A Direct Role for c-fos in AP-1-dependent Gene Transcription

Roman Herrera, Sadhana Agarwal, Kevin Walton, Brett Satterberg, Robert J. Distel, Richard Goodman, Bruce M. Spiegelman, and Thomas M. Roberts

Dana-Farber Cancer Institute [R. H., S. A., B. S., R. J. D., B. M. S., T. M. R.], Department of Biological Chemistry and Molecular Pharmacology [R. H., R. J. D., B. M. S.], and Department of Pathology, [S. A., T. M. R.], Harvard Medical School, Boston, Massachusetts 02115, and Division of Molecular Medicine, Tufts-New England Medical Center, Boston, Massachusetts 02111 [K. W., R. G.]

Abstract
Transcription factor activator protein 1 (AP-1) is a protein fraction that contains c-fos, c-jun, and several other related proteins. Although this protein fraction can stimulate transcription in vitro, the relative contributions of c-fos and c-jun to the transcriptional effect of AP-1 are not clear. In order to approach this question, we have overexpressed both proteins using a baculovirus-mediated expression system and defined their DNA-binding and transcriptional enhancement activities in vitro. Gel mobility-shift and DNase I footprinting assays showed that c-jun protein specifically binds to DNA through an AP-1 binding site. Under the same conditions, no detectable binding of c-fos protein was observed. However, when the DNA binding assays were performed in the presence of both c-jun and c-fos, a marked increase in the affinity of c-jun for the AP-1 site was observed.

An AP-1-dependent transcription assay was used to test the capability of both proteins to stimulate correctly initiated RNA synthesis in vitro. Under our conditions, c-jun protein was capable of stimulating specific RNA transcription in an AP-1 site-dependent manner. In contrast, c-fos protein showed no detectable transcriptional activation by itself. However, a transcription assay carried out in the presence of both c-fos and c-jun proteins showed that the c-fos/c-jun complex was more active as a transcriptional regulator than c-jun protein alone. These experimental results indicate that c-fos and c-jun proteins are required to reconstitute full AP-1-dependent transcriptional activation and directly demonstrate that c-fos is a regulator of gene expression.

Introduction
The mechanisms of signal transduction that are involved

in the regulation of eukaryotic gene expression are poorly understood. A model system that has been very useful in studying the molecular steps involved in the stimulation of gene expression by extracellular stimuli is that of phorbol ester-induced genes. Current evidence indicates that tumor promoter agents, such as 12-O-tetradecanoylphorbol-13-acetate, exert the majority of their biological effects by activating the cellular enzyme protein kinase C (1).

Several genes that are induced transcriptionally by TPA\(^2\) have been identified. Analysis of the 5' flanking sequences of some of these genes (human metallothionein Ila, collagenase) led to the identification of a short DNA sequence (TGACTCA) that behaves as a TPA-induced enhancer (2). This TPA-responsive element was shown to be identical to the recognition sequence of a previously identified protein fraction designated activator protein 1. Purified AP-1 transcription factor has been shown to interact with the TRE sequence and to stimulate transcription in vitro of the human metallothionein Ila gene (3). Both activities were shown to be associated with a M. 47,000 protein present in the purified AP-1 preparation (3).

A crucial observation that related the activity of AP-1 to a previously identified oncogene (v-jun) was the fact that the recognition sequence for AP-1 was very similar to the recognition sequence of GCN4 (4, 5), a yeast transcriptional factor with extensive sequence homology to v-jun (6). The logical hypothesis was to suggest that AP-1 represented the cellular homologue of the v-jun gene. Indeed, Bohmann et al. (7) and Angel et al. (8) have shown that the protooncogene c-jun encodes a DNA-binding protein with structural and functional properties identical to AP-1. Similarly, the oncogenic form of c-jun (v-jun) also possesses the enhancer-binding properties of AP-1 (9). Thus, the above observations represent one of the mechanisms that is thought to be involved in cellular transformation, i.e., direct transcriptional activation of cellular genes.

