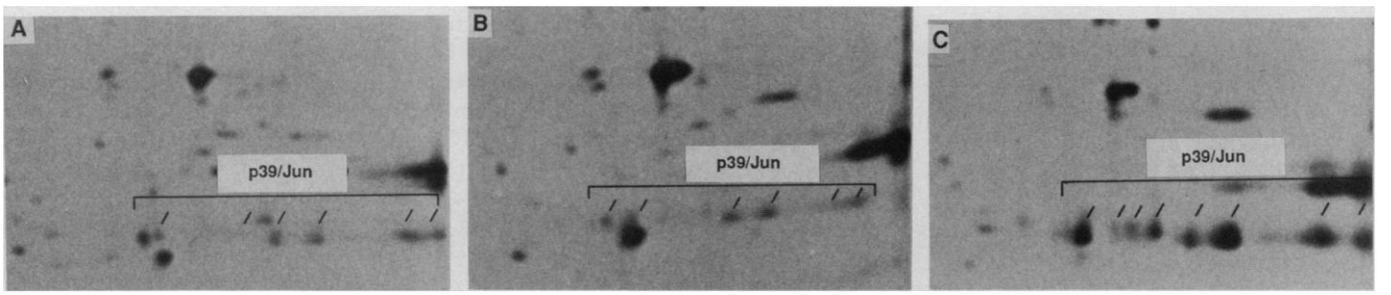


Fig. 1. Comigration of proteins immunoprecipated by anti-Jun and p39 isolated in DNAP assays with oligonucleotides containing AP-1 binding sites. H9 cells (1×10^8) were stimulated by the addition of Ca^{2+} ionophore A23187 to a final concentration of $10^{-6}M$ for 20 minutes; [^{35}S]methionine (2.0 mCi) was then added. Cells were harvested 30 minutes later; extracts were prepared and examined by immunoprecipitation (A) or by DNAP assays (B and C). Recovered proteins were separated on 2D gels, and autoradiography was performed as described (*21, 22*). The first-dimension gel was an isoelectric focusing gel, *p*H 3.5 to 10 (acidic proteins to the left), and the second was a 10 percent polyacrylamide SDS gel (apparent molecular size decreasing from top to bottom). Only the portion of the gel in which p39 migrates is presented. (**A**) H9 cells were treated with A23187 labeled with [^{35}S]methionine as described above, and then cells were harvested and prepared for immunoprecipitation (*22*). Cell extracts were incubated with anti-PEP-1 (*15*). Immune complexes were recovered with Pansorbin, a *Staphylococcus aureus* protein A preparation, and analyzed on 2D gels. (**B**) DNAP analysis with biotinylated DFSE2 oligonucleotide. (**C**) DNAP analysis with the HIV (−357 to −316) oligonucleotide. Both oligonucleotides contain AP-1 binding sites and in DNAP assays bind Fos, Fos-related antigens, and p39 (*22*). The hash marks on each panel indicate the number of forms of p39 that were resolved on the 2D gels.

LTR, and the human metallothionein promoter (*24*) were shown to bind the Fos complex (*22, 23*). Furthermore, DNAP assays in conjunction with high resolution two-dimensional (2D) gel electrophoresis demonstrated that several FRA's and FAP p39, induced in human lymphoblast cells by calcium ionophore (A23187), also bind to the AP-1 site (*22*). One of the questions posed by these observations is which of the many polypeptides present in a complex with AP-1 sites is Jun/AP-1?

Comigration of p39 with Jun/AP-1 on high resolution two-dimensional gels. The proteins in an extract from H9 (*25*) cells identified by DNAP analysis with an oligonucleotide containing sequences related to the AP-1 site were compared to those immunoprecipitated from the same extract by an antibody to the c-*jun* product (anti-Jun) (Fig. 1A). The oligonucleotides used represent the FSE2 element (Fig. 1B) (*20*) and the region −357 to −316 from the HIV LTR (Fig. 1C) (*22*). The antibodies were produced with a synthetic peptide (PEP-1) corresponding to amino acids 209 to 225 of the v-*jun* product (*15*). Only the region of the gel in which p39 migrated is shown. As described previously (*22*), p39 identified by DNAP was detected in the basic region of the 2D gel as a heterogeneous array of polypeptides (designated by hash marks), differing primarily by charge probably as a consequence of phosphorylation (Fig. 1, B and C). The predominant proteins precipitated by anti-Jun, but not by serum from an unimmunized rabbit, migrated with a pattern similar to that of p39 (Fig. 1A). This observation led to the hypothesis that the protein product of c-*jun* was p39 Fos–associated protein.

To further investigate the possibility that p39 was the product of c-*jun*, we used a cell line (RS2) transformed by the FBJ-MSV retrovirus because the Fos-p39 complex is readily detectable in these cells (*3, 10*). ^{35}S-Methionine–labeled cell extracts from RS2 cells were prepared for immunoprecipitation analysis. Antibodies to a synthetic peptide corresponding to Fos amino acids 127 to 152 (M-peptide) (anti-M) (*6*) and antibodies to PEP-1 (anti-PEP-1) (*14, 15*) were used in the analysis of RS2 extracts (Fig. 2). The p55$^{v\text{-}fos}$ protein migrated as a collection of polypeptides in the acidic region of 2D gels (Fig. 2, A and B). The p55$^{v\text{-}fos}$ protein was detected in precipitates from native (Fig. 2A) as well as denatured (by boiling in SDS) (Fig. 2B) cell extracts, whereas p39 was only present in anti-Fos antibody immunoprecipitates from nondenatured cell extracts (Fig. 2A). The anti-PEP-1 immunoprecipitate from a denatured RS2 cell extract contained a specific protein that migrated in the same region of the 2D gel as p39 (Fig. 2C). A similar pattern was obtained with native cell extracts or with an antibody (anti-PEP-2) to a peptide corresponding to v-Jun amino acids 73 to 87 (PEP-2) (*15*). To confirm the apparent comigration of p39 and the protein precipitated by anti-PEP-1, equivalent amounts of each immunoprecipitate were mixed before resolution on the gel (Fig. 2D). In the mixed sample, a single major protein was detected in the p39 region of the gel. On longer exposures of the autoradiograph several of the modified forms of p39 detected with anti-PEP-1 also comigrated with those precipitated by anti-Fos. Because of the very high resolution provided by this 2D gel system (*26*), we can infer from these data that (i) the p39 protein precipitated indirectly with anti-Fos, (ii) the p39 protein detected by its binding to different elements containing AP-1 sites, and (iii) the cellular protein recognized by anti-PEP-1 and anti-PEP-2 are identical. Therefore, FAP p39 was considered a probable candidate product of the *jun* proto-oncogene. Since this protein was immunoprecipitated by both the anti-PEP-1 and anti-PEP-2, it is unlikely to be the product of a recently described *jun*-related gene (*jun*-B) in that the *jun*-B product does not contain the PEP-2 amino acid sequence (*27*).

