MOLECULAR AND CELLULAR BIOLOGY, May 1996, p. 1881–1888 Vol. 16, No. 5
0270-7306/96/$04.00+0

Stepwise Transformation of Rat Embryo Fibroblasts: c-Jun, JunB, or JunD Can Cooperate with Ras for Focus Formation, but a c-Jun-Containing Heterodimer Is Required for Immortalization

LAURENCE VANDEL,[1] NICOLE MONTREAU,[2] EMMANUEL VIAL,[1] CURT M. PFARR,[3] BERNARD BINETRUY,[2] AND MARC CASTELLAZZI[1]*

Unité de Virologie Humaine, INSERM-U412, Ecole Normale Supérieure, 69364 Lyon Cedex 07,[1] Institut de Recherche sur le Cancer, CNRS-ER 105, 94801 Villejuif,[2] and Unité des Virus Oncogènes, CNRS-URA 1644, Département des Biotechnologies, Institut Pasteur, 75724 Paris Cedex,[3] France

Received 10 November 1995/Returned for modification 8 January 1996/Accepted 30 January 1996

Among the Jun family of transcription factors, only c-Jun displays full transforming potential in cooperation with activated c-Ha-Ras in primary rat embryo fibroblasts. c-Jun in combination with Ras can both induce foci of transformed cells from rat embryo fibroblast monolayers and promote the establishment of these foci as tumoral cell lines. JunB can also cooperate with Ras to induce foci but is unable to promote immortalization. We report here that JunD, in cooperation with Ras, induces foci with an efficiency similar to that of JunB. Artificial Jun/eb1 derivatives from each of the three Jun proteins were also analyzed. These constructs carry a heterologous homodimerization domain from the viral EB1 transcription factor and are thought to form only homodimers in the cell. We show here that these Jun/eb1 chimeras are potent transactivators of AP1 sites and that they can cooperate with c-Ha-Ras to induce foci. However, among all the Ras-Jun and Ras-Jun/eb1 combinations tested, only foci from Ras–c-Jun can be efficiently expanded and maintained as long-term growing cultures. Therefore, we suggest that a heterodimer containing c-Jun might be required for in vitro establishment of these primary mammalian cells.

In mammals, the Jun family of transcription factors consists of three closely related proteins designated c-Jun, JunB, and JunD which are classified as bZIP DNA-binding proteins (4, 45). These factors bind to the consensus sequence 5′-TGAC/GTCA-3′ through an α-helical, bipartite domain consisting of a basic, DNA-binding domain (b) followed by a dimerization domain called the leucine zipper (ZIP) (15, 18). Members of the Jun family form either Jun-Jun homodimers or heterodimers or combine with other, more distantly related bZIP proteins such as the members of the Fos and ATF protein families (16, 40). The collection of dimeric bZIP transcription factors which includes all of the various dimer combinations among the Jun, Fos, and ATF proteins is referred to as the AP1 complex (2, 6). AP1 is involved in converting numerous external signals generated by growth factors, cytokines, tumor promoters, or genotoxic agents into longer-lasting changes in the transcription of cellular target genes (33). In particular, AP1 is thought to play an important role in cell proliferation and the development of cancer (4, 44), in the response to genotoxic stress (12, 41), and in the induction of apoptosis (13, 17).

Numerous independent reports suggest that each dimeric combination involving c-Jun, JunB, and/or JunD might display a unique pattern of properties such as dimer stability, DNA binding specificity (29, 35), transactivating capacities (28), interaction with nuclear receptors (31), and interaction with tissue-specific transcription factors (24). At a given time, the net AP1 activity of this mixture of homo- and heterodimeric factors is the result of all of the individual activities of the dimers present in the cell. An important question concerns the specific contribution to the transformed phenotype of each homodimeric and heterodimeric combination involving c-Jun, JunB, and JunD. Oncogenic transformation of primary cultures of rat embryo fibroblasts (REFs) classically needs the cooperation of two oncogenes (10, 46). This requirement corresponds to the acquisition of two biological properties: (i) long-term growth capacity (i.e., establishment or immortalization) and (ii) focus formation and tumor induction in vivo (i.e., transformation). The first property can be the result of the activity of single oncogenes such as *myc* (immortalizing genes), whereas the second one requires, in addition to an immortalizing gene, the action of genes such as *ras* (transforming genes) (23, 34).

c-Jun has been found to cooperate with c-Ha-Ras to induce foci and to immortalize REFs. Since Ras alone has no effect on REF cells and although the immortalizing potential of c-*jun* has not been tested by transfection as a single oncogene, the efficient long-term establishment of c-Jun–Ras cell lines is most probably the consequence of c-Jun overexpression (36, 37). The cotransformation assay requires the N-terminal transactivating domain of c-Jun (1). More specifically, Ha-Ras has been shown to stimulate phosphorylation of the N-terminal mouse c-Jun residues Ser-63 and Ser-73, and these posttranslational modifications in turn are necessary for enhanced transactivation and oncogene cooperation (5, 39). To study the involvement of c-Jun homodimers in cotransformation of REF cells, Oliviero et al. (30) constructed a chimeric c-Jun/gcn4 derivative in which the natural dimerization ZIP domain was replaced by the heterologous homodimerization domain from the yeast transcription factor GCN4. Although both the c-Jun/gcn4 protein and the natural GCN4 protein were able to trans-

* Corresponding author. Mailing address: Unité de Virologie Humaine, INSERM-U412, Ecole Normale Supérieure, 46 allée d'Italie, 69364 Lyon Cedex 07, France. Phone: (33) 72 72 81 65. Fax: (33) 72 72 86 86. Electronic mail address: Marc.Castellazzi@cri.ens-lyon.fr.

activate through AP1 sites in REF cells, only c-Jun/gcn4 was able to cooperate with Ras to transform REF cells as tested by focus formation assays (30). These results demonstrated that transcriptional activation through AP1 sites is not sufficient for cellular transformation and that c-Jun functioning as a homodimer retains biological activity. However, in these experiments the c-Jun/gcn4-induced foci were not tested for their ability to grow as established transformed cell lines. Therefore, and because of the intrinsic ability of c-Jun to form heterodimers, the specific contribution of c-Jun homodimers to the immortalizing potential is still unknown.

JunB has also been shown to cotransform REF cells by inducing foci, but this transcription factor was less potent than c-Jun and was unable to immortalize efficiently (37). Interestingly, an inhibitory effect of JunB on c-Jun-mediated focus formation was also reported (37). This antagonism between c-Jun and JunB could be related to the preferential formation of c-Jun–JunB heterodimers, which are less active in transactivation activity (11). The present work is the first study of the oncogenic potential of JunD in this cotransformation assay. This protein was previously reported to efficiently suppress transformation by an activated *ras* gene in immortalized 3T3 cells (32).

