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Partial oncogenic transformation of chicken embryo fibroblasts by Jun dimerization protein 2, a negative regulator of TRE- and CRE-dependent transcription

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Jun dimerization protein 2 (JDP2) was identified as a bZIP protein that forms dimers with Jun proteins. JDP2 represses transcriptional activation of reporter constructs containing 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-responsive elements (TRE) or cyclic AMP responsive elements (CRE). JDP2, overexpressed by the avian retroviral vector RCAS, induces partial oncogenic transformation of chicken embryo fibroblasts. JDP2-expressing cells form multilayered foci in monolayer cultures but do not show anchorage-independent growth. Both the carboxyl and the amino terminus of JDP2 are required for the transforming activity. Chimeric constructs of JDP2 carrying the leucine zipper domain of Fos, GCN4 or EB1 fail to transform CEF. The leucine zipper of Fos mediates only heterodimerization; it cannot homodimerize. In contrast, the leucine zippers of GCN4 and of EB1 exclusively homodimerize and do not form dimers with other bZip proteins. The results with the JDP2 chimeras suggest that the JDP2 homodimer and the JDP2/Jun heterodimer (or other bZip heterodimers formed with the Fos leucine zipper) are nontransforming, leaving as possible transforming combination the JDP2/Fos heterodimer. The unexpected transforming activity of a negative regulator of TRE- and CRE-dependent transcription raises an important question concerning the mechanisms of transformation by the related bZIP proteins Jun and Fos that address the same target sequences.
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Introduction

The activator protein-1 (AP-1) transcription factor complex is composed of homodimers and heterodimers of Jun proteins (c-Jun, JunB and JunD), Fos proteins (c-Fos, FosB, Fra1 and Fra2) and members of the CREB/ATF family (ATF2, LRF1/ATF3 and B-ATF) (Karin *et al*., 1997). Jun can also heterodimerize with some Maf proteins that form a related family of bZIP transcription factors (Kataoka *et al*., 1994). Jun homodimers and Jun-Fos heterodimers bind the TPA-responsive element (TRE, TGACTCA), and Jun–CREB/ATF dimers prefer the cyclic AMP-responsive element (CRE, TGACGTCA) (van Dam and Castellazzi, 2001). Jun–Maf heterodimers bind equally well to the TRE and CRE consensus sequences (Kataoka *et al*., 1994).

AP-1 activity is induced by various stimuli including TPA, serum, growth factors and oncoproteins (Karin *et al*., 1997). Three components of the AP-1 complex, Jun, Fos and Maf, were originally identified as oncoproteins encoded by a cellular insert in the genome of an acutely transforming retrovirus (Curran *et al*., 1982; Maki *et al*., 1987; Nishizawa *et al*., 1989). The AP-1 complex transmits normal growth signals but also has oncogenic potential (Karin *et al*., 1997; Shaulian and Karin, 2001; Vogt, 2001, 2002).

JDP2 was identified as a binding partner of c-Jun in a yeast two-hybrid screen based on the recruitment of the Sos system (Aronheim *et al*., 1997; Broder *et al*., 1998). JDP2 heterodimerizes with c-Jun and represses AP-1-mediated transcriptional activation (Aronheim *et al*., 1997). It also interacts with ATF-2, a member of the CREB/ATF family, repressing CRE-dependent transcription mediated by ATF-2 (Jin *et al*., 2001). JDP2 is post-transcriptionally induced by UV and inhibits UV-induced apoptosis by downregulating the transcription of p53 (Piu *et al*., 2001). Given the roles of AP-1 in cellular transformation and the reported repressing activity of JDP2 on Jun and ATF-2-mediated transcription, JDP2 was expected to inhibit oncogenic transformation induced by Jun. Jun-induced oncogenic transformation depends on its transcriptional regulatory activity (Morgan *et al*., 1992; Vogt, 2001). Inhibition of transcription from TRE or CRE sites should then have an antioncogenic effect. Dominant-negative Jun constructs interfere with transformation in several cell systems (Brown *et al*., 1993; Domann *et al*., 1994;

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Brown *et al*., 1996). Yet contrary to expectation, overexpression of JDP2 stimulates cell growth and induces a partial oncogenic transformation in CEF.

Results

Overexpression of JDP2 induces focus formation in CEF

JDP2 inhibits transcriptional activation by Jun. Therefore, we expected overexpression of JDP2 to interfere with Jun-induced transformation. To test this possibility, JDP*2* was cloned into the avian retroviral vector RCAS (R-JDP2) and transfected into chicken embryo fibroblasts (CEF) (Figure 1). Surprisingly, the transfected cultures became transformed; cells assumed an elongated shape and grew in multiple layers. Higher dilutions of infectious R-JDP2 virus induced discrete foci of transformed CEF. The latent period of transformation was about 3 weeks (Figure 2). The RCAS vector alone is not transforming. However, JDP2-transformed cells failed to grow into colonies in nutrient agar (data not shown); JDP2-induced transformation does therefore not include anchorage-independent growth. Injection of the R-JDP2 viruses into wing webs of young chickens also did not cause tumors (data not shown). These results suggest that JDP2 induces a partial transformation of CEF marked by a characteristic cell shape and the ability to grow in multiple layers. RCAS is a replication-competent retroviral vector, and there is a chance that the JDP2 sequences mutate during replication in a retrovirus. The transforming ability of R-JDP2 could then have resulted from mutation; transforming mutants may have a selective advantage and could rapidly establish a majority in the virus population. In order to examine this possibility, three individual foci induced by the R-JDP2 virus were picked under the dissecting microscope, and the cells were expanded in culture. The JDP2 inserts of the R-JDP2 provirus integrated in the genomes of these cells were subcloned by PCR with primers from adjacent vector sequences. The JDP2 inserts were sequenced, and no mutations were found, suggesting that wild-type JDP2 causes the observed transformation of CEF.