A second level of regulation of the AP-1 transcription factor was delineated by the discovery that a protein complex containing the protooncogene product c-fos and related proteins had sequence-specific DNA-binding activity (10-13). A combination of studies involving DNA-affinity precipitation, antibody neutralization, specific competition, and mutagenesis revealed that the sequence recognized by this fos-containing protein complex was identical to the consensus sequence recognized by transcription factor AP-1 (11-13). This observation raised the possibility that the c-fos protein may be a

\(^{1}\) This work was supported by NIH Grant HD24926 (B. M. S.) and CA43803 (T. M. R.). R. H. is a Damon Runyon-Walter Winchell postdoctoral fellow (DRG-936). R. J. D. is a Medical Foundation postdoctoral fellow. B. M. S. is an Established Investigator of the American Heart Association.

\(^{2}\) To whom requests for reprints should be addressed, at Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115.

\(^{3}\) The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; TRE, TPA-responsive element; AP-1, activator protein 1; kb, kilobase(s); cDNA, complementary DNA; NF-40, Nonidet P-40; SDS, sodium dodecyl sulfate; AcNPV, Autographa californica nuclear polyhedrosis virus; SF9, Spodoptera frugiperda.
functional component of the AP-1 transcription factor.

Earlier studies carried out on c-fos protein showed that this nuclear protein formed a complex with an M_68,000 protein (p39) present in many cell types (14–16). Subsequent studies identified p39 as the product of the c-jun protooncogene (13, 17, 18). These results led to the conclusion that both fos and jun proteins form part of a complex capable of specific DNA-binding activity through interaction with an AP-1 binding site.

Other indirect evidence supported the notion that a fos/jun complex is involved in the functional activity of an AP-1 site. Cotransfection experiments designed to study activation of gene expression by c-jun have shown that c-jun activity can be amplified by coexpression of c-fos protein (17, 18). In a separate experiment, it was shown that fos-mediated transactivation of gene expression required a functional AP-1 site (19, 20). Similarly, induction of AP-1-responsive target genes by TPA and oncogenes such as ras and src required a fos/jun interaction (20).

The transcriptional activation mediated by an AP-1 binding site has been clearly demonstrated both in vivo and in vitro. However, the transcriptional activity of various components of the AP-1 protein complex has not been dissected. The AP-1 protein complex is a family of related proteins of which c-fos and c-jun are the best characterized. Although previous studies have shown that c-jun protein is capable of both binding to DNA and stimulating transcription (8, 9), the role of c-fos protein as part of the AP-1 complex is not clear. Purified preparations of AP-1 contain not only fos and jun, but a set of proteins named fos-related antigens (12, 13) as well as an M_68,000 protein that is capable of binding to an AP-1 site (13). Thus, given the above-mentioned facts, it is necessary to define whether the various species present in AP-1 complex are not only capable of binding to DNA but also able to stimulate transcription through an AP-1 binding site. We have approached this question by directly testing the transcriptional activities of the two main components of the AP-1 transcription complex, namely c-fos and c-jun. We demonstrate that baculovirus-expressed c-fos protein stimulates transcription in vitro in a c-jun-dependent fashion.

Results

Production of c-fos and c-jun Proteins in Insect Cells.

The helper-independent baculovirus-based expression system has been widely used as a means to obtain functionally active proteins of diverse biological activities (21). We have used this system to express the protooncogenes c-fos and c-jun in order to investigate the nature of fos/jun interaction and to study the functional relationship of these proteins in relation to gene regulation mediated by the AP-1 binding site.

