Heterodimerization with c-Fos Is Not Required for Cell Transformation of Chicken Embryo Fibroblasts by Jun

Mark Hughes, Anil Sehgal, Martin Hadman, and Timothy Bos
Department of Microbiology and Immunology, Eastern Virginia Medical School, Norfolk, Virginia 23501

Abstract

c-Jun belongs to a family of proteins that require dimerization for activity. Dimerization occurs through a leucine-rich region near the carboxy terminus called the leucine zipper. Jun can form dimeric complexes with other Jun family as well as Fos family members. The relative proportion of these different dimeric complexes is determined by the relative abundance of each family member at a particular time. Overexpression of v-Jun or c-Jun alone will lead to cell transformation of chicken embryo fibroblasts, albeit with varying efficiencies. Upon overexpression, v-Jun or c-Jun presumably becomes the predominant AP-1 component in the cell. Theoretically, this should lead to a larger proportion of homodimers than heterodimers. It is not clear what role, if any, the other Jun and Fos family proteins play during cell transformation. We have examined the ability of Jun to induce cell transformation in chicken embryo fibroblasts in the absence of interaction with other Jun or Fos family proteins. To this end, we have constructed a chicken v-Jun mutant that is incapable of heterodimerization. This was accomplished by replacing the leucine zipper region of Jun with that of the yeast transcription factor GCN4. This chimeric protein, VJ-GLZ, contains all of the DNA binding and transcriptional activation domains of v-Jun. As expected, in vitro translated VJ-GLZ was found to be incapable of forming heterodimers with c-Fos, FosB, and JunD. Expression of VJ-GLZ from an avian retroviral expression vector in chicken embryo fibroblasts resulted in the induction of cell transformation as measured by focus formation and the ability to grow and form soft agar colonies. We conclude that the Jun homodimer is sufficient to induce cell transformation and that heterodimerization with c-Fos- or Fos-related antigens is not required.

Introduction

v-Jun, the oncogenic effector of AVS 17, induces sarcomas in chickens and transforms CEFs3 in culture (1–3). Overexpression of its cellular counterpart, c-Jun, also induces cell transformation, both as a single gene (CEF and RAT 1) (4–6) or in cooperation with activated Ras (rat embryo fibroblasts) (6). Although c-Jun overexpression will result in cell transformation, the efficiency is much lower than overexpression of the oncogenic v-Jun (4). This difference in efficiency has been mapped to a number of structural alterations in both coding and noncoding sequences. Cell transformation by Jun has been shown to require intact dimerization, DNA-binding, and transcriptional activation domains (7). However, the mechanism(s) by which Jun induces cell transformation are still not clear.

Jun is one of the major components of the AP-1 transcription complex (reviewed in Ref. 8). It is a sequence-specific DNA-binding protein that recognizes the consensus target sequence TGACTCA (9, 10). Both v-Jun and c-Jun have a strict dependence upon dimerization for DNA binding as well as subsequent transactivation activity (11–14). Dimerization of the Jun protein is a function of a specific region of the protein near the carboxy terminus termed the leucine zipper (15–23). The leucine zipper motif, first identified by Landschulz et al. (24), consists of 4 to 5 leucine residues spaced at 7 amino acid intervals such that, in an α-helical structure, the leucine residues will line up in the same rotational plane. A number of proteins, known as the B-Zip family of proteins, contain, in addition to a leucine zipper, a highly basic region of amino acids directly adjacent to the leucine zipper motif. This basic region is directly responsible for DNA binding (13, 14, 25, 26). A number of cellular proteins belong to this general family of proteins. These include the Jun family (c-Jun, JunB, and JunD), the Fos family (c-Fos, FosB, Fra-1, Fra-2), the CREB family (CRE BP-1 and CRE BP-2), the yeast transcription factor GCN4, C/EBP, and a number of others. This shared structural motif allows the formation of dimeric complexes between certain members of this general family. The presence of a leucine zipper, however, is not in itself sufficient to allow indiscriminate dimerization between two leucine zipper-containing proteins. For instance, c-Fos and Fos family members, although fully competent as dimerization partners for the Jun family of proteins, do not self-dimerize or heterodimerize with each other (18, 20, 22, 23, 27). Conversely, the yeast GCN4 protein contains a leucine zipper motif that is restricted to the formation of homodimers and will not heterodimerize with the Jun or Fos family of proteins (27, 28). c-Jun, on the other hand, not only will form homodimers but also has the ability to heterodimerize with other Jun, Fos, and CREB family proteins (15, 18, 19, 21–23, 29–31).