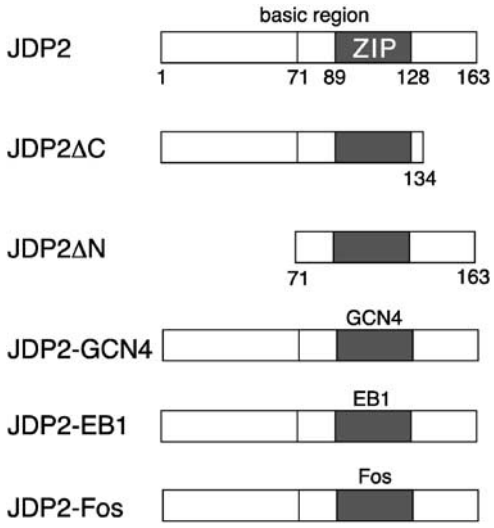

Figure 1 Schematic diagram of JDP2 constructs used in this study. The basic and leucine zipper regions are marked. JDP2–GCN4, JDP2–EB1 and JDP2–Fos are chimeric constructs in which the leucine zipper region of JDP2 is replaced with that of the indicated protein

DNA-binding properties of JDP2

JDP2 binds to the TRE as a homodimer and as a heterodimer with Jun and other bZip proteins (Aronheim *et al*., 1997). It also binds to the CRE (Jin *et al*., 2001). We tested DNA binding of JDP2 with nuclear extracts from R-JDP2-transformed CEF. JDP2 translated *in vitro* with rabbit reticulocyte lysate was also investigated. In EMSA, JDP2 bound strongly to CRE and to TRE sequences (Figure 3). The binding was barely visible when nuclear extracts from RCAS-transfected CEF were used (data not shown). Supershift experiments with antibodies against JDP2 and Jun identified both JDP2–JDP2 homodimers and Jun–JDP2 heterodimers in the TRE- and CRE-binding complexes.

The carboxyl-terminal region of JDP2 contains an active repression domain

JDP2 is known to repress AP-1 activity on TRE and CRE sites. We examined JDP2-induced repression in transient transfection with luciferase reporters that contained TRE or CRE sequences. The assays were performed in the human JEG-3 choriocarcinoma cell line that has low endogenous AP1 activity (Kruse *et al*., 1997). JDP2 strongly repressed both basal and v-Jun-induced transcription from CRE and TRE reporters (Figure 4). Repression of the endogenous transcription from these reporters was also observed in the DF-1 chicken embryo fibroblast cell line (data not shown). The extent of that repression suggests that besides competing with components of AP-1, JDP2 may contain an active repression domain. To examine this possibility, we made fusion constructs of JDP2 and the DNA-binding domain of the yeast GAL4 protein. Full-length JDP2 (163 aa) or fragments containing the amino terminus (98 aa) or carboxyl terminus (36 aa) were cloned into the pCMV-BD vector and expressed as GAL4 fusion proteins in HEK293 cells (Figure 5a). A GAL4-luciferase reporter construct and an internal control reporter were cotransfected with these constructs. Full-length JDP2 repressed the GAL4-dependent promoter efficiently, comparable to the strong repressor domain of the v-Qin oncoprotein (Figure 5b). The 36 amino acids carboxyl terminal to the bZIP domain JDP2(129–163) also induced strong repression, whereas the amino-terminal fragment containing the basic region but not the zipper domain, JDP2(21–88), did not repress. These results suggest that JDP2 harbors an active, transferable repression domain within the carboxyl-terminal portion of the protein.

