Biol. Chem. Hoppe-Seyler, Vol. 376, pp. 321–325, May 1995

Short Communication

The Leucine Zipper of c-Jun Binds to Ribosomal Protein L18a: A Role in Jun Protein Regulation?

Kosi Gramatikoff, Walter Schaffner*, and Oleg Georgiev

Institut für Molekularbiologie II der Universität Zürich, Winterthurerstr. 190, CH-8057 Zürich, Switzerland

* Corresponding author

Recently we developed a method called direct interaction rescue (DIRE) for selective cloning in filamentous phage. The rescue is effected by the interaction of two heterologous proteins, one fused to the N-terminus of gene 3 adhesion protein, the other fused to the C-terminus. When heterologous fusion proteins interact with each other, gene 3 protein activity is restored thereby rescuing phage infectivity. We have used the leucine zipper of c-Jun protein as a 'bait' to select for interacting proteins from a human cDNA library. Two interacting clones were isolated, one coding for ribosomal protein L18a, a component of the large ribosomal subunit, and the other for tropomyosin, a component of the cytoskeleton. L18a contains two zipper-like domains which probably interact with c-Jun. We consider it possible that L18a (and tropomyosin) are involved in the cellular regulation of Jun protein levels.

Key words: DIRE baits method / Interaction cloning / Jun / L18a / Oncogenic transformation / Phage display / Phagemid / Protein-protein interaction.

The transcription factor c-Jun is a key regulator of cell proliferation (Angel and Karin, 1991; Busch and Sassone-Corsi, 1990). Depending on the DNA binding site and the cell type, it has the ability to dimerize with itself or with related Jun proteins (Jun-B or Jun-D) or with other factors such as c-Fos and Fra. The best known example is the c-Jun/c-Fos interaction at AP-1 sites TGA(C/G)TCA (Gutman and Wasylyk, 1991; Hirai and Yaniv, 1989; Smith and Bohmann, 1992 and references therein). Homo- or heterodimers are formed via a protein interaction domain, the so-called leucine zipper. This is a protein motif with alpha-helical structure revealing a periodic repetition of leucine residues at every seventh position over a distance covering eight helical turns (Landschulz *et al.*, 1988; reviewed in Hurst, 1994). DNA binding factors with a leucine zipper have another peculiar feature: The leucine zipper region is preceded by a basic region with affinity for the specific DNA site. DNA binding is prevented if one of the partners, while containing a zipper, lacks the basic region. Such configurations give rise to dominant negative factors. Complex gene regulation can be effected by various combinations of homo- and heterodimers of these zipper factors. Nevertheless leucine-zippers are not promiscuous in their interaction: although many zipper proteins are known, typically the interaction of each of them seems to be restricted to a few partner molecules. For example, c-Fos can readily form a heterodimer with c-Jun but can not homodimerize (Smeal *et al.*, 1989).

We have recently developed a new technique for isolating specific cDNA clones by direct interaction rescue (DIRE) from a cDNA library in filamentous phage (Gramatikoff *et al.*, 1994). Filamentous phage have been used previously to display protein domain libraries of great complexity, from which the desired domains can be selected *in vitro* by using either matrix-bound protein or DNA (Blond-Elguindi *et al.*, 1993; Gragerov *et al.*, 1994; Rebar and Pabo, 1994). Since the phage encodes the protein displayed on its surface, the DNA can be readily analysed. This analysis is facilitated by the use of chimeric phage/plasmid ('phagemid') that is maintained as plasmid after infection. We have modified this phage technique as follows: Firstly, we expressed the N-terminal and C-terminal halves of the phage gene 3 protein each from a separate LacZ promoter. (Gene 3 protein attaches the phage to the bacterial pili and is thus essential for infectivity). Secondly, the C-terminal half was fused to a 'bait' encoding a domain for protein-protein interaction. Thirdly, the N-terminal half was fused to members of a cDNA library to provide possible interaction partners for the 'bait'. When a protein-protein interaction occurs, the N- and C-terminus of gene 3 protein (Figure 1) are joined, thus restoring phage infectivity (Figure 1) (Gramatikoff *et al.*, 1994; see also Duenas and Borrebaeck, 1994). Since the Jun-Fos leucine-zipper interaction (K_d approx. 100 nM; Pernelle *et al.*, 1993) was known to work on the surface of a filamentous phage, we decided to use the zipper of c-Jun as a 'bait' to fish for interacting clones in a cDNA library derived from an Epstein-Barr virus-immortalised human B-lymphocyte line (Durfee *et al.*, 1993). Our selection yielded several phagemid clones, one of which turned out to encode cytoskeletal tropomyosin, a filament-stabilizing protein. Interestingly, tropomyosin was also identified by others to specifically interact with c-Jun using a different protein-protein interaction assay, namely the yeast two-hybrid system (Chevray and Nathans, 1992). Two further clones contained identical inserts coding for a human homologue of

