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Genetics

Mutations in the Jun Delta region suggest an inverse correlation between transformation and transcriptional activation

(Jun/activator protein 1/transactivation/oncogenic transformation)

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ABSTRACT **The viral Jun protein (v-Jun) transforms chicken embryo fibroblasts (CEF) more effectively than its cellular counterpart (c-Jun). In certain cell types v-Jun is also a stronger transcriptional activator than c-Jun. These functional differences between v-Jun and c-Jun result from a deletion in v-Jun (referred to as "delta deletion") that seems to weaken the interaction of Jun with a negative cellular regulator molecule. These observations suggested that the oncogenicity of v-Jun may be due to an enhanced ability to activate transcription of target genes. To test this hypothesis, we constructed several deletions in the delta domain of chicken c-Jun and determined their transforming and transactivating properties. Surprisingly, we found an inverse correlation between the ability of the mutants to transform CEF and to transactivate the collagenase and transin promoters in CEF. In contrast, there was no significant effect of the delta mutations in c-Jun on transactivation in F9 murine embryonal carcinoma cells. The function of the delta region is therefore cell-type specific. The inverse correlation between transformation and transactivation in CEF suggests that the strong growth-promoting effect of v-Jun may be related to a failure to activate the transcription of growth attenuating genes.**

The product of the proto-oncogene c-*jun* is a major component of the activator protein 1 (AP-1) transcription factor family (1, 2). c-*jun* is an "immediate early" gene and is rapidly induced by a great variety of extracellular stimuli (3–7). The Jun protein functions as a homodimer or as a heterodimer together with other members of the AP-1 family to regulate the expression of genes containing AP-1-responsive elements (8, 9). Viral *jun* (v-*jun*), the oncogene of the avian sarcoma virus ASV-17 (10), also codes for a protein that is able to bind to and regulate transcription from AP-1 sites (11). The oncogenicity of v-*jun* presumably derives from its ability to induce changes in the expression of critical growth-controlling genes. A comparison of the v- and c-Jun proteins has demonstrated that the greater oncogenic potential of the viral protein in chicken embryo fibroblasts (CEF) is due mainly to a 27-amino acid deletion that defines the "δ region" of Jun (12). This observation suggests that the delta region contains an important control element. In an *in vitro* transcription system derived from HeLa cells, bacterially synthesized v-Jun functions as a more potent transcriptional activator than similarly produced human c-Jun (13). Genetic evidence obtained with hybrid molecules consisting of portions of Jun fused to heterologous DNA binding domains suggest the existence of a cell type-specific inhibitor that interacts with the delta region and down-regulates transcriptional activation by c-Jun (14). Therefore, the absence of the delta region from v-Jun could explain the greater transactivation induced by this protein (14). These observations are compatible with the hypothesis that the oncogenic activation of Jun results from the generation of a more active transcription factor. To assess the role of the delta region in transformation and transcriptional activation, we constructed several chicken c-*jun*-expressing plasmids containing mutations and deletions within the delta region. Unexpectedly, we found an inverse correlation between the transformation properties of these mutants in CEF and their ability to transactivate the collagenase and transin promoters in the same cells. We also found that the differences between the transactivation potentials of the mutants are cell-type specific.

MATERIALS AND METHODS

***In Vitro* Mutagenesis and Construction of Mutants and Plasmids.** The properties of the expression plasmid RCAS and the construction of RCAS CJ-3 and RCAS VC-3 have been described (12). Mutagenesis to create unique restriction sites in the delta region of c-*jun* was carried out with the T7-GEN *in vitro* mutagenesis kit supplied by United States Biochemical. The 1134-base-pair (bp) CJ-3 *Cla* I fragment from RCAS CJ-3 was inserted into the *Cla* I site of pGEM 7Z(+) to generate the parent plasmid for subsequent mutagenesis. Single-stranded pGEM CJ-3 DNA was prepared by infection with the M13 K07 helper phage. By using three different mismatched oligonucleotides, the three unique restriction sites *Hpa* I, *Eco*RV, and *Stu* I were introduced simultaneously into the delta region of c-*jun*. The mutations were introduced at nucleotide positions 401–406 (*Hpa* I), 438–441 (*Eco*RV), and 464–466 (*Stu* I). These changes resulted in a substitution in the encoded amino acids from Leu-Lys to Val-Asn, from Ala-Ser to Asp-Ile, and from Asn to Pro, respectively. The mutant containing these five amino acid substitutions was termed CJ-3-234. Subsequently, three delta-region deletion mutants CJ-3-23, CJ-3-34, and CJ-3-24 were constructed by digesting the pGEM CJ-3-234 plasmid with *Hpa* I/*Eco*RV, *Eco*RV/*Stu* I, and *Hpa* I/*Stu* I, respectively. All mutants were analyzed for the presence of the mutated sequence by restriction enzyme digestion followed by nucleotide sequence determination. The *Cla* I fragments of CJ-3-23, CJ-3-24, CJ-3-34, and CJ-3-234 were subcloned into the RCAS expression vector. The RCAS vector is derived from the genome of the replication-competent Prague strain of Rous sarcoma virus (RSV) (15, 16). These constructs were used for the transformation assays. Similarly, the *Xba* I fragments of CJ-3, VC-3, CJ-3-23, CJ-3-24, CJ-3-34, and CJ-3-234 were subcloned into the pRc/RSV (Invitrogen) expression vector and used together with the parent Rc/RSV