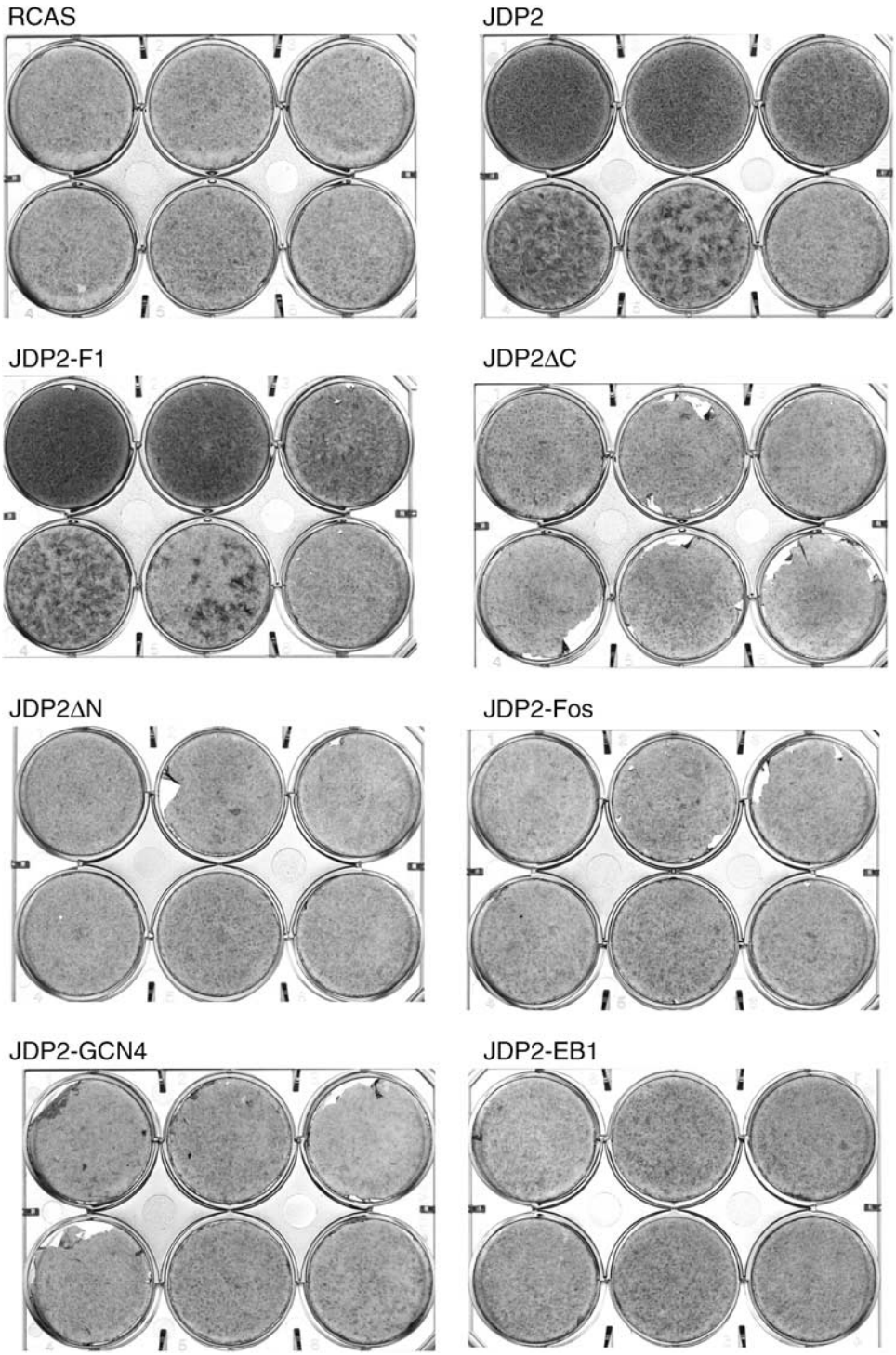

Figure 2 Transformation of CEF induced by JDP2. CEF were infected with viruses containing the indicated JDP2 constructs. Each plate was infected with 100 μl of the virus stocks diluted to 10^{-1} (top left well), 10^{-2} (top center well), 10^{-3} (top right well), 10^{-4} (bottom left well), 10^{-5} (bottom center well) or with no virus (bottom right well). The cells were overlaid with nutrient agar for 21 days and then fixed and stained with crystal violet. JDP2-F1 is the virus stock obtained from culture of a single focus induced by the R-JDP2 virus

Transforming activity of deletion and leucine zipper mutants of JDP2

Deletion mutants of JDP2 were prepared in order to elucidate the role of the amino-terminal and carboxyl-terminal region in the oncogenicity of JDP2. JDP2ΔC lacks the carboxyl-terminal 29 amino acids, and JDPΔN lacks the amino-terminal 70 amino acids, but both contain the bZIP domain. These two constructs were expressed in CEF using the RCAS vector and tested for the transforming potential. Both constructs failed to induce focus formation (Figure 1). These results suggest

