Journal of Biochemistry and Molecular Biology, Vol. 32, No. 6, November 1999, pp. 594–598

JOURNAL OF
Biochemistry
and
Molecular Biology

Quantitative Assay for the Binding of Jun-Fos Dimer and Activator Protein-1 Site

Sangkyou Lee, Seyeon Park, Gyo Jun[1], Eun-Ryeong Hahm, Dug-Keun Lee, and Chul-Hak Yang*
Department of Chemistry, Seoul National University, Seoul 151-742, Korea
[1] Ka-Chun Medical School, Kyunggi-Do, Korea

Received 28 June 1999, Accepted 8 September 1999

The Jun and Fos families of eukaryotic transcription factors form heterodimers capable of binding to their cognate DNA enhancer elements. We are interested in searching for inhibitors or antagonists of the binding of the Jun-Fos heterodimer to the activator protein-1 (AP-1) site. The basic-region leucine zipper (bZIP) domain of c-Fos was expressed as a fusion protein with glutathione S-transferase, and allowed to form a heterodimer with the bZIP domain of c-Jun. The heterodimer was bound to glutathione-agarose, to which were added radiolabeled AP-1 nucleotides. After thorough washing, the gel-bound radioactivity was counted. The assay is faster than the conventional electrophoretic mobility shift assay because the gel electrophoresis step and the autoradiography step are eliminated. Moreover, the assay is very sensitive, allowing the detection of picomolar quantities of nucleotides, and is not affected by up to 50% dimethylsulfoxide, a solvent for hydrophobic inhibitors. Curcumin and dihydroguaiaretic acid, recently known inhibitors of Jun-Fos-DNA complex formation, were applied to this Jun-GST-fused Fos system and revealed to decrease the dimer-DNA binding.

Keywords: AP-1, Curcumin, Dihydroguaiaretic acid, Glutathione agarose, GST-fused Fos.

Introduction

Transcription factors are known to be intricately involved in proliferation, differentiation, and tumorigenesis. One such factor, Activator Protein 1 (AP-1), is a homo- or hetero-dimer formed by the products of the *fos* and *jun* proto-oncogene families. The feature of Fos and Jun proteins is the almost identical amino acid sequences that comprise the basic DNA binding sequence (B) and the adjacent leucine zipper region (Zip), by which the proteins dimerize with each other (Curran and Franza, 1988; Mitchell and Tjian, 1987; Johnson and Mcknight, 1989). The AP-1 transcription factor binds specifically to the DNA sequence 5′-TGAG/CTCA, referred to as the AP-1 site (Lee *et al.*, 1987). The expression of *fos* and *jun* is autoregulated. The transcription of *jun* is stimulated by its own product in contrast to *fos*, which is negatively autoregulated (Angel *et al.*, 1988; Sassone-Corsi *et al.*, 1988; Angel and Karin, 1991). The c-*jun* gene may be permanently activated or overexpressed, which could lead to neoplastic transformation (Schutte *et al.*, 1988; Bossy-Wetzel *et al.*, 1992). In addition, enhanced levels of expression of c-*jun* and c-*fos*, as well as of AP-1-dependent genes, is found in tumors derived from *in vivo* and *in vitro* transformation (Liehr *et al.*, 1992; Huang *et al.*, 1997). Activation of c-*jun* is probably crucial in transmitting the cancer-promoting signals. Disruption of Fos-Jun dimerization has been shown to impair the transcriptional activation and cell transformation regulated by these proteins (Baichwal and Tjian, 1990; Persson *et al.*, 1996).

Recently, an immunoassay was developed to determine the dissociation constant for the dimer formation of Jun and Fos, and to detect the leucine zipper domain of Jun and Fos (Heuer *et al.*, 1996). For the assay of the interaction between AP-1 and DNA, however, the conventional electrophoretic mobility shift assay (EMSA) has been used (Dent and Latchman, 1993). The EMSA gives clear-cut results because the mobility on the gel of a DNA duplex is greatly changed when bound to the Jun-Fos heterodimer or Jun-Jun homodimer. Since crude cell extracts are used as sources of Jun or Fos proteins in most cases, however, nonspecific binding of DNA makes it

To whom correspondence should be addressed.
Tel: 82-(2)-878-8545; Fax: 82-(2)-878-8545
E-mail: chulyang@plaza.snu.ac.kr

difficult to obtain a quantitative result from EMSA. In this report, we describe a new quantitative assay that is faster than the conventional method.