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Abbreviations: CAT, chloramphenicol acetyltransferase; CEF, chick embryo fibroblasts; AP-1, activator protein-1; RSV, Rous sarcoma virus.

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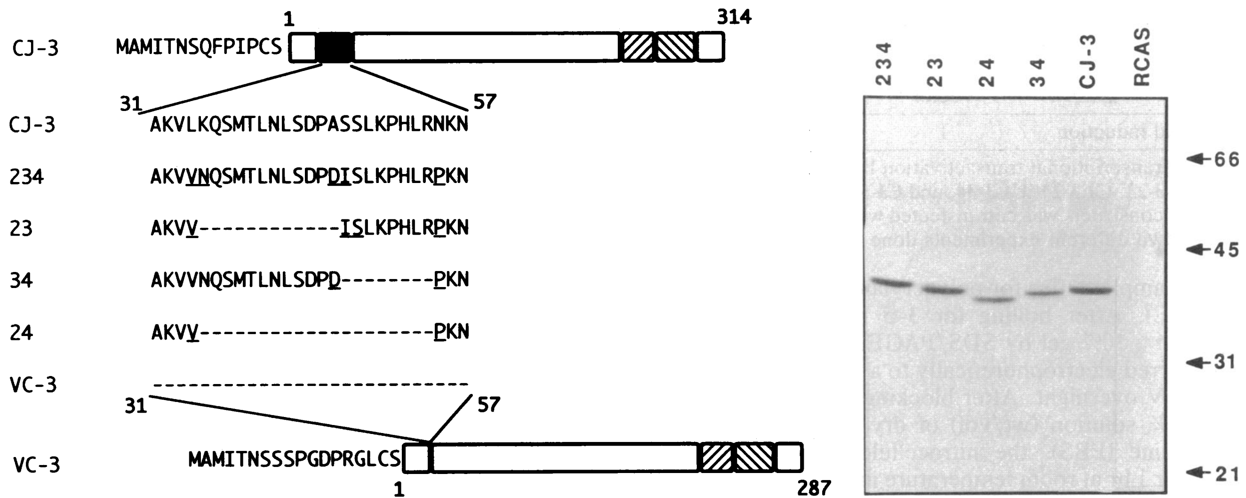


FIG. 1. (*Left*) Structure of c-Jun delta region mutants. Schematic representation of c-Jun (CJ-3), a v-Jun/c-Jun chimera (VC-3), and the c-Jun delta region mutants (CJ-3-234, CJ-3-23, CJ-3-34, and CJ-3-24). The black box is a 27-amino acid region called the delta region, which is deleted from v-Jun. The small hatched boxes near the carboxyl terminus are the DNA contact region and the leucine zipper dimerization domain, respectively. Amino acid changes are underlined, and deletions are represented by dashed lines. The CJ-3 constructs have 14 and the VC-3 construct has 18 amino acids added at the extreme amino terminus before the first jun-encoded methionine. (*Right*) Western blot analysis of c-Jun (CJ-3) and the delta region mutants of c-Jun (CJ-3-24, CJ-3-23, CJ-3-24, and CJ-3-34) expressed from RCAS vectors in CEF. Proteins were separated on a SDS/10% PAGE and probed with a polyclonal rabbit antiserum made against the carboxyl-terminal 100 amino acids of Jun. Expression from RCAS of the VC-3 construct has been demonstrated previously (12).

for transient expression of mutant and wild-type proteins in chloramphenicol acetyltransferase (CAT) assays. Construction of Col-CAT reporter plasmids in which transcription of the CAT gene is driven by the collagenase gene promoter, −73 Col-CAT (AP-1^+) and −63 Col-CAT (AP-1^-), and of the transin-CAT reporter has been described (17, 18).