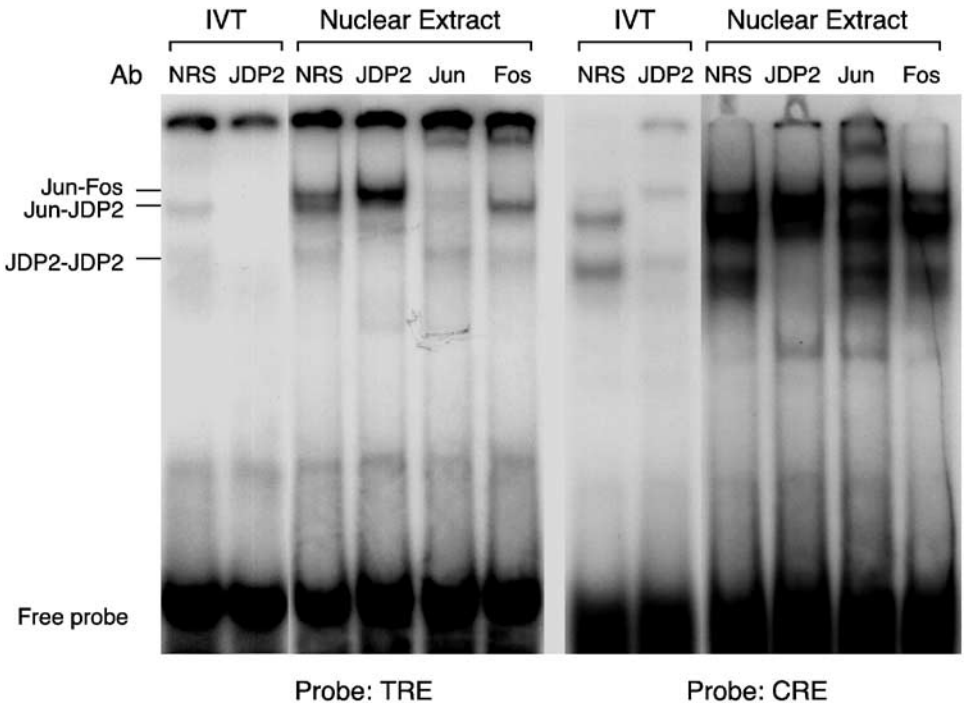

Figure 3 Electrophoretic mobility shift assay (EMSA) of JDP2 binding to TRE and CRE sequences. JDP2 protein and c-Jun protein were synthesized *in vitro* in rabbit reticulocyte lysates. Nuclear extracts were prepared from CEF transfected with R-JDP and incubated with ^{32}P-labeled oligonucleotides. Reactions were preincubated with antibodies specific for JDP2, Jun or Fos, or with normal rabbit serum (NRS) to identify the components in the shifted bands. The bands representing the JDP2–Jun heterodimers, JDP2 homodimers and JDP2–Fos heterodimers, as judged by the supershift by the antibodies, are indicated

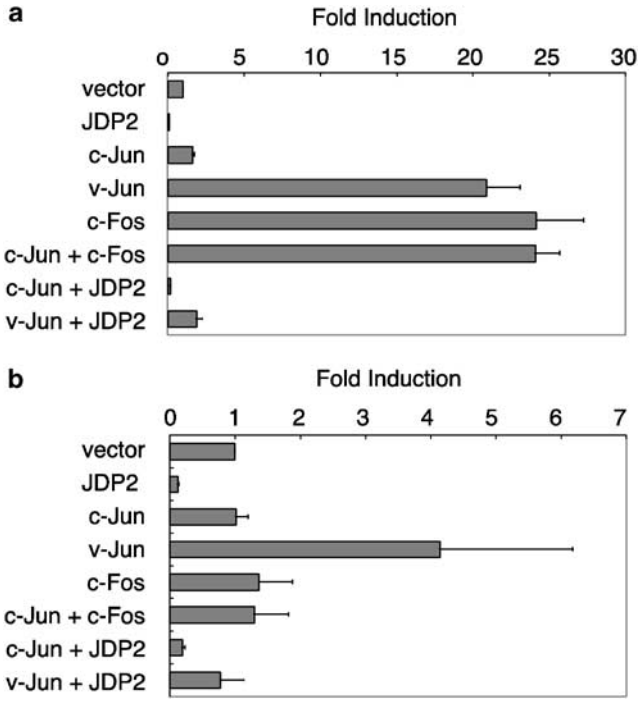

Figure 4 Inhibition of Jun-induced transactivation by JDP2. Human JEG-3 choriocarcinoma cells were transfected with the firefly luciferase reporter constructs regulated by TRE (panel A) or CRE (panel B) sequences together with the pHygEF2 expression plasmids containing indicated constructs. Luciferase activities were determined as described in Materials and methods and are expressed as fold induction over the reporter gene activity obtained with the empty pHygEF2 vector

that both the amino- and carboxyl-terminal regions are necessary for transformation by JDP2.

JDP2 forms homodimers and also heterodimerizes with Jun or other components of AP-1. To gain insight into the roles of JDP2 dimerization partners in transformation, three chimeric constructs were generated. In JDP2–GCN4 and JDP2–EB1, the leucine zipper region of JDP2 was replaced by that of GCN4, a yeast transcription factor, or that of EB1, a transcription factor encoded by Epstein–Barr virus. GCN4 and EB1 form only homodimers; they do not interact with any other known bZIP protein. The chimeric constructs JDP2–GCN4 and JDP2–EB1 are therefore expected to form only homodimers. In JDP2–Fos, the zipper region was replaced with that of Fos. This leucine zipper cannot homodimerize but heterodimerizes with Jun or CREB/ATF proteins. None of these three chimeric constructs induced transformation in CEF (Figure 2). Expression of the deletion mutants and zipper mutants in CEF was confirmed by Western blotting, and nuclear localization was documented by immunofluorescence (Figure 6). These results suggest that JDP2 homodimers are not transforming and that JDP2–Jun or JDP2–CREB/ATF heterodimers are also not transforming. Since wild-type JDP2 can form both homodimers and heterodimers, it is possible that its transforming activity depends on the complementing action of these two different dimeric forms in the same cell. To test this possibility, we coexpressed JDP2–GCN4 and JDP2–Fos in CEF using RCAS vectors of different subgroups. Infection of RCAS(B) JDP2–GCN4-transfected cells