This wide range of potential protein-protein interactions is likely to promote some of the diverse effects of Jun on cell growth and differentiation. Each Jun/Jun or Jun/Fos family complex is likely to regulate, in addition to a number of common target genes, a number of unique ones. Interestingly, the relative proportion of each Jun family-Fos family complex is governed by the relative abundance of each at any one time (32). Presum-

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2 To whom requests for reprints should be addressed, at Department of Microbiology and Immunology, Eastern Virginia Medical School, P.O. Box 1980, Norfolk, VA 23501.

3 The abbreviations used are: CEF, chicken embryo fibroblast; PCR, polymerase chain reaction; TBS, Tris-buffered saline.
ably, each of the various Jun family-Fos family complexes exerts subtle differences in DNA-binding or transcriptional regulatory specificities. When c-Jun or v-Jun is overexpressed, a shift in the type and abundance of different complexes that bind to AP-1 target sequences occurs. Thus, v-Jun- and c-Jun-infected CEFs contain a different complement of proteins capable of binding to AP-1 target sequences than do normal CEFs. Overexpression of v-Jun or c-Jun is likely to favor an increase in homodimerization. This had led us to investigate the role, if any, that other endogenous Jun and Fos proteins play during cell transformation. Although coexpression of c-Fos appears to enhance transformation by Jun (7, 33), it is not clear whether it is required. To address this question, we have generated a v-Jun mutant that is incapable of heterodimerization with other Jun or Fos family proteins. This was accomplished by replacing the leucine zipper region of jun, which is responsible for dimerization, with that of the yeast transcription factor GCN4. GCN4 has previously been shown to form only homodimers and to be incapable of forming dimers with c-Jun and c-Fos (27, 28). The chimeric protein VJ-GLZ retains all of the functional domains of v-Jun, including the basic DNA-binding domain and transactivation domains, but contains the dimerization specificity of GCN4. We have expressed this chimeric protein in CEFs and found that it will induce cell transformation. Thus, heterodimerization with endogenous Fos and Jun family proteins does not appear to be required in the process of cell transformation by Jun.

Results
Construction of Jun-GCN4 Chimera. In order to assay the role that endogenous Jun and Fos proteins play in cell transformation, we constructed a v-Jun mutant that was incapable of heterodimerization. This was accomplished by taking advantage of the fact that the yeast GCN4 protein is incapable of forming heterodimers with Jun and Fos as well as other Jun and Fos family proteins (27, 28). A chimera between Jun and GCN4 was constructed in which the leucine zipper portion of Jun was replaced with that of GCN4 (Fig. 1). The construction was made such that the GCN4 region in VJ-GLZ spans precisely from the first leucine to the last leucine in the zipper with no extra or altered amino acids being introduced (see “Materials and Methods”). The VJ-GLZ chimera retains all of the functional domains of v-Jun including the basic DNA-binding region and the transactivation domains as well as the extreme carboxy terminus. The chimeric VJ-GLZ DNA fragment was cloned into pGEM 3Z, which contains an SP-6 and T7 promoter for in vitro transcription. In vitro translated proteins were generated to assay dimerization specificity. In addition, the VJ-GLZ fragment was cloned into an avian retroviral expression vector, RCAS (34, 35), for subsequent cell transformation assays.