Transformation Assays. CEF were prepared as described (19). CEF were grown at 37°C overnight in 35-mm six-well plates at a density of 1×10^6 cells per well. The cells were transfected with 0.5 μg of the RCAS-Jun constructs by using the dimethyl sulfoxide/Polybrene method (20). Transfected cells were overlaid with nutrient agar the day after transfection and thereafter every other day until foci appeared (12). The numbers of foci in each well were counted after 14 days. Agar colony assays were performed by the method of Bister *et al.* (21).

Transfection and CAT Assays. CEF were plated in Dulbecco's modified Eagle's medium (DMEM) containing 10% or 3% (vol/vol) fetal calf serum at 2×10^6 cells per 60-mm tissue culture dish 24 hr before DNA transfection. The cells were transfected with 0.2 μg of Col-CAT reporter plasmid and 1 μg of the pRC/RSV-Jun expression plasmid by using the calcium phosphate coprecipitation technique. With the transin-CAT reporter, 0.4 μg and 2 μg, respectively, were used. F9 mouse embryonal carcinoma cells were plated in DMEM supplemented with 10% fetal calf serum at 1×10^6 cells per 100-mm tissue culture dish. After 24 hr they were transfected with 5 μg of the Col-CAT reporter and 5 μg of the pRC/RSV-Jun expression vector. Four hours and 16 hr after transfection of CEF and F9 cells, respectively, the cells were shocked with 15% (vol/vol) glycerol in DMEM, and 24 hr later the cells were harvested. CAT activity in cell extracts containing equal amounts of protein was determined by standard techniques (22). Following thin-layer chromatography, the total cpm in the acetylated and nonacetylated forms was determined with an AMBIS Radioanalytic Imaging Detector.

Immunoblotting (Western Blotting). CEF were seeded at 3×10^6 cells per 60-mm tissue culture dish and transfected with the RCAS-Jun plasmids. After 4 days under agar, the cells were transferred to liquid medium. The following day the cells were lysed in 350 μl of sodium dodecyl sulfate (SDS)-

Table 1. Transformation of CEF by delta region mutants of Jun

Construct	Assay: Focus, no. of foci per μg of DNA transfected	Assay: Agar colony, no. of colonies per 1000 cells seeded
CEF	0	0
RCAS	0	0
CJ-3	9 ± 6	76
VC-3	64 ± 12	50
CJ-3-23	2 ± 2	4
CJ-3-24	31 ± 14	41
CJ-3-34	60 ± 11	78
CJ-3-234	35 ± 7	59

Effect of deletions and mutations in the delta region of c-Jun on focus formation and anchorage-independent growth. The numbers in the focus assays are an average of three experiments done in duplicate. The numbers in the agar colony assays are an average of two experiments done in duplicate.

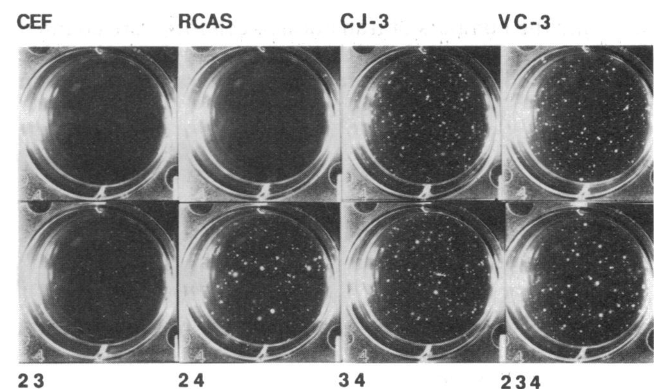


FIG. 2. CEF transformed by Jun mutants form colonies in nutrient agar. After transfection cells were grown for 2 weeks under agar, the cells were trypsinized and reseeded in soft agar at a density of 1×10^3 cells per well. CEF indicates nontransfected cells, RCAS indicates expression vector alone, CJ-3 indicates wild-type chicken c-Jun protein, and VC-3, 23, 24, 34, and 234 indicate the different delta region mutants shown in Fig. 1.