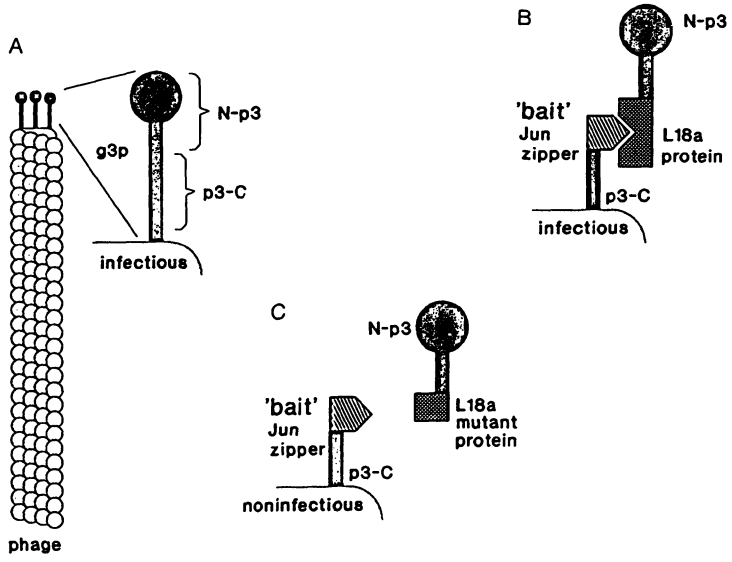

Fig. 1 Principle of **D**irect **I**nteraction **R**escue (DIRE).
The minor coat protein, g3p can be functionally separated into two parts, an N-terminal domain (N-p3) binding to the F' pili and allowing the infection of *E. coli*, and a C-terminal domain (p3-C), anchored at the tip of the phage body. The phage also contains information to be maintained as a plasmid ('phagemid') after infection of host bacteria (A). The interaction of the Jun zipper (as a 'bait') with the L18a protein can place the N-terminal part of the minor coat protein N-p3 on the surface of the phage. This restores phage infectivity (B). In contrast, the mutant L18a does not interact with the 'bait', and the truncated gene 3 protein (p3-C) is not sufficient for infectivity (C).