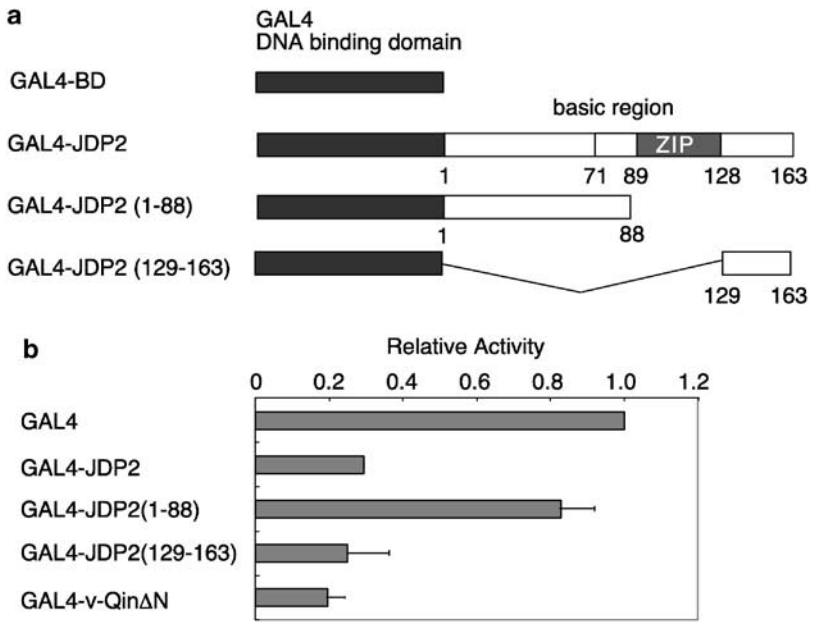

Figure 5 The carboxyl terminus of JDP2 contains a modular repression domain. The full-length, the amino terminus (amino acid 1–88) and the carboxyl terminus of JDP2 (amino acid 129–163) of JDP2 were fused to the DNA-binding domain of GAL4. HEK293 cells were transfected with the pGL23CMV + Gal4-LM reporter plasmid and expression plasmids for GAL4 fusion constructs. Reporter gene activity obtained with the empty GAL4 vector was arbitrarily set as 1

with RCAS(A) JDP2–Fos virus did not induce transformation (data not shown).

Possible explanations for these results are considered in the Discussion section.

The deletions and chimeric constructs were also tested in reporter assays in JEG-3 cells. JDP2–GCN4 and JDP2–EB1 have lost much of the repression activity toward the endogenous transcription activity as compared to JDP2 (Figure 7a and b). JDP2–Fos did not repress, and the two deletion mutants JDP2ΔN and JDPΔC also did not repress the endogenous activity (Figure 7a and b). Lack of repression activity in JDPΔN appears to be inconsistent with the results of GAL4 fusion experiments, which suggested the presence of a repression domain in the carboxyl-terminal region of JDP2 (Figure 5). However, JDP2ΔN significantly repressed the v-Jun-induced transactivation while JDP2ΔC did not repress (Figure 7c). There are several possible explanations for this relatively weak repressor activity of JDP2ΔN. Without the amino-terminal domain, JDP2 may not fold properly and hence may be functionally impaired. The amino-terminal region may also affect dimerization efficiency or may be essential for proper subcellular localization. These results show a correlation between transformation and strong transcriptional repression and suggest that repression may be required for transformation.

Discussion

JDP2 represses transcription from TRE- and CRE-containing promoters. *Prima facie*, it is a negative regulator of AP-1 activity. It is therefore surprising that JDP2 is able to induce a partial oncogenic transformation. Transformation by the viral Jun protein requires the presence of the amino-terminal transactivation domain (Morgan *et al*., 1992). Transdominant negative Jun is able to abrogate oncogenic transformation in various cell systems (Brown *et al*., 1993; Domann *et al*., 1994; Brown *et al*., 1996). However, even with the oncogenic Jun protein, there is evidence of transcriptional repression activity. In CEF, viral Jun represses reporters with a TRE consensus sequence but activates CRE-like reporters (Gao *et al*., 1996). v-Jun-transformed cells show downregulation of numerous genes. Two of these, AKAP12 and MARCKS, appear to be essential for oncogenic transformation (Cohen *et al*., 2001, T Berg, J Iacovoni, S Cohen and PK Vogt, 2002, unpublished observations). The re-expression of these genes induces a reversion of transformed to normal cellular phenotype. Jun also represses other tumor suppressors and growth-inhibitory genes, including p53, p21Cip1 and p16Ink4 (Schreiber *et al*., 1999; Passegue and Wagner, 2000; Shaulian *et al*., 2000; Shaulian and Karin, 2002). It will be important to determine the expression levels of these Jun-repressed genes as well as Jun-induced genes in JDP2-transformed cells. Our preliminary results suggest that expression of HB-EGF, a Jun target gene whose expression is correlated well with Jun-induced transformation (Fu *et al*., 1999), is not upregulated in JDP2-transformed CEF.

Recently the bZIP protein ATF3, a close relative of JDP2, was shown to induce partial transformation in cultures of CEF (Perez *et al*., 2001). ATF3 shares with JDP2 the ability to repress CRE-dependent transcription but, unlike JDP2, it activates TRE-containing promoters (Hai and Curran, 1991; Hsu *et al*., 1991;