Determination of Dimerization Specificity of VJ-GLZ. The dimerization specificity of VJ-GLZ was determined using proteins generated by in vitro transcription and translation. c-Fos, v-Jun (VJ-1), and VJ-GLZ were translated or cotranslated in rabbit reticulocyte lysates in the presence of [35S]methionine and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to confirm protein production. c-Fos was cotranslated with either VJ-GLZ or VJ-1. The cotranslated products were then immunoprecipitated with either c-Fos or c-Jun (PEP 1) antibody. Anti-c-Fos does not recognize c-Jun, and anti-PEP 1 does not cross-react with c-Fos (data not shown) (16). Coprecipitation of c-Fos with VJ-1 or VJ-GLZ is an indicator of dimerization. The results of this assay are presented in Fig. 2A. It is clear that VJ-1 and c-Fos coprecipitate with both anti-c-Fos and anti-c-Jun antibody, indicating that they readily form dimers. However, anti-Fos antibody does not coprecipitate VJ-GLZ with c-Fos, and anti-Jun antibody does not coprecipitate c-Fos with VJ-GLZ. This indicates that, as expected, VJ-GLZ does not dimerize with c-Fos. A similar analysis (Fig. 2B) was performed on in vitro translated JunD and FosB. Again, VJ-GLZ was cotranslated with either JunD or FosB. Immunoprecipitation was carried out with antibodies specific for FosB, JunD, or v-Jun. No coprecipitation was detected between JunD and VJ-GLZ or between FosB and VJ-GLZ, indicating that they also do not form heterodimers.

We have also examined the dimerization specificity of VJ-GLZ in vivo by immunoprecipitation of 35S-labeled protein lysates prepared from RCAS VJ-GLZ-transformed cells (see below). As expected, only endogenous c-Jun and VJ-GLZ are precipitated with the Jun-specific antiserum PEP 1 (Fig. 2C), with no coprecipitated proteins detectable. The level of c-Jun immunoprecipitated in Fig. 2C is similar to that seen in normal uninfected CEFs but greater than that normally seen in RCAS-infected CEFs (data not shown). No proteins, including VJ-GLZ, were precipitated with antisera directed against c-Fos, FosB, Fra-2, or JunD, suggesting that these Fos and Jun family proteins are present in extremely low amounts in these cells. We therefore cannot formally prove lack of dimerization to the low endogenous levels of these proteins with VJ-GLZ in vivo. We have, however, shown that VJ-GLZ does not dimerize to c-Fos FosB, or JunD when coexpressed to roughly the same levels in vitro. This, coupled to the finding that no other coprecipitated protein could be detected in vivo, strongly suggests that VJ-GLZ is restricted to self-dimerization. From these data, we speculate that, in an in vivo situation, VJ-GLZ will not

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* T. Bos, M. Hadman, and M. Loo, submitted for publication.
Fig. 2. In vitro dimerization assays between VI-GLZ and c-Fos, FosB, and JunD. A, in vitro cotranslated v-Jun and c-Fos or VI-GLZ and c-Fos are immunoprecipitated with antibody specific for c-Jun (PEP 1) or c-Fos (Ab-2). B, Lanes 1–4, in vitro cotranslated VI-GLZ and FosB or JunD are immunoprecipitated with antibody specific for v-Jun (PEP 1), FosB, or JunD. Lanes 5–7, in vitro translated JunD, FosB, and VI-GLZ are immunoprecipitated with their respective antibodies to show relative position on gel. C, immunoprecipitation of 35S-labeled proteins in vivo from VI-GLZ-expressing CTEs. Lane 1, lysate with Protein A only; Lane 2, lysate plus normal rabbit serum; Lane 3, lysate plus anti-Jun PEP 1; Lane 4, lysate plus anti-c-Fos; Lane 5, lysate plus anti-FosB; Lane 6, lysate plus anti-JunD; Lane 7, lysate plus anti-Fra-2. Equivalent amounts of c-Jun are seen in uninfected growing CTEs (not shown). The bands present in Lanes 5 and 6 often appear in normal serum control (not shown) and are thought to be an artifact.
heterodimerize with any of the normal dimerization partners of Jun.