The cDNAs of c-jun (1.7 kb) and c-fos (1.3 kb) were

![Fig. 1. Baculovirus-mediated expression of c-fos and c-jun proteins in insect cells. Immunoblot of cellular extracts prepared from mock (A and B, Lane 1), wild-type (WT; A and B, Lane 2), Bac-fos (A, Lane 3), and Bac-jun (B, Lane 3) infected Sf9 cells. Total cellular extracts were prepared 40 h postinfection, and the proteins were resolved on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with antipeptide antibodies directed against either c-fos (A) or c-jun (B), as described in "Materials and Methods." The sizes of the molecular weight markers are indicated on the left in kD.](image-url)
subcloned into the expression vectors pVL941 and pAC373, respectively, and transected into Sf9 cells in order to obtain recombinant viruses by in vitro recombination of wild-type viral DNA with the transfer vector carrying the c-fos or c-jun cDNA. Recombinant baculoviruses carrying either the fos gene (Bac-fos) or the jun gene (Bac-jun) were isolated, and their ability to direct the synthesis of either c-fos or c-jun proteins was tested by immunoblotting of total cellular extracts prepared from infected cells. Correct expression of the inserted cDNA fragments in Bac-fos or Bac-jun in infected Sf9 cells should result in proteins of M, 55,000–62,000 or M, 39,000–40,000, respectively. As shown in Fig. 1, cell extracts derived from either Bac-fos (Fig. 1A) or Bac-jun (Fig. 1B) infected cells contain significant amounts of expressed protein of approximately the expected molecular weight. The proteins are specifically recognized by antibodies generated against the synthetic peptide Pep1 of the c-jun protein (9) (Fig. 1B) or by affinity-purified antibodies generated against the M peptide (22) of c-fos (Fig. 1A). These proteins are not detectable in extracts of uninfected cells or of cells infected with the wild-type virus (Fig. 1A and B, Lanes 1 and 2). As it has been observed for mammalian c-fos, baculovirus-expressed c-fos is heterogeneous in size. This is due, in part, to extensive phosphorylation, since treatment of this protein with potato acid phosphatase reduces the degree of heterogeneity (data not shown). The expression of these proteins followed a time course characteristic of the activation of the viral polyhedron promoter (21). Both proteins can be recovered in a soluble form from total cell lysates. We estimate that each represents approximately 1% of total cellular protein.

**DNA-binding Activity of c-fos and c-jun Proteins.** The DNA-binding activity of c-jun has been clearly demonstrated using bacterially expressed protein (7, 8). Recent results obtained with protein translated in vitro have shown that c-jun is capable of forming a homodimer which is the functional species binding to DNA (23–26). It has also been shown using gel retardation assays that in vitro-translated c-fos and jun proteins can form a heterodimer that binds to DNA with higher affinity than the jun homodimer (23–27), although it has been claimed that this effect cannot be observed in other assays (27). The formation of the dimer requires a conserved leucine-rich sequence present not only in fos and jun but also in other DNA-binding proteins such as C/EBP and GCN4 (23, 24, 26, 28). We have tested whether the baculovirus-expressed c-fos and c-jun proteins are capable of associating with DNA, using both the gel retardation and DNase I footprinting assays. Cellular extracts prepared from Bac-jun-, Bac-fos-, or wild-type-infected cells were incubated with a 32P-labeled oligonucleotide containing an AP-1 binding site, and the products of the reaction were analyzed in a low-ionic-strength polyacrylamide gel. As shown in Fig. 2A, a specific DNA-protein complex (arrowhead) is formed only when the source of protein is a Bac-jun-infected cell extract. Neither Bac-fos- nor wild-type-infected cell extracts showed detectable specific DNA-binding activity since the protein-DNA complexes formed with these extracts are not competed away by unlabeled oligonucleotide. Thus, under these conditions, only c-jun is capable of forming a stable DNA-protein complex.

A gel retardation analysis was carried out in the presence or absence of c-fos using different levels of input c-jun protein to test the effect of c-fos on the DNA-binding activity of c-jun protein. As shown in Fig. 2 (compare Lanes 3 and 8), and previously by others (23–27), the presence of c-fos in the gel retardation binding
assay dramatically increased the ability of c-jun to bind to the AP-1 site. The specificity of the DNA-protein complex formed in the presence of both c-fos and c-jun was determined by competition experiments using homologous or heterologous oligonucleotides and neutralizing antibodies. As shown in Fig. 3. Lanes 1–3, the c-fos/c-jun-DNA complex specifically recognizes the AP-1 site since the binding could not be competed out by an oligonucleotide that does not contain an intact AP-1 site. That c-fos and c-jun are components of the DNA-protein complex was shown by the ability of anti-fos and anti-jun antibodies to disrupt or neutralize the formation of this DNA-protein complex (Fig. 3, Lanes 4–7). This effect was not seen when the antibodies were first neutralized with their cognate peptide.