In the present study the oncogenic potentials of chimeric c-Jun, JunB, and JunD homodimers in REF cells were examined by focus formation assays and subsequent establishment as cell lines. Previously constructed *jun/eb1* hybrid genes carrying the homodimerization domain of another bZIP protein (the Epstein-Barr virus transcription factor EB1) were used (42). Cotransfections of the various wild-type and artificial *jun* genes with an activated c-Ha-*ras* gene were performed. The effects of the chimeric Jun/eb1 proteins on focus formation and on immortalization were compared with those of the natural c-Jun, JunB, and JunD proteins.

MATERIALS AND METHODS

Cell culture. Primary cultures of REFs were prepared from 14-day Wistar rat embryos. The embryos were pooled, minced, washed with Dulbecco's modified Eagle's medium (Gibco), and then trypsinized. The resulting mixture was resuspended in regular medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1 mM glutamine, and penicillin-streptomycin) and plated. Cells were grown to near confluency and frozen as aliquots in liquid nitrogen. REF cultures were used for focus formation or chloramphenicol acetyltransferase (CAT) assays at their second passage after thawing.

Construction of jun vectors. The various *jun* derivatives were expressed from the same pLMPN1 plasmid (25). In this plasmid the coding sequences were cloned downstream of the Moloney murine leukemia virus 5′ long terminal repeat by insertion into a polylinker which carries the following sites from 5′ to 3′ in the sequence: *Bam*HI, *Hin*dIII, *Eco*RI, *Xho*I, *Not*I, *Stu*I, and *Xba*I. c-*jun* and *junD* were inserted as *Hin*dIII-*Eco*RI and *Eco*RI-*Eco*RI fragments, respectively (32). The coding sequences for c-*jun*/eb1, *junB*, *junB/eb1*, *junD/eb1*, and *junD/gcn4* were recovered as *Bam*HI-*Eco*RI fragments from the previously described RCAS derivatives (42) and inserted into pLMPN1 by using these restriction sites.

Cotransformation and immortalization assays. The following plasmids were used: pSV-c-myc-1, which carries a rearranged form of the human c-*myc* gene; pEJ6.6, containing the human c-Ha-*ras* gene (27); and the different pLMPN1-jun derivatives. Ten micrograms of each plasmid was transfected overnight into subconfluent REF cultures as previously described (5). Cells were replated at a density of 10^5 per plate and incubated in normal medium for 12 to 15 days. The cells were then fixed and stained with Giemsa stain, and the foci were counted. Individual foci were then plated separately and cultivated for 1 month to test for immortalization.

CAT assay. The various *jun* sequences were expressed from the Rous sarcoma virus long terminal repeat sequence in a pDP plasmid (42). The COL(−73/+63)-CAT reporter plasmid (3) contains a portion of the human collagenase promoter placed upstream of the CAT gene. At the fourth passage, 0.5×10^6 REF cells were seeded per 60-mm-diameter plate, and the cells were transfected 16 h later by standard calcium phosphate coprecipitation. Two micrograms of COL(−73/+63)-CAT reporter plasmid, 1 μg of pDP-jun, and 2 μg of either pZIPneo or pZIPneoRas (Leu-61) (5) were used, and the total amount of DNA was adjusted to 12 μg per plate with pUC18. Eight hours after transfection, the cells were washed twice with phosphate-buffered saline (PBS) and further incubated in normal medium (supplemented with 10% serum). Cell lysates were prepared 48 h after transfection, and CAT activity was measured by using an enzyme immunoassay (Boehringer CAT-ELISA; no. 1363 727). Plates were analyzed in duplicate.

RNA analysis. RNA was prepared and analyzed as previously described (26). Briefly, total RNA (20 μg) isolated from individual transfected cell lines was electrophoretically separated on agarose gels, transferred to nitrocellulose filters, and hybridized to ^{32}P-labelled DNA probes. The c-*jun*, *junB*, and *junD* coding sequences used as DNA probes were recovered from the pLMPN1 plasmids with the same specific restriction sites used for cloning (listed above). The S26 probe was used as a control for equivalent loading (43).

Protein analysis. Subconfluent cultures were washed with PBS, recovered by scraping, centrifuged, and resuspended in lysis buffer [50 mM Tris (pH 7.2), 150 mM NaCl, 1 mM dithiothreitol, 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1% sodium dodecyl sulfate], boiled for 5 min, and frozen in liquid nitrogen. Thawed aliquots were treated with DNase I, and the protein concentration was measured with the Bradford reagent (Bio-Rad). Proteins from each sample (30 μg per lane) were separated on a 10% polyacrylamide gel and transferred to a nitrocellulose filter (BAS 85; Schleicher & Schuell). Nonspecific binding was reduced by overnight incubation in 5% powdered milk in PBS supplemented with 0.1% Tween 20. This medium was also used for the subsequent incubations with the different antibodies. Anti-Jun and anti-EB1 antibodies were rabbit polyclonal antibodies. Specific antibodies were raised against glutathione *S*-transferase fusion proteins which included amino acids 1 to 58 of murine c-Jun (cJ1 antibodies), amino acids 1 to 75 of murine JunB (JB1), amino acids 329 to 341 of murine JunD (JD2) (32), and amino acids 192 to 245 of EB1. Anti-Ras monoclonal antibodies were directed against mammalian c-Ha-Ras p21 (Oncogene Science). Antibody-antigen complexes were detected with a peroxidase-labelled goat anti-rabbit or anti-mouse immunoglobulin G antibody and the ECL Western blotting (immunoblotting) analysis system (Amersham). Quantification of the signals was done with the ImageQuant software from Molecular Dynamics.

RESULTS

Focus formation. We first compared the transforming capacities of the three *jun* genes. REFs were cotransfected with an activated c-Ha-*ras* expression plasmid and each of the *jun*-containing pLMPN1 expression vectors. As shown in Table 1, *ras* alone did not induce a significant number of foci. *myc* or the different *jun* genes alone were also incapable of inducing foci (data not shown). By contrast, a combination of *ras* and *myc* or *ras* and c-*jun* resulted in a marked increase in focus formation (averages of 106 and 84 foci, respectively). Foci were also observed with *ras* plus *junB*, although at a slightly lower level (69% of the value for c-*jun* plus *ras*). These values are in good agreement with published data on c-*jun*, *junB*, and *myc* cotransformation with *ras* (27, 36, 37). Surprisingly, we found that *junD* was also capable of inducing foci, with an efficiency only slightly lower than that of *junB* (60% of the c-*jun*–*ras* value). These results demonstrate that each mouse *jun* gene is able to cooperate with *ras* to induce foci in REF cells, with c-*jun* being the most efficient.

