course of kindling, before development of generalized seizures and, more generally, after synchronous activation of neural pathways, is consistent with the hypothesis that abnormal activity can induce structural reorganization that promotes epileptogenesis. Repeated seizure-induced alterations in hippocampal connectivity could also cause neuronal and behavioral dysfunction in chronic epileptic disorders.

Our observations have been confined to the hippocampal mossy fiber pathway, but similar phenomena may occur in other pathways of the brain.

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12. Rats kindled via the olfactory bulb and amygdala were implanted with permanent electrodes and were stimulated by the methods used for perforant path kindling [see Fig. 1 for details] (*3*). Rats receiving olfactory bulb implants experienced a kindled generalized (class 5) seizure after 7.6 ± 3.6 afterdischarges. Rats receiving amygdala implants experienced class 5 seizures after 9.0 ± 2.8 afterdischarges.
13. Electron micrographs of the dentate gyrus were obtained from rats kindled by perforant path, olfactory bulb, and amygdala stimulation. Eighteen hours after the last kindled generalized seizure, the rats were perfused in accordance with the Timm method as modified for electron microscopy (*10*).
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The Fos Complex and Fos-Related Antigens Recognize Sequence Elements That Contain AP-1 Binding Sites

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The Fos protein complex and several Fos-related antigens bind directly or indirectly to a common sequence element that is similar to the consensus binding site for HeLa cell activator protein 1 (AP-1). This element is present in a negative regulatory sequence in the differentiation-sensitive adipocyte gene, aP2; in a transcriptional enhancer for the Gibbon ape leukemia virus; and in a region of the human immunodeficiency virus (HIV) long terminal repeat partially characterized as a negative regulatory element. The protein level and binding activity of Fos and Fos-related antigens increase rapidly after calcium ionophore treatment of a CD4$^+$ human lymphoblast cell line, H9. These data suggest that several proteins may associate with the AP-1 binding site. Moreover, temporally regulated control of the level of each protein could represent a mechanism for modulation of these putative mediators of gene expression.

THE PROTO-ONCOGENE c-*fos* encodes a nuclear phosphoprotein (Fos) that is associated with chromatin and that displays DNA binding in vitro (*1*). Fos is expressed at relatively low levels in the majority of cell types; however, it can be rapidly and transiently induced by a great variety of extracellular stimuli (*2*). It has been proposed that Fos might function as a nuclear "third messenger" molecule in coupling short-term signals elicited by cell-surface stimulation to long-term alteration in cellular phenotypes by regulating expression of specific target genes (*3*). Indeed, v-*fos* has been shown to stimulate transcription of selected promoters in trans (*4*). Recently, evidence was presented that Fos participates in a nuclear protein complex with a sequence element in a control region of the adipocyte (3T3-F442A) differentiation-sensitive gene, aP2 (*5*). We noticed considerable similarity between the results of oligonucleotide gel retention assays done with the fat-specific element 2 (FSE2) by Distel *et al.* (*5*) to those of Quinn *et al.* (*6*) with an enhancer element from the Gibbon ape leukemia virus (GALV) long terminal repeat (LTR). These two oligonucleotides share a sequence motif within the region (−357 to −278) of the HIV-LTR that acts as a negative regulatory element (NRE) (*7*). This common sequence element is remarkably similar to the consensus binding site AP-1 (*8–10*) (see Fig. 1). To investigate a possible association of Fos with these nucleotide sequences we have applied an assay system that permits direct analysis of cellular proteins that interact with specific nucleic

Fig. 1. Sequences of synthetic oligonucleotides used in DNAP assays. DFSE2 is a two-site iteration of the aP2 gene control element (bases −122 to −98 from transcription start site) as described by Distel *et al.* (*5*). DMFSE2 is a two-site iteration of a mutant of FSE2. The sites of mutation are −111 and −109. This mutant was designed to minimally disrupt the AP-1 site of FSE2. DGALVEN is a two-site iteration of the region identified in the GALV Seato strain LTR as sufficient for enhancer activity by Quinn *et al.* (*6*). HIV −(357/316) is a single iteration of these bases in the HIV-LTR as described previously (*7*). The * designates bases of significant sequence similarity to the reported AP-1 consensus (*8–10*). The underlined bases indicate the two-base insertion between the two sequence elements. Oligonucleotides were synthesized, purified, and biotinylated as described (*11*). Under the complementary strand arrows indicate the 5′ to 3′ orientation of the AP-1 related sequences.

