Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 4236–4239, May 1992
Biochemistry

Transformation and transactivation suppressor activity of the c-Jun leucine zipper fused to a bacterial repressor

(gene regulation/oncogenes/Ras/Fos/LexA)

MICHÈLE GRANGER-SCHNARR†, ENRICO BENUSIGLIO‡, MANFRED SCHNARR†, AND PAOLO SASSONE-CORSI‡§

‡Laboratoire de Génétique Moléculaire des Eucaryotes du Centre Nationale de la Recherche Scientifique, U184 de l'Institut Nationale de la Santé et de la Recherche Médicale, Faculté de Médecine, 11, rue Humann, 67085 Strasbourg, France; and †Institut de Biologie Moléculaire et Cellulaire du Centre Nationale de la Recherche Scientifique, 15, rue R. Descartes, 67084 Strasbourg, France

Communicated by William J. Rutter, February 5, 1992 (received for review December 2, 1991)

ABSTRACT **Transcription factor c-Jun appears to be a nuclear target of the Ras-induced signal transduction pathway. In fact, some experiments show that transforming forms of the Ras protein cooperate with Jun in transcriptional activation mediated by an AP-1 site and others indicate that the two oncoproteins cooperate in cellular transformation. Although it is likely that intracellular signaling systems activated by Ras might act directly on c-Jun by inducing specific phosphorylation, it is unclear how c-Jun participates in the transformation process. Here, we present results obtained with a LexA–Jun zipper fusion that lacks both the transcriptional activation domains and the basic region of the DNA-binding domain of c-Jun and contains only the intact leucine-zipper domain. This fusion product has a dominant negative effect on the transcriptional activation elicited by phorbol esters, c-Jun, c-Fos, Ras and E1A on an AP-1-responsive site. An analogous LexA–Fos zipper fusion has similar effects on transcriptional induction. The LexA–Jun zipper fusion acts further as a transformation suppressor, since it causes the generation of nontransformed revertants of *ras*-transformed cells. This effect is likely to be elicited by the dimerization potential of the Jun leucine zipper trapping cellular Jun and/or Fos in a protein complex unable to bind to DNA. These data implicate further that Ras-mediated transformation involves functional transcription factor AP-1 and that it is possible to interfere with cell transformation by interfering simply with the dimerization of transcription factors involved in the transformation process.**

Previous results indicated that transforming *ras* products are able to elicit transcriptional activation mediated by an AP-1 site (1–4), the target of Fos/Jun nuclear oncoproteins (5–7). There is a remarkable correlation between transformation potential by *ras* and the activation of an AP-1 site as shown by the use of a large panel of *ras* mutants (4). Furthermore, additional data indicate that *ras* transformation potential is augmented by coexpression of *jun* (8, 9). *ras* enhances c-Jun-mediated transactivation by inducing an intracellular protein kinase cascade that results in the hyperphosphorylation of specific sites in the activation domain of c-Jun (10), and a v-Jun protein harboring an intact DNA-binding domain and the proline-rich activation domain A2 but lacking activation domain A1 may suppress the transformation activity of *ras* (11). This suppressor activity may be due to the saturation of AP-1 binding sites with one or two truncated Jun monomers unable to promote efficient transcriptional activation and/or due to the titration of a protein being part of or cooperating with cellular AP-1 compounds.

To define the minimal c-Jun segment sufficient to act as a suppressor of *ras* transformation and AP-1 transactivation, we constructed a LexA–Jun zipper fusion protein that contains the amino-terminal domain of the *Escherichia coli* LexA repressor linked to the leucine-zipper part of c-Jun (Fig. 1*A*), which bears the information necessary for the formation of Jun/Jun homo- and Jun/Fos heterodimers (5–7). This construct efficiently represses gene expression in a prokaryotic environment, and the dimerization domain is essential for DNA binding of the fusion protein (12). The LexA–Jun zipper protein efficiently dimerizes with wild-type Jun and Fos leucine-zipper peptides, as expected by the conservation of the required domains in the fusion (12). Thus, we reasoned that the LexA–Jun zipper fusion could also affect the transcriptional enhancement exerted by several effectors on an AP-1 site in eukaryotic cells.