p39, Fos, and several Fos-related antigens are present in affinity-purified preparations of AP-1. Oligonucleotides containing AP-1 sites have been used for affinity purification of transcription factors. We examined such AP-1 preparations for the presence of Fos and FRA's for the following reasons: (i) We were able to identify Fos and several FRA's in DNAP assays (*22*) and observed gel-shift activity inhibited by anti-Fos (*23*), which was dependent on DNA sequences related to the AP-1 binding site; (ii) if the Fos-associated protein p39 is Jun or AP-1, we would expect to find Fos in nondenatured AP-1 preparations. A whole cell extract from HeLa cells was used to indicate apparent molecular sizes of the detected proteins (Fig. 3A, lane 1). [A HeLa cell extract was chosen for molecular size approximations because the AP-1 was derived from these cells (*14*) (Fig. 3A, lane 1).] Using the DNAP assay with an AP-1 probe (Fig. 3A, lane 3), we recovered every protein detectable (by silver stain) in the starting material (Fig. 3A, lane 2). These proteins were compared to those detected in an immunoblot of the same material with anti-Fos (Fig. 3B). The major protein recognized by anti-c-Jun (*14*) is indicated as p39/Jun in the silver stain (Fig. 3A). It migrates between two anti-Fos cross-reactive bands (Fig. 3B, lanes 1 and 2). A smaller protein (approximately 35 kD) was detected by anti-Fos on the immunoblot and by silver staining. A fuzzy band representing forms of c-Fos was seen in the immunoblot and was barely detectable by silver stain. Clearly, it is less

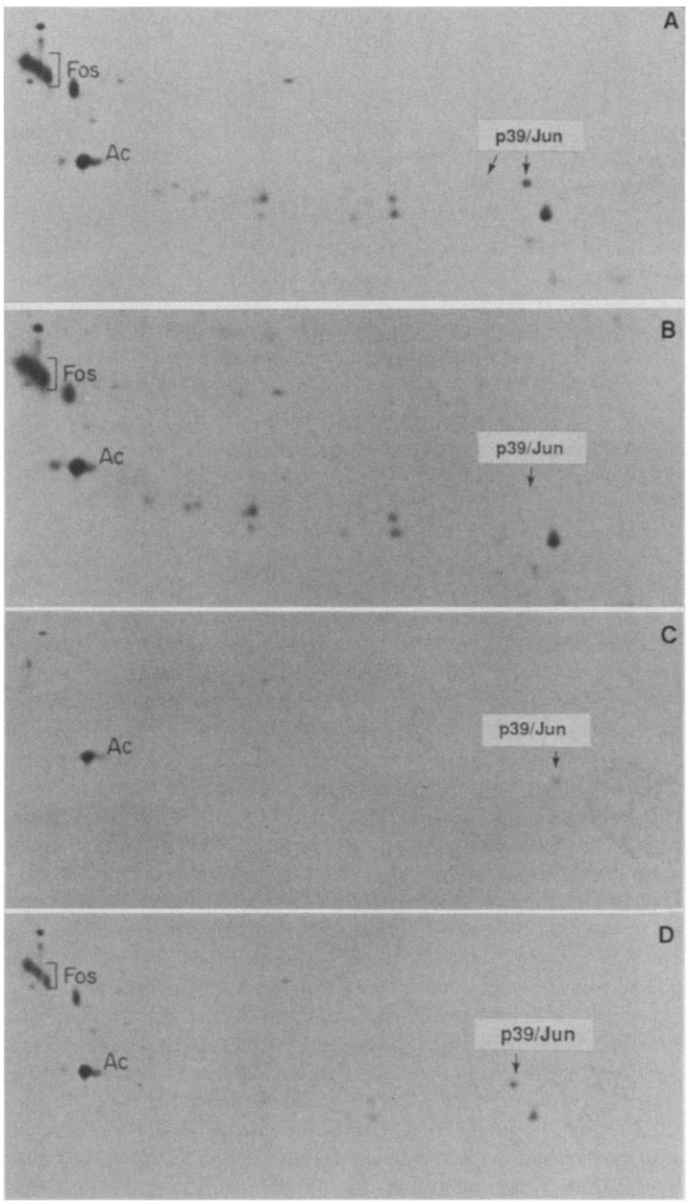


Fig. 2. Detection of p39/Jun in v-*fos*–transformed cells. Subconfluent FBJ-MSV–transformed RS2 cells (*3*) in 35-mm dishes were incubated in growth medium without methionine for 20 minutes; [^{35}S]methionine (0.5 to 1.0 mCi) was added, and incubation was continued for 30 minutes. The medium was then removed, cells were lysed by addition of (1 ml per dish) RIPA buffer [150 m*M* NaCl, 10 m*M* tris-HCl, *p*H 7.4, 0.1 percent SDS, 1 percent Triton X100, 1 percent sodium deoxycholate, 1 m*M* EDTA, leupeptin at 2 μg/ml, aprotinin at 5 μg/ml, and 0.1 m*M* phenylmethylsufonylfluoride (PMSF)]. Extracts were clarified by centrifugation at 80,000*g* for 30 minutes and then incubated with antibody. If extracts were to be denatured before addition of antibody, the ^{35}S-containing medium was removed and 200 μl of denaturation buffer [0.5 percent SDS, 1.0 m*M* dithiothreitol (DTT), 50 m*M* tris-HCl, *p*H 7.4, 1 m*M* EDTA, leupeptin at 2 μg/ml, aprotinin at 5 μg/ml, and 0.1 m*M* PMSF)] was added. Cells were scraped into a 1.5-ml microfuge tube and extracts were boiled for 5 minutes. Then 800 μl of RIPA buffer without SDS was added, and the denatured lysate was centrifuged at 80,000*g*. The immune complexes were collected with Pansorbin and prepared for 2D gel analysis (*21*). (**A**) Proteins precipitated from native RS2 cell lysates by anti-M (*6*). (**B**) Proteins precipitated from denatured RS2 cell lysates with anti-M. (**C**) Proteins precipitated from denatured RS2 cell extracts with anti-PEP-2 (*15*). (**D**) Approximately equal amounts of ^{35}S-labeled material recovered from the immunoprecipitates shown in (A) and (C) were combined, and the mixture was resolved on a 2D gel. The multiple forms arising from posttranslational modification of Fos are marked, as are p39/Jun and actin.