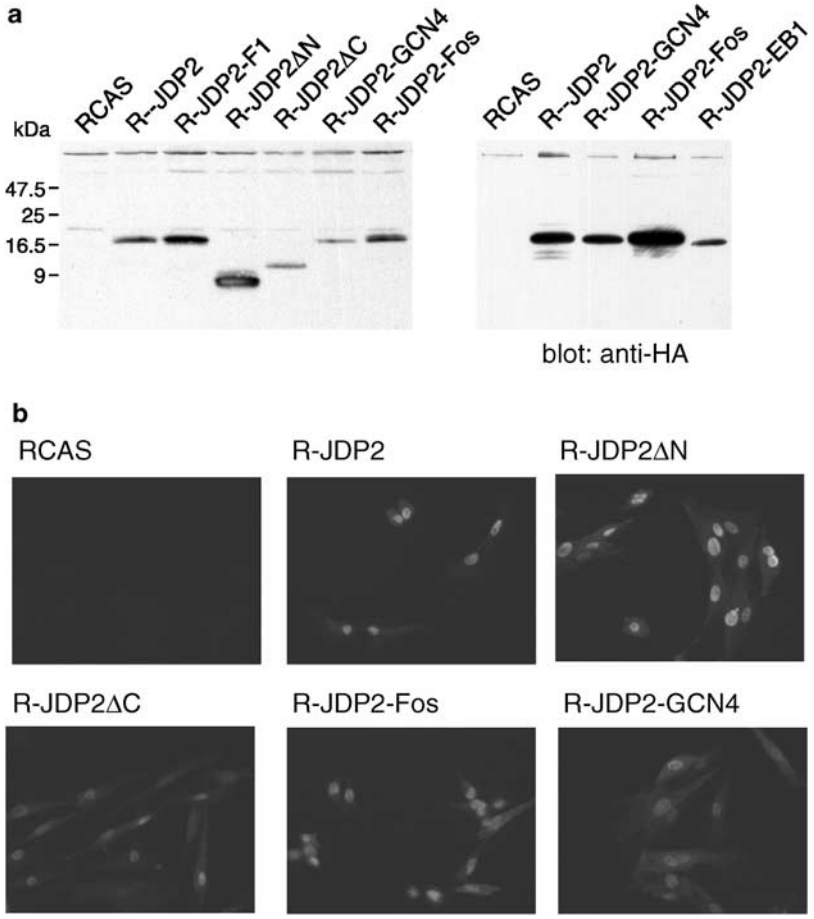

Figure 6 Expression of the JDP2 constructs in CEF. (**a**) Western blot analysis of the JDP2 proteins. Lysates of CEF transfected with the RCAS vector containing indicated JDP2 constructs were probed with anti-JDP2 antibody (left panel) or with anti-HA antibody (right panel). The faint band at 20 kDa in all lanes of the left panel is possibly endogenous chicken JDP2, recognized by the antibody against rat JDP2. (**b**) Immunofluorescence analysis for subcelluar localization of the JDP2 proteins. CEF transfected with the indicated constructs were fixed with 3% paraformaldehyde and permeabilized with Triton X-100. They were then incubated with the rabbit anti-JDP2 antibody and a fluorescein isothiocyanate-conjugated secondary antibody

Perez *et al*., 2001). It is not known whether the mechanisms of transformation by ATF3 and JDP2 share common elements.

An independent connection between JDP2 and oncogenesis was established recently when insertional retroviral mutagenesis in the mouse identified JDP2 as a potential oncogene in the induction of lymphomas (Hwang *et al*., 2002). Bcl6 downregulates AP-1 in human lymphoma/myeloma cell lines establishing another connection between cancer and loss of AP-1 function (Vasanwala *et al*., 2002). However, in Hodgkin's lymphoma cell lines and biopsy samples, Jun is regularly overexpressed (Mathas *et al*., 2002). The effect of this overexpression on differential regulation of target genes remains to be determined.

Our observations suggest the presence of a repression domain in the carboxyl-terminal region of JDP2. The inhibition of AP-1-dependent transcriptional activation by JDP2 is therefore probably not due to the mere replacement of active AP-1 complexes with JDP2-containing inactive ones. Rather, JDP2 may function as an active repressor. This repression is not likely to be due to transrepression via squelching because GAL4–JDP2 (129–163) did not repress TRE-dependent transcription while GAL4–JDP2 did repress, suggesting that repression requires DNA binding (data not shown). JDP2 recruits histone deacetylase 3, and this complex can reduce the retinoic acid-induced transcription of Jun (Jin *et al*., 2002). The precise role of histone deacetylase in JDP2-induced repression requires further study.

The tests with JDP2 chimeric constructs suggest that neither JDP2 homodimers nor heterodimers between JDP2 and other bZIP proteins except Fos can induce oncogenic transformation of CEF. By exclusion, this leaves the JDP2–Fos heterodimer as the likely active candidate. A test of this suggestion would require a leucine zipper that does not homodimerize but heterodimerizes exclusively with Fos. At present such a leucine zipper is not known. We also concede the possibility that the chimeras may not faithfully reflect JDP2 function in the cell. Their repression activity is significantly reduced and this, not partner selection, may be the reason for their failure to transform.