In order to analyze the contribution of Jun-Jun homodimers to transformation, we next analyzed the activities of the three *jun/eb1* derivatives carrying the homodimerization domain of the EB1 transcription factor. In cooperation with *ras*, each of the *jun/eb1* hybrids induced foci in REFs with frequencies comparable to those of the corresponding natural genes (Table 1). There was no obvious difference in size or morphology between the foci induced by *jun/eb1*, *myc*, or the natural *jun* genes (data not shown). Focus formation by c-*jun/eb1* was expected from results reported by Oliviero et al. (30). These authors reported that a c-*jun/gcn4* hybrid (containing a heterologous homodimerization domain from the yeast transcription factor GCN4) also induced foci in REF cells. We have extended this observation to the *junB* and *junD* genes. To eliminate the possibility that the EB1 dimerization domain per se was responsible for transformation by *junD/eb1*, we also tested a *junD/gcn4* construct (42). The JunD/gcn4 protein was as efficient as JunD and JunD/eb1 in focus formation (Table 1). Together, these data demonstrate that each Jun protein

TABLE 1. Cotransformation by *ras* and the different *jun* genes

Gene cotransfected with *ras*	Total no. of foci in expt[a]:										Mean no. of foci (%[b]) in expt:	
	I	II	III	IV	V	VI	VII	VIII	IX	X	I–VII	VIII–X
None	0	0	5	0	0	0	2	0	0	0	1	0
c-*myc*	—[c]	104	168	88	82	62	—	112	—	—	101	112
c-*jun*	16	12	50	40	56	16	34	130	120	154	32 (100)	135 (100)
c-*jun*/*eb1*	8	12	15	22	34	—	—	—	86	44	20.7 (65)	
junB	12	8	35	20	36	—	—	—	—	—	22.2 (69)	
junB/*eb1*	—	24	25	28	44	—	—	—	—	—	30.3 (95)	
junD	8	16	40	10	26	8	24	80	80	62	19 (60)	74 (55)
junD/*eb1*	—	16	15	20	28	10	—	40	—	—	18 (56)	40 (30)
junD/*gcn4*	—	—		—	—	8	30	60	42	82	19 (60)	58 (43)

[a] Experiments I to VII and VIII to X were performed with two independent primary bulk cultures frozen as aliquots in liquid nitrogen.
[b] The values for *ras*–c-*jun* were taken as 100%.
[c] —, not done.

possesses an intrinsic transforming potential as defined by the focus-forming assay with *ras*-cotransfected REF cells.

Immortalization. Immortalization constitutes a second step, or more advanced stage, in the transformation of REF cells. It has previously been reported that one-third of the foci from cells transfected with *ras* plus c-*jun* could be expanded into continuous cell lines, whereas foci from transfections with *ras* plus *junB* could not be expanded (37). We have investigated the growth potentials of the foci induced by *junD* and by the different *jun*/*eb1* derivatives. As shown in Table 2, individual foci were recovered from several independent transfections and tested for long-term growth. In keeping with the previous reports, immortalization by *ras* plus c-*myc* was very efficient (six of seven foci tested; clone M). Immortalization by *ras* plus c-*jun* was also detectable (7 of 20 foci tested; clones C1 to C7). Surprisingly, we were unable to immortalize any of 26 foci subcloned from several *ras*–*c-jun*/*eb1* transfections, demonstrating that c-Jun homodimers are deficient in immortalization. A much lower frequency of establishment of long-term cultures was observed for foci subcloned from transfections with *ras* plus either *junB* (clones B1 and B2), *junB*/*eb1* (clone BE1), *junD* (clone D1 and D2), or *junD*/*eb1* (clone DE1). Together these results strongly suggest that only wild-type c-Jun has an immortalizing potential, whereas JunB, JunD, and all of the chimeras are deficient in this activity.

TABLE 2. Immortalization by the different *jun* genes

Gene cotransfected with *ras*	No. of foci tested[a]	No. of continuous cell lines	No. (names) of cell lines overexpressing expected Jun product
c-*myc*	7	6	
c-*jun*	20	7	5 of 5 (C1, C2, C3, C4, C5)
c-*jun*/*eb1*	26	0	
junB	20	2	—[b]
junB/*eb1*	20	1	0
junD	20	2	1 (D2)
junD/*eb1*	20	1	1 (DE1)

[a] Foci were from several independent experiments from those described for Table 1.
[b] —, all *ras-jun* transfectants showed an enhanced accumulation of JunB compared with normal REFs and *ras-myc* transfectants (see Results and Fig. 1).

The different established clones grew with doubling times of 18 to 24 h and were morphologically altered, with many cells exhibiting a rounded shape and being only loosely adherent to the petri dish (data not shown). They could not be distinguished as c-*jun*, *junB*, or *junD* transfectants on the basis of growth capacities or morphological alterations.

Transactivation of an AP1 reporter construct by the different Jun and Jun/eb1 proteins. To test whether the behaviors of the different Jun proteins in transformation and immortalization assays were due to a lack of function in an REF background, we checked their transactivating properties in the presence or absence of an activated c-Ha-*ras* gene. In vitro transfection assays with REF cells and the human collagenase promoter COL(−73/+63) upstream of the CAT gene as a reporter plasmid were performed. This collagenase promoter element contains a single consensus AP1 site, also described as a TPA-responsive element (3). The data reported in Fig. 1 were obtained from four independent transfections with different REF cultures and plasmid preparations. Although the absolute values from each experiment varied in the range of 1 to 5, the relative values were very close from one experiment to another for the different *jun* and *ras-jun* combinations. Each of the natural Jun proteins displayed a clear transactivation capacity. However, c-Jun was more potent than JunB and JunD by a factor of two- to threefold. These variations in activities are in agreement with previous data on single AP1 sites for c-*jun* compared with *junB* (8, 11) and *junD* (20). Cotransfection with c-Ha-*ras* stimulated transactivation, corresponding to a 2.4-fold increase with c-*jun*, a 2-fold increase with *junB*, and a 1.5-fold increase with *junD*. These data also agree with independent data on the Ras-mediated stimulation of c-Jun (5).

In the same experiments, the Jun/eb1 derivatives were consistently more active than the corresponding natural Jun. The fold enhancement was 2.3 for c-*jun*/*eb1* versus c-*jun*, 3.6 for *junB*/*eb1* versus *junB*, and 3.8 for *junD*/*eb1* versus *junD*. Again, c-Ha-*ras* stimulated transactivation, corresponding to a 1.7-fold increase with c-*jun*/*eb1*, a 2.9-fold increase with *junB*/*eb1*, and a 2.9-fold increase with *junD*/*eb1*. Clearly, these data indicate that the Jun/eb1 products are strong transactivators through AP1 sites in REF cells. They are also sensitive to stimulation by c-Ha-Ras.