Recently, experimental results reported by Turner and Tjian (27) have challenged the conclusion that c-fos increases the affinity of c-jun for DNA on the basis of their experiments examining the chromatographic properties of c-jun homodimers or c-fos/c-jun heterodimers on sequence affinity columns. In order to further investigate the above conclusion, we have used a DNase I-based assay to detect DNA-protein interactions in solution. A 32P-labeled DNA fragment containing an AP-1 site derived from the 5' flanking sequence of the aP2 gene (10, 29) was incubated with different concentrations of c-jun protein in the presence or absence of c-fos and was then subjected to DNase I digestion. As shown in Fig. 4, the ability of c-jun to protect the AP-1 site against DNase I digestion is enhanced dramatically by the presence of c-fos. The amount of c-jun protein required to obtain significant protection of the AP-1 site against DNase I digestion is lowered by at least 5-fold in the presence of
c-fos protein (compare Lanes 4, 9, and 10 in Fig. 4), in close agreement with the result obtained in the experiment depicted in Fig. 2B. This effect is specific since this DNA template contains other protein-binding sites located close to the AP-1 site that are not protected against DNase I digestion (29).

Transcriptional Activation by c-fos and c-jun Proteins. As described earlier, the transcriptional activity associated with an AP-1 fraction purified from cells has previously been characterized (3). Since the AP-1 protein complex is composed of several components, a defined role for c-fos in the transcriptional activity of AP-1 has never been determined. Therefore, we have carried out a direct assay to test whether transcriptional activation via the AP-1 site can be conferred by c-fos alone, by c-jun alone, or by a c-fos/c-jun complex.

The ability of either c-fos or c-jun to activate correctly initiated RNA transcription from the collagenase promoter, which contains an AP-1 site (at -70) was examined in a transcription reaction in vitro. HeLa cell nuclear extracts were prepared and made deficient in AP-1-binding activity by specific affinity adsorption using a Sepharose-linked AP-1 oligonucleotide. Correctly initiated RNA transcription was measured by primer extension of the collagenase-CAT fusion gene. The initial characterization of the transcriptional activity of the AP-1-depleted HeLa extracts showed that it was 43% of the activity of the undepleted extract, and this activity was stimulated (122% relative to the activity of whole extract) by the addition of extract prepared from Bac-jun-infected cells but was not stimulated by Bac-fos extracts (data not shown). This confirms the transcriptional activity observed with bacterially expressed c-jun protein (7).

The ability of c-fos and c-jun to cooperate in stimulation of in vitro transcription is shown in Fig. 5. The AP-1-depleted transcription system was supplemented with a fixed amount of extract prepared from either wild-type or Bac-fos-infected cells. To these, increasing amounts of c-jun-containing extract were added. As shown in Fig. 5A, the level of transcription of the collagenase promoter (Fig. 5A, large arrow) in the depleted extract was slightly increased (1.7-fold over the internal control, RSV-CAT fusion gene that lacks any AP-1 site. small arrow) by the addition of the highest concentration of c-jun extract (4 μg) in the presence of wild-type extract. However, the transcription of the collagenase promotor was greatly increased (4.1-fold over the control) when the c-jun protein was added to a transcription system containing the c-fos extract. The increased transcription due to the Bac-fos extract was seen at every concentration of the Bac-jun extract used (Fig. 5B). That this effect of the c-fos-containing extract was a direct result of the c-fos protein was demonstrated by pretreating these extracts with an antibody to c-fos. This returned the level of transcriptional activity to that of c-jun alone (data not shown). Thus, these results demonstrate directly that c-fos protein stimulates both the ability of c-jun to bind to DNA and its activity as a transcriptional factor.