MATERIALS AND METHODS

Construction of Leucine-Zipper Fusion Proteins. The LexA–Jun zipper protein was constructed as previously described (12). The LexA–Fos zipper protein was constructed by the same procedure. The amino-terminal domain of LexA (amino acids 1–87) was fused to the Fos leucine zipper. The DNA coding for this peptide was chemically synthesized, annealed, ligated, and purified by electrophoresis in an agarose gel. The amino-terminal domain of LexA, obtained from an *Xmn* I/*Pst* I cleavage of plasmid pJLW70 (16), was fused to the leucine-zipper fragment via a short *Xho* I linker, which contributes to a valine in position 88 of the hybrid protein. As previously described for the LexA–Jun zipper protein (12), we checked that the LexA–Fos zipper protein is functional as a repressor in a bacterial tester strain (data not shown). Both fusion proteins were introduced separately into a eukaryotic expression vector (pSG5) containing the simian virus 40 early promoter (15). As controls we cloned into this expression vector the entire *lexA* gene as well as an antisense LexA–Fos zipper construct.

DNA Transfection and Transient Expression Assays. Cells were plated at 50% confluence and transfected by the calcium phosphate coprecipitation technique with 1–5 μg of plasmid DNA, depending on the experiment. Colonies were individually picked and expanded as described (17). The variability in CAT activity among several experiments was <10%. Cells used were NIH 3T3 mouse fibroblasts and JEG-3 human choriocarcinoma cells. Results were analogous in both cell types.

The Ha-*ras* mutants used in this study (indicated as Ha-ras*) contain either the Arg-12 or the Leu-61 substitution (4). Results obtained with the two mutants were analogous.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: CAT, chloramphenicol acetyltransferase; TPA, "12-*O*-tetradecanoylphorbol 13-acetate" (phorbol 12-myristate 13-acetate); TRE, TPA-responsive element; DSE, dyad symmetry element; tk, thymidine kinase.

§To whom reprint requests should be addressed.

