v-Jun Represses c-jun Proto-Oncogene Expression in Vivo through a 12-O-Tetradecanoylphorbol-13-acetate-responsive Element in the Proximal Gene Promoter

Shakeel Hussain, Anna Kilbey, and David A. F. Gillespie
 Beatson Institute for Cancer Research, Cancer Research Campaign Beatson Laboratories, Bearsden, Glasgow G61 1BD, United Kingdom

Abstract

c-jun proto-oncogene expression is extinguished in cells transformed by v-Jun; however, the mechanistic basis of this phenomenon has not been elucidated. c-jun mRNA levels are greatly reduced in v-Jun-transformed cells, and we show that this reduction is associated with a similar decrease in the rate of c-jun transcription. Transcriptional down-regulation was also evident in functional assays in which the c-jun gene promoter was -10-fold less active in v-Jun-transformed cells than it was in normal cells. This reduction was due largely to a conserved 12-O-tetradecanoylphorbol-13-acetate-responsive element (TRE)-like motif at position -72 (the proximal junTRE) that was essential for efficient basal expression in normal cells but that conferred little, if any, detectable transcriptional activity in v-Jun-transformed cells.

DNA-binding analysis showed that this element was recognized by a mixture of c-Jun/Fra and cyclic AMP-responsive element-binding protein/activating transcription factor-like complexes of normal cells but that v-Jun/Fra heterodimers predominated in v-Jun-transformed cells. Furthermore, ectopic expression of v-Jun repressed c-jun promoter activity in normal cells through the proximal junTRE. Thus, the defect in transcription mediated by the junTRE correlates with and is most likely attributable to binding of v-Jun to this element in vivo. We also find that the c-jun promoter is refractory to induction via the stress-activated protein kinase/c-jun NH2-terminal kinase pathway in v-Jun-transformed cells, suggesting that v-Jun interferes with signal-regulated gene expression.

Therefore, c-jun is an example of a cellular gene, the transcription of which is regulated negatively by v-Jun in vivo.

Introduction

The c-jun proto-oncogene, together with its relatives junB and junD, and the fos family of proto-oncogenes collectively encode the components of the generic transcription factor AP-1, which binds to and activates transcription through TREs3 (1). Each of these genes is subject to complex and distinct patterns of regulation, with the result that the precise subunit composition of AP-1 varies, not only between cells of different types but also according to the growth or differentiation state of individual cells (2). As a result, the regulation of the c-jun and c-fos proto-oncogenes in particular has been studied in considerable detail.

c-jun expression can be induced in response to a wide range of environmental signals, including growth factors, cytokines, and various forms of radiant or chemical stress, such as UV light (reviewed in Refs. 1 and 2). Interestingly, the protein encoded by c-jun, p39 c-Jun, is thought to play a role in mediating induction of its own gene in response to such signals through two TRE-like sites within the gene promoter (3, 4). c-Jun can interact with one or both of these sites in association with ATF-2 or Fos-related partner proteins (4, 5) and is thought to mediate gene induction in two stages. First, posttranslational activation of DNA-bound c-Jun and/or ATF-2 through phosphorylation by signal-regulated kinases such as SAPK and JNK leads to a rapid and protein synthesis-independent increase in c-jun transcription (4, 5). Subsequently, the resulting increase in biosynthesis and, hence, intracellular concentration of p39 c-Jun protein is thought to sustain elevated c-jun expression for a prolonged period (3).

Although many aspects of this positive autoregulatory model have been confirmed experimentally (3–5), a number of issues remain unresolved. In particular, because c-jun expression is typically induced only transiently in response to cell stimulation, it is evident that this positive feedback loop must be limited or governed by some mechanism to prevent an uncontrolled escalation of c-jun expression. Because current thinking suggests that both posttranslational mechanisms and increases in p39 c-Jun levels can enhance c-jun transcription, how this "repression phase" is imposed is unclear (5).