Discussion

The experiments described in this paper were designed to answer two related questions: (a) Does c-fos protein stimulate the DNA-binding activity of c-jun? and (b) Can the transcriptional activity of an AP-1 complex be recon-
can be dramatically modulated by interaction with c-fos. Two different DNA binding assays performed with c-jun protein in the presence of c-fos protein show an enhanced DNA-binding activity of c-jun (Fig. 2) over the binding activity obtained with c-jun alone. Under these DNA-binding conditions, the association of baculovirus-produced c-fos and c-jun monomer is a rapid ($t_{1/2} \approx 5$ min) process. It has previously been shown by gel electrophoresis assays that the enhanced affinity of c-jun for DNA in the presence of c-fos is accompanied by physical association of the two proteins, resulting in a complex whose rate of dissociation from DNA is severalfold slower than the c-jun homodimer (24). It is now clear that this effect is not limited to the electrophoresis assays (27), since c-fos effect can also be observed in a solution-based assay (Fig. 4).

Although c-fos protein lacks DNA-binding activity by itself, association with c-jun protein enables it to participate directly in a specific DNA-protein complex. The role of c-fos in this complex is not a passive one. Several lines of evidence support the conclusion that c-fos is a functional player in the c-fos/c-jun complex. First, as mentioned above, the affinity of the c-fos/c-jun heterodimer for DNA is severalfold higher than the affinity of the c-jun homodimer. Second, the DNA-protein complex formed by the c-fos/c-jun dimer can be totally disrupted by specific c-fos antibodies (Fig. 3). This effect is not restricted to the properties of the formed protein complex between c-fos and c-jun in vitro, since the protein complex isolated from preadipocytes (which is capable of binding to an AP-1 site) was also disrupted by anti-fos antibodies (10). The antibodies are effective either by disrupting a preformed DNA-protein complex or by preventing its formation. Finally, careful dissection of the c-fos protein has shown that specific domains (not involved in the dimerization process) are required to form a complex with DNA (26, 27), indicating that c-fos protein is a functional DNA-binding entity as part of the dimer.

A role for c-fos (or its oncogenic form, v-fos) in transcriptional regulation has been inferred from several experimental observations. For example, it has been reported that v-fos could transactivate the type III collagen promoter in cotransfection experiments (30). Similarly, overexpression of c-fos leads to transactivation of TRE-containing reporter genes (19, 20). However, when the capability of c-fos to transactivate TRE-containing promoter was tested in F9 embryonic carcinoma cells, there was little activity obtained by c-fos alone (17). More direct evidence linking c-fos to transcriptional regulation was provided by Lech et al. (31). Hybrid proteins containing the DNA-binding domain of the bacterial LexA repressor and c-fos are capable of efficient activation of gene transcription in yeast cells (31), suggesting that c-fos contains a domain specifically involved in transcriptional activation. However, many different, apparently nonspecific, regions can be active in this assay.

In spite of these data, it has never been shown that c-fos directly participates in activation of gene transcription. The in vitro transcription activation obtained with purified preparations of AP-1 could not define the role of individual components since AP-1 preparations are composed of c-fos, c-jun, and fos-related antigens (4, 7). The availability of both c-fos and c-jun proteins made it possible to measure the transcriptional contribution of the individual proteins in a transcription assay in vitro.

The data presented in Fig. 4 show that c-fos protein does not activate transcription by itself. However, the association of c-fos with c-jun brings about a potent transcriptional activation, well above the level obtained by c-jun protein alone at a comparable dose. Since the transcription system used in these experiments was depleted of AP-1-binding activity, the above results mean either that c-fos and c-jun are sufficient to restore an AP-1 function or that c-fos and c-jun are required for full functionality of an AP-1 complex. Although the transcription system was depleted of AP-1 activity, it is still a very complex mixture of components. Therefore, it is also possible that the activation of transcription, obtained by the addition of c-fos, was mediated by the interaction of c-fos with some other component of this system. Nevertheless, the results clearly demonstrate that c-fos protein is directly involved in this activation. This conclusion is supported by the fact that neutralization of c-fos protein prior to the assay resulted in decreased AP-1-dependent transcription.