Table 2. Transactivation of the collagenase promoter by delta region mutants of Jun in F9 cells

	Construct						
	Vector	CJ-3	VC-3	CJ-3-23	CJ-3-24	CJ-3-34	CJ-3-234
Fold induction	1	7.8	5.3	5.1	4.7	5.4	7.6

Transcriptional transactivation by wild-type Jun protein (CJ-3) and different c-Jun delta region mutant proteins (VC-3, CJ-3-23, CJ-3-24, CJ-3-34, and CJ-3-234) carried out in F9 cells. The pRC/RSV expression vector containing the different jun constructs was cotransfected with the −73 Col-CAT reporter plasmid. Induction of transcription represents the average of two different experiments done in duplicate.

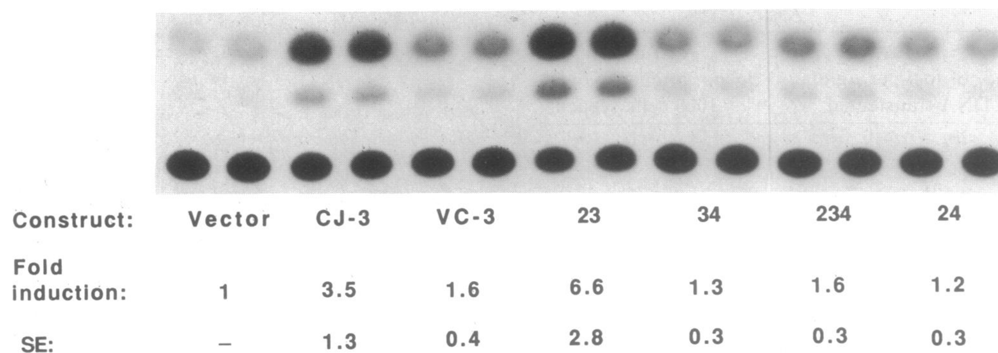
containing sample buffer for polyacrylamide gel electrophoresis (PAGE). After boiling for 3–5 min, proteins were separated on a 10% gel by SDS/PAGE. The proteins were then transferred electrophoretically to a nitrocellulose membrane at 40 V overnight. After blocking unreacted sites (30 min) in a 3% solution (wt/vol) of dry milk in phosphate-buffered saline (PBS), the nitrocellulose membrane was incubated for 1 hr at room temperature in a 1:2500 dilution of a rabbit antiserum in 0.05% Tween/PBS. The rabbit antiserum was prepared against the conserved carboxyl-terminal part of Jun. The membrane was washed three times for 10 min each in PBS and incubated with a 1:2500 dilution of goat anti-rabbit IgG-alkaline phosphatase conjugate (Bio-Rad) for 1 hr. After washing five times for 10 min each in PBS, the membrane was developed with a Bio-Rad alkaline phosphatase detection system.

RESULTS

Construction and Expression of Delta Region Deletion Mutants. The deletion mutants of the chicken c-Jun delta region are shown in Fig. 1 (*Left*). After verification of the expected nucleotide sequences, the mutants were cloned into expression vectors for transformation and transcription assays as described. To determine whether the mutants produced proteins of the appropriate size, they were cloned into the RCAS plasmid (15, 16). These plasmids were then transfected into CEF. Western blots were carried out with protein extracts from the transfected cultures and showed that the mutants encoded proteins of the predicted sizes and that the levels of protein expression were similar for all constructs (Fig. 1 *Right*). These proteins were located exclusively in the nucleus as determined by an immunofluorescence assay.

Transformation of CEF by c-Jun and Mutant Proteins. It has been shown (12) that v-Jun is a more potent transforming protein than c-Jun in CEF because of the deletion of the delta region. To analyze this region further, RCAS constructs of the mutants detailed in Fig. 1 (*Left*) were transfected into CEF, and the numbers of transformed cell foci were counted after 2 weeks. The results of these assays are shown in Table 1. As expected, the VC-3 protein was more transforming than CJ-3, and the foci observed appeared 3–5 days sooner with VC-3 than with CJ-3. Deletions within the delta region had diverse effects upon the transforming property of c-Jun. Removing eight amino acids from the carboxyl-terminal part of the delta region increased the transforming potential of chicken c-Jun (CJ-3-34). Deletion of 12 amino acids from the amino-terminal region of the delta region (CJ-3-23) resulted in a significant decrease in the ability of c-Jun to transform CEF. Several point mutations (CJ-3-234) or a deletion of 20 amino acids (CJ-3-24) within the delta region resulted in a moderate increase in the transforming potential of the c-Jun protein. To test for anchorage-independent growth, CEF expressing the mutant proteins were cloned in agar. The results are shown in Table 1 and Fig. 2. Jun proteins that were moderately or highly transforming in focus assays conferred onto CEF about equal cloning efficiencies in agar. However, the CJ-3-23 protein was a poor inducer of agar colonies, as it was also weakly transforming in focus assays.