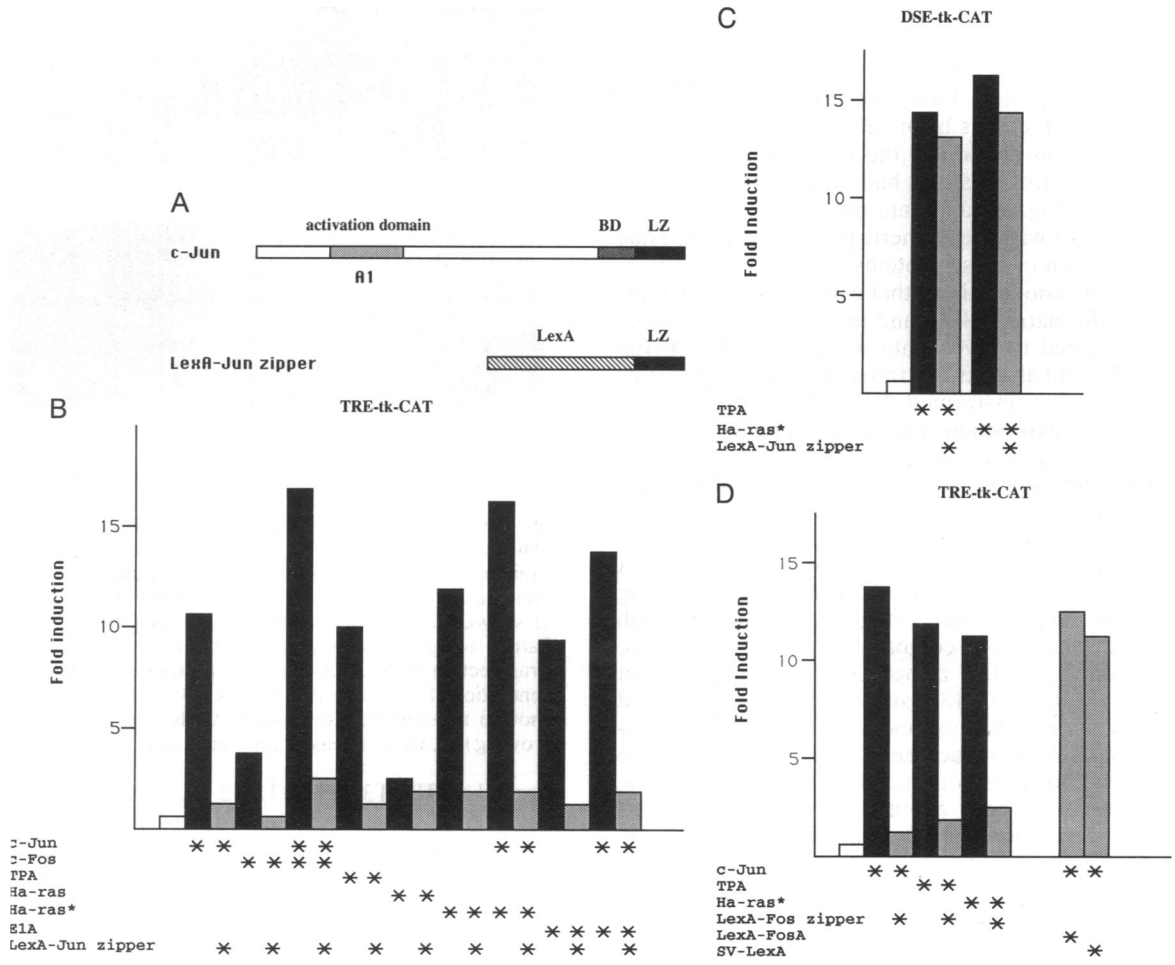

FIG. 1. The LexA–Jun zipper protein is a trans-dominant negative regulator of TRE-mediated activation. [TRE, TPA response element; TPA, "12-*O*-tetradecanoylphorbol 13-acetate" (phorbol 12-myristate 13-acetate).] (*A*) Structure of the LexA–Jun zipper expression vector. It encodes only the Jun leucine zipper (LZ, amino acids 280–312) linked to the *E. coli* LexA repressor (12). The resulting fusion product lacks the two c-Jun activation domains (13), one of which is also the site of regulatory phosphorylations (14). This fusion product is functional in a prokaryotic cell system and dimerizes with Jun and Fos leucine-zipper peptides (12). BD, binding domain. (*B*) Data compiled from several transfections in cultured cells showing that LexA–Jun zipper protein blocks TRE-mediated activation. Various degrees of activation of a TRE–tk–CAT reporter plasmid (tk, herpes simplex virus thymidine kinase promoter; CAT, chloramphenicol acetyltransferase; refs. 5–7) were obtained by treating the cells with TPA (40 μg/ml) or by cotransfection of vectors expressing c-Jun and c-Fos (5–7), normal or transforming Ras (Ha-ras and Ha-ras*, respectively, ref. 4), and adenovirus 2 E1A (15). The histogram presents the basal expression level of TRE–tk–CAT (white bar) with respect to fold induction of the inducers (black bars) and of the inducers with LexA–Jun (gray bars). (*C*) Cotransfection of LexA–Jun zipper protein has no effect on the basal and induced transcription level of DSE–tk–CAT (4). This reporter plasmid contains the c-*fos* dyad symmetry element (DSE) at the same position as the TRE in TRE–tk–CAT. There was no decrease of TPA- and Ras-mediated induction of the DSE by LexA–Jun zipper protein. (*D*) Effect of a LexA–Fos zipper fusion on TRE–tk–CAT activation by c-Jun, TPA, and Ha-ras*. LexA–FosA is equivalent to LexA–Fos zipper except that the coding sequence corresponding to the fusion protein is inserted in the antisense orientation. SV–LexA indicates an expression vector in which the entire LexA sequence has been inserted in the simian virus 40-based eukaryotic expression vector pSG5.