abundant after high-pressure liquid chromatography (HPLC) fractionation of the affinity-purified preparation (Fig. 3B). Conversely, an 80-kD protein was detected by silver staining but not by immunoblotting. Three proteins detected by silver staining were also detected by a DNA affinity blot analysis (*28*) with a ^{32}P-labeled nucleic acid probe containing Ap-1 sites (Fig. 3A, lane 4). These proteins include the 80-kD protein, a protein that comigrates with the upper FRA band, and one that is coincident with the p39/Jun band. Control reactions demonstrated that a ^{32}P-labeled HIV enhancer element oligonucleotide (*21*) did not bind to any protein in the AP-1 preparation. Thus, Fos, several Fos-related proteins, and p39/Jun are present in AP-1 preparations and the DNA affinity blot data suggest that certain of these polypeptides bind to AP-1 sites directly.

AP-1 has been described as a 47-kD protein on SDS-polyacrylamide gels (*29*); however, this apparent molecular size refers to the collection of proteins shown in Fig. 3A, lane 2. We now suggest that p39 is one of the major proteins detected by silver staining and DNA affinity blot analysis in AP-1 oligonucleotide affinity chromatography preparations of HeLa nuclear extracts. Originally p39 was reported to have an apparent molecular size of 39 kD (*3*), but this migration is dependent, in part, on the percentage of acrylamide in the gel and the degree of posttranslational modification of p39. Pulsed labeling studies show some forms of p39 migrating between 39 and 42 kD with the most highly modified forms comigrating with actin (*7, 11*). Thus, depending on the growth state of cells at the time of protein isolation, the apparent molecular size of the most abundant protein in an AP-1 preparation may vary. Contributing to this variation is the fact that certain Fos-related antigens, found in AP-1 preparations, bound directly to AP-1 sites as was the case for p39/Jun. A further contribution to the heterogeneity is that although direct binding of Fos itself to the AP-1 probe was not detected by DNA affinity blot analyses, Fos is present in these preparations. The presence of Fos and FRA's in AP-1 preparations may have a functional significance because anti-Fos inhibits a portion of the footprinting and transcriptional activities of AP-1 (*30*). Whether Fos require p39/Jun for specific association with AP-1 sites is not yet known. The functional significance, if any, and further biochemical characterization of the 80-kD AP-1 oligonucleotide-binding protein remain to be determined.

Structural comparison of p39 and Jun. To confirm that the Fos-associated protein p39 and the c-*jun* product are the same polypeptides, we prepared tryptic peptide maps. Equal amounts of a ^{35}S-methionine–labeled RS2 cell extract were treated with anti-Fos or anti-Jun, and the immunoprecipitates were separated on one-dimensional gels. Protein bands (Fig. 4A, lanes 1 and 3, indicated by arrowheads) were excised; the proteins were eluted and digested with trypsin and the resulting peptides were resolved in two dimensions (*10, 32*). The identity of p39 in anti-Fos immunoprecipitates was confirmed by comparison with immunoprecipitates from denatured extracts that lack p39 (Fig. 4A, lane 2). The array of ^{35}S-methionine–labeled tryptic peptides obtained from p39 digests (Fig. 4B) was similar to that obtained from Jun (Fig. 4C). Indeed, when the digests were mixed and resolved together on the same thin-layer cellulose plate, all of the peptides detected comigrated (Fig. 4D). This observation provides compelling evidence for the identification of p39 as Jun. A two-dimensional tryptic peptide map of in vitro translated Jun derived from mRNA synthesized in vitro from a c-*jun* clone was indistinguishable from the maps shown in Fig. 4 (*31*).

p39/Jun exists in both bound and free states in v-*fos*–transformed cells. Our results indicate that Fos forms a complex with p39/Jun. However, the data in Fig. 2 suggest that some p39/Jun may not be complexed with Fos. Specifically, although anti-Fos

precipitated p39/Jun because of the association with Fos, neither anti-PEP-1 nor anti-PEP-2 precipitated equivalent amounts of Fos in a complex with p39/Jun (Fig. 2). On long exposures of the autoradiographs a small amount of Fos was detected in anti-PEP-1 and anti-PEP-2 immunoprecipitates, indicating that some interaction with Jun persisted. It was conceivable that some form of posttranslational modification of p39/Jun was required for complex formation so that the complex could not be detected in the short periods used for the labeling experiments shown in Figs. 2 and 4. Therefore, we performed a pulse-chase experiment to determine whether a special form of p39/Jun was required for association with Fos. Both $p55^{v\text{-}fos}$ and p39/Jun were detected in immunoprecipitates from nondenatured cell extracts either made from cells labeled for a 30-minute period (Fig. 5A, lane 1) or from cells labeled for a 30-minute period followed by a 60-minute incubation (chase) with unlabeled methionine (lane 2). Fos-containing immunoprecipitates from denatured cell extracts contained $p55^{v\text{-}fos}$ but no p39/Jun (Fig. 5A, lanes 5 and 6). The apparent molecular size of $p55^{v\text{-}fos}$ changed from approximately 52 to 55 kD and that of p39 from 39 to 41 kD during the chase period. Although the p39/Jun protein precipitated by anti-PEP-2 from nondenatured extracts exhibited the same shift in molecular size after chase, no Fos was detected (Fig. 5A, lane 4). One possible explanation for these results is that p39/Jun bound to Fos cannot be recognized by anti-PEP-1 or anti-PEP-2 antibodies. To further address this possibility, we carried out sequential immunoprecipitation analysis (Fig. 5B). Comparable cell extracts were treated first with anti-Fos and the immune complexes were collected (Fig. 5B, lane 1). Then the remaining soluble proteins were treated with anti-PEP-2 and the immune complexes were collected (lane 2). The experiment was also carried out in reverse, clearing first with anti-PEP-2 (lane 3) before precipitation with anti-Fos (lane 4). After all of the immunoprecipitable Fos complex was removed from the RS2 cell extract, a population of free p39/Jun remained. Control experiments consisting of three sequential immunoprecipitations with anti-Fos showed that essentially all of the detectable $p55^{v\text{-}fos}$ was removed from the extract in the first precipitation. Similarly, after cell extracts were treated with anti-Jun, the $p55^{v\text{-}fos}$/p39 complex could still be precipitated with anti-Fos. However, in this case repeated treatments with anti-PEP-2 continued to precipitate small amounts of p39/Jun. The result of three sequential precipitations with anti-PEP-2 was a diminution in the