The ability of c-fos and c-jun to form a heterodimer brings about a second level of regulation of their DNA-binding activities. It is known that both c-fos and c-jun are members of a family of related genes (32, 33). Therefore, the possibility of cross-association among different fos-related and jun-related proteins adds a further level of complexity. Indeed, it has already been shown that fosB, a growth factor-regulated gene, is capable of combining with either c-jun or junB (34). Similarly, junB and junD are capable of interacting with c-fos (23). More interesting is the fact that the oncogenic form of jun, v-jun, is also capable of forming a heterodimer with c-jun and may have important implications for the mechanism of oncogenic transformation by v-jun. In intact cells, both fos and jun proteins undergo extensive posttranslational modifications, but the effect of these events on their overall activities is not known. The biochemical system that we describe herein will be most useful as a tool to answer this question.

Materials and Methods

Cells and Virus. The wild-type baculovirus, Autographa californica nuclear polyhedrosis virus, and the recombinant virus that carries either the c-fos gene (Bac-fos) or the c-jun cDNA (Bac-jun) were used to infect Spodoptera frugiperda cells at a multiplicity of infection of 2–10 plaque-forming units for protein production and 0.1–1 plaque-forming unit for virus amplification as described (35). SF9 cells were grown and maintained in Grace’s medium supplemented with 10% fetal calf serum. The baculovirus AcNPV, the plasmid transfer vectors pAC373 and pVL941, and SF9 cells were generously provided by Dr. M. Summers, Texas A&M University.

Construction and Isolation of Recombinant Bac-jun and Bac-fos. The human c-jun cDNA (1.7 kb) (8) was isolated from the vector p718 jun (constructed by Bohmann and Tjian), ligated to BamH1 linkers, and subcloned into the transfer vector pVL941 using currently available protocols (36). A 1275-base pair Nael fragment containing the entire coding region of the rat c-fos cDNA was isolated from the plasmid pc-Fos (rat)-1, which was a gift from T. Curran (22). This fragment was ligated to BamH1 linkers and cloned into the transfer vector pAC373. Transfer vectors containing single copies of each gene in
the correct orientation were isolated and characterized by restriction endonuclease mapping. Transfer of the subcloned fragments to the AcNPV genomic sequence was achieved by homologous recombination into the resident polyhedrin gene after cotransfection of both wild-type AcNPV genomic DNA and the corresponding transfer vector (35). Using plaque hybridization and visual screening (35), several recombinant vectors were isolated and characterized. A representative virus expressing either c-fos or c-jun was used in the studies described herein.

**Protein Production and Analysis**

SF9 cells were infected with wild-type baculovirus or with recombinant baculovirus expressing either c-fos or c-jun. At 40 h postinfection, the cells were washed once in phosphate-buffered saline and then lysed in an NP-40 buffer (20 mM Tris-Cl, pH 8.0, 130 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 10% glycerol, 1% NP-40) containing 5 mM phenylmethylsulfonyl fluoride, 0.2 unit/ml aprotinin, 0.02 mM sodium leupeptin, and 0.5 µg/ml pepstatin A. The lysates were cleared by centrifugation. Protein concentration was determined using the BioRad laboratory protein assay kit. Protein analysis was performed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting as described (37). Protocols and reagents of the Protoblot system from Promega Biotec were used for the immunoblots.