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acid sequences, referred to as a microscale DNA-affinity precipitation (DNAP) assay (*11*). In conjunction with high-resolution, two-dimensional (HR2D) gel electrophoresis the assay has detected proteins that interact with an HIV enhancer, including a protein whose detection in certain cell lysates is dependent on the cells having been stimulated by the T-lymphoblast activator, phytohemagglutinin (PHA) (*9*).

Many agents that induce transcription of latent, proviral DNA or reporter genes linked to the HIV-LTR (*12*) also induce a substantial level of Fos expression (*13*). While no causal link between these two phenomena has yet been demonstrated, lymphocyte cell lines provide a useful source to the study of regulated Fos expression. Here we have used the human $CD4^+$ lymphoblast line H9 (*14*), which supports HIV replication, as a source of Fos protein complexes and Fos-related antigens for use in the microscale DNA affinity precipitation assays.

The antibody used to identify Fos in H9 cell extracts was raised against c-*fos* amino acids 127 to 152 (*15*). This antibody (anti-Fos) precipitates Fos in the form of a protein complex with a set of cellular proteins (*15, 16*). In addition, several Fos-related proteins are also immunoprecipitated from extracts made from cells in which Fos has been induced. These related antigens appear to be encoded by c-*fos*–related genes that contain an evolutionarily conserved epitope (*16*); for a description of terminology used see (*17*). Figure 2a shows that Fos and two Fos-related antigens (FRA1 and FRA2) immunoprecipitated from H9 cell extracts can be resolved on HR2D gels. After 50 minutes of treatment with the calcium ionophore A23187 substantial increases in the levels of Fos, Fos-related antigens (FRAs), and a Fos-associated protein (FAP p39) were observed (Fig. 2b).

Similar extracts from untreated and A23187-treated H9 cells were used to investigate protein binding to an oligonucleotide containing two iterations of the FSE2 element (DFSE2; Fig. 1) by means of the DNAP assay. Figure 3A, panel a, shows an HR2D gel containing proteins from uninduced H9 cells recovered from a control reaction that contained all components except biotinylated-DFSE2 (b-DFSE2). Addition of b-DFSE2 to an identical reaction with uninduced H9 cellular extract resulted in recovery of four classes of proteins (compare Fig. 3A, panels a and b). They are: (i) approximately equivalent amounts of certain proteins seen in the control reaction such as actin (Ac) and tubulin (T), referred to as nonenriched, background proteins; (ii) Fos; (iii) FAP p39; and (iv) proteins not observed in immunoprecipitations of similar H9 cell extracts with the anti-Fos. Proteins in class iv are designated A, B, C, D, and E. Recovery of A, C, D, and E polypeptides was significantly enriched by the DFSE2 synthetic biotinylated oligonucleotide. Recovery of B was enriched by b-DFSE2 but also by several other biotinylated oligonucleotides (*18*). In A23187-induced H9 cells, the four classes of proteins described above were observed, as well as a fifth class composed largely of FRAs (Fig. 3A, panels c and d). There was a marked increase in the amount of Fos and FAP p39 recovered after stimulation, consistent with the immunoprecipitation analysis of A23187-induced H9 cells. The recovered amounts of A, B, C, D, and E reflect an apparent insensitivity of these polypeptides to 50 minutes of exposure to A23187. Clearly the Fos complex and Fos-related antigens enriched by immunoprecipitation by anti-Fos (Fig. 2b) are also significantly enriched in the FSE2 binding assay.