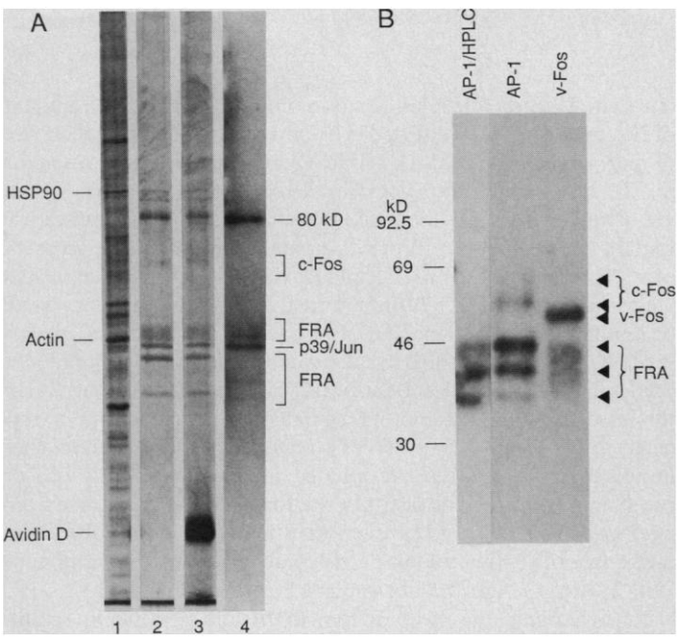


Fig. 3. Purified AP-1 preparations contain Fos and Fos-related antigens. (**A**) HeLa cell nuclear extracts were used to purify proteins that bound to oligonucleotide affinity columns containing AP-1 binding sites (*14*). Equal amounts of material obtained after three successive passages over the column were then resolved on 9 percent SDS-polyacrylamide gels. Resulting proteins were either silver-stained (lane 2), or DNAP assays were performed (lane 3), or they were transferred to nitrocellulose and probed with ^{32}P-labeled AP-1 oligonucleotides in a DNA-affinity blot (*28*) (lane 4). For molecular sizing, a whole cell extract of HeLa cells was placed on the same gel and visualized by silver stain (lane 1). The positions of Fos, FRA's, and p39/Jun were determined by immunoblot analysis of regions of the same gel with anti-M or anti-PEP-2 (*31*). Actin, HSP90, Fos, FRA's, and p39/Jun are indicated. (**B**) Fos and Fos-related proteins were detected in AP-1 or anti-PEP-2 preparations by immunoblot analysis with anti-M. Fos-immunoreactive proteins were visualized by subsequent reaction with ^{125}I-labeled protein A (30 μCi/mg; Amersham), followed by autoradiography. (Lane 1) HPLC-purified AP-1 (*14*); (lane 2) affinity-purified AP-1 prior to HPLC separation (*14*); (lane 3) extract of FBJ-MSV–transformed fibroblasts (RS2). The positions of the c-Fos and v-Fos proteins and several FRA's are indicated. The position of p39/Jun was determined by immunoblot analysis of the same gel with anti-PEP-2 (*31*). In addition, [^{14}C]methylated molecular markers (Amersham) were subjected to electrophoresis on the same gel.

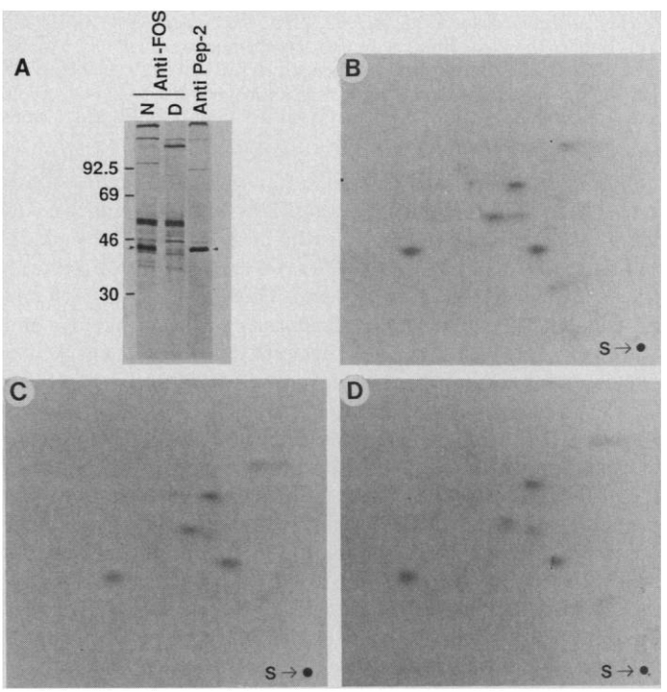


Fig. 4. Tryptic peptide mapping analysis of p39 and Jun. v-*fos*–transformed RS2 cells (six dishes) were labeled with [^{35}S]methionine, harvested, and nondenatured lysates were prepared as for Fig. 1. The clarified lysate from each dish was divided into two portions and one was incubated with anti-M and the other with anti-PEP-2. Immunoprecipitates were recovered with Pansorbin and resolved on one-dimensional SDS-polyacrylamide gels. (**A**) Examples of immunoprecipitates used for peptide map analyses. (Lane 1) Native RS2 lysate precipitated with Fos M-peptide antibodies. (Lane 2) Denatured RS2 lysate precipitated with anti-M. (Lane 3) Native RS2 lysates precipitated with anti-PEP-2. The protein bands corresponding to p39/Jun (see arrowheads) were rehydrated, and proteins eluted from the gel slices were processed for tryptic peptide mapping (*10, 32*). Proteins were precipitated with trichloroacetic acid, oxidized, and then cleaved with TPCK-treated trypsin (Worthington). Peptide mixturęs were spotted onto cellulose thin-layer chromatography plates (E. Merck, Darmstadt) and electrophoresis was conducted for 30 minutes at 1000 V in 2.5 percent pyridine and 2.5 percent acetic acid (*p*H 4.5). Chromatography was performed in the direction perpendicular to electrophoresis in a buffer consisting of *n*-butanol, acetic acid, water, and pyridine. Plates were dried and subjected to autoradiography for 12 days. (**B**) Tryptic peptides of c-Jun isolated with anti-PEP-2. (**C**) Tyrptic peptides of p39 isolated with anti-M. (**D**) A 1:1 mixture of peptides used in (B) and (C). The black spot in the lower right-hand corner indicates the origin of electrophoresis.