**VJ-GLZ Binds Specifically to AP-1 Target DNA.** Jun/Fos heterodimers are more stable than Jun homodimers (12). This increased stability results in more stable DNA binding of heterodimers compared to homodimers. Because the leucine zipper of GCN4 is thought to be more stable than that of Jun, we have examined the possibility that the VJ-GLZ homodimers may bind DNA with a higher affinity than Jun homodimers and thus mimic Jun/Fos heterodimerization. We have examined DNA binding by VJ-GLZ and found that, like Jun homodimers, DNA binding by VJ-GLZ is hard to detect (Fig. 3). DNA binding by in vitro translated VJ-1, VJ-GLZ, or cotranslated VJ-1/c-Fos is shown in Fig. 3 (Lanes 1, 2, and 3, respectively). Clearly, the v-Jun/Fos heterodimer has a higher binding affinity than either of the homodimers. In order to detect DNA binding by the VJ-1 homodimer or the VJ-GLZ homodimer, we had to express each in large quantities in a bacterial system (see “Materials and Methods”). The bacterial extracts, containing 5 histidine residues at the amino terminus, were purified by passage over a ProBond resin (Invitrogen). The resulting purified VJ-1 (Fig. 3, Lanes 4 and 5) and VJ-GLZ (Fig. 3, Lanes 6 and 7) proteins were assayed for DNA-binding activity and were found to have nearly equal affinities. Thus, both v-Jun and VJ-GLZ homodimers have much lower DNA binding affinities than Jun/Fos heterodimers.

**Expression of VJ-GLZ in CEFs.** Overexpression of v-Jun in CEFs has been shown to result in formation of a characteristic focus morphology as well as anchorage-independent growth (4). In order to test the biological activity of the VJ-GLZ mutant, we cloned it into a replication-competent avian retroviral expression vector, RCAS (34, 35). This was then transfected into primary CEFs and assayed for protein production and transforming activity. To ensure that VJ-GLZ was being expressed efficiently from this vector, protein was isolated and assayed by immunoblot analysis. Fig. 4 shows the results of this assay. CEFs transfected with RCAS VJ-1 or RCAS VJ-GLZ express large amounts of VJ-1 (Fig. 4, Lane 1), and VJ-GLZ (Fig. 4, Lanes 3–6) protein, respectively, whereas CEFs infected with vector alone (RCAS) have very little endogenous c-Jun (Fig. 4, Lane 2). Bands of the expected size and in roughly equal amounts can be detected. The level of endogenous c-Jun in RCAS VJ-GLZ-infected cells varies (Fig. 4, Lanes 3–6) and is not always detectable (data not shown). Fig. 5 shows results of immunoperoxidase staining of the same cells, clearly showing nuclear localization of both VJ-1 and VJ-GLZ proteins. Again, endogenous c-Jun levels are very low, as indicated by the staining pattern of RCAS. Thus, VJ-GLZ is not only expressed to the same levels as VJ-1 but is also properly localized to the nucleus.

**Cell Transformation by VJ-GLZ.** Cell transformation was assessed by focus formation and ability to grow in soft agar. Transfected CEFs were examined 14 days post-transfection for the appearance of foci. Both VJ-1 and VJ-GLZ transfectants produced transformed foci, whereas no foci were evident in the RCAS vector-transfected controls (Fig. 6; Table 1). The numbers of foci generated in response to overexpression of VJ-GLZ were similar to those obtained with VJ-1 (Table 1). Interestingly, VJ-GLZ-transformed cells have a different morphology than VJ-1-transformed cells, being more rounded and random in nature rather than elongated and in parallel arrays. This
morphological distinction may indicate that in classical v-Jun transformation, heterodimerization, although not strictly necessary for transformation, may play some role in the transformed phenotype.

Anchorage-independent growth properties were also assayed on VJ-GLZ transfectants. We find that the agar colonies formed are consistently larger in VJ-GLZ-transfected CEFs compared to Vj-1-transfected CEFs (Fig. 6). Although there were slight differences in focus morphology and agar colony size, the numbers of transformed foci and agar colonies generated as a result of transfection with Vj-1 or VJ-GLZ are similar (Table 1). We conclude that heterodimerization with other Jun or Fos family proteins is not necessary for induction of cell transformation; however, they may contribute to the transformed phenotype.

**Discussion**

Jun is capable of dimerizing with a host of other B-zip proteins. These multiple interactions are no doubt involved in many of the diverse effects that Jun expression has on cell growth, differentiation, and tumorigenesis. The focus of this study was to determine the role, if any, that heterodimerization with endogenous Jun and Fos family proteins plays in cell transformation by v-Jun. To this end, we have generated a v-Jun mutant that cannot heterodimerize with other Jun and Fos family members by replacing the leucine zipper dimerization region of v-Jun with that of the yeast transcription factor GCN4. Dimerization specificity was assayed by commmunoprecipitation of in vitro cotranslated Vj-GLZ with Fos and Jun family proteins. As expected, the mutant Jun protein is incapable of heterodimerization with all of the Jun and Fos proteins tested. c-Fos, FosB, JunD, and Fra-2 were undetectable in VJ-GLZ cells and are likely to be expressed only in very limited amounts. We did observe that c-Jun appears to be expressed at higher levels in VJ-GLZ-transformed cells than in RCAS-infected or VJ-1-transformed CEFs. The level of c-Jun, however, varied in different VJ-GLZ CEF extracts, and it was not always present.