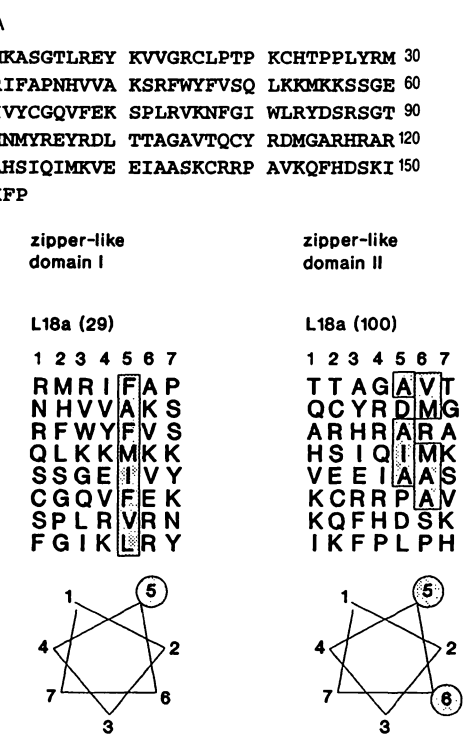

Fig. 2 Sequence of Human L18a Protein.
(A) L18a protein sequence, deduced from our cDNA clone. Our protein sequence matches perfectly with a human L18a sequence deposited in the EMBL data library (accession number L05093), except for 20 extra C-terminal amino acids in the latter sequence. These 20 aa are also highly divergent from the C terminal L18a sequences of rat (Aoyama *et al.*, 1989) and *Drosophila* (Ntwasa *et al.*, 1994). The alignment between cDNA-deduced L18a proteins of human, rat and *Drosophila* was done with UWGCG's BestFit and Pretty programs (not shown). (B) Helical wheel analysis of the two zipper-like motifs of the human L18a ribosomal protein. The numbers in parentheses indicate the position of the first amino acid residue of the helix. The amino acid sequence is aligned in segments of seven amino acid residues (above) and the positions of the arrays containing the putative hydrophobic zippers (boxed) are marked on a helical wheel (below). Here, we refer to these two zipper-like structures as 'hydrophobic-zippers' because they are composed of various hydrophobic amino acid residues besides leucine (Tsurugi and Mitsui, 1991; Chan *et al.*, 1994). Domain I displays similarity to a potential zipper element of rat ribosomal protein L37a which is located at approximately the same position in the protein (Chan *et al.*, 1994). It is of particular interest that in domain II the hydrophobic zipper is 'bilateral', similar to zippers in some yeast ribosomal proteins (Tsurugi and Mitsui, 1991). The helical and zipper predictions were performed by using UWGCG's PeptideStructure and Pegnet programs.

rat and *Drosophila* ribosomal protein L18a, a component of the large ribosomal subunit (Aoyama *et al.*, 1989; Ntwasa *et al.*, 1994) (Figure 2A).

Ribosomal proteins are synthesized in the cytoplasm and transported to the nucleolus, where they associate with ribosomal RNA to form the ribosomal subunits. At least for some of the ribosomal proteins in bacteria, yeast and vertebrates, there are intricate regulation mechanisms to ensure equimolar production in relation to the other ribosomal proteins (Pierandrei-Amaldi *et al.*, 1985; Mager, 1988). Surprisingly, in mitogen-activated B-lymphocytes (Hammerich *et al.*, 1993) and in certain colon cancers (Barnard *et al.*, 1991; Chester *et al.*, 1989; Pogue-Geile *et al.*, 1991; Barnard *et al.*, 1992; Chiao *et al.*, 1992), a subset of ribosomal proteins are strongly upregulated relative to others (see also below).

An inspection of the human L18a sequence revealed two potential hydrophobic zippers, domain I (aa 40–80)

and domain II (100–150) which are candidates for the interaction with c-Jun. According to a Chou-Fasman algorithm (Chou and Fasman, 1978) (Figure 2B) they could form alpha helices, similar to those found in some other ribosomal proteins (Tsurugi and Mitsui, 1991; Chan *et al.*, 1994). The overall specificity of the interaction was confirmed by omitting the L18a cDNA, or by generating a truncation/frameshift at amino acid residue 29 which eliminates both of the putative hydrophobic-zippers (this clone was designated FS; Figure 3). Such clones were unable to restore activity of the c-Jun phagemid (Figure 4). Conversely, the isolated zipper-like domain I (Figure 2B) still gave considerable infectivity, suggesting that this domain is involved in the c-Jun interaction (Figure 4). We obtained further evidence for involvement of a zipper structure by probing with a zipper/coil-specific monoclonal antibody (MAb). This antibody is directed against a homodimeric synthetic leucine-zipper peptide (MAb:29AB12B3; a kind gift of L. Leder and H.R. Bosshard; Leder *et al.*, 1994). When we tested this MAb in a solid-phase ELISA it cross-reacted with phage produced from phagemids containing the c-Fos zipper, L18a, or the zipper-like domain I of L18a, but not with wild-type phage or with the frameshift (FS) mutant phage (data not shown). In a more direct test for infectivity, phage produced from the phagemid with L18a were mixed with a 5-fold molar excess of the antibody. In addition, this phage/antibody mixture was treated with 0.3 M NaCl at pH 7.5 to destabilize electrostatically the Jun/L18a dimers. Since Jun protein can form homodimers (Jun/Jun, Pernelle *et al.*, 1993), the MAb which recognizes dimerized zippers, could either favour formation of such homodimer phage or directly block the Jun/L18a interaction, thus abolishing phage infectivity. Infectivity of the treated phage samples was tested and the colonies counted (Figure 4). A one hundred fold decrease of infectivity was found after treatment of the phage with the antibody in presence of salt, compared to the controls. Such an effect was not observed with nonreactive control antibody (anti-Fab mouse antibody, Sigma A-2179; data not shown).