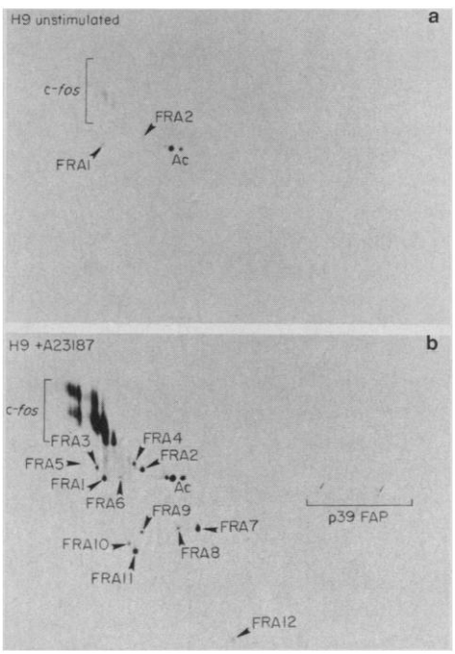


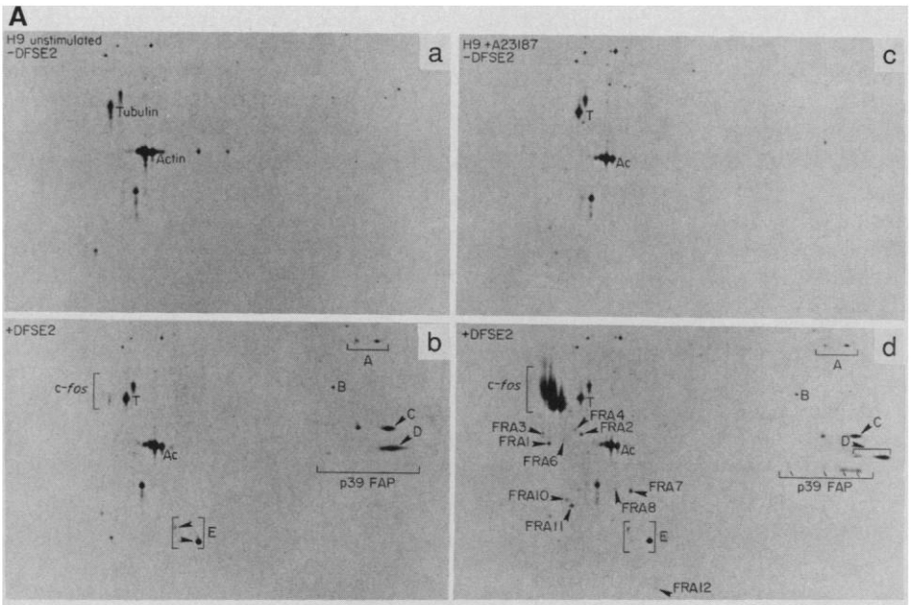
Fig. 2. Immunoprecipitation analysis of H9 cellular extracts stimulated by Ca^{2+} ionophore A23187. H9 cells (5×10^7) were either used as controls or stimulated by the addition of A23187 to a final concentration of $10^{-6}M$. After 15 minutes, 1.0 mCi [^{35}S]methionine was added to each reaction, and 30 minutes later the cells were harvested and immunoprecipitated as described (*16*). The Fos antibody used had been raised against a synthetic peptide (c-*fos* amino acids 127 to 152) (*15*). Preparation of immune complexes for HR2D gel analysis was as described (*16*). (**a**) Proteins immunoprecipitated from unstimulated H9 cells and resolved on a *p*H 3.5 to 10 isoelectric focusing gel (acidic proteins oriented to the left) and a second-dimension 10% polyacrylamide-SDS gel (apparent molecular size decreasing from top to bottom of the image). Only a portion of the HR2D gel image is presented in this and subsequent figures. (**b**) The same region of a HR2D gel analysis of the A23187-stimulated H9 cells. Multiple forms of Fos arising from post-translational modification are labeled c-*fos*; FRAs, FAP p39, and actin (Ac) are marked accordingly. The FRA numbering system is arbitrary, and is used to facilitate comparisons in this report.

To indicate the specificity of the affinity precipitation analysis of DFSE2 binding of cellular proteins, an oligonucleotide was synthesized that had only two bases of the FSE2 native site altered (Fig. 3; compare DFSE2 with DMFSE2). The two-base alteration was selected to determine if a minimal alteration of the AP-1–related sequence in FSE2 would result in any reduction in the interaction with Fos. A substantial reduction in Fos and FRA1 recovery occurred when the mutant construct was used (Fig. 3B, panel b) instead of the wild-type FSE2 oligonucleotide (Fig. 3B, panel c). The actual recovery of Fos and FRA1 is indicated by comparing the region of the HR2D gel containing the starting material (Fig. 3B, panel a) with the wild-type FSE2 binding reaction (Fig. 3B, panel c). Studies with mutants of the AP-1 site including alterations of the 5′ TG (TGACTCA) indicated a >90% reduction of Fos-complex association (*18*).