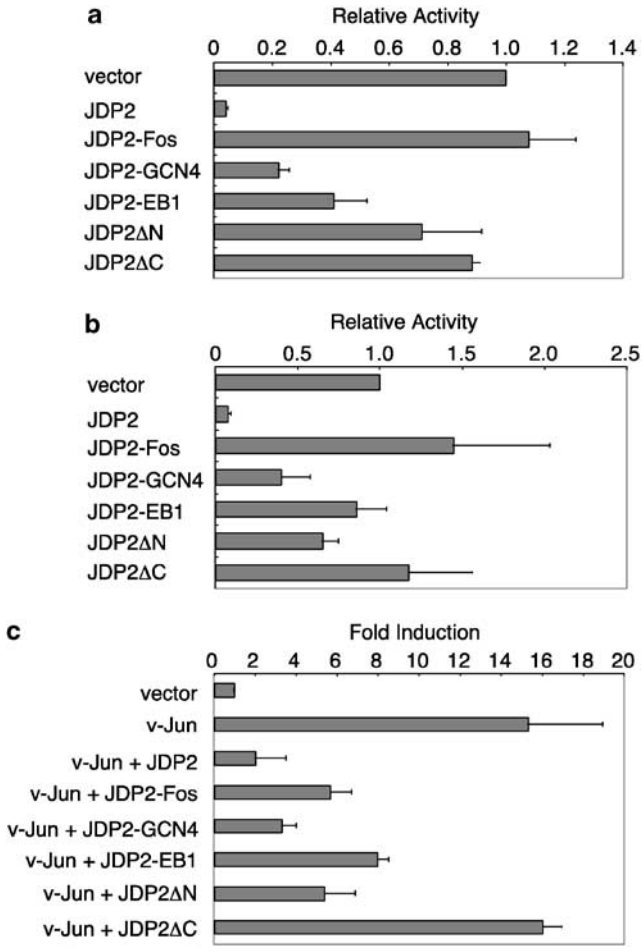

Figure 7 Repression activity of leucine zipper swap mutants and deletion mutants of JDP2. Human JEG-3 choriocarcinoma cells were transfected with the firefly luciferase reporter constructs regulated by TRE (panels a and c) or CRE (panel b) sequences together with the pHygEF2 expression plasmids containing JDP2 mutants. In panel c, the JDP2 mutants were cotransfected with pHygEF2-v-Jun plasmid. Reporter gene activity in control cells transfected with the reporter construct and the empty pHygEF vector was arbitrarily set as 1

Further studies on the mechanism of JDP2-induced transformation should help clarify the role of transcriptional repression in AP-1-induced oncogenesis.

Materials and methods

JDP2 constructs

An HA epitope tag was added to the carboxyl terminus of JDP2 by PCR, and the JDP2HA sequence was subcloned into the pBSFI adaptor plasmid (Aoki *et al*., 1998) at *Xba*I and *Eco*RI sites. The zipper mutants, JDP2–GCN4, JDP2–FOS, and JDP2–EB1, were generated using the Seamless Cloning Kit of Stratagene. Briefly, the recipient DNA, JDP2HA lacking the leucine zipper region (amino acids 99–127), was generated by PCR using primers JDP2–ΔZIP–FWD and JDP2–ΔZIP–REV on the pBSFI–JDP2HA template. The GCN4 leucine zipper insert was amplified with primers GCN4–ZIP–FWD and GCN4–ZIP–REV from the pGCN4 template plasmid (Kataoka *et al*., 2001). The Fos leucine zipper insert was amplified with primers FOS–ZIP–FWD and FOS–ZIP–REV from the pc-fos template plasmid. The EB1 leucine zipper insert was generated with primers EB1–ZIP–FWD and EB1–ZIP–REV on pET11d-EBVcZ (a generous gift from Dr George Miller). The sequences of the primers are as follows: JDP2HA-ΔZIP-FWD, 5′-AGCTCTTCGATCCTGATGCTCAACCGCCAC; JDP2HA-ΔZIP-REV, 5′-GCCTCTTCGGAACTCCGTGCGCTCCTTCTT; GCN4-ZIP Fwd 5′-AGCTCTTCGTTCCTCGAGGACAAGGTTGAAGAATT; GCN4-ZIP-REV, 5′-AGCTCTTCGGATGCGTTCGCCAACTAATTTCTTTAA; FOS-ZIP-FWD, 5′-AGCTCTTCGTTCCTCGAGGCGGAGACGGACCA; FOS-ZIP REV, 5′-AGCTCTTCGGATCAGCTTCTCCTTCTCCTTCAGC; EB1-ZIP-FWD, 5′-AGTTACTCTTCAAAGCAACTGCTGCAGCACTACC; EB1-ZIP-REV, 5′-AGTTACTCTTCACAGGCTTGGGCACATCTGCTT. All constructs generated by PCR were sequenced and then transferred to RCAS.SFI vector using *Sfi*I. Deletion mutants of JDP2 were also generated by PCR. JDP2ΔC was amplified by PCR using primers JDP2-Forward and bZIP Reverse, and JDP2ΔN was amplified using primers bZIP-Forward and JDP2-Reverse. The PCR products were digested with *Bam*HI and *Xho*I, and then ligated to pBSFI-AU1, the pBSFI vector containing the AU1 tag on the amino terminus followed by cloning sites. After confirming successful construction, the *Sfi*I fragments were subcloned into RCAS.Sfi. To generate GAL4 fusion products, JDP2 sequences were amplified using primers JDP2-Forward and JDP2-Reverse (GAL4-JDP2), JDP2-Forward and N-terminus Reverse (GAL4-JDP2ΔC), or C-Terminus Forward and JDP2-Reverse (GAL4-JDP2ΔN). The PCR products were digested with *Bam*HI and *Xho*I, and then ligated to pCMV-BD (Stratagene). The primer sequences are as follows: JDP2-Forward, 5′-GCTCGGATCCCCTGGGCAGATCCCAGACC; N-Terminus Reverse, 5′-GAGCCTCGAGTCATTCCTCATCTAGCTCACTC; C-Terminus Forward, 5′-GCTCGGATCCATCCTGATGCTCAACCGCCAC; JDP2 Reverse, 5′-CTCGCTCGAGTCACTTCTTGTCCAGCTGCTC; bZIP Forward, 5′-GCTCGGATCCGAAGAGCGAAGGAAAAGGCGC; bZIP Reverse, 5′-CTCGCTCGAGTCAGTGGCGGTTGAGCATCAGGA.

Cell culture and virus infection

Primary CEF cultures were prepared from White Leghorn embryos supplied by SPAFAS (CT) and maintained by standard procedures (Vogt, 1969). Focus assays using the RCAS virus stocks were performed as described previously (Bos *et al*., 1990). The assay plates were stained with crystal violet and scanned. The human choriocarcinoma cell line JEG-3 was obtained from the American Type Culture Collection and maintained as previously described (Kruse *et al*., 1997).