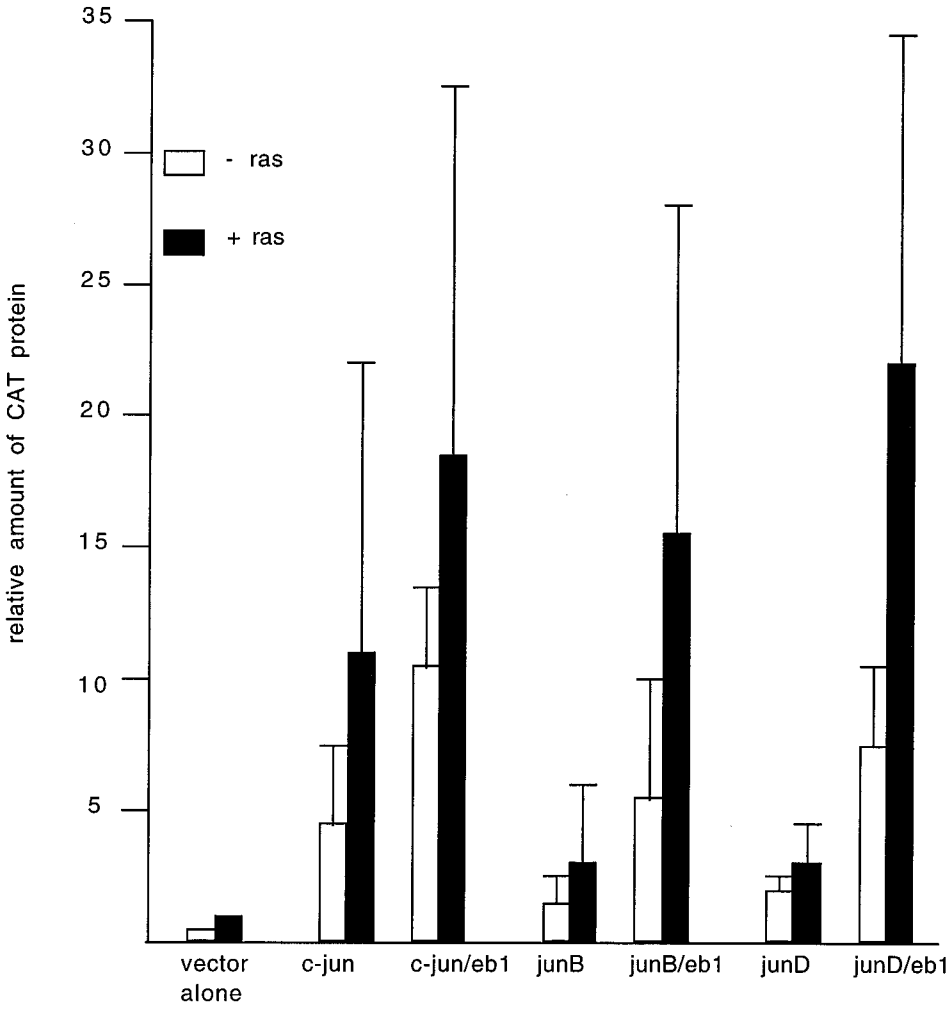

FIG. 1. Transactivation potentials of the different *jun* and *jun/eb1* derivatives in the presence or absence of c-Ha-*ras*. The values represent averages from four independent experiments; error bars indicate the largest amount of CAT protein obtained (see text).

Finally, although the Jun/eb1 hybrids were better transactivators than the corresponding natural Jun proteins, they could not induce foci more efficiently. Conversely, JunB and JunD were poor transactivators of the collagenase promoter but still induced foci with high efficiency (compare the relative values in Fig. 1 and Table 1). This situation might reflect the fact that the COL(−73/+63) promoter used in this study is not a fair representative of the oncogenically relevant target(s) of Jun. In any case, these results demonstrate that the various Jun constructs are expressed and transactivate in a REF background.

Patterns of expression of Ras and Jun in the immortalized lines. To determine whether the established cultures accumulated the proteins encoded by the transfected genes, Western blots were probed with specific antibodies directed against the Ras and Jun proteins. Extracts from normal REFs and from a *ras-myc* clone (M) were included, as well as extracts from chicken embryo fibroblasts (CEFs) which expressed JunB/eb1 or JunD/eb1 (42). The level of accumulation of each protein was estimated by densitometric scanning of the specific signals. In extracts from normal REF cells, Ras was not detectable. By contrast, each Jun protein was easily detected, either as a single band for c-Jun, as a doublet for JunD, or as two or three bands for Jun-B (see also reference 17 for the detection of the different Jun proteins in PC12 rat cells). These bands migrated at the expected molecular masses (Fig. 2 and 3, lanes R).

In the extracts from *ras-jun*-transfected cells, Ras was clearly detected as a single band of approximately 21 kDa. Surprisingly, a high level of JunB was found in each of the established cell lines. There is a good correlation between the levels of expression of JunB and Ras, as represented in Fig. 4. The primary REF cultures which did not show any detectable level of Ras had the lowest level of JunB. Cell lines recovered after transfection with *ras* plus c-*jun* or *ras* plus *junD* had either intermediate levels of JunB (C2, D1, and C4) or high levels of this protein (C5, C3, D2, and C1). Finally, the two *ras-junB*-transfected cell lines, B1 and B2, also displayed high JunB levels. These observations are consistent with the fact that an exogenous *junB* mRNA is expressed in these two lines (Fig. 5). The increased levels of JunB protein in *ras* cells which we observed agree with earlier reports showing that transfected c-Ha-*ras* stimulates the transcription of the *junB* promoter in rodent cells (9, 38). The *ras-myc* cell line (M) was the only line which did not fit this correlation; although the level of Ras was elevated, the amount of JunB remained close to that found in nonimmortalized REF cultures.

(i) The *ras*–c-*jun*-transfected cell lines (C1 to C5). In addition to Ras and JunB, cell lines C1 to C5 also accumulated c-Jun at high levels (Fig. 2 and 3). The amounts of c-Jun in the C1, C2, C3, C4, and C5 lines were 7.6-, 26.6-, 25.1-, 21.9-, and 39.1-fold, respectively, the amount present in the REF cells. In