One of the features of DNAP analysis is the ability to compare different sequence elements to see if they bind similar or unique proteins. Synthetic constructs that mimic the GALV enhancer sequence and the region from −357 to −316 of the HIV-LTR [−(357/316)] region were therefore compared to FSE2 by a combination of DNAP analysis and Western blotting with anti-Fos (*13*). H9 cells stimulated with A23187 for 50 minutes were extracted and recovered by affinity precipitation analysis with either no biotinylated oligonucleotide (Control), b-DFSE2, b-GALVEN, or b-HIV −(357/316). The recovered proteins were resolved on a one-dimensional polyacrylamide gel and then transferred to nitrocellulose (*19*). Induction of Fos and FRA occurred as evidenced by comparing the recovered proteins from stimulated and unstimulated cellular extracts (Fig. 4A). A difference in the amount of Fos and FRA recovered with each biotinylated construct is evident in both the stimulated and unstimulated samples. A likely explanation for why the HIV −(357/316) construct recovers less Fos and FRA relative to the other constructs is that it is not a multimerized set of binding sites. Also, the close approximation of the two AP-1–related sequences may not permit double occupancy as would be seen with multimerized, farther-spaced sets of binding sites such as those in DFSE2 and DGALVEN. However, it is clear from this study that Fos is recovered, and that A23187 induction augments this recovery for each

construct analyzed. A construct containing the other AP-1–like site (−291 to −299) in the HIV-LTR also binds the Fos complex and Fos-related antigens (*18*).

A direct comparison of A23187-stimulated H9 cellular proteins that associate with either the b-DFSE2 or b-HIV −(357/316) construct is shown in Fig. 4B. As seen in the Western blot (Fig. 4A), the b-DFSE2 probe is more efficient than the b-HIV −357/316) probe in complexing Fos and FRAs. Specific proteins complexed with b-