Recovery of JDP2 from foci

Three individual foci of JDP-transformed cells were picked and expanded. Genomic DNA was prepared using QIAamp DNA Mini Kit (QIAGEN), and JDP2 inserts were amplified from the genomic DNA by PCR using the following primers: 5′-TGGTGGTATAGCGCTTGCGA and 5′-CTCAGATACGCGTATATCTGG. The PCR products were subcloned using Zero Blunt TOPO PCR cloning kit (Invitrogen) and sequenced.

Luciferase reporter assays

Chicken c-*jun*, rat c-*fos*, and rat *JDP2* cDNAs were cloned into the pHygEF2 vector containing a modified elongation factor 1

promoter and used as effector plasmids. The JDP2 mutants described above were also subcloned into pHygEF2.Sfi containing the *Sfi*I cloning sites. The reporter constructs with TRE or CRE sequences upstream of the basal promoter controlling expression of luciferase cDNA have been previously described (Kataoka *et al*., 1996). As an internal control, pRL-Rluc DNA with renilla luciferase under the control of a modified CMV promoter (a cryptic AP-1 site was destroyed) was used (Makoto Nishizawa, personal communication). JEG-3 cells seeded onto MP-24 16-mm-well plates were transfected with 240 ng reporter-DNA, 240 ng effector-DNA and 1 ng pRL-Rluc using the Lipofectamine reagent (Invitrogen) according to the manufacturer's protocol. The firefly and renilla luciferase activities were measured using the Dual Luciferase Assay Kit (Promega) in Berthold Lumat model LB9501 illuminometer.

Nuclear extracts, in vitro *translation, and electrophoretic mobility shift assay*

Nuclear extracts were prepared from cells infected with RCAS-JDP2 or with RCAS as previously described (Schreiber *et al*., 1989). For the preparation of *in vitro* translated JDP2, the TNT Coupled Reticulocyte Lysate system (Promega, CA, USA) was used with pBSFI-JDP2 as a template. c-Jun was transcribed and translated *in vitro* with the TNT Quick Coupled Reticulocyte Lysate system (Promega) from the *Bam*HI linearized pGEM-cJ3 plasmid containing the full-length chicken c-*jun*.

Electrophoretic mobility shift assays were performed using nuclear extracts (6 μg) or products of *in vitro* translation (6 μl).

The probe was prepared by labeling col-TRE containing the TRE sequences of the collagenase promoter or the CRE oligonucleotide with T4 polynucleotide kinase and [^{32}P]-γ-ATP (NEN, MA, USA). The labeled oligonucleotides (30 000 cpm) and the proteins were incubated in a 20 μl total volume of binding buffer (20 mM HEPES (pH 7.9), 1 mM EDTA, 20 mM KCl, 4 mM $MgCl_2$, 5 mM dithiothreitol, 0.2 mg/ml poly (dI-dC), 0.1 mg/ml BSA, 10% glycerol) at RT for 30 min. For supershift experiments, antibody was included in the binding reaction. The samples were resolved in 6% polyacrylamide gels, and the results were visualized by autoradiography on X-Omat film (Kodak). Sequences of the pairs of oligonucleotides used as probes are as follows: CRE, 5′-TCGAGCTCCG-GAATCAATGACGTCATTGTTACTC and 5′- CGAGAG-TAACAATGACGTCATTGATTCCGAGCT; mutated CRE 5′-TCGAGCTCCGGAATTGAGGACGTCCTCATTACTC and 5′-CGAGAGTAATGAGGACGTCCTCAATTCCGGA-GCT; collagenase (col)-TRE, 5′-GCCAGAGGTGTCTGACT-CATGCTTTATAA and 5′-GTTATAAAGCATGAGTCAGA-CACCTCTGG.

GAL4-fusion constructs

The luciferase reporter vector pGL3-CMV + GAL4 was created from pGL3-Basic (Promega). A CMV minimal promoter was cloned into the *Hind*III/*Xho*I site. Five GAL4 binding sites were cloned as an *Xba*I/*Kpn*I-Fragment into the *Nhe*/*Kpn*I site located 5′ of the CMV promoter. Fragments of *JDP2* were cloned in frame into the *Bam*HI/*Sal*I site of pCMV-BD (Stratagene). pCMV-BD-vQin137–395 was created by cloning amino acids 137–395 of v-Qin into the *Eco*RI/*Sal*I site of pCMV-BD. HEK293 (human kidney epithelial cell line) cells were transfected with pGL3-CMV + GAL4 reporter and pCMV-BD constructs using the Lipofectamine reagent.

Western blots

Cells were lysed in lysis buffer (Aoki *et al*., 1998). Lysates containing 40 μg of protein were separated by 15% SDS/PAGE and transferred to an Immobilon-P membrane (Millipore, MA, USA). The membrane was probed with rabbit polyclonal anti-JDP2 antibody or with anti-HA monoclonal antibody HA-11 (Covance, CA, USA) followed by horseradish peroxidase-conjugated secondary antibody (Sigma, MO, USA). JDP2 proteins were visualized by incubation with chemiluminescent substrate (PIERCE, IL, USA).

Immunofluorescence

Cells grown on glass coverslips were washed with PBS and fixed with 3.7% formaldehyde for 30 min. After a wash with PBS, cells were permeabilized with 0.1% Triton X-100 for 30 min. The coverslips were then incubated with anti-JDP2 antibody for 30 min at room temperature in a humidified container. Three washes with PBS were followed by incubation with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Sigma) for 30 min. The coverslips were again washed three times with PBS and mounted on glass slides with Slowfade mounting medium (Molecular Probes, OR, USA).

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