RESULTS AND DISCUSSION

We investigated the negative effect of the LexA–Jun zipper (Fig. 1*B*) and LexA–Fos zipper (Fig. 1*D*) fusion proteins on transcriptional activation of a promoter harboring an AP-1 binding site. For these experiments a reporter plasmid containing the canonical AP-1 binding site (TRE) from the human collagenase gene linked to the herpes simplex virus tk promoter (−109 to +57) was used (5–7). Transcriptional activation of this reporter is obtained by treatment of the transfected cells with TPA or by cotransfection with expression vectors for c-*jun*, c-*fos*, Ha-*ras*, and E1A (refs. 5–9 and 15; Fig. 1). To obtain efficient TRE activation, the Ras protein needs to be mutated at position 12 or 61 (4), mutations required for efficient transformation (17). Only transforming Ras cooperates with c-Jun in the activation of the TRE site (Fig. 1 and ref. 4). Coexpression of the LexA–Jun zipper fusion protein in transfection experiments resulted in a dramatic down-regulation of the transcriptional activation obtained by all the treatments described above (Fig. 1*B*). The effect is specific as indicated by experiments performed with a DSE-tk-CAT reporter, a plasmid that contains the DSE of the c-*fos* promoter (4). The DSE is a target, in the *fos* protooncogene, of the activation by TPA (18, 19) and Ras (4), but it binds transcription factor SRF and not Fos/Jun (18, 19). DSE-mediated transcription was not affected by cotransfection of LexA–Jun zipper protein (Fig. 1*C*). These results demonstrated a trans-dominant negative effect of the LexA–Jun zipper fusion product on the activation of a TRE by various agents.

In addition we performed experiments with a LexA–Fos zipper fusion, a construct analogous to the LexA–Jun zipper protein (see Fig. 1*A*), in which the Fos leucine zipper is substituted for the equivalent Jun zipper region. The LexA–Fos zipper protein exerted the same effect as the LexA–Jun zipper protein on the transcriptional activation elicited from an AP-1 site (Fig. 1*D*). As controls for these experiments we used an antisense LexA–Fos zipper construct and a con-

struct, SV-LexA, containing the entire *lexA* gene alone inserted in the same mammalian expression vector. SV–LexA differs from LexA–Jun zipper or LexA–Fos zipper in that the SV–LexA protein bears the original dimerization domain of LexA, which has been substituted for the Jun or the Fos dimerization domain in the cases of the chimeric proteins. These control plasmids had no effect in our transactivation assays (Fig. 1*D* and data not shown). Therefore, the observed effects with the chimeric proteins cannot be due to the LexA portion of these proteins.

Considering the cooperativity that c-Jun and Ras proteins exhibit in transformation (8, 9) and in the light of our data (Fig. 1), we decided to investigate whether the LexA–Jun zipper product might also act as a transformation suppressor. We transfected *ras*-transformed NIH 3T3 cells with the LexA–Jun zipper expression vector to score for possible revertants. In the same set of experiments we also used a Krev-1 expression vector; Krev-1 is a protein of the Ras superfamily that has been shown to act as a suppressor of Ras-induced transformation (20). Cells ($\approx 10^6$) were transfected with 5 μg of plasmid DNA or control vector DNA (pBluescript, Stratagene) and selected in medium containing G418 at 1 mg/ml. The efficiencies of *neo*-marker transfer with the various plasmids were comparable (data not shown). However, when LexA–Jun zipper DNA was used, about two-thirds of the total G418-resistant colonies, observed under a phase-contrast microscope after 6–7 days of selection, were relatively compact and appeared dark. These colonies consisted of cells with increased attachment to the substrate. After 2–3 weeks about a third of the colonies overgrew and detached from the substrate, whereas the remaining colonies consisted of very flat cells (Table 1). In summary, about 70% of the LexA–Jun zipper transfectants were flat cells and the remainder were partially flat cells. In contrast, no colonies with flat morphology were observed in the cultures transfected with control DNA, such as pBluescript plasmid and the SV–LexA construct, a plasmid equivalent to the LexA–Jun zipper construct (see Fig. 1*A*) but harboring the entire *lexA* gene and lacking the Jun leucine zipper (Table 1). Interestingly, transfection of pKrev-1 generated the expected number of flat and partially flat transfectants (20), which appeared to be significantly lower than with LexA–Jun zipper (Table 1).