We have reported the inhibition of Jun-Fos-DNA complex formation by dihydroguaiaretic acid (DHGA) isolated from *Myristica fragrans* and curcumin (Park *et al.*, 1998). DHGA and curcumin were assayed using this Jun-GST-fused Fos system.

Materials and Methods

Materials Cloning vectors (pLM1) containing the c-*fos* and the c-*jun* gene sequences and used to encode the B-Zip region were provided by J.N. Mark Glover, Harvard University. Dithiothreitol, glutathione-agarose, and curcumin were purchased from Sigma Chemical Co.; AP-1 consensus oligonucleotide, poly (dI-dC), and isopropyl-D-thiogalactopyranoside (IPTG) were from Promega; and polymerase chain reaction (PCR) primers were from Bioneer (Taejeon, Korea). DHGA was purified from *Myristica fragrans*.

Subcloning of the *fos* bZIP gene To produce the *fos* bZIP gene, primer I was designed to have a *Bam*HI site at the 5′-end, and primer II to have an *Eco*RI site at the 5′-end (Primer I: 5′-CGGATCCATGAAAC GTCGCATCCGC-3′; Primer II: 5′-CGGAATTCTCATTAGTGTGCCGCCAG-3′). The *fos* bZIP gene was produced by PCR using pLM1-fos as the template DNA with 1 mM $MgCl_2$ at an annealing temperature of 45°C. The produced *fos* bZIP gene was doubly digested with *Bam*HI and *Eco*RI and cloned into the pGEX-2T plasmid to enable the expression of *fos* bZIP as a fusion protein with glutathione S-transferase (GST). The final plasmid clones (DH5α/pGEX-2T-*fos* bZIP) were selected by *Pst*I restriction digestion.

Preparation of gel-bound Jun bZIP-Fos bZIP heterodimer The fusion protein *fos* bZIP-GST was overexpressed in *E. coli* DH5α with 0.1 mM IPTG, and Jun bZIP was overexpressed in *E. coli* BL21 (DE3) with 1 mM IPTG (Lee *et al.*, 1993; Kim *et al.*, 1998). The two kinds of cells were mixed together and sonicated. After centrifugation, the crude cell extract was applied onto a glutathione-agarose column to bind Jun bZIP/ *fos* bZIP-GST to the gel. For a negative control, glutathione-agarose gel to which only *fos* bZIP-GST was bound was also prepared.

Preparation of ^{32}P-labeled probe DNA Labeled DNA probe was prepared by combining 1.75 pmol of AP-1 consensus oligonucleotide with 10 μCi [γ-^{32}P] ATP and T4 polynucleotide kinase. The mixture was incubated at 37°C for 30 min and quenched by 0.5 M EDTA and then diluted to 50 μl.

Binding assay of Jun-Fos and AP-1 nucleotides Typically, 1 μl of radiolabeled AP-1 nucleotide was added to an Eppendorf tube containing 10 μl of glutathione-agarose gel with Jun-Fos heterodimers bound and 3 μl of shift buffer (the 5× solution contains 10 mM Tris·HCl, pH 7.5, 50 mM NaCl, 0.05 mg/ml poly(dI-dC), 1 mM EDTA, 0.5 mM DTT, 4% glycerol) (Sassone-Corsi *et al.*, 1988). The final volume was adjusted to 15 μl by adding 1 μl of H_2O. In the case of the inhibition assay, DHGA or curcumin was dissolved in DMSO and diluted with water and then added to the reaction solution. The unbound AP-1 nucleotides were removed by washing the gel three times with 300 μl of the shift buffer after the reaction mixture was allowed to stand still for 10 min at the ambient temperature. The radioactivity of the washed gel was measured with a scintillation counter.

Results

The *fos* gene was produced by PCR as a 213 bp nucleotide, subcloned into the pGEX-2T plasmid, and transformed into *E. coli* DH5α. Transformed clones were selected by *Pst*I restriction digestion (Fig. 1), and the fusion protein Fos bZIP-GST was overexpressed. Jun bZIP was also over-expressed in a similar manner in *E. coli* BL21(DE3). The Jun-Fos coiled-coil was formed in the mixed cell extract of the two *E. coli* strains, and attached to glutathione-agarose by the affinity between glutathione and GST. This gel is capable of binding an AP-1 nucleotide and was called the positive-control gel. A negative-control gel was also prepared by attaching only Fos bZIP-GST to the glutathione-agarose gel.