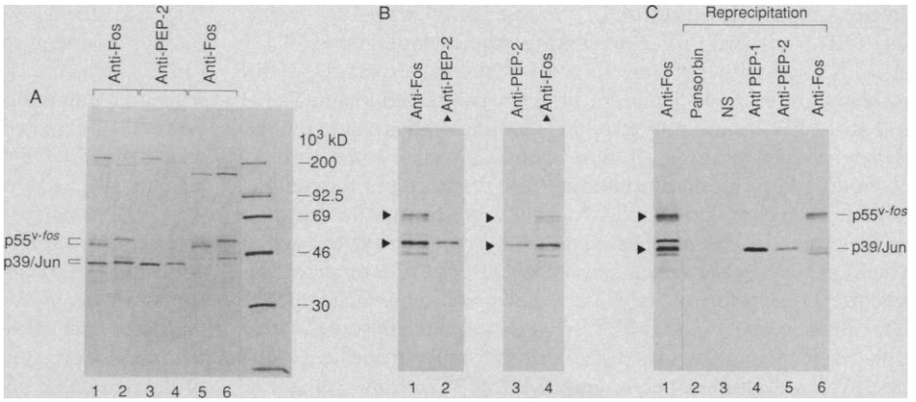


Fig. 5. Pulse-chase and sequential precipitation analysis of p39/Jun in RS2 cells. (**A**) RS2 cultures were labeled for 30 minutes with [^{35}S]methionine and then lysed in RIPA buffer (lanes 1, 3, and 5) or were labeled for 30 minutes and then the medium was changed to Dulbecco's minimum essential medium (DMEM) plus 10 percent fetal calf serum for 60 minutes (lanes 2, 4, and 6). Clarified extracts were precipitated with anti-Fos (lanes 1, 2, 5, and 6) or anti-PEP-2 (lanes 3 and 4). In lanes 5 and 6, cells lysates were denatured (as in legend to Fig. 2) before antibody incubation. Immunoprecipitates were resolved by SDS-PAGE (polyacrylamide gel electrophoresis) and the gel was processed for autoradiography (*6, 7*). The positions of the variously modified forms of $p55^{v\text{-}fos}$ and p39/Jun are indicated. (**B**) Sequential immunoprecipitation from native RS2 extracts. A native lysate from ^{35}S-labeled RS2 cells was incubated with anti-Fos (lane 1). Pansorbin was added, and the mixtures were centrifuged; the supernatant was removed and incubated with anti-PEP-2 (lane 2). The procedure for lanes 3 and 4 was identical except that anti-PEP-2 (lane 3) was used before anti-Fos (lane 4). (**C**) Reprecipitation of Fos and Jun. A subconfluent 100-mm dish of RS2 cells was labeled with [^{35}S]methionine (2.0 μCi) for 30 minutes and a native RIPA lysate was prepared. After incubation with anti-M and Pansorbin absorption, the precipitate was washed four times with RIPA buffer. The Pansorbin-containing absorbed immune complex was then collected by centrifugation and resuspended in 200 μl of RIPA, 0.5 percent SDS, and 5 m*M* mercaptoethanol. A portion (one-sixth volume) of the resuspension was removed and processed for SDS-PAGE. This sample represented the total proteins in the immunoprecipitate before denaturation (lane 1). The remainder of the immune complexes on Pansorbin were boiled for 5 minutes, centrifuged at 10,000*g* twice for 5 minutes, and the supernatant was removed. RIPA buffer was added to the supernatant to a total volume of 1 ml, and 200-μl samples of this mixture were placed in Eppendorf tubes. The soluble proteins were then reprecipitated by addition of the following. (Lane 2) Pansorbin alone (as a control for renaturation of the anti-Fos); (lane 3) pre-immune serum; (lane 4) anti-PEP-1; (lane 5) anti-PEP-2; (lane 6) anti-Fos. Reprecipitated proteins were collected on Pansorbin, washed, and analyzed by SDS-PAGE. The arrowheads indicate the position of $p55^{v\text{-}fos}$ (upper) and p39/Jun (lower).

amount of p39 recovered in association with $p55^{v\text{-}fos}$ in a fourth precipitation with anti-Fos antibodies. This result suggests that anti-PEP-2 may have some ability to disrupt the $p55^{v\text{-}fos}$/p39 complex by binding to p39/Jun. No loss of p39 from the Fos complex occurred on extended incubation with anti-Fos. Thus, p39/Jun exists in both free and bound states in cells transformed by v-*fos*. Because anti-PEP-1 and anti-PEP-2 do not precipitate the Fos complex, it is formally possible that the bound form of p39/Jun is structurally different from the free p39/Jun (*33*).

To further address the issue, we performed experiments in which we used anti-Fos to precipitate the Fos complex from ^{35}S-methionine–labeled lysates. The immune complexes were then denatured by boiling in 0.5 percent SDS, and the proteins were precipitated with either nonimmune serum (Fig. 5C, lane 3), anti-PEP-1 (lane 4), anti-PEP-2 (lane 5), or anti-Fos (lane 6). A portion of the starting material was immunoprecipitated with anti-Fos before denaturation (Fig. 5C, lane 1). Both anti-PEP-1 and anti-PEP-2 precipitated p39/Jun from denatured extracts, whereas reprecipitation with anti-Fos yielded only the anti-Fos immunoreactive proteins present in the original cell extract. This experiment established that p39 present in the Fos complex can be recognized by anti-Jun. Surprising as it may be, the PEP-1 and PEP-2 epitopes, although one is close to the NH_2-terminus and one is close to the COOH-terminus of Jun, appear to be inacessible in the complex of Fos with p39/Jun.