Taken together, these findings argue for the formation of a zipper-like structure between c-Jun and the ribosomal protein L18a. It remains to be seen whether L18a (or tropomyosin) interacts with all members of the Jun family (including Jun-D or Jun-B) or with c-Jun only. In our selection experiments, no c-Fos clones were isolated from this library, presumably due to the very low level of c-Fos expression in unstimulated B-lymphocytes. However, in reconstruction experiments we observed a strong selection for mixed-in Fos clones (Gramatikoff *et al.*, 1994).

The question arises whether this interaction of the c-Jun zipper with the ribosomal protein L18a, and also tropomyosin, is a mere artefact, or whether it has some deeper meaning. Both of these proteins would qualify as 'house-keeping' proteins and at first glance seem not to be suited for interaction with a regulator of cell proliferation such as c-Jun. However, our data suggest that the interaction is specific, and it seems worth mentioning that some other seemingly erratic interactions between cellular components make biological sense. For example, it is known since decades that DNase I is specifically bound by depolymerized, cytoplasmic actin as is abundant in the mitosis phase of the cell cycle (Sheterline and Sparrow, 1994). A distorted cell regulation, due to untimely expression of a proto-oncogene, unleashes the DNase and results in programmed cell death/apoptosis (Peitsch *et al.*, 1993). The activity of transcription factors can be specifically blocked by protein-protein interactions with ubiquitous cellular proteins, for example between glucocorticoid receptor and heat shock proteins 90 (Picard *et al.*, 1988;

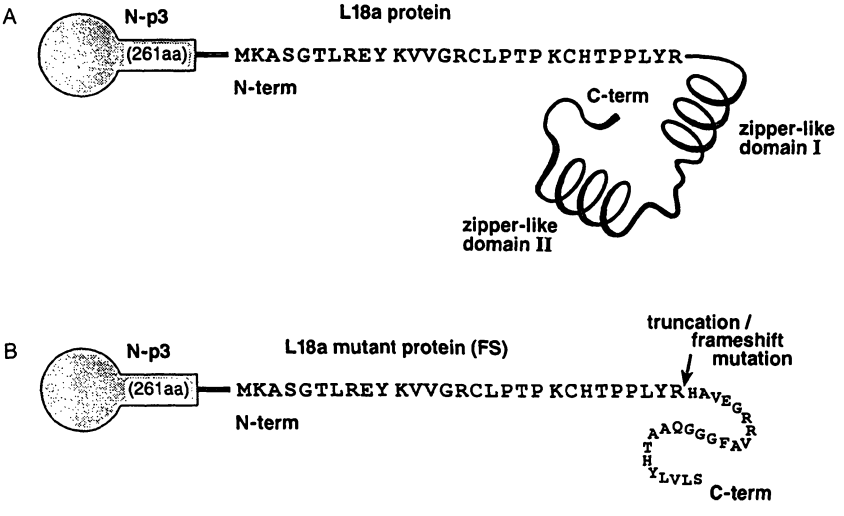

Fig. 3 Human Ribosomal Protein L18a (A) and L18a-Mutant (B) as Fusion Proteins to the N-Terminal Half (N-p3) of the Phage Protein g3p. The 261 amino acids long N-terminus of the g3p is indicated on the left hand site. The 29 N-terminal residues of the L18a protein are shown in the center. The remaining portion of the protein is indicated schematically by a line starting from the site of mutation at position 29. With a frame shift mutation at the *SphI* site, the mutant L18a protein does not contain the predicted zipper-like domains. This resulted in a loss of binding to the Jun zipper (Figure 4).

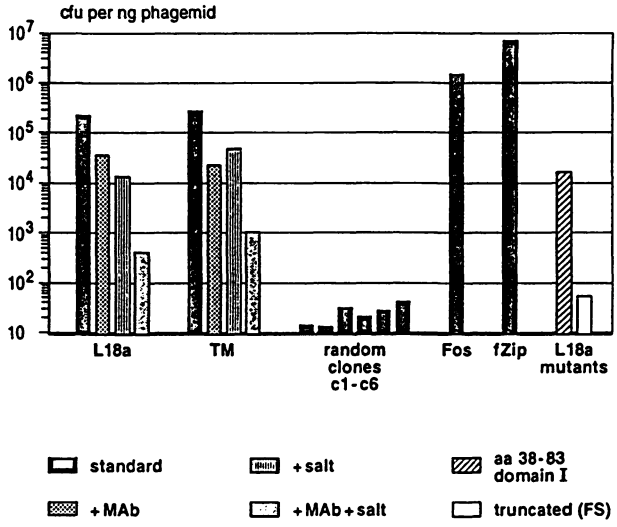

Fig. 4 Infectivity of Phage Obtained from Different Phage-Rescue Experiments.