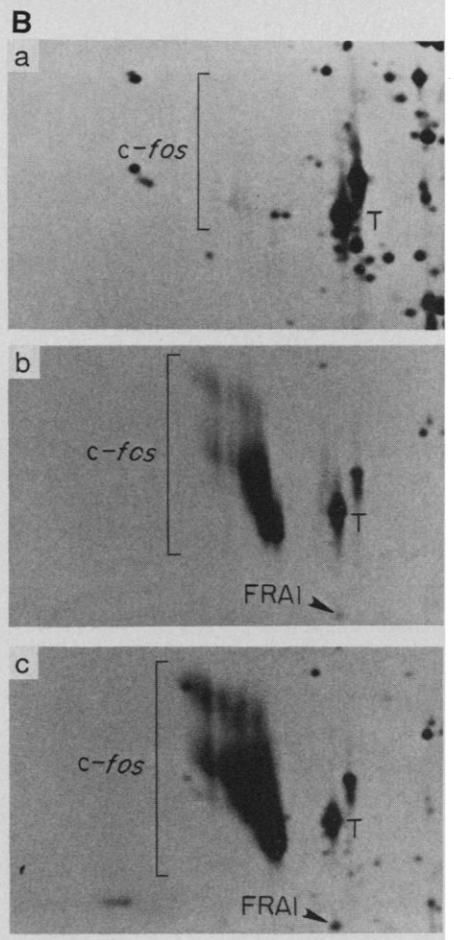


Fig. 3. (**A**) DNAP analysis of H9 cellular proteins that bind the DFSE2 oligonucleotide. H9 cells (1×10^8) were either used as controls or stimulated by the addition of A23187 to a final concentration of $10^{-6}M$. Cells had been removed from their normal growth media [RPMI + 15% calf serum] and resuspended in D-methionine prior to adding the A23187. After 20 minutes 2.0 mCi of [^{35}S]methionine was added to each reaction and 30 minutes later the cells were harvested. All extractions (whole cell), DNAP assays, and gel separation procedures were done as described (*11*). The final potassium chloride (KCl) concentration for extraction was 300 m*M* and sodium fluoride (NaF) was 50 m*M*. The binding reactions were done with 50 m*M* KCl and 1.5 m*M* $MgCl_2$ final concentrations, and 40×, by mass, poly(dI-dC)-(dI-dC). The amount loaded onto each HR2D gel represents approximately 20% of each reaction. The exposure was for 17 days on XAR film at −70°C. Panel a, proteins from unstimulated cells bound to the streptavidin-agarose (SAA) matrix in the absence of any biotinylated (b-) oligonucleotide. Panel b, proteins recovered by addition of 100 pmol of b-DFSE2 to the reaction. Panel c, proteins from A23187-stimulated cells that bound to the SAA matrix in the absence of b-oligonucleotide. Panel d, proteins from A23187-stimulated cells that are recovered by addition of 100 pmol of b-DFSE2. The multiple forms of Fos arising from post-translational modification (labeled) c-*fos*, FRAs, FAP p39, and other b-DFSE2–recovered proteins (A, B, C, D, and E) are designated, as well as actin (Ac) and tubulin (T). The small hatch marks in the FAP p39 region indicate the number of forms of this protein resolved on HR2D gels. (**B**) DNAP analysis of H9 cellular proteins that bind the DFSE2 and DMFSE2 oligonucleotides. All conditions including numbers of cells used for these assays are identical to those described in (A). Exposure was for 17 days on XAR film at −70°C. Panel a, a region from the HR2D gel image of the whole-cell lysate (H9 + A23187) used for the DNAP analysis; approximately 100,000 dpm were loaded. Panel b, proteins recovered from A23187-stimulated H9 cells by addition of 100 pmol of b-DMFSE2. Panel c, the proteins recovered from A23187-stimulated H9 cells by addition of 100 pmol of b-DFSE2. Approximately 20% of each reaction loaded onto the HR2D gels.

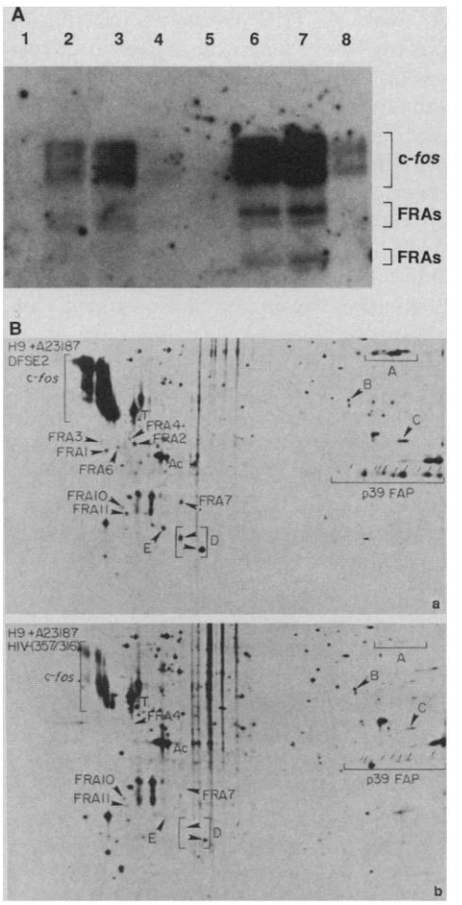


Fig. 4. (**A**) Western blot analysis of DNAP-recovered proteins. All conditions for A23187 treatment of cells (including transferring cells to D-methionine media) were as described in Fig. 3. Proteins extracted from the SAA matrix were resolved on a one-dimensional 9% polyacrylamide gel and then transferred to nitrocellulose for immunoblotting (*19*). Immunoblotting was done with anti-Fos and detected by subsequent reaction with ^{125}I-labeled protein A (30 μCi/mg; Amersham) followed by autoradiography for approximately 6 days. H9, lanes 1 to 4; H9 + A23187, lanes 5 to 8. Lanes 1 and 5, no b-oligonucleotide (control); lanes 2 and 6, b-DFSE2; lanes 3 and 7, b-DGALVEN; lanes 4 and 8; b-HIV −(357/316). (**B**). DNAP analysis of A23187-stimulated H9 cells reacted with b-DFSE2 or b-HIV −(357/316). All conditions as in legend to Fig. 3, except that twice as many cells were used for each reaction. Panel a represents a region from the HR2D gel image of the A23187-stimulated H9 proteins that were recovered from a reaction containing 100 pmol of b-DFES2. Panel b represents the proteins recovered from a reaction containing 100 pmol of b-HIV −(357/316). Approximately 40% of the reactions were loaded onto the HR2D gels. Exposure was for 20 days on XAR film at −70°C. Multiple forms of Fos (labeled c-*fos*); FRA; FAP p39; A, B, C, D, and E proteins; actin (Ac); and tubulin (T) are shown. The small hatch marks in the FAP p39 region indicate the number of forms of this protein are resolved on HR2D gels.