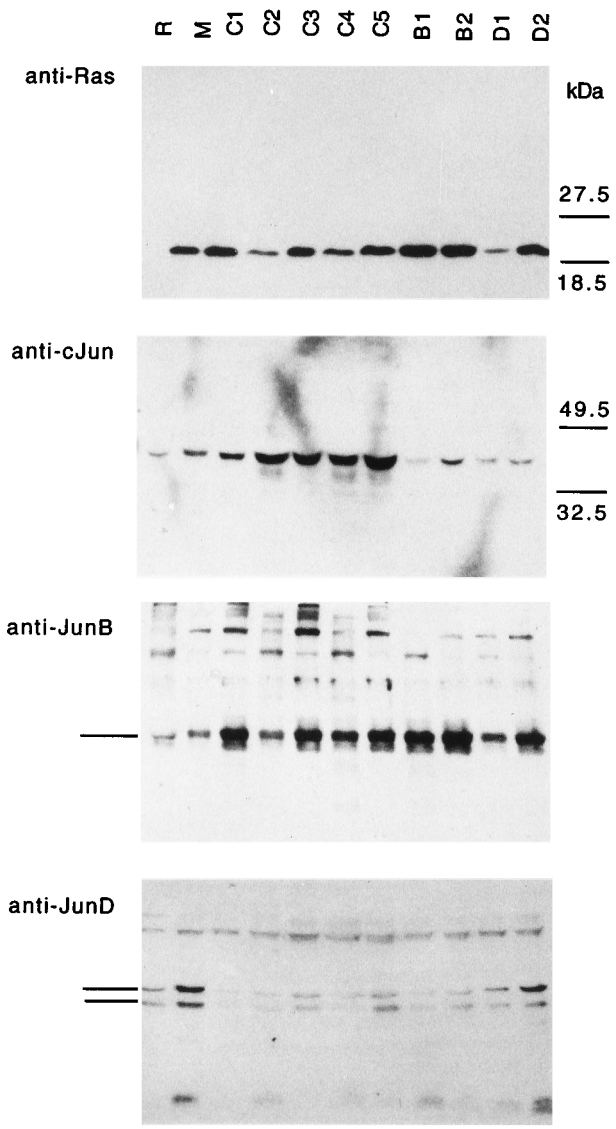

FIG. 2. Western blot analyses of the Ras and Jun products accumulated in the different cell lines. Total extracts were prepared from nontransfected primary REF cells (lane R) and from established REF cell lines isolated from *ras-myc* (lane M), *ras*–c-*jun* (lanes C1 to C5), *ras-junB* (lanes B1 and B2), and *ras-junD* (lanes D1 and D2) transfections. The specific antibodies used are indicated on the left. The positions of the endogenous JunB and JunD from REFs are indicated by solid lines.

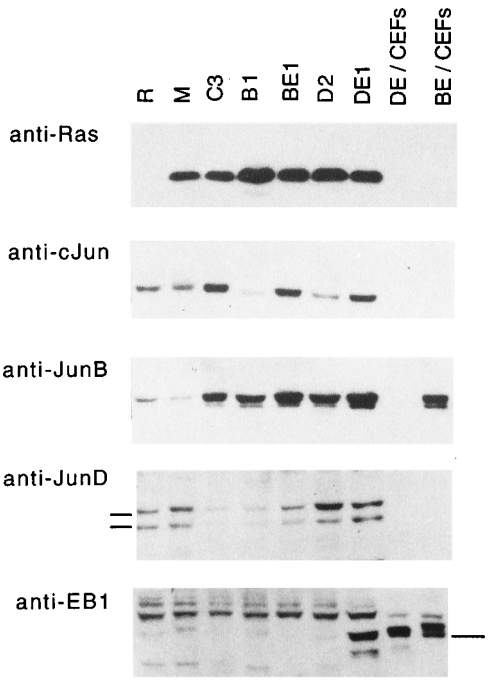

FIG. 3. Western blot analyses of the Jun/eb1 products. As for Fig. 2, total extracts were prepared from the R, M, C3, B1, and D2 cell lines. Additional extracts were prepared from cell lines obtained from *ras-junB/eb1* (lane BE1) and *ras-junD/eb1* (lane DE1) transfections, as well as from CEFs accumulating JunD/eb1 (lane DE/CEFs) or JunB/eb1 (lane BE/CEFs) (42). The positions of the endogenous JunD from REFs and JunD/eb1 from CEFs are indicated by solid lines.

agreement with this observation, an exogenous c-*jun* mRNA of about 4.5 kb was detected in each of these cell lines in addition to the endogenous c-*jun* mRNA (Fig. 5). The levels of JunD in the various cell lines were either close to the level in normal REF cells (C3, 72% of the normal Jun-D levels, and C5, 108%) or lower (C1, 7%; C2, 26%; and C4, 40%) (data not shown).

(ii) The *ras-junB*- and *ras-junB/eb1*-transfected cell lines (B1, B2, and BE1). Clones B1, B2, and BE1 all accumulated high levels of JunB. The amounts of JunB in the B1, B2, and BE1 lines were 13.9, 14.3, and 25.0 times that found in the REF cells, respectively. Clones B2 and BE1 also accumulated c-Jun (Fig. 2 and 3). However, because all of the *ras-jun*-transfected lines were found to accumulate JunB, the specific contributions of the exogenous *junB* gene in these lines are uncertain. Such a contribution might take place in B1 and B2 (which expressed an exogenous *junB* mRNA [Fig. 5]) but probably not in BE1 (in which no JunB/eb1 hybrid protein could be detected with anti-EB1 antibodies [Fig. 3]).

(iii) The *ras-junD*- and *ras-junD/eb1*-transfected cell lines (D1, D2, and DE1). Clone D1 did not accumulate JunD (Fig. 2), and there was no detectable exogeneous *junD* mRNA (Fig. 5). By contrast, clones D2 and DE1 accumulated a JunD product and a JunD/eb1 product, respectively. The amounts of JunD in the D2 and DE1 lines were 4.7- and 3.2-fold, respectively, the amount in the REF cells. These products were likely to be produced by the transfected genes because (i) there is an exogeneous *junD* mRNA in D2 (Fig. 5) and (ii) anti-EB1 antibodies detected a Jun/eb1 product in DE1 (Fig. 3). DE1 also accumulated c-Jun (Fig. 3) at a level 3.9-fold over the level in REF cells (data not shown).

In conclusion, all of the lines obtained from the *ras*–c-*jun* transfections were shown to overexpress the transfected c-*jun* gene. By contrast, among the few lines recovered from the other transfections, only two lines, D2 and DE1, displayed a convincing correlation between the transfected genes and the pattern of mRNA and/or Jun accumulation (Table 2). The reason for the survival of these few cell lines is unclear, and at least some of them might correspond to *ras*-transformed REF lines, as explained in Discussion.