Rescue phage containing L18a or tropomyosin (TM) were also treated with a monoclonal antibody (MAb) against a dimeric zipper/coil structure (a kind gift of L. Leder and H.R. Bosshard; Leder *et al.*, 1994), with NaCl at pH 7.5 (salt), and with a combination of them (MAb+salt). The infectivity of six randomly chosen phage clones of the cDNA library (c1–c6), or with c-Fos and the isolated Fos zipper (fZip) as positive controls, is presented on the right-hand side. The isolated zipper-like domain I of L18a (hatched bar) still yields 10% phagemid infectivity compared to the entire L18a, while truncation to the N-terminal 29 aa yields only background infectivity (open bar).

Methods: Most procedures were done as described in Gramatikoff *et al.* (1994). The human B-cell cDNA library (Lib-II) was excised from pAct (Durfee *et al.*, 1993) by *Bgl*II and replaced the fZip. Clones isolated from the library screen were sequenced with two flanking primers. The first one (IRD: 5′-CCGGTGATTTTGATTATGAAGG-3′), was designed to anneal at the very end of N-p3, for sequencing of the 5′ ends of the library clones. The second primer (Comb2: 5′-CGACGTTGTAAAACGACGGCC-3′), was designed to anneal downstream of the library insertion site for sequencing of the 3′ library ends. After identifying the human L18a ribosomal clone, four additional primers were designed for sequencing of the entire L18a clone:

1. Up: (5′-GTCGCCAAGTCCCGCTTCTGG-3′);
2. Down: (5′-TAACTGAGATACA-AAGTACCAG-3′);
3. Rsa: (5′-CACGCTACGAGAGTACAAGG-3′); and
4. Acc: (5′-GAGATTGTCTACTGTGGGCAGG-3′).

The L18a domain-I was PCR-amplified by using the following primers: Pst-up: (5′-TCGACTGCAGGTCGTCGCCAAGTCCCGC-3′) and Bgl-down: (5′-GAAGATCTGCAGCGCAGCCAGATCCCG-3′).

Green and Chambon, 1988; Scherrer *et al.*, 1993), and between helix-loop-helix (bHLH) factors and the ubiquitous Ca^{2+}-binding protein calmodulin (Corneliussen *et al.*, 1994). Thus we consider it possible that ribosomal protein L18a (and/or tropomyosin) bind to c-Jun and other zipper-containing factors to regulate their activity within the cell. As judged from phage infectivity, both L18a and tropomyosin have a lower affinity to c-Jun, as compared to c-Fos (Figure 4). However, this does not necessarily rule out a role in c-Jun regulation, since both L18a and tropomyosin are two to three orders of magnitude more abundant in the cell than c-Jun and c-Fos (D. Bohmann, personal communication; see also Mager, 1988; Sheterline and Sparrow, 1994).

There are a few findings that may indirectly relate to that concept. Both tropomyosin and the ribosomal protein L7a, which also contains a potential zipper domain, have been found in chromosome translocations to be fused to oncogenic protein kinases (Martin-Zanca *et al.*, 1986; Ziemiecki *et al.*, 1990). In these cases, ectopic docking of the kinase via tropomyosin or L7a moiety to a transcription factor may result in constitutive activation. In addition, as mentioned before, a subset of ribosomal proteins is specifically overexpressed in colon cancer, including L18 (L18 and L18a are different proteins but have the same electrophoretic mobility; Aoyama *et al.*, 1989). It is conceivable that proteins (including ribosomal ones) overexpressed in cancer cells are titrating others, like L18a which then would no longer be able to regulate c-Jun. According to this scenario, L18a would qualify as an 'auxiliary' anti-oncogene with a primary role in ribosomal function. Taken together, we consider it an interesting possibility that many proteins of a basal cellular function are also involved in cellular growth control.

Acknowledgements

We are indebted to Mr. Lukas Leder and Prof. Hans Rudolf Bosshard (Biochemistry Institute, University of Zürich) for providing us with the monoclonal antibody 29AB12B3, to Dirk Bohmann (EMBL) for communicating unpublished results, and to Drs. Ilian Jelesarov (Biochemistry Institute), Philippe Douville, Kristina Rother, and John Silke for critical reading of the manuscript. This work was supported by the Schweizerischer Nationalfonds and the Kanton Zürich.