We next isolated and expanded typical LexA–Jun zipper colonies and analyzed their growth properties and morphology. In Fig. 2 we present micrographs that compare the modified morphology of the revertant clones of both LexA–Jun zipper and Krev-1 with respect to the transformed aspect of the controls. The revertant clones presented flat cells with a higher degree of adherence with respect to the *ras*-transformed cells. Growth rates of the transfectants were significantly lower than that of control *ras*-transformed NIH 3T3 cells, and in one case (R8-7) even lower than that of

Table 1. Distribution of colony types after transfection of *ras*-transformed cells

Transfected plasmid	% total G418-resistant colonies		
	Flat	Partially flat	Transformed
pBluescript	0	0	100
pKrev-1	27	55	18
LexA–Jun zipper	71	29	0
SV–LexA	0	0	100

Values show percentages of G418-resistant colonies of various morphologies after transfection of control plasmids (pBluescript, Stratagene; SV–LexA; see legend to Fig. 1), pKrev-1, or LexA–Jun zipper. The efficiencies of *neo*-marker transfer with the various plasmids were comparable. Five micrograms of each plasmid was transfected in each 100-mm plate. Under these conditions, LexA–Jun zipper generated nearly 3-fold more flat revertants than pKrev-1.

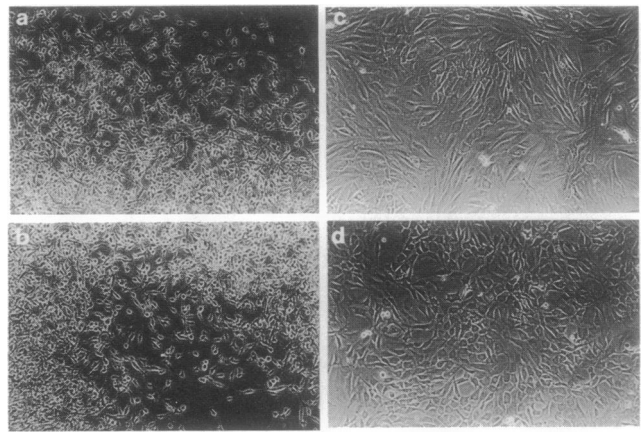

FIG. 2. Morphological aspect of *ras*-transformed cells and revertants generated after transfection of Krev-1 and LexA–Jun zipper. (*a*) *ras*-transformed fibroblasts. (*b*) Transfection of pBluescript plasmid DNA does not alter the aspect of the transformed cells. (*c*) Transfection with Krev-1 induced revertant clones, which were individually expanded and cultivated. Some of the clones (see Table 1) showed a mutated morphology, in which cells appeared to be flatter, to grow slowly, and to show increased adherence. (*d*) Transfection with LexA–Jun zipper expression plasmid caused the generation of revertants (see Tables 1–3). Individually isolated clones showed a revertant morphology. Cells appeared nontransformed, growing flat, with increased adherence and diminished proliferation.