For the assay to be successful, a good washing buffer had to be selected to remove unbound DNA from the agarose gel. We tested varying concentrations of phosphate-buffered saline (PBS) as washing buffers: (1) distilled water, (2) 0.05× PBS, (3) 0.1× PBS, (4) 0.2× PBS, (5) 0.5× PBS, (6) 1× PBS, (7) 1× PBS with 5% glycerol. When radiolabeled DNA is added to the agarose gel with Fos bZIP only, an ideal washing buffer

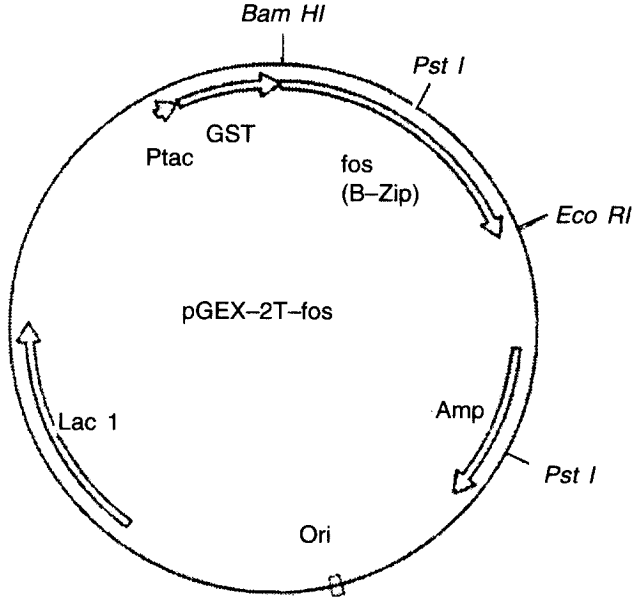

Fig. 1. Subcloning of pGEX-2T-*fos*(B-Zip). The cleavage of plasmids with *Pst*I endonuclease to confirm the insertion of the *fos* gene. Because each of fos gene and pGEX-2T has a *Pst*I cleavage site, the clones harboring the *fos* gene give rise to two DNA fragments of 4 kb and 1 kb by *Pst*I cleavage.

should be able to wash away the entire radioactivity, because this negative-control gel with only Fos bZIP is unable to bind any DNA duplex. Among the seven solutions tested, all except distilled water were acceptable as washing buffers, with the remaining radioactivities of 192–429 cpm (Table 1). We also tested the following four solutions to compare its efficiency as washing buffer for positive controls: (1) 0.1× PBS, (2) 0.1× PBS with 5% glycerol, (3) 0.5× PBS, (4) 0.5× PBS with 5% glycerol. For positive-control gels, an ideal washing buffer should be able to wash away unbound radioactivity without disturbing the interaction between Jun-Fos and DNA. The four solutions showed no significant differences, with the remaining radioactivity counts of 13,557-14,261 (Table 2), which was approximately 60–70 times higher than the background level. Neither PBS concentrations in the range from 0.05× PBS to 1× PBS nor 5% glycerol resulted in any significant difference. However, 0.5× PBS was better than others by a slight margin, and was used for subsequent experiments.

When the amount of AP-1 nucleotide added to the agarose gel was varied, the gel-bound radioactivity increased linearly (Fig. 2). The linearity is expected when the capacity of the gel is considered. When we prepared the gel, we typically applied cell lysate containing Jun bZIP/Fos bZIP-GST to 18 mM of glutathione-agarose. If saturated, 10 μl of the gel used for one assay contains approximately 180 nmol of the Jun-Fos heterodimer, which is in high excess to the amount of the AP-1 nucleotide used for the linearity test. Since the linearity was observed in concentration ranges of lower than 0.1 nmol AP-1 nucleotide, this assay is as sensitive as the conventional EMSA method, which yielded a meaningful band after overnight exposure of an x-ray film.

We also tested the effect of dimethylsulfoxide (DMSO), which is often used as a solvent for hydrophobic inhibitors. The assay was not affected at all even with up to 50% DMSO (Fig. 3). This result indicated that DMSO does not interfere with the leucine zipper interaction or the AP-1 nucleotide binding.