References

Angel, P., and Karin, M. (1991). The role of Jun, Fos and the AP1 complex in cell-proliferation and transformation. Biochem. Biophys. Acta *1072*, 129–157.

Aoyama, Y., Chan, Y.-L., Meyuhas, O., and Wool, I.G. (1989). The primary structure of rat ribosomal protein L18a. FEBS Lett. *247*, 242–246.

Barnard, G.F., Staniunas, R.J., Bao, S., Mafune, K.-I., Steele, G.D., Jr., Gollan, J.L., and Chen, L.B. (1992). Increased expression of human ribosomal phosphoprotein PO messenger RNA in hepatocellular carcinoma and colon carcinoma. Cancer Res. *52*, 3067–3072.

Barnard, G.F., Staniunas, R.J., Steele, G.D., Jr., and Chen, L.B. (1991). Increased mRNA expression of ribosomal proteins L18, L37, S6 in human colon carcinoma metastasized to primary hepatoma. J. Cell Biol. *115*, 1523A.

Blond-Elguindi, S., Cwirla, S.E., Dower, W.J., Lipshultz, R.J., Sprang, S.R., Sambrook, J.F., and Gelthing, M.-J.H. (1993). Affinity panning of library of peptides displayed on bacteriophages reveals the binding specificity of BIP. Cell *75*, 717–728.

Busch, S.J., and Sassone-Corsi, P. (1990). Dimers, leucine zippers and DNA-binding domains. Trends Genet. *6*, 36–40.

Chan, Y.-L., Olivera, J., Glück, A., and Wool, I.G. (1994). A leucine zipper-like motif and basic region-leucine zipper-like element in rat ribosomal protein L13a. J. Biol. Chem. *269*, 5589–5594.

Chester, K.A., Robson, L., Begent, R.H.J., Talbot, I.C., Pringle, J.H., Primrose, L., MacPherson, A.J.S., Boxer, G., Southall, P., and Malcolm, D.B. (1989). Identification of a human ribosomal mRNA with increased expression in colorectal tumours. Biochem. Biophys. Acta *1009*, 297–300.

Chevray, P.M., and Nathans, D. (1992). Protein interaction cloning in yeast: identification of mammalian proteins that react with the leuzine zipper of Jun. Proc. Natl. Acad. Sci. USA *89*, 5789–5793.

Chiao, P.J., Shin, D.M., Sacks, P.G., Hong, W.K., and Tainsky, M.A. (1992). Elevated expression of the ribosomal protein S2 gene in human tumors. Mol. Carcinog. *5*, 219–231.

Chou, P.Y., and Fasman, G.D. (1978). Empirical predictions of protein conformation. Ann. Rev. Biochem. *47*, 251–276.

Corneliussen, B., Holm, M., Waltersson, Y., Onions, J., Hallberg, B., Thornell, A., and Grundstrom, T. (1994). Calcium/Calmodulin inhibition of basic-helix-loop-helix transcription factor domains. Nature *368*, 760–764.

Duenas, M., and Borrebaeck, C.A.K. (1994). Clonal selection and amplyfication of phage displayed antibodies by linking antigen recognition and phage replication. Bio/Technology *12*, 999–1002.

Durfee, T., Becherer, K., Chen, P.-L., Yeh, S.-H., Yang, Y., Kilburn, A.E., Lee, W.-H., and Elledge, S.J. (1993). The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. Genes Dev. *7*, 555–569.

Gragerov, A., Zeng, L., Zhao, X., Burkholder, W., and Gottesman, M.E. (1994). Specificity of DnaK-peptide binding. J. Mol. Biol. *235*, 848–854.

Gramatikoff, K., Georgiev, O., and Schaffner, W. (1994). Direct interaction rescue, a novel filamentous phage technique to study protein-protein interactions. Nucleic Acids Res. *22*, 5761–5762.

Green, S., and Chambon, P. (1988). Nuclear receptors enhance our understanding of transcription regulation. Trends Genet. *4*, 309–313.

Gutman, A., and Wasylyk, B. (1991). Nuclear targets for transcriptional regulation by oncogenes. Trends Genet. *7*, 49–54.

Hammerich, P., Mikecz, A., Neumann, F., Sözeri, O., Wolf-Vorbeck, G., Zoebelein, R., and Krawinkel, U. (1993). Structural and functional properties of ribosomal protein L7 from humans and rodents. Nucleic Acids Res. *21*, 223–231.