DISCUSSION

In the process of in vitro transformation of primary cultures of rat embryo fibroblasts, two steps can be experimentally defined: focus formation on top of REF monolayers and subsequent cell line establishment, or immortalization, of these

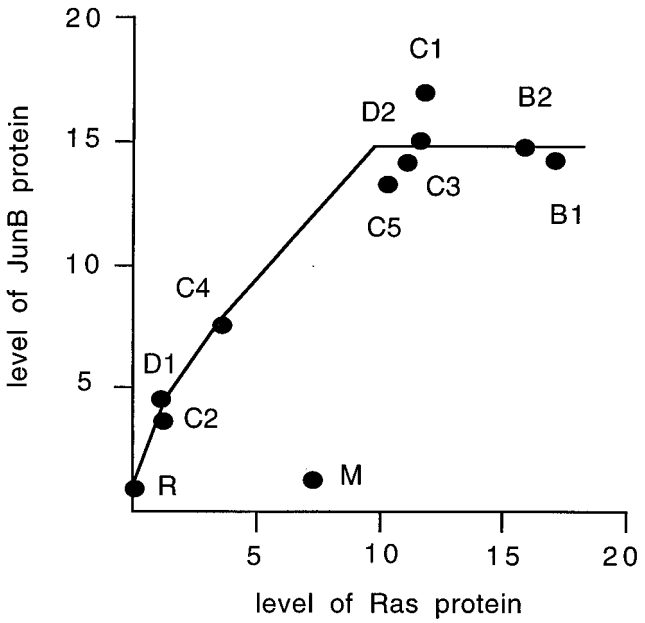

FIG. 4. Correlation between Ras and JunB protein levels in the c-Ha-*ras*-transfected cell lines. The relative values were obtained by densitometric scanning of the different blots from Fig. 2. Ras protein was undetectable in the primary REF culture (cell line R).

foci. We have shown here that (i) each member of the mouse Jun family can induce focus formation in cooperation with activated c-Ha-Ras, (ii) Jun/eb1 hybrids which are limited to forming only homodimers also can induce foci, and (iii) a specific c-Jun-containing heterodimer is involved in the further step of immortalizing the cells.

In cotransformation assays, c-*jun* was 20 to 50% more efficient than either *junB*, *junD*, or any of the three artificial *jun/eb1* derivatives. With respect to focus formation, these data demonstrate that in a REF background, each Jun protein, even when limited to functioning as a homodimer, displays a transforming potential. Overexpression of the mouse *jun* and *jun/eb1* genes in CEFs produced results slightly different from these obtained with REF cells. In CEFs, c-*jun* transforms efficiently, whereas *junB* transforms only weakly and *junD* does not transform at all (7). The lack of oncogenicity exhibited by *junD* was independently confirmed with genes from both avian (19) and mouse (21) origins. In CEFs, introduction of the homodimerization domain of EB1 enhances transformation by *junB* and turns *junD* into a mildly transforming gene (42). With these *junB/eb1* and *junD/eb1* hybrid genes, enhanced transformation was correlated with enhanced transactivating potential through AP1 sites, possibly resulting from an increase in dimer stability. Because JunD/eb1 homodimers are transforming in both avian and rodent cells, we hypothesize that the lower oncogenic potential of the naturally occurring JunD protein is related to its cell-type-specific dimerization partners. Thus, depending on the cellular context, JunD might be involved in different heterodimer combinations which can have different effects on cell growth and transformation. This idea could explain why JunD has recently been found to antagonize Ras-mediated transformation in mouse NIH 3T3 cells; presumably, the available dimer partners for JunD can facilitate a negative growth signal in these established cells (32). Together these results demonstrate that each mouse Jun protein possesses a specific biological activity depending upon the cellular system

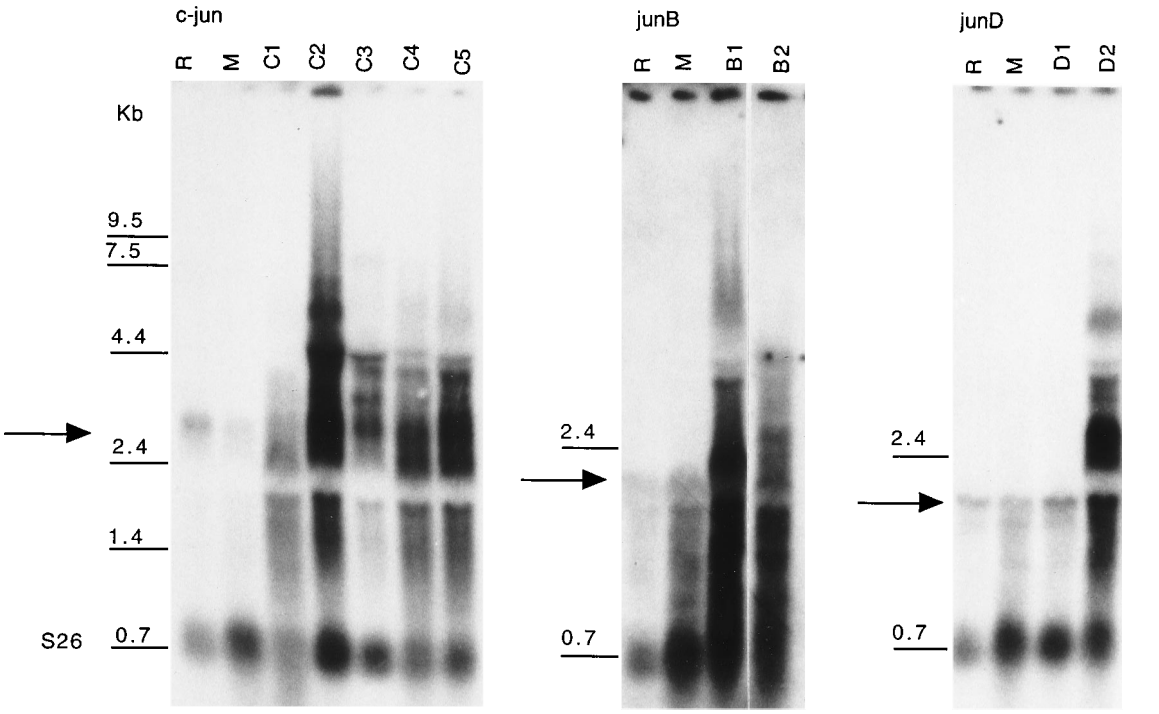

FIG. 5. Northern (RNA) blot hybridization analysis of nontransfected primary REFs (lanes R), and established cell lines from *ras-myc* (lanes M), *ras*–c-*jun* (lanes C1 to C5), *ras-junB* (lanes B1 and B2), and *ras-junD* (lanes D1 and D2) transfections. Each panel shows a different blot probed with c-*jun*, *junB*, or *junD*, as indicated. The arrows indicate the positions of the endogenous messengers: about 2.5 to 3 kb for c-*jun*, about 2 kb for *junB*, and about 1.8 kb for *junD*. The exogenous *jun* messengers are expressed from the pLMPN1 viral structure as a 4.4- to 4.6-kb messenger. RNAs from each lane were quantified by using the S26 probe, which recognizes a 0.7-kb messenger (43).

and suggest that variation of this activity can occur through the presence of cell-type-specific dimerization partners.