Transcriptional Properties of the Jun Proteins. The transactivation properties of Jun proteins have been analyzed previously in the murine embryonal carcinoma cell line F9 (14, 17, 23–25). This cell line has a low level of endogenous AP-1 activity, making it ideal for such studies (26, 27). We also determined the transcriptional regulator activities of the delta region mutants in F9 cells (Table 2). The reporter plasmid used was the AP-1 binding site-containing −73 Col-CAT (17), which has the collagenase promoter driving the transcription of the CAT gene. This construct has been used in several previous studies (14, 17, 24). All of the assayed Jun proteins functioned as transactivators in F9 cells. There were only minor differences between the mutants and the wild type. This pattern contrasted with the results obtained in the CEF transformation assays (Table 1). Therefore, we determined the transcriptional properties of the mutants also in CEF. Again the −73 Col-CAT plasmid served as a reporter. The results are summarized in Fig. 3. In CEF the transcriptional activator properties of the Jun proteins were not the same. The poorest transforming protein, CJ-3-23, was the best transactivator of the collagenase promoter, while the most potent transforming proteins (VC-3 and CJ-3-34) failed to transactivate. The moderately transforming



Construct:	Vector	CJ-3	VC-3	23	34	234	24
Fold induction:	1	3.5	1.6	6.6	1.3	1.6	1.2
SE:	–	1.3	0.4	2.8	0.3	0.3	0.3

FIG. 3. Activation of the collagenase promoter by Jun (CJ-3) and different delta region mutants of Jun (VC-3, CJ-3-23, CJ-3-24, CJ-3-34, and CJ-3-234). CEF were cotransfected with the pRC-RSV expression vector containing the different Jun constructs and the −73 Col-CAT reporter plasmid. Induction of transcription represents the average of five experiments done in duplicate and is expressed relative to the value obtained with the pRC/RSV expression vector alone. Results from a representative experiment are reproduced in the upper panel.

Table 3. Transactivation of the transin promoter by delta region mutants of Jun in CEF

	Construct						
	Vector	CJ-3	VC-3	CJ-3-23	CJ-3-24	CJ-3-34	CJ-3-234
Fold induction	1.0 ± 0.1	1.3 ± 0.1	0.7 ± 0.1	4.1 ± 0.4	0.5 ± 0.1	0.6 ± 0.1	0.8 ± 0.1

Transcriptional transactivation by wild-type Jun protein (CJ-3) and different c-Jun delta region mutant proteins (VC-3, CJ-3-23, CJ-3-24, CJ-3-34, and CJ-3-234) carried out in CEF. The pRC/RSV expression vector containing the different jun sequences was cotransfected with a transin-CAT plasmid containing 754 bp of upstream promoter sequence.

proteins (CJ-3-234 and CJ-3-24) also seemed to lack the ability to transactivate, while the relatively weakly transforming wild-type protein (CJ-3) was a transactivator, although not as strong as the CJ-3-23 protein. Control experiments with the Col-CAT reporter plasmid from which the AP-1 site had been deleted showed no transactivation with any of the Jun proteins. To determine whether this inverse correlation between transformation and transactivation was promoter specific, other promoter-CAT constructs were tested. Several were inactive in CEF, whereas a 5xTRE tk-CAT construct (tk = thymidine kinase gene) showed a pattern similar to Col-CAT but with smaller differences. In Table 3 the results obtained with a transin-CAT reporter (18) are shown. The results were similar to those obtained with the Col-CAT construct. Although transin and collagenase genes are members of the same family (28), the data demonstrate that the inverse correlation between transformation and transactivation is not unique to the collagenase promoter. Fig. 4 summarizes the relationship between transactivation and transforming properties of the Jun proteins in CEF and suggests an inverse correlation of these two parameters.