Table 1. Remaining radioactivity of negative-control gels after washing.

Washing buffer	Remaining radioactivity (cpm)[a]
Distilled H_2O	1954 ± 211
0.05× PBS	429 ± 101
0.1× PBS	351 ± 87
0.2× PBS	359 ± 122
0.5× PBS	192 ± 109
1× PBS	230 ± 72
1× PBS with 5% glycerol	403 ± 81

[a] averages of three independent values ± SD.

Table 2. Remaining radioactivity of positive-control gels after washing.

Washing buffer	Remaining radioactivity (cpm)[a]
0.1× PBS	13,692 ± 1121
0.1× PBS with 5% glycerol	13,557 ± 796
0.5× PBS	14,261 ± 1051
0.5× PBS with 5% glycerol	13,651 ± 2111

[a] averages of three independent values ± SD.

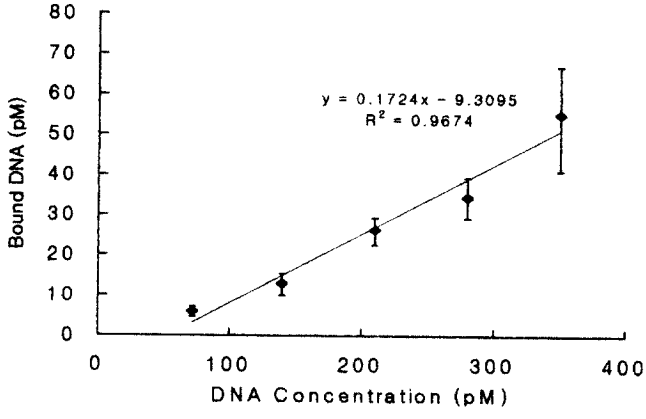

Fig. 2. Amounts of the gel-bound radioactivity with varying concentrations of AP-1 nucleotide. In each reaction tube, radiolabeled nucleotide was added to 15 μl of suspended glutathione-agarose gel with Jun/Fos dimers and 5× shift buffer. The final volume was adjusted to 25 μl by adding distilled water. After 10 min, the gel was washed 3 times with 300 μl of 0.5× PBS each time.

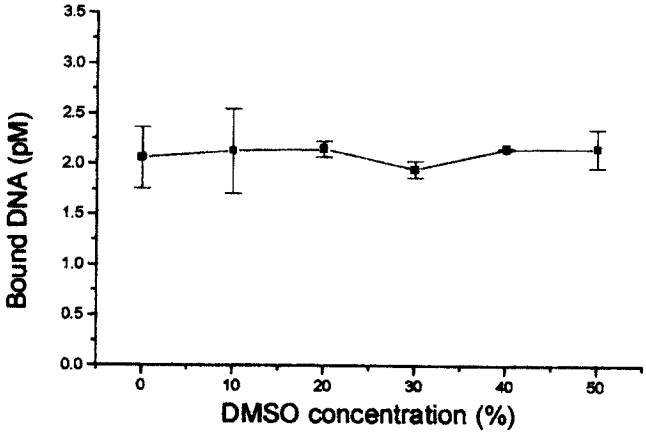

Fig. 3. Effect of DMSO concentration on the assay. In each reaction tube, the agarose gel, 5× shift buffer, and radiolabeled DNA were mixed, and DMSO was added to (1) 0%, (2) 10%, (3) 20%, (4) 30%, (5) 40%, and (6) 50%.The final volume was adjusted to 50 μl by adding distilled water. After 10 min, the gel was washed 3 times with 0.5× PBS containing each concentration of DMSO.

Figure 4 shows the effect of dihydroguaiaretic acid (DHGA) on the complex formation of DNA with dimer. The concentration of bound DNA decreased at increasing concentration of DHGA with an IC_{50} of 10.2 μmol/ml. Measured by EMSA, the IC_{50} of DHGA represented 8.4 μmol/ml.

Another inhibitor of Jun-Fos-DNA complex formation, curcumin, showed an IC_{50} of 0.34 μmol/ml in this Jun-GST-fused Fos system. Curcumin has shown an IC_{50} of 0.28 μmol/ml in EMSA. The experimental data revealed that the value measured via EMSA was close to that obtained by the newly constructed assay system. Therefore, it is provided that this assay is adaptable for investigating inhibitors. As shown in Fig. 5, the slope of [Jun bZIP/Fos bZIP-GST-DNA]/$[DNA]_{tot}$, which represents the bound fraction, was determined with an inhibitor from linear regression. The experimental value of the bound fraction was decreased from 0.17 (without an inhibitor: Fig. 2) to 0.11 and 0.034 by treatment of curcumin. This approach allowed the comparison of the relative affinities of dimer and DNA by treatment with an inhibitor. It is supposed that the inhibitor decreases the number of binding site of DNA.