In agreement with previous reports (36, 37), more than 30% of foci from transfections with *ras* plus c-*jun* could be expanded into long-term cultures, whereas only 10% of foci from *ras*-*junB* transfections were established. Immortalization occurred with a lower frequency in the case of *ras* plus *junD*, *ras* plus *junB*/*eb1*, and *ras* plus *junD*/*eb1* (one or two lines in each case from 20 foci tested) and was not detected at all in *ras*–c-*jun*/*eb1* transfections (no line could be recovered from 26 foci tested). Furthermore, all of the *ras*–c-*jun* established cell lines expressed exogeneous c-*jun* transcripts and accumulated high levels of c-Jun protein. In contrast, the few other lines recovered from *junB*, *junB*/*eb1*, *junD*, and *junD*/*eb1* transfections did not always exhibit relevant patterns of Jun proteins or the expected exogenous mRNAs. The establishment of these lines is likely to be due primarily to the action of *ras*. Indeed, oncogenic conversion by *ras* alone has been reported to take place at a low frequency in REF cells (22). Therefore, the different *jun* genes can be distinguished by their capacities to promote the immortalization; most of the foci induced by *ras* plus *junB* or *junD* stop growing after isolation. Such a situation is not restricted to the combination of *jun* plus *ras* in oncogenic cooperation. For example, a mutated polyomavirus large T-antigen gene which interacts poorly with the retinoblastoma protein Rb is unable to promote the immortalization step following focus formation (14). Our results indicate that c-Jun, JunB, or JunD homodimers are also deficient in inducing the immortalization step. Moreover, the absence of immortalization by c-*jun*/*eb1* markedly contrasts with the efficient immortalization by the natural c-*jun* gene. The incapacity of c-Jun/eb1 to immortalize is not due to a lack of function, since the hybrid protein caused efficient focus formation and strongly transactivated the COL(−73/+63)-CAT reporter construct. In regard to oncogene cooperation in REF cells, these findings demonstrate that c-Jun displays two distinct and molecularly separable biological properties: focus formation in cooperation with Ras and immortalization. Furthermore, these results suggest that immortalization by c-Jun requires the presence of a specific c-Jun-containing heterodimer. This heterodimer should involve another bZIP partner. Because neither JunB nor JunD, in cooperation with Ras, could immortalize, it is very likely that neither of these two proteins is the right partner for c-Jun. This view is also supported by the finding that JunB–c-Jun heterodimers are weak in both DNA binding and transactivation (11). The involvement of c-Fos or FosB is also unlikely, since these proteins were undetectable in the c-Jun-overexpressing clones (data not shown). Other members of the Fos and ATF families of transcription factors can heterodimerize efficiently with c-Jun and are potential candidates for participation in immortalization. Further studies are necessary to identify the specific c-Jun partner within the immortalization-promoting heterodimer. Finally, the characterization of relevant target genes differentially regulated by c-Jun/eb1 and c-Jun will also help towards understanding how c-Jun, and more generally AP1, controls immortalization of these primary mammalian cells.

ACKNOWLEDGMENTS

This work was supported by grants from the ARC, FNCLCC, INSERM, and MGEN to M.C. and by a grant from the ARC to B.B. L.V. was supported by a fellowship from the ARC.

REFERENCES

1. **Alani, R., P. Brown, B. Binétruy, H. Dosaka, R. K. Rosenberg, P. Angel, M. Karin, and M. J. Birrer.** 1991. The transactivating domain of the c-Jun proto-oncoprotein is required for cotransformation of rat embryo cells. Mol. Cell. Biol. **11:**6286–6295.
2. **Angel, P., E. A. Allegretto, S. T. Okino, K. Hattori, W. J. Boyle, T. Hunter, and M. Karin.** 1988. Oncogene jun encodes a sequence-specific trans-activator similar to AP-1. Nature (London) **332:**166–171.
3. **Angel, P., I. Bauman, B. Stein, H. Delius, H. J. Rahmsdorf, and P. Herrlich.** 1987. 12-*O*-Tetradecanoyl-phorbol-13-acetate induction of the human collagenase gene is mediated by an inducible enhancer element located in the 5′-flanking region. Mol. Cell. Biol. **7:**2256–2266.
4. **Angel, P., and M. Karin.** 1991. The role of Jun, Fos and the AP-1 complex in cell proliferation and transformation. Biochim. Biophys. Acta **1072:**129–157.
5. **Binétruy, B., T. Smeal, and M. Karin.** 1991. Ha-Ras augments c-Jun activity and stimulates phosphorylation of its activation domain. Nature (London) **351:**122–127.
6. **Bohmann, D., T. J. Bos, A. Admon, T. Nishimura, P. K. Vogt, and R. Tjian.** 1987. Human proto-oncogene c-jun encodes a DNA binding protein with structural and functional properties of transcriptional factor AP-1. Science **238:**1386–1392.
7. **Castellazzi, M., G. Spyrou, N. La Vista, J. P. Dangy, F. Piu, M. Yaniv, and G. Brun.** 1991. Overexpression of c-jun, junB and junD affects cell growth differently. Proc. Natl. Acad. Sci. USA **88:**8890–8894.
8. **Chiu, R., P. Angel, and M. Karin.** 1989. JunB differs in its biological properties from and is a negative regulator of c-Jun. Cell **59:**979–986.
9. **Coffer, P., M. de Jonge, A. Mettouchi, B. Binétruy, J. Ghysdael, and W. Kruijer.** 1994. junB promoter regulation: Ras mediated transactivation by c-Ets-1 and c-Ets-2. Oncogene **9:**911–921.
10. **Cuzin, F.** 1984. The polyoma virus oncogenes. Coordinated functions of three distinct proteins in the transformation of rodent cells in culture. Biochim. Biophys. Acta **781:**193–204.
11. **Deng, T., and M. Karin.** 1993. JunB differs from c-Jun in its DNA-binding and dimerization domains, and represses c-Jun by formation of inactive heterodimers. Genes Dev. **7:**479–490.
12. **Devary, Y., R. A. Gottlieb, T. Smeal, and M. Karin.** 1992. The mammalian ultraviolet response is triggered by activation of Src tyrosine kinases. Cell **71:**1081–1091.
13. **Estus, S., W. J. Zaks, R. S. Freeman, M. Gruda, R. Bravo, and E. M. J. Johnson.** 1994. Altered gene expression in neurons during programmed cell death: identification of c-jun as necessary for neuronal apoptosis. J. Cell. Biol. **127:**1717–1727.
14. **Freund, R., R. T. Bronson, and T. L. Benjamin.** 1992. Separation of immortalization from tumor induction with polyoma large T mutants that fail to bind the retinoblastoma gene product. Oncogene **7:**1979–1987.
15. **Glover, J. N. M., and S. C. Harrison.** 1995. Crystal structure of the heterodimeric bZIP transcription factor c-Fos-c-Jun bound to DNA. Nature (London) **373:**257–261.
16. **Hai, T., and T. Curran.** 1991. Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. Proc. Natl. Acad. Sci. USA **88:**3720–3724.
17. **Ham, J., C. Babij, J. Whitfield, C. M. Pfarr, D. Lallemand, M. Yaniv, and L. L. Rubin.** 1995. A c-Jun dominant negative mutant protects sympathetic neurons against programmed death. Neuron **14:**927–939.
18. **Harrison, S. C.** 1991. A structural taxonomy of DNA-binding domains. Nature (London) **363:**715–719.
19. **Hartl, M., and P. K. Vogt.** 1992. A rearranged JunD transforms chicken embryo fibroblasts. Cell Growth Differ. **3:**909–918.
20. **Hirai, S. I., R. P. Ryseck, F. Mechta, R. Bravo, and M. Yaniv.** 1989. Characterization of junD: a new member of the jun proto-oncogene family. EMBO J. **8:**1433–1439.
21. **Kameda, T., A. Akahori, M. H. Sonobe, T. Suzuki, T. Endo, and H. Iba.** 1993. JunD mutants with spontaneously acquired transforming potential have enhanced transactivating activity in combination with Fra-2. Proc. Natl. Acad. Sci. USA **90:**9369–9373.
22. **Land, H., A. C. Chen, J. P. Morgenstern, L. F. Parada, and R. A. Weinberg.** 1986. Behavior of *myc* and *ras* oncogenes in transformation of rat embryo fibroblasts. Mol. Cell. Biol. **6:**1917–1925.
23. **Land, H., L. F. Parada, and R. A. Weinberg.** 1983. Cellular oncogenes and multistep carcinogenesis. Science **222:**771–778.
24. **Li, L., J. C. Chambard, M. Karin, and E. N. Olson.** 1992. Fos and Jun repress transcriptional activation by myogenin and MyoD: the amino-terminus of Jun can mediate repression. Genes Dev. **6:**676–689.
25. **Marty, L., P. Roux, M. Royer, and M. Piechaczyk.** 1990. MoMLV derived cells inactivating retroviral vectors possessing multiple cloning sites and expressing resistance to either G418 or hygromycin B. Biochimie **72:**885–887.
26. **Mettouchi, A., F. Cabon, N. Montreau, P. Vernier, G. Mercier, D. Blangy, H. Tricoire, P. Vigier, and B. Binetruy.** 1994. SPARC and thrombospondin genes are repressed by the *c-jun* oncogene in rat embryo fibroblasts. EMBO J. **13:**5668–5678.
27. **Mougneau, E., L. Lemieux, M. Rassoulzadegan, and F. Cuzin.** 1984. Biological activities of *v-myc* and rearranged *c-myc* oncogenes in rat fibroblasts in culture. Proc. Natl. Acad. Sci. USA **81:**5758–5762.
28. **Nakabeppu, Y., and D. Nathans.** 1991. A naturally truncated form of FosB