Overexpression of VI-GLZ in CEFs results in cell transformation, as measured by focus formation and ability to grow in soft agar. We have detected differences in focus morphology as well as agar colony size in the VJ-GLZ-
expressing CEFs compared to VI-1 CEFs (Fig. 6), which may indicate a minor role for Fos or Fos family members in some aspects of transformation by Jun. Hartl and Vogt (36) have obtained analogous results with similar but not identical chimeras.

Jun homodimers have been shown to be less stable than Jun-Fos heterodimers. However, when v-Jun is overexpressed, it presumably becomes the most predominant AP-1 species in the cell. This leads to the formation of a greater proportion of homodimers than is normally found. Interestingly, in vivo complexes formed in response to overexpression of v-Jun also appear to recognize different subsets of "AP-1 and CREB-like" targets than the complexes found in c-Jun-overexpressing or normal CEFs, suggesting an altered target recognition specificity. Ryseck and Bravo (37) have also seen differences in target recognition between Jun homodimers and Jun-Jun family or Jun-Fos family heterodimers on a number of different targets. Transcriptional activity of target genes may also be differentially regulated by Jun homodimers compared to Jun/Fos heterodimers. For instance, the collagenase and transin promoters—which are normally up-regulated by Jun/Fos—are down-regulated upon overexpression of v-Jun in CEF (38).

c-Fos has been shown to enhance cell transformation by c-Jun in both rodent and chicken cells (7,33); however, the mechanism for this enhancement is not clear. Coexpression of c-Fos in CEFs increases the transforming efficiency of c-Jun (which normally shows moderate to low oncogenic potential) while exerting no effect on the highly oncogenic v-Jun (7). In addition, overexpression of c-Fos (in the absence of exogenous c-Jun expression)
will induce transformation, although at a reduced efficiency. We have recently found differences in target recognition by in vivo complexes formed in response to overexpression of v-Jun to c-Jun. It is possible that these differences in target recognition play an important role in determining the oncogenic potential of Jun. Coexpression of c-Fos with c-Jun (which is weakly transforming) may act to compensate for some of these differences through the formation of c-Jun-c-Fos or even JunB or JunD/c-Fos heterodimers. Whether transformation via Jun homodimers or Jun-Fos heterodimers occurs through the deregulation of the same or different sets of target genes is currently not known.

Our results suggest that Jun homodimers are sufficient to induce cell transformation and that heterodimerization with c-Fos or other Jun or Fos family endogenous proteins is not required. This raises the possibility that target subsets deregulated by Jun homodimers are directly involved in inducing cell transformation. Identification of the target genes regulated by these homodimers is the subject of further investigation.