normal NIH 3T3 cells (Table 2). Southern and Northern analyses to score for the expression of both *ras* and LexA–Jun zipper were performed for the transfectants as well as in control cell lines. The results of these analyses showed the expected pattern of expression (Table 2), with some limited variability among the transfectants, not correlated with the morphology of the cells. To assess whether the morphological change in the transformed phenotype and the decreased growth rate could correlate with a mutated proliferation potential, we chose eight clones at random from the LexA–Jun zipper colonies and tested their growth in soft agar. The efficiency of colony formation and the size of the colonies of the LexA–Jun zipper transfectants were compared with those of control cell lines and a control SV–LexA transfectant. All of the LexA–Jun zipper transfectants analyzed had a much lower efficiency of colony formation than the *ras*-transformed cells (Table 3). The transformed morphology of the control SV–LexA transfectants (Table 1) correlated with their efficiency of colony formation in soft agar (Table 3). These results indicate that the c-Jun leucine zipper was responsible for the reversion of the transformed phenotype. In summary, the LexA–Jun zipper fusion, when expressed in *ras*-transformed

Table 2. Properties of isolated colonies

Cell line	Morphology	Doubling time, hr	Ras expression	LexA–Jun expression
NIH 3T3	Flat	20	–	–
NIH 3T3/*ras*	Transformed	10	+	–
Transfectants				
R8-2	Flat	23	+	+
R8-4	Partially flat	17	+	+
R8-7	Flat	26	+	+
R8-8	Flat	21	+	+

Properties of isolated transfectants compared with normal and *ras*-transformed NIH 3T3 cells. Data from four isolated LexA–Jun zipper colonies are presented. Results on soft-agar colony formation for these transfectants are presented in Table 3. All cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Expression was determined by Northern blot hybridization.

Table 3. Growth in soft agar

Cell type	No. of colonies		
	3–20*	21–100*	>100*
NIH 3T3	0	0	0
NIH 3T3-*ras*	>115	>250	>650
NIH 3T3/*ras*/Krev-1	16	5	2
R8-1	23	7	0
R8-2	5	0	0
R8-3	11	2	1
R8-4	24	10	2
R8-5	13	2	0
R8-6	3	0	0
R8-7	2	0	0
R8-8	9	1	0
SV–LexA	>110	>240	>550

Cells (200,000) were resuspended in Dulbecco's modified Eagle's medium containing 5% calf serum, 0.2% Noble agar, and with a base layer of 0.6% Noble agar in 60-mm plates. Colonies were scored after 2 weeks. R8-1 to R8-8 are eight randomly selected individual clones obtained after transfection of LexA–Jun zipper into *ras*-transformed NIH 3T3 cells.

*Diameter of colonies as expressed by the number of cells lined up across the colonies, determined on day 14.

cells, induces reversion of morphology, reduction in growth rate, and suppression of colony-forming ability in soft agar.

Our results indicate that the mechanism of negative dominance by the LexA–Jun zipper protein is likely to be due to its potential to dimerize with cellular proteins of the Jun and Fos families, since the only Jun domain present in the fusion protein is the leucine zipper (Fig. 1*A*). The fact that SV-LexA, comprising the entire LexA protein but lacking the Jun dimerization domain, has no effect in *ras*-mediated transactivation and transformation strongly supports this model. Examples of products that may function in a similar fashion are the ΔFosB, Id, and CREM proteins, natural antagonists of various transcriptional activators (13, 21–23). Alternatively, the identification of putative AP-1 inhibitors (24, 25) might indicate that LexA–Jun could titrate them or escape from their regulation, thus causing an unbalanced situation. It is also conceivable that the effect generated by the LexA–Jun product could be due to the lack of regulated phosphorylation, which is known to occur at sites adjacent to the activation domain that has been removed in the fusion protein (10, 14).

Our results indicate further that c-Jun is a molecular effector of *ras*-mediated transformation. Although it is likely that other nuclear factors would act in an analogous way, these findings will help our understanding of the molecular mechanisms of oncogene cooperation (26).