DFSE2 that did not bind b-HIV −(357/316) and the converse is also true, indicating the potential contribution of sites other than AP-1 in each synthetic construct to the recovery of other, specific cellular proteins. The amount of the protein designated C was approximately twofold greater in the b-DFSE2 assay. This protein is particularly interesting in that it migrates between 47 and 45 kD. The apparent molecular size of AP-1 has been reported to be 47 kD (*10*). The spot designated C is different from Fos and the Fos-related antigens in that it does not appear to be sensitive to A23187 in H9 cells as determined by the amounts recovered in DNAP analysis with b-DFSE2 (Fig. 3A, compare panel b and d). A 12-hour stimulation with phorbol 12-myristic 13-acetate (PMA) induces transcription of reporter genes linked to synthetic constructs containing either the AP-1 consensus sequence or AP-1 related sequences from different genes (*9, 10*). It will be interesting to see if the protein designated C that interacts with the DFSE2 oligonucleotide is sensitive to PMA to the same extent as Fos and Fos-related antigens (*18*). It may be that indirect assays such as gel retardation or nuclease protection have not discriminated multiple cellular proteins, some of which are inducible, that interact with AP-1 sites.

In DNAP analysis of A23187-stimulated H9 cells in which Fos was clearly detected in association with b-HIV −(357/316), and with b-DFSE2 and not detected in association with an HIV enhancer element oligonucleotide, b-HIVEN3c (*11, 20*). Therefore the interaction of Fos and FRAs is dependent on nucleic acid sequences unique to the FSE2 and HIV −(357/316) oligonucleotides.

The significance of the Fos complex and Fos-related antigen interaction with the aP2 element, the GALV enhancer element, and a portion of the NRE in the HIV-LTR remains to be determined. The results of our studies suggest that the Fos complex and Fos-related antigens represent a class of inducible cellular proteins that could modulate the expression of genes containing the AP-1 binding sites. It is probable, given that each element studied contains a sequence related to the AP-1 consensus, that a number of cellular proteins may interact directly or indirectly (through protein-protein contact) with AP-1 sites. It is most likely that the AP-1–like site in each element contributes to the recovery of the Fos complex and Fos-related antigens in that Fos interacts with synthetic AP-1 consensus oligonucleotides (*18*). These data imply that several different cellular proteins can interact with the same regulatory element. The consensus binding site for the yeast transcription factor GCN4 (*21*) and the v-*jun* oncogene (*22*) are identical to the AP-1 motif (*8–10*). Recently, a similarity between the predicted amino acid sequence of a partial c-*jun* complementary DNA clone and the amino acid sequences of certain AP-1 peptides has led to the suggestion that c-*jun* encodes AP-1 (*23*). A low level of sequence similarity between v-*jun* and v-*fos* has also been reported (*22*). Furthermore, one of the Fos-related antigens displays maximal homology to Fos in the GCN4/jun/AP-1–related region (*24*). Although this conserved region corresponds to the portion of GCN4 thought to be involved in DNA binding (*21*), the data presented here cannot distinguish among the following possibilities: (i) Fos and Fos-related antigens bind directly to the AP-1 site; (ii) they bind by way of interaction with FAPs such as p39; or (iii) they bind to an AP-1 protein.

When more than one protein is suspected of interacting with a specific sequence element, and at least one of these proteins is inducible, the scenario exists for that sequence element to play several roles in gene regulation depending on the status of the cell. It is possible that positive and negative effects on gene expression could be mediated by the same element depending on the particular cellular proteins to which it is associated. A comparative and quantitative study, employing microscale DNA affinity precipitation analysis, can now be used to monitor the sequence-specific binding proteins present in cells at any time following application of extracellular stimuli. Such analysis is anticipated to reveal the temporal changes in protein–nucleic acid interaction required for the exquisite modulation of gene expression responsible for control of growth and development.

Note added in proof: A report providing further evidence for Fos being associated with AP-1–similar sites in FSE2 has recently been accepted for publication (*25*).

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