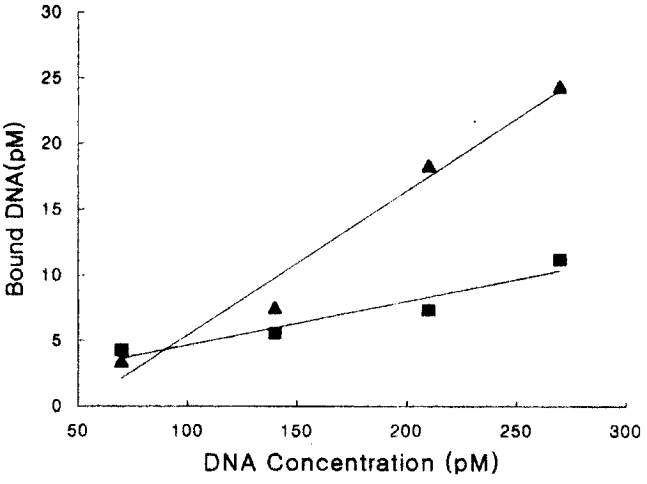

Fig. 5. Effect of curcumin on binding of the dimer with varying concentrations of AP-1 nucleotide. In each reaction tube, radiolabeled nucleotide was added to the suspended glutathione-agarose gel with Jun/Fos dimers and 5× shift buffer. The final volume was adjusted to 25 μl by adding distilled water. After 10 min, the gel was washed 3 times with 300 μl of 0.5× PBS each time. (▲, 0.21 μmol/ml of curcumin; ■, 0.67 μmol/ml of curcumin).

Discussion

In need of a faster and more efficient assay method to screen a number of potential antagonists against the interaction of Jun-Fos transcription factors and AP-1 nucleotides, we developed a new assay that requires smaller amounts of materials than the conventional method, EMSA. This new assay relies on the affinity between gel-bound glutathione and GST, and on the leucine zipper interaction between Fos-GST and Jun. The method avoids gel electrophoresis and autoradiography, making it much faster than EMSA. Moreover, this assay is very sensitive, exhibiting linearity with a very small amount of AP-1 nucleotides. In addition, the assay is functional with high concentrations of an organic solvent, DMSO. As long as the ionic strength is kept to a minimum, a potent assay method for the interaction between Jun-Fos and AP-1 nucleotides is possible. Validated inhibitors, DHGA and curcumin, were used for the determination of a reliable inhibitory effect. Thus, this approach may be applicable to the screening of other inhibitors on Jun-Fos-DNA complex formation.

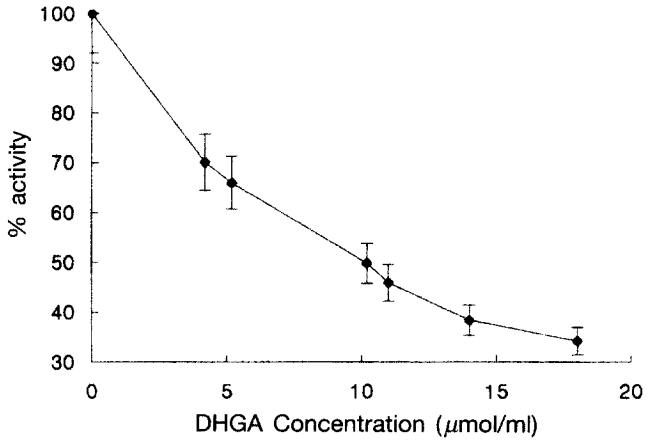

Fig. 4. Effect of DHGA on binding of DNA. In each reaction tube, radiolabeled nucleotide was added to the suspended glutathione-agarose gel with Jun/Fos dimers and 5× shift buffer. DHGA was added at various concentrations. The final volume was adjusted to 25 μl by adding distilled water. After 10 min, the gel was washed 3 times with 300 μl of 0.5× PBS each time. The value of inhibitor-treated samples was compared with that of positive controls.