DISCUSSION

Jun is a transcriptional regulator; it can activate as well as repress transcription (26, 27, 29–32). It is an immediate early gene, responsive to mitogenic signals (3–7). Increased expression of *jun* leads to enhanced AP-1 activity often found in actively growing cells (for reviews, see refs. 33 and 34). Therefore, oncogenicity of Jun was expected to be correlated with an enhanced transactivation potential. This possibility was supported by initial experiments. The increase of oncogenic potential seen with v-Jun was correlated with enhanced transcriptional activation in a HeLa cell system *in vitro* (13). These greater transformation and transcription activities were shown to result from a 27-amino acid deletion in v-Jun that marks the delta region (12–14). Because of this importance of the delta region in determining the properties of Jun, we have carried out a mutational study of that domain. Our results confirm some of the previous work. (*i*) Strongly and weakly transforming Jun proteins differ in their transcriptional properties. (*ii*) The differences in transcriptional activation are cell type specific and are not evident in F9 cells. It follows from *i* and *ii* that certain cell types contain a

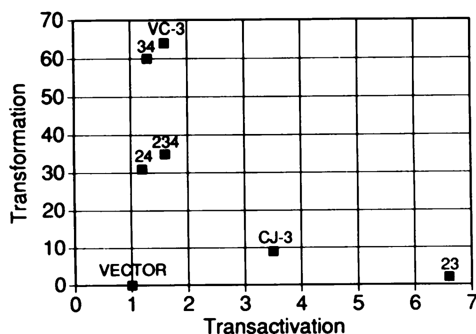


FIG. 4. Transformation (number of foci induced by 0.5 μg of DNA) plotted against transactivation of the collagenase promoter in CEF (vector ≡ 1).

regulatory protein whose interaction with Jun is influenced by the delta region. This "delta-tropic" protein could be an inhibitor as suggested (13, 14) or an enzyme such as a kinase or a phosphatase that modifies the Jun protein and its function. For instance, *ras* expression increases the phosphorylation of c-Jun (35) and increases its transcriptional properties (36). Among the delta region mutants, the poorest transformer is the best transactivator, and the high transformers are poor transactivators. Since transformation and transactivation were measured in the same cell type, CEF, the inverse correlation between these two functions may be significant for the explanation of Jun-induced oncogenesis. Indeed, enhanced AP-1 activity is not invariably associated with cell growth but also has been found to induce terminal differentiation (for a review, see ref. 33). It is conceivable that oncogenic Jun, rather than activating the transcription of growth-promoting genes, fails to activate the transcription of tumor suppressor genes and thus effectively silences negative-growth regulation. In this context it is of interest that overexpressed v-Jun reduces the expression of endogenous c-Jun as detected by a specific antibody directed against a peptide within the delta region (T. Bos, F. Monteclaro, and P.K.V., unpublished data). Therefore, the transactivation of negative growth regulators by endogenous c-Jun may also be down-regulated by v-Jun. An example for such a situation is the interaction of Jun and the transcriptional regulator protein MyoD that drives and determines muscle differentiation. Transforming Jun interferes with myogenesis in cell culture; it inhibits fusion of myoblasts into myotubes and induces extended cell division in myogenic cultures (37, 38). This growth-promoting effect of transforming Jun is correlated with a down-regulation of MyoD at the transcriptional and posttranscriptional levels (J. Li, H. Su, and P.K.V., unpublished data; I. M. Verma, personal communication). Although two promoter constructs gave the same results in the transactivation tests of the Jun mutants, it is possible that the differences between highly and poorly transforming Jun proteins are promoter dependent. The ability of Jun to form complexes with diverse transcriptional regulators broadens the spectrum of DNA target sequences to include several non-AP-1 binding sites (for review, see ref. 39). It is conceivable that transcriptional control of such sites by delta mutants of Jun is different from or even opposite to the effects described here. Other transcription factor oncoproteins have recently been shown to cause transformation by down-regulating transcription. The oncogenic versions of p53 and *rel* activate transcription less effectively than their normal cellular counterparts and, in the case of *rel*, interference with transcriptional activation was found (40–42). More work is needed to determine whether the inverse relationship between transformation and transactivation in CEF applies generally to all mutant and wild-type Jun proteins. The identification of target genes relevant for the oncogenic process induced by jun emerges as an urgent immediate task.

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