Serum-stimulation increases complexed but not free p39/Jun. To examine the association of p39/Jun with Fos, we used serum-stimulated NIH 3T3 cells as the source of Fos complex. The levels of p39/Jun and Fos in subconfluent proliferating NIH 3T3 cells were determined by immunoprecipitation with anti-Fos (Fig. 6A, lane 1) and PEP-2 (Fig. 6A, lane 2). Fos was not detected in the autoradiograph exposure in Fig. 6A, lane 1. Although Fos is present in very small amounts in unstimulated cells, detection is complicated because heterogeneous posttranslational modification spreads the signal over a large molecular size range (55 to 62 kD). However, in unstimulated cell extracts p39/Jun was detected with anti-Fos, as were several FRA's (lane 1). When somewhate lower amounts of p39/Jun were detected with anti-PEP-2 (lane 2). There are two possible explanations for the p39/Jun signal in the anti-Fos precipitate (lane 1). Either p39/Jun forms a complex with the small amount of Fos present in unstimulated cells or it forms a complex with one or more of the FRA's. In the NIH 3T3 cells that were serum-starved for 24 hours and were then stimulated with 20 percent serum, induction of Fos and the 46-kD Fos-related antigen was clearly evident (lane 3). Also evident was a parallel increase in the recovery of p39/Jun in the anti-Fos precipitates. In an identical lysate treated with anti-PEP-2, more p39/Jun was detected (lane 4) than in a lysate of uninduced cells (lane 2). The identity of p39/Jun in Fos precipitates was confirmed by denaturation of the serum-induced cell lysate before addition of antibody (Fig. 6A, lanes 7 and 8). In this case, p39/Jun was not precipitated by anti-Fos, but it was detected with anti-PEP-2. Tryptic peptide mapping analysis confirmed that the p39/Jun precipitated in association with Fos or precipitated directly with anti-Jun was identical to that isolated from v-*fos*–transformed cells (*31*). Even after the 60-minute incubation in media free of [^{35}S]methionine (chase), immunoprecipitates of anti-PEP-2 with p39/Jun did not contain Fos (lanes 5 and 6).

To investigate the ratio of free to bound p39/Jun in serum-stimulated cells we performed sequential immunoprecipitations as described for v-*fos* extracts in Fig. 5B. As shown (Fig. 6B, lane 1), Fos, Fos-related antigens, and p39/Jun were precipitated by anti-Fos. The supernatant from this precipitate contained no detectable PEP-2 immunoprecipitable material (lane 2). Thus, essentially all of the p39/Jun detected in the 30-minute labeling period was complexed with Fos (or Fos plus FRA's) in serum-stimulated cells. Sequential immunoprecipitation carried out in reverse order (lanes 3 and 4) showed that significant amounts of p39/Jun could be precipitated by anti-PEP-2 and that subsequently anti-Fos could still precipitate Fos, FRA's, and p39/Jun from the cleared extract. Three successive precipitations with anti-PEP-2 resulted in a reduction of the amount of p39/Jun in the Fos complex as described for v-*fos*. Thus, anti-Jun appears capable of partially disrupting the complex between $p62^{c\text{-}fos}$ and p39/Jun.

We then ascertained that the increase in p39/Jun was the result of induction of c-*jun* transcription. The appreciable increase in p39/Jun after serum stimulation (Fig. 6A) was compared to similar increases in p39/Jun in extracts of H9 cells treated with calcium ionophore.

For determining whether this response reflects an increase in protein or in an accumulation of RNA, RNA from untreated H9 cells and RNA from H9 cells treated for 30 minutes with A23187 was analyzed by RNA transfer and hybridization with a c-*jun* probe (Fig. 7). A substantial increase in the 2.7-kb c-*jun* mRNA was detected in A23187-stimulated cells.

Congregation of proto-oncogene products at AP-1 sites. The *fos* proto-oncogene is a target for many of the second messenger signals ellicited by extracellular stimulation (*2*). Our data indicate that its product (Fos) forms a complex with the protein encoded by the *jun* proto-oncogene. This complex exhibits sequence-specific DNA binding to regulatory regions of genes that contain AP-1 binding sequences. Thus, Fos may exert its oncogenic potential in part by forming complexes with cellular transcription factors and thereby influencing gene expression.

Several lines of evidence support the conclusion that the Fos-associated protein p39 is encoded by the c-*jun* proto-oncogene. (i) p39 isolated as a result of its association with Fos or by binding to AP-1 sites comigrates with the proteins precipitated by anti-Jun. (ii) Jun and p39 isolated from v-*fos*–transformed cells display identical 2D tryptic peptide maps and are modified similarly in timed labeling experiments. (iii) Two-dimensional tryptic peptide maps of protein translated from a c-*jun* in vitro transcribed mRNA and p39 are essentially identical. (iv) p39 purifed with anti-Fos reacts with antibodies against two distinct epitopes on Jun. Thus, two distinct epitopes on Jun, one encompassing the putative DNA binding domain, are conserved in the p39/Fos–associated protein.

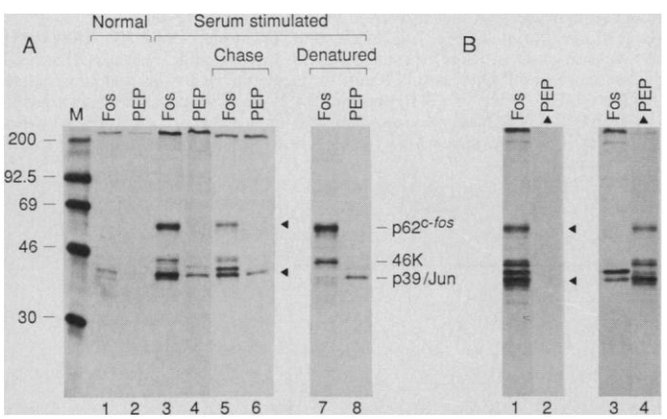
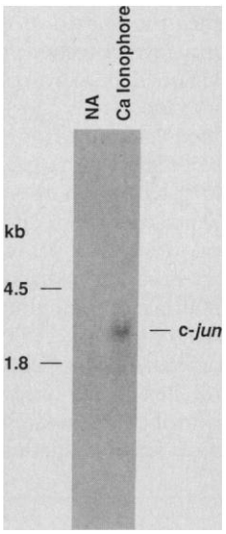