Materials and Methods

Construction of Jun-GCN4 Chimeras. The Jun-GCN4 chimeras were constructed by PCR cloning. v-Jun DNA sequences between nucleotides 1 and 705 were PCR amplified using two artificially synthesized primers, BS-1 and BS-2 (nucleotide numbering starts at the first AUG in v-Jun). The 5' primer (BS-1: 5' GATTTACAGGACACTGTCGCA 3'), corresponds to the bases surrounding the upstream XbaI site in pAVI-1 (4). The 3' primer (BS-2: 5' CCTGCGAATCTTTTACACT 3') is complementary with the v-Jun nucleotides immediately adjacent to the first leucine in the leucine zipper. PCR amplification was performed using standard procedures with VENT polymerase (New England Biolabatories). Briefly, 10 pmol each of BS-1 and BS-2 were added to EcoRl linearized pAVI-1 (4) template in the presence of 10 mm KCl, 10 mm (NH4)2SO4, 20 mm Tris-HCl (pH 8.8), 2 mm MgSO4, 0.1% Triton X-100, 0.2 mm each of dATP, dCTP, dGTP, and dTTP, and 10 μg bovine serum albumin and VENT polymerase. PCR conditions were 45 s at 96°C, 1.5 min at 57°C, and 3 min at 72°C for 30 cycles. Amplification of the sequences corresponding to the leucine zipper of GCN4 (GLZ fragment) was carried out as above except for the use of GCN4 template DNA (Ycp88-GCN4 (39), kindly provided by Kevin Struhl) and BS-3 and BS-5 primers. The primers (BS-3: 5' CCTGAGAAAGTTGAAGATTCG 3' and BS-5: 5' ACCCTTAACTCCTCGCAACCTTCCAATCCT 3') span a region of GCN4 from the first leucine of the leucine zipper to the last leucine of the leucine zipper. The first 9 nucleotides of BS-5 are not complementary to the GCN4 leucine zipper sequence. Nucleotides 7–9 of BS-5 alter the native leucine codon TTA to the leucine codon CTT, forming an artificial AluI site. This alteration facilitated replacement of the v-Jun leucine zipper coding region with that of GCN4. The PCR products were treated with mung bean nuclease, restricted with either XbaI (Jun fragment) or AluI (GCN4 fragment), phosphorylated with T4 kinase, and then isolated on low melt agarose gels.

The v-Jun-GCN4 chimera was placed into the adapter plasmid CLA12NCO (35) by ligation of an XbaI to AluI fragment of pAVI-1 (4) to the Jun and GCN4 PCR fragments described above, to yield pAVI-GLZ. A ClaI fragment containing the entire coding sequence of VJ-GLZ from pAVI-GLZ was excised, made blunt with DNA polymerase I (Klenow), digested with XbaI, and ligated between XbaI and Smal in pGEM 3Z to yield pGVJ-GLZ, which was used in the in vitro transcription and translation assays. Similarly, the ClaI fragment of pGVJ-GLZ was placed into the ClaI site of the avian retroviral expression vector RCAS (34, 35) to yield RCAS VJ-GLZ, which was used in the transformation assays.

Dimerization Assays. Dimerization assays were carried out as previously described (16). Briefly, plasmids containing v-Jun (pGVJ-1), VJ-GLZ (pGVJ-GLZ), c-Fos (pSP65-c-fos), FosB (in pGEM I), and JunD (in Bluescript KS+) were linearized with EcoRI (v-Jun and VJ-GLZ), BamHI, EcoRV, and XbaI, respectively (Fos B and JunD plasmids were provided by Rodrigo Bravo; c-Fos plasmid was obtained from Tom Curran). Linearized plasmids were transcribed using the manufacturer's recommended conditions with SP-6 (c-Fos, VJ-GLZ), T7 (v-Jun and FosB), or T3 (JunD) polymerases. The in vitro transcribed RNA was then translated in a rabbit reticulocyte lysate in the presence of [35S]methionine. For the purpose of the dimerization assays, v-Jun or VJ-GLZ was cotranslated with the c-Fos, FosB, and JunD proteins. Four μl of cotranslated protein lysate were used in immunoprecipitation reactions as previously described (40). Antibodies used were: Jun antibody, PEP 1 (9, 40) (Oncogene Science); Fos antibody, Ab-2 (Oncogene Science); and FosB and JunD antibodies (kindly provided by Rodrigo Bravo).

Cell Culture and Transformation Assays. Primary CEFs were generated as previously described (4). Transfections of RCAS VJ-1, RCAS VJ-GLZ, and RCAS were performed by the method of Kawai and Nishizawa (41), using 1 μg of double CsCl banded DNA in a 60-mm subconfluent dish of CEFs. Transfected CEFs were overlaid within 16 h of transfection and incubated at 37°C for 14 days until foci were clearly developed. Agar was removed, and cells were transferred to fresh plates for isolation of protein for immunoblotting. Alternatively, cells were transferred to plates with coverslips for subsequent immunoperoxidase assays. Agar colony assays were performed as described (42). Agar colonies were generated by transfection of CEFs with the appropriate plasmids followed by solution culture for 4–6 days. Transfected cells were then plated into soft agar at densities ranging from 104 cells to 105 cells in a 35-mm dish. Agar colonies were very apparent after about 14–18 days.