Acknowledgments This research was supported by a grant from the Ministry of Science & Technology (Grant No. 08-01-15).

References

Angel, P., Hattori, K., Smeal, T. and Karin, M. (1988) The *jun* proto-oncogene is positively autoregulated by its product, Jun/AP-1. *Cell* **55**, 875–885.

Angel, P. and Karin, M. (1991) The role of Jun, Fos and the AP-1 complex in cell proliferation and transformation. *Biochem. Biophys. Acta* **1072**, 129–157.

Baichwal, V. R. and Tjian, R. (1990) Control of c-Jun activity by interaction of a cell-specific inhibitor with regulatory domain δ: Differences between v- and c-Jun. *Cell* **63**, 815–825.

Bossy-Wetzel, E., Bravo, R. and Hanahan, D. (1992) Transcription factors junB and c-jun are selectively up-regulated and functionally implicated in fibrosarcoma development. *Genes Devel.* **6**, 2340–2351.

Curran, T. and Franza, R. (1988) Fos and Jun: The AP-1 connection. *Cell* **55**, 395.

Dent, C. L. and Latchman, D. D. (1993) *Transcription Factors; A Practical Approach*, Latchman, D. S. (ed.), pp. 1–26, IRL Press, Oxford.

Huang, M. T., Ma, W., Yen, P., Xie, J. G., Han, J., Frenkel, K., Grunberger, D. and Conney A. H. (1997) Inhibitory effects of topical application of low doses of curcumin on 12-O-tetradecanoylphorbol-13-acetate-induced tumor promotion and oxidized DNA bases in mouse epidermis. *Carcinogenesis* **18**, 83–88.

Heuer, K. H., Mackay, J. P., Podzebenko, P., Bains, N. P. S., Weiss, A. S., King, G. F. and Easterbrook-Smith, S. B. (1996) *Biochemistry* **35**, 9069–9075.

Johnson, P. F. and McKnight, S. L. (1989) Eukaryotic transcriptional regulatory proteins. *Annu. Rev. Biochem.* **58**, 799.

Kim, H. K., Kim, Y. and Yang, C. H. (1998) An efficient system for the expression and purification of yeast geranylgeranyl protein transferase type I. *J. Biochem. Mol. Biol.* (formerly *Korean Biochem. J.*) **31**, 77–82.

Lee, E. K., Ahn, S., Hwang, S. H. and Yang, C. H. (1993) Purification and characterization of the catalytic domain of protein tyrosine kinase of v-*yes* oncogene expressed in *E. coli*. *Korean Biochem. J.* (presently *J. Biochem. Mol. Biol.*) **26**, 609–613.

Lee, W., Mitchell, P. and Tjian, R. (1987) Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. *Cell* **49**, 741–752.

Liehr, J. G., Chiappeta, C., Roy, D. and Dtancel, G.M. (1992) Elevation of protooncogene messenger RNAs in estrogen-induced tumors in the kidney. *Carcinogenesis* **13**, 601–604.

Mitchell, P. J. and Tjian, R. (1989) Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* **245**, 371.

Park, S., Lee, D.-K. and Yang, C.-H. (1998) Inhibition of *fos-jun*-DNA complex formation by dihydroguaiaretic acid and *in vitro* cytotoxic effects on cancer cells. *Cancer Letters* **127**, 23–28.

Persson, A. E., Pontén, I., Cotgreave, I. and Jernström, B. (1996) Inhibitory effects on the DNA binding of AP-1 transcription factor to an AP-1 binding site modified by benzo[α]pyrene 7,8-dihydrodiol 9,10-epoxide diasteromers. *Carcinogenesis* **17**, 1963–1969.

Sassone-Corsi, P. and Verma, I. M. (1987) Modulation of c-*fos* gene transcription by negative and positive cellular factors. *Nature* **326**, 507–510.

Sassone-Corsi, P., Lamph, W. W., Kamps, M., and Verma, I. M. (1988) Fos-associated cellular p39 is related to nuclear transcription factor AP-1. *Cell* **54**, 553–560.

Schutte, J., Nau, M., Birrer, M., Thomas, F., Gazdar, A. and Minna, J. (1988) Constitutive expression of multiple mRNA forms of the *c-jun* oncogene in human lung cancer cell lines. *Pro. Am. Assoc. Cancer Res.* **1808**, 455.