Fig. 6. Induction of p39/Jun in serum-stimulated NIH 3T3 cells. NIH 3T3 cells were seeded in 35-mm dishes and 24 hours later growth medium was changed from 10 percent fetal calf serum (FCS) to 0.5 percent FCS. Twenty-four hours later, medium was removed and replaced with DMEM medium without methionine, but with 20 percent dialyzed FCS. After 30 minutes, 0.5 to 1.0 mCi of [^{35}S]methionine was added, and incubation was continued for 15 minutes. The medium was removed immediately, and fresh growth medium containing 10 percent FCS was added for 60 minutes. Cells that were not serum-stimulated (lanes 1 and 2) were maintained in normal growth medium containing 10 percent FCS. These cell cultures were incubated with DMEM without methionine for 30 minutes before they were labeled as described for serum-stimulated cells. Native or denatured extracts were prepared as described for RS2 cells, and precipitated with anti-Fos or anti-PEP-2. (**A**) (Lanes 1 and 2) Unstimulated NIH 3T3 cells pulse-labeled; (lanes 3 and 4) pulse-labeled serum-stimulated NIH 3T3 cells; (lanes 5 and 6) serum-stimulated NIH 3T3 cells pulse-labeled followed by a 60-minute chase period; (lanes 7 and 8) denatured extracts from serum-stimulated, pulse-labeled NIH 3T3 cells. (**B**) Sequential immunoprecipitation from serum-stimulated NIH 3T3 cell lysate labeled for 30 minutes with [^{35}S]methionine. After preparation of native cell lysates, sequential immunoprecipitations from the same lysate were performed exactly as described in Fig. 4 for RS2 cells. Either anti-Fos was added first (lane 1) and then anti-PEP-2 (lane 2), or anti-PEP-2 was added first (lane 3) and then anti-Fos was added (lane 4). The locations of p62$^{c\text{-}fos}$, the Fos-related antigen 46 kD, and p39/Jun are indicated by the arrowheads.

Fig. 7. Accumulation of p39/c-Jun mRNA in A23187-treated H9 cells. H9 cells were treated with A23187 for 30 minutes as described in the legend to Fig. 1, and RNA was prepared by the method of Auffray and Rougeon (*40*) as modified in (*41*). Electrophoresis was carried out using 4 μg of RNA from either control (lane 1) or A23187 treated (lane 2) cells in 0.8% agarose-formaldehyde mini-gels. After separation RNAs were transferred to nitrocellulose for northern blot analysis. A human c-*jun* genomic DNA fragment (*14*) was nick-translated to a specific activity of 1×10^9 cpm/μg and used to probe the nitrocellulose filter. Hybridization and washing conditions were as described (*41*). The positions of the 28*S* and 18*S* ribosomal RNA's indicated were determined by ethidium bromide staining of the gel before transfer. p39/Jun was detected as a 2.7-kb RNA species in A23187-stimulated H9 cells (lane 2).



A combination of DNAP analysis, gel retardation, mutational studies, and antibody inhibition experiments demonstrated that Fos complexes recognize the AP-1 consensus sequence (*22, 23*). Thus, as would be predicted, affinity-purification with oligonucleotides containing AP-1 sites results in copurification of Fos and several Fos-related antigens along with Jun/AP-1. Of particular interest is whether Fos and FRA's are recovered from AP-1 columns because of interaction with the Jun or because of sequence-specific DNA binding. The following observations are pertinent to these issues. (i) Certain FRA's have now been shown to bind to AP-1 site probes by DNA affinity blotting. (ii) Although Fos was present in the protein preparations, it did not react with the AP-1 probe. (iii) The Fos obtained by in vitro translation of full-length RNA's in reticulocyte lysates and the Fos isolated from COS cells transiently expressing a transfected c-*fos* complementary DNA (cDNA) both fail to bind AP-1 oligonucleotides in gel shift assays. However, Fos from reticulocyte lysates does bind to DNA cellulose. Therefore, it appears that the Fos association with specific regulatory control elements such as AP-1 sites may require the presence of p39/Jun or certain FRA's (or both). Reconstitution experiments with purified proteins are necessary to clarify these issues.

Some of the Fos-related proteins are induced with a slightly delayed kinetics with respect to Fos (*9*). Indeed, serum-stimulation of fibroblasts results in an increase in c-*fos* mRNA, which reaches a peak at 30 to 45 minutes after the cells are stimulated, whereas RNA encoded by *fra*-1 is first detected at 60 minutes and reaches a peak between 90 to 120 minutes (*12*). Thus, a rather complicated scenario could exist involving the interaction or competition of several inducible proteins, including Fos and Jun, for similar DNA regulatory elements. Together with the effects of extensive phosphorylation these interactions could form a fine-tuned system to control gene expression.

It is possible to combine our data with the available information on the complex interactions that occur among proto-oncogenes into a generalized hypothesis. Many proto-oncogene products participate in signal transduction cascades. Intracellular transmission of growth and differentiation signals is stimulated by polypeptide messenger molecules such as platelet-derived growth factor (c-*sis* product) (*34*). Information is received by specific cell-surface receptors such as the epidermal growth factor receptor (c-*erb*^{B} product) (*35*) or colony-simulating factor 1 receptor (c-*fms* product) (*36*). Membrane signals may be transduced by G proteins, like the c-*ras* product, since receptor activation events can be bypassed by microinjection of p21^{ras} (*37*). The mechanism whereby information is

then transmitted to the nucleus is not yet well understood, but it may involve tryosine (c-*src* family) (*38*) or serine (c-*raf*) (*34*) protein kinases. The arrival of the signal in the nucleus precipitates a rapid increase in Fos and Jun. These proteins form a complex that may modulate transcription via specific regulatory elements in the control regions of target genes and thereby effect both short-term and long-term responses to extracellular stimuli such as nerve cell depolarization or differentiation. The components of the biochemical pathway (or pathways) of signal transduction in mammalian cells are being defined and integrated into a model that already suggests multiple opportunities for even subtle alterations to effect deregulation of growth control and development. One consequence of such deregulation is neoplasia. The study of this disease has already resulted in the uncovering of numerous oncogenes that probably contribute to its cause, and simultaneously has provided molecular tools for investigating the normal processes of signal transduction.