Immunoblotting, Immunoprecipitation, and Immunoperoxidase Staining of Jun Proteins. Proteins isolated from RCAS VJ-1, RCAS VJ-GLZ, or RCAS-transfected CEFs were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel, transferred to nitrocellulose, and probed with the Jun antibody PEP 1 at a 1:1000 dilution. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Fisher Scientific) at 1:1000 dilution was used as the secondary antibody, and bands were visualized using the ECL detection system (Amersham).

35S-labeled proteins from VJ-GLZ-transformed CEFs were prepared by preincubation in methionine-free medium for 1 h followed by incubation for 2 h with 100 μCi/ml [35S]methionine. Cell extracts were prepared as described (40). Immunoprecipitation of lysates containing 6 × 106 cpm were preformed as previously described (40), except that Protein A-agarose was used in place of Pansorbin. Antibodies used were: PEP 1 (Oncogene Science), c-Fos (AB-2) (Oncogene Science), FosB and JunD
(provided by Rodrigo Bravo), and Fra-2 (Santa Cruz Biotechnology).

For immunoperoxidase staining, 3 × 10^5 cells were plated on glass coverslips precoated with 0.2% gelatin in 35-mm dishes. After 24 h, cells were fixed with 3.7% formaldehyde for 10 min at room temperature. After washing two times with 1× TBS at -20°C, acetone was added for 2 min. Cells were then washed three times with 1× TBS and then incubated with PEP 1 or PEP 2 (1:100 diluted in 1× phosphate-buffered saline-1% bovine serum albumin) anti-Jun antibodies for 60 min at 37°C. Following washing, secondary antibody (biotinylated anti-rabbit IgG, 1:100) was added and further incubated at 37°C for 60 min. Coverslips were washed three times as above, and streptavidin horseradish peroxidase complex was added (1:100) for an additional hour. Diaminobenzidine solution (3 ml 1× phosphate-buffered saline-1 ml 2% diaminobenzidine-1.2 μl 30% H2O2) was added and incubated at room temperature for 10 min. Coverslips were then washed in 1× TBS three times, dried for 24 h, and mounted in cytoseal media.

DNA Binding Assays. Gel retardation assays were performed using a probe generated by 5′ end labeling a PCR-generated DNA fragment containing the consensus AP-1 site, TGACTCA. Binding conditions were as previously described by Nakabeppe et al. (43) with minor modifications. The binding buffer contained: 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 8.0), 17.5% glycerol (v/v), 0.1 mM EDTA, 10 mM NaCl, 4 mM MgCl2, 2 mM dithiothreitol, and 2 mM spermidine. Three μl of in vitro translated v-Jun, VJ-GLZ, or cotranslated v-Jun and Fos, or 0.4-0.8 μg of purified bacterially expressed v-jun or VJ-GLZ (see below), were mixed with 1 μg polydeoxyinosinoc-deoxyctydylid acid in binding buffer for 15 min on ice. 32P-labeled probe (50,000 cpm) was added, incubated at room temperature for 15 min, and electrophoresed onto 5% (59:1) acrylamide/bisacrylamide gels with 1X Tris-borate-EDTA running buffer at 30 mA. Gels were then dried and exposed for autoradiography.

Preparation of Bacterially Expressed v-Jun and VJ-GLZ Proteins. Bacterially expressed v-Jun and VJ-GLZ were prepared after isopropyl thiogalactoside induction for 2 h of Escherichia coli transformed with pTrchis VJ-1 or pTrchis VJ-GLZ, respectively. pTrchis VJ-1 was constructed by placing the BamHI to EcoRI fragment of pGVI-1 (16) into the BamHI to EcoRI-digested pTrchis B vector (Invitrogen). pTrchis VJ-GLZ was constructed by inserting the BamHI to EcoRI fragment of pGVI-GLZ into the same vector. Protein isolation and purification were performed according to the manufacturer's instructions using the Xpress Protein Purification System (Invitrogen). Purified VJ-1 or VJ-GLZ proteins collected from the ProBond resin were dialyzed against 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.9)-20% glycerol-0.1 M KCl-0.2 mM EDTA-1 mM phenylmethylsulfonyl fluoride-0.5 mM dithiothreitol for 4 h at 4°C before use in DNA binding studies.

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