We thank Y. Ikawa and M. Noda for the gift of a Krev-1 expression vector, C. Der for *ras* mutants, and A. Fusco for the ras-transformed NIH 3T3 cells. We thank N. S. Foulkes for discussions, B. Binétruy and M. Karin for communicating data before publication, and F. Schlotter for excellent technical assistance. This work was supported by grants from the Centre Nationale de la Recherche Scientifique, Institut National de la Santé et de la Recherche Médicale, European Economic Community, Association pour la Recherche contre le Cancer, and Rhone-Poulenc-Rorer (France).

1. Sistonen, L., Holtta, E., Makela, T. P., Keski-Oja, J. & Alitalo, K. (1989) *EMBO J.* **8**, 815–822.
2. Imler, J. L., Schatz, C., Wasylyk, C., Chatton, B. & Wasylyk, B. (1988) *Nature (London)* **332**, 275–278.
3. Murakami, Y., Asano, M., Satake, M. & Ito, Y. (1990) *Oncogene* **5**, 5–13.
4. Sassone-Corsi, P., Der, C. J. & Verma, I. M. (1989) *Mol. Cell. Biol.* **9**, 3174–3183.
5. Curran, T. & Franza, B. R. (1988) *Cell* **55**, 395–397.
6. Sassone-Corsi, P., Lamph, W. W., Kamps, M. & Verma, I. M. (1988) *Cell* **54**, 553–560.
7. Chiu, R., Boyle, W., Meek, J., Smeal, T., Hunter, T. & Karin, M. (1988) *Cell* **54**, 541–552.
8. Schutte, J., Minna, J. D. & Birrer, M. J. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 2257–2261.
9. Schutte, J., Viallet, J., Nau, M., Segal, S., Fedorko, J. & Minna, J. D. (1989) *Cell* **59**, 987–997.
10. Binétruy, B., Smeal, T. & Karin, M. (1991) *Nature (London)* **351**, 122–127.
11. Lloyd, A., Yancheva, N. & Wasylyk, B. (1991) *Nature (London)* **352**, 635–638.
12. Schmidt-Dörr, P., Oertel-Buchheit, P., Pernelle, C., Bracco, L., Schnarr, M. & Granger-Schnarr, M. (1991) *Biochemistry* **30**, 9657–9664.
13. Nakabeppu, Y. & Nathans, D. (1991) *Cell* **64**, 751–759.
14. Boyle, W. J., Smeal, T., Defize, L. H. L., Angel, P., Woodgett, J. R., Karin, M. & Hunter, T. (1991) *Cell* **64**, 573–584.
15. DeGroot, R., Foulkes, N. S., Mulder, M., Kruijer, W. & Sassone-Corsi, P. (1991) *Mol. Cell. Biol.* **11**, 192–201.
16. Little, J. M. & Hill, S. A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 2301–2305.
17. Der, C. J., Finkel, T. & Cooper, G. M. (1986) *Cell* **44**, 167–176.
18. Treisman, R. (1986) *Cell* **46**, 567–574.
19. Stacey, D. W., Watson, T., Kung, H. F. & Curran, T. (1987) *Mol. Cell. Biol.* **7**, 523–527.
20. Kitayama, H., Sugimoto, Y., Matsuzaki, T., Ikawa, Y. & Noda, M. (1989) *Cell* **56**, 77–84.
21. Benezra, R., Davis, R. L., Lockshon, D., Turner, D. L. & Weintraub, H. (1990) *Cell* **61**, 49–59.
22. Foulkes, N. S., Borrelli, E. & Sassone-Corsi, P. (1991) *Cell* **64**, 739–749.
23. Foulkes, N. S. & Sassone-Corsi, P. (1992) *Cell* **68**, 411–414.
24. Auwerx, J. & Sassone-Corsi, P. (1991) *Cell* **64**, 983–993.
25. Baichwal, V. R., Park, A. & Tjian, R. (1991) *Nature (London)* **352**, 165–168.
26. Hunter, T. (1991) *Cell* **64**, 249–270.