REFERENCES AND NOTES

1. C. H. Heldin and B. Westermark, *Cell* **37**, 9 (1984); J. M. Bishop, *ibid.* **42**, 23 (1985); T. Curran, R. Bravo, R. Muller, *Cancer Surv.* **4**, 655 (1985); A. G. Gilman, *Annu. Rev. Biochem.* **56**, 615 (1987).
2. T. Curran, in *The Oncogene Handbook*, E. P. Reddy, A. M. Skalka, T. Curran, Eds. (Elsevier, Amsterdam, 1988).
3. T. Curran and N. M. Teich, *Virology* **116**, 221 (1982).
4. M. P. Finkel, B. O. Biskis, P. B. Jinkins, *Science* **151**, 698 (1966); T. Curran *et al.*, *J. Virol.* **443**, 674 (1982); M. P. Finkel, C. A. Reilly, Jr., B. O. Biskis, I. L. Greco, "Bone tumer viruses," in *Colston Papers, Proceedings of the 24th Symposium of the Colston Research Society* (Butterworths, London, 1973), p. 353.
5. C. Van Beveren *et al.*, *Cell* **32**, 1241 (1983).
6. T. Curran, C. Van Beveren, N. Ling, I. M. Verma, *Mol. Cell. Biol.* **5**, 167 (1985).
7. T. Curran, A. D. Miller, L. Zokas, I. M. Verma, *Cell* **36**, 159 (1984).
8. J. R. Barber and I. M. Verma, *Mol. Cell. Biol.* **7**, 2201 (1987).
9. B. R. Franza, Jr., L. C. Sambucetti, D. R. Cohen, T. Curran, *Oncogene* **1**, 213 (1987).
10. T. Curran and N. M. Teich, *J. Virol.* **42**, 114 (1982).
11. R. Muller, R. Bravo, J. Burckhardt, T. Curran, *Nature* **312**, 716 (1984); T. Curran and J. I. Morgan, *Science* **229**, 1265 (1985); *Proc. Natl. Acad. Sci. U.S.A.* **83**, 8521 (1986); J. I. Morgan and T. Curran, *Nature* **322**, 552 (1986).
12. D. R. Cohen and T. Curran, *Mol. Cell. Biol.*, in press.
13. P. K. Vogt, T. J. Bos, R. F. Doolittle, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 3316 (1987).
14. D. Bohmann *et al.*, *Science* **238**, 1386 (1987).
15. T. J. Bos, D. Bohmann, H. Tsuchie, R. Tjian, P. K. Vogt, *Cell* **52**, 705 (1988); T. Curran and B. R. Franza, unpublished results.
16. P. Angel *et al.*, *Nature* **332**, 166 (1988).
17. L. C. Sambucetti and T. Curran, *Science* **234**, 1417 (1986).
18. C. R. Setoyama *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 3213 (1987).
19. T. Curran and J. I. Morgan, *Science* **229**, 1265 (1986); *Bioessays* 7, 255 (1987).
20. R. J. Distel, H. S. Ro, B. S. Rosen, D. L. Groves, B. M. Spiegelman, *Cell* **49**, 835 (1987).
21. B. R. Franza, S. F. Josephs, M. Z. Gilman, W. Ryan, B. Clarkson, *Nature* **330**, 391 (1987).
22. B. R. Franza, F. J. Rauscher III, S. F. Josephs, T. Curran, *Science* **239**, 1150 (1988).
23. F. J. Rauscher III, L. C. Sambucetti, T. Curran, R. J. Distel, B. M. Spiegelman, *Cell* **52**, 471 (1988).
24. W. Lee, A. Haslinger, M. Karin, R. Tjian, *Nature* **325**, 368 (1987).
25. H9 cells are a $CD4^+$ human lymphoblast cell line that supports replication of HIV-I [M. Popovic, M. G. Sarngadharan, E. Reed, R. C. Gallo, *Science* **224**, 497 (1984)].
26. J. Garrels and B. R. Franza, *J. Biol. Chem.*, in press; *ibid.*, in press.
27. K. Ryder, L. F. Lau, D. Nathans, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 1487 (1988).
28. W. K. Miskimins, *ibid.* **82**, 6741 (1985).
29. P. Angel *et al.*, *Cell* **49**, 729 (1987); W. Lee, P. Mitchell, R. Tjian, *ibid.*, p. 741.
30. D. Bohman and R. Tjian, unpublished data.
31. F. J. Rauscher III *et al.*, unpublished results.
32. C. Dickson and M. Atterwill, *J. Virol.* **26**, 660 (1978).
33. No structural differences between complexed and free p39/Jun have been detected by peptide mapping or 2D gel analysis (R. Franza and T. Curran, unpublished results.
34. R. F. Doolittle *et al.*, *Science* **221**, 275 (1983); M. D. Waterfield *et al.*, *Nature* **304**, 35 (1983).
35. J. Downward *et al.*, *Nature* **307**, 521 (1984).
36. C. J. Sherr *et al.*, *Cell* **41**, 655 (1985).
37. D. W. Stacey and H.-F. Kung, *Nature* **310**, 508 (1984); J. R. Feramisco *et al.*, *Cell* **38**, 109 (1984); D. W. Stacey, T. Wilson, H.-F. Kung, T. Curran, *Mol. Cell. Biol.* **7**, 523 (1987).
38. For review see J. Brugge, in *The Oncogene Handbook*, E. P. Reddy, A. M. Skalka, T. Curran, Eds. (Elsevier, Amsterdam, 1988).
39. K. Moelling *et al.*, *Nature* **312**, 558 (1984).
40. C. Auffray and F. Rougeon, *Eur. J. Biochem.* **107**, 303 (1980).
41. J. Morgan, D. R. Cohen, J. L. Hempstead, T. Curran, *Science* **237**, 192 (1987).

42. We thank J. D. Watson for encouragement, J. Ross and P. Voulalas for technical assistance, and J. Duffy and P. Renna for preparing the artwork and photography. The QUEST facility at CSHL prepared the 2D gels. Supported by NIH grant CA 42564 (P.K.V.); National Cancer Institute grant CA40512 (B.R.F); the Cancer Research Institute of New York (B.R.F.); and the American Foundation for AIDS research (B.R.F.).

5 April 1988; accepted 25 April 1988