that inhibits Fos/Jun transcriptional activity. Cell **64:**751–759.
29. **Nakabeppu, Y., K. Ryder, and D. Nathans.** 1988. DNA binding activities of three murine Jun proteins: stimulation by Fos. Cell **55:**907–915.
30. **Oliviero, S., G. S. Robinson, K. Struhl, and B. M. Spiegelman.** 1992. Yeast GCN4 as a probe for oncogenesis by AP-1 transcription factors—transcriptional activation through AP-1 sites is not sufficient for cellular transformation. Genes Dev. **6:**1799–1809.
31. **Pfahl, M.** 1993. Nuclear receptor/AP-1 interaction. Endocrine Rev. **14:**651–657.
32. **Pfarr, C. M., F. Mechta, G. Spyrou, D. Lallemand, S. Carillo, and M. Yaniv.** 1994. Mouse JunD negatively regulates fibroblast growth and antagonizes transformation by ras. Cell **76:**747–760.
33. **Radler-Pohl, A., S. Gebel, C. Sachsenmaier, H. Konig, M. Kramer, T. Oehler, M. Streile, H. Ponta, U. Rapp, H. J. Rahmsdorf, A. C. B. Cato, P. Angel, and P. Herrlich.** 1993. The activation and activity control of AP-1 (Fos/Jun). Ann. N. Y. Acad. Sci. **684:**127–148.
34. **Ruley, H. E.** 1983. Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature (London) **304:**602–606.
35. **Ryseck, R., and R. Bravo.** 1991. c-JUN, JUN B, and JUN D differ in their binding affinities to AP-1 and CRE consensus sequences: effect of FOS proteins. Oncogene **6:**533–542.
36. **Schütte, J., J. D. Minna, and M. Birrer.** 1989. Deregulated expression of human c-jun transforms primary rat embryo cells in cooperation with an activated c-Ha-ras gene and transforms Rat-1 cells as a single gene. Proc. Natl. Acad. Sci. USA **86:**2257–2261.
37. **Schütte, J., J. Viallet, M. Nau, S. Segal, J. Fedorko, and J. Minna.** 1989. Jun B inhibits and c-fos stimulates the transforming and transactivating activities of c-jun. Cell **59:**987–997.
38. **Sistonen, L., E. Holtta, T. P. Makela, J. Keski-Oja, and K. Alitalo.** 1989. The cellular response to induction of the p21 c-Ha-ras oncoprotein includes stimulation of jun gene expression. EMBO J. **8:**815–822.
39. **Smeal, T., B. Binetruy, D. A. Mercola, M. Birrer, and M. Karin.** 1991. Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of c-Jun on serines 63 and 73. Nature (London) **354:**494–496.
40. **van Dam, H., M. Duyndam, R. Rottier, A. Bosch, L. Devriessmits, P. Herrlich, A. Zantema, P. Angel, and A. J. van der Eb.** 1993. Heterodimer formation of cJun and ATF-2 is responsible for induction of c-jun by the 243-amino acid adenovirus E1A protein. EMBO J. **12:**479–487.
41. **van Dam, H., D. Wilhelm, I. Herr, A. Steffen, P. Herrlich, and P. Angel.** 1995. ATF-2 is preferentially activated by stress-activated kinases to mediate c-jun induction in response to genotoxic agents. EMBO J. **14:**1798–1811.
42. **Vandel, L., C. M. Pfarr, S. Huguier, L. Loiseau, A. Sergeant, and M. Castellazzi.** 1995. Increased transforming activity of JunB and JunD by introduction of an heterologous homodimerization domain. Oncogene **10:**495–507.
43. **Vincent, S., L. Marty, and P. Fort.** 1993. S26 ribosomal protein RNA: an invariant control for gene regulation experiments in eucaryotic cells and tissues. Nucleic Acids Res. **21:**1498.
44. **Vogt, P. K.** 1994. Oncogenic transformation by Jun, p. 203–219. *In* P. E. Angel and P. Herrlich (ed.), The Fos and Jun family of transcription factors. CRC Press, Boca Raton, Fla.
45. **Vogt, P. K., and T. Bos.** 1990. Jun: oncogene and transcription factor. Adv. Cancer Res. **55:**1–35.
46. **Weinberg, R. A.** 1985. The action of oncogenes in the cytoplasm and nucleus. Science **230:**770–776.