Hirai, S., and Yaniv, M. (1989). Jun DNA-binding is modulated by mutations between the leucines or by direct interaction of *fos* with the TGACTCA sequence. New Biol. *1*, 181–191.

Hurst, H. (1994). Transcription factors 1: bZIP proteins. Protein Profile Vol *1*, Issue 2.

Landschulz, W.H., Johnson, P.F., and McKnight, S.L. (1988). The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science *240*, 1759–1764.

Leder, L., Went, H., Schawab, C., Jelesarov, I., Bornhauser, S., Ackermann, F., and Bosshard, H.R. (1994). Genuine and apparent cross-reaction of polyclonal antibodies to proteins and peptides. Eur. J. Biochem. *219*, 73–81.

Mager, W.H. (1988). Control of ribosomal protein gene expression. Biochem. Biophys. Acta *949*, 1–15.

Martin-Zanca, D., Hughes, S.H., and Barbacid, M. (1986). A human oncogene formed by fusion of truncated tropomyosin and protein tyrosine kinase sequences. Nature *319*, 743–748.

Ntwasa, M., Buchanan, S.G.S.C., and Gay, N.J. (1994). *Drosophila* ribosomal protein L18a: cDNA sequence, expression and chromosomal localization of the gene. Biochem. Biophys. Acta *1228*, 210–212.

Peitsch, M.C., Polzar, B., Stephan, H., Crompton, T., MacDonald, H.R., Mannherz, H.G., and Tschopp, J. (1993). Characterization of the endogenious deoxyribonuclease involved in nuclear DNA degradation during apoptosis (programmed cell death). EMBO J. *12*, 371–377.

Pernelle, C., Clerc, F., Dureuil, C., Bracco, L., and Tocque, B. (1993). An efficient screening assay for the rapid and precise determination of affinities between leucine zipper domains. Biochemistry *32*, 11682–11687.

Picard, D., Salser, S., and Yamamoto, K.R. (1988). A movable and regulable inactivation function within the steroid binding domain of the glucocorticoid receptor. Cell *54*, 1073–1080.

Pierandrei-Amaldi, P., Beccari, E., Bozzoni, I., and Amaldi, F. (1985). Ribozomal protein production in normal and anucleolate *Xenopus* embryos: regulation at the posttranscriptional and translational levels. Cell *42*, 317–323.

Pogue-Geile, K., Geiser, J.R., Shu, M., Miller, C., Wool, I.G., Meisler, A.I., and Pipas, J.M. (1991). Ribosomal protein genes are overexpressed in colorectal cancer: isolation of a cDNA clone encoding the human S3 ribosomal protein. Mol. Cell. Biol. *11*, 3842–3849.

Rebar, E.J., and Pabo, C.O. (1994). Zinc finger phage: affinity selection of fingers with new DNA-binding specificities. Science *263*, 671–673.

Scherrer, L.C., Picard, D., Massa, E., Harmon, J.M., Simons, S.S., Jr., and Yamamoto, K.R. (1993). Evidence that the hormon binding domain of steroid receptors confers hormonal control on chimeric proteins by determining their hormone-regulated binding to heat-shock protein 90. Biochemistry *32*, 5381–5386.

Sheterline, P., and Sparrow, J. (1994). Actin. Protein Profile Vol.*1*, Issue 1.

Smeal, T., Angel, P., Meek, J., and Karin, M. (1989). Different requirements for formation of Jun:Jun and Jun:Fos complexes. Genes Dev. *3*, 2091–2100.

Smith, S.E., and Bohmann, D. (1992). Functional dissection of AP-1 transcription factors using the Gal4 adaptor assay. Cell. Growth. Differ. *3*, 523–529.

Tsurugi, T., and Mitsui, K. (1991). Bilateral hydrophobic zipper as a hypothetical structure which binds acidic ribosomal protein family together on ribosomes in yeast *Saccharaomyces cerevisie*. Biochem. Biophys. Res. Commun. *174*, 1318–1323.

Ziemiecki, A., Müller, R.G., Xiao-Chang, F., Hynes, N.E., and Kozma, S. (1990). Oncogenic activation of the human trk protooncogene by recombination with the ribosomal large subunit protein L7a. EMBO J. *9*, 191–196.

Received December 27, 1994; accepted February 28, 1995