**DNA Binding and DNase I Footprinting Analysis**

Total cell extracts (0.5–2 µg) were prepared as described above and incubated with 32P-labeled oligonucleotides (10) in the presence of 150 µg/ml polydeoxyinosinic-deoxyinosinic acid-tetradecyl acid-10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.5–100 mM NaCl-5 mM MgCl₂-1 mM dithiothreitol for 10 min at 20°C. The DNA-protein complexes were separated by polyacrylamide gel electrophoresis as described previously (10). For DNase I footprinting, the extracts were incubated with a 32P-labeled DNA fragment from the ap2 gene promoter (–168 to –79), which contains binding sites for several nuclear factors such as AP-1 and C/EBP (29), and subjected to DNase I treatment as previously described (29). The products of the reaction were analyzed on an 8% polyacrylamide DNA-sequencing gel (30).

**Transcription in Vitro**

HeLa nuclear extracts were prepared by the method of Dignam et al. (38) with protein concentrations ranging from 5 to 10 mg/ml. Extracts were depleted of AP-1-binding activity by incubation at 4°C for 1 h with an equal volume of Sepharose resin derivatized with an oligonucleotide containing an AP-1 site (39). The 0.3-kb collagenase-CAT DNA fragment was derived from pBLCAT2 (40) (a gift from P. Herrlich) as follows. The ScaI to BamH1 fragment was isolated, containing sequences from –300 to +63 of the collagenase promoter, and cloned into pBLCAT2, which was cut with HindIII, blunted with Klenow polymerase, and then cut with BamHI. This eliminated all sequences upstream of –300 in the collagenase promoter. The transcription reactions were performed in a final volume of 50 µl containing 14 µl nuclear extract, 8 µl baculovirus lysis buffer or extract, 0.5 mM each ribonucleotide triphosphate, 1.0 mM spermidine, 3 mM MgCl₂, 22 mM NaCl, 38 mM KCl, 7.6 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.9, 3 mM Tris, pH 8, 9% glycerol, 0.2% NP-40, 10 units RNAsin (Promega), and the indicated amount of plasmid DNA (generally 50–150 ng). The reaction was incubated at 30°C for 1 h, followed by the addition of 20 µl of 1.5 mg/ml protease K in 0.3% SDS, and further incubated at 37°C for 15 min. Fifty µl stop buffer (0.1 mM sodium acetate-C, 1 mg/ml RNAseA-1% SDS) were added, and the mixture was then phenol/isoamyl/chloroform extracted, precipitated with 300 µl of 0.75 mM ammonium acetate, and reprecipitated with sodium acetate and ethanol. The message was resuspended in 10 µl of 625 mM KCl-10 mM Tris, pH 7.9–0.5 mM EDTA containing 5 µM primer (labeled with T4 kinase and 7000 mCi/mmol γ-ATP), heated at 85°C for 3 min, and then allowed to anneal at 37°C for 1 h. The primer is specific for the CAT gene at bases +48 to +68. The reverse transcription reaction was performed in a total volume of 75 µl, containing 60 mM Tris, pH 7.9–10 mM dithiothreitol-4.5 mM MgCl₂, 0.5 mM each deoxyribonucleotide triphosphate, 10 units RNasin, and 100 units Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories). The reaction was incubated at 45°C for 1 h, 10 µl of reverse transcription stop were added (700 µM NaOH-140 mM EDTA), and the mixture was incubated at 65°C for 10 min. The reaction was extracted once again with phenol/chloroform, brought to 0.3 mM sodium acetate, and precipitated with 200 µl ethanol. The pellet was resuspended in 6 µl formamide and analyzed on a 10% polyacrylamide-urea gel at 55°C. The gel was dried and exposed to Kodak XAR film for the indicated amount of time.

**Acknowledgments**

We thank Dr. Tom Curran for the rat c-fos cDNA clone and Dr. Robert Tjian for the cDNA clone for human c-jun. We also thank Adah Levens for expert assistance in the preparation of this manuscript.

**References**


26. Schuermann, M., Neuberg, M., Hunter, J. B., Jenuwein, T., Ryseck, R., Bravo, R., and Muller, R. The leucine repeat motif in fos protein mediates complex formation with Jun/AP-1 and is required for transfor-