Received 4/17/98; revised 6/24/98; accepted 6/25/98.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by the Cancer Research Campaign and Medical Research Council of the United Kingdom.

2 To whom requests for reprints should be addressed, at Beatson Institute for Cancer Research, Cancer Research Campaign Beatson Laboratories, Garscube Estate, Switchback Road, Bearsden, Glasgow G61 1BD, United Kingdom. Phone: 0141-942-9361; Fax: 0141-942-6521; E-mail d.gillespie@beatson.gla.ac.uk.

3 The abbreviations used are: TRE, 12-O-tetradecanoylphorbol-13-acetate-responsive element; ATF, activating transcription factor; SAPK, stress-activated protein kinase; JNK, c-jun NH2-terminal kinase; UTR, untranslated region; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CAT, chloramphenicol acetyltransferase; MEK, mitogen-activated protein kinase kinase; CRE, cyclic AMP-responsive element; CREB, CRE binding protein; CEF, chicken embryo fibroblast; MEKK, mitogen-activated protein kinase kinase kinase; WCE, whole-cell extract.
Oncogenic transformation by the v-Jun oncoprotein requires both overexpression and activating mutations that alter the structure of the protein (6, 7). Although it was initially assumed that the v-Jun oncoprotein would act in the presence of its normal cellular counterpart, several studies have reported that endogenous p39 c-Jun protein expression is, in fact, undetectable in v-Jun-transformed cells (8–10). This is not simply a consequence of cell transformation because cells transformed by other oncogenes, such as Myc or Src, express normal or in some cases modestly enhanced levels of p39 c-Jun (11).

Loss of p39 c-Jun expression as a consequence of v-Jun transformation was unexpected because it has generally been considered that the combination of overexpression and activating mutations confers increased transcriptional potential on v-Jun (12, 13). It might, therefore, be expected that v-Jun would stimulate endogenous c-jun gene expression in transformed cells by the positive autoregulatory mechanisms discussed above. These considerations suggested that an analysis of the molecular mechanism through which p39 c-Jun expression is extinguished in v-Jun-transformed cells might provide insights into not only the regulation of c-jun gene expression but also into how v-Jun may affect the expression of certain cellular target genes.

Here, we show that c-jun transcription is down-regulated in v-Jun-transformed cells and that this is, in large part, attributable to a deficit in transcriptional activity mediated by a specific element in the c-jun promoter, the proximal TRE. Furthermore, this deficit correlates with and is most likely due to binding of the v-Jun oncoprotein to this element at the expense of c-Jun and/or certain CREB/ATF-like factors that interact with this site in c-jun-expressing cells. Our results suggest that rather than acting as a "super-activator" of transcription, v-Jun can repress the expression of at least some cellular genes through specific TRE elements.

**Results**

*c-jun* Transcription Is Down-Regulated in v-Jun-transformed Cells. Several studies have documented loss of p39 c-Jun protein expression in v-Jun-transformed cells (an example is shown in Fig. 1a; see also Refs. 8–10). To establish the molecular basis of this phenomenon, we first quantitated the levels of c-jun mRNA in normal and v-Jun-transformed cells by Northern blotting using a 3' UTR probe specific for the endogenous c-jun transcript. As shown in Fig. 1b, the level of c-jun mRNA seen in normal avian fibroblasts was reduced to essentially undetectable levels after infection and transformation of these cells with a retrovirus encoding v-Jun (ASV17; Ref. 14). Control experiments using GAPDH and a jun coding region probe, which detects both the endogenous c-jun and exogenous retrovirally encoded v-jun mRNA tran-
scripts, confirmed equivalent RNA loading and efficient infection of the cultures with the transforming retrovirus (Fig. 1b).

A reduction in steady-state mRNA levels could reflect differences either in transcription rate or in mRNA stability. To distinguish between these alternatives, we performed a run-on analysis using nuclei isolated from normal and v-Jun-transformed cells and the 3'-UTR and coding region probes used in Fig. 1b. This analysis revealed that, although jun coding region transcription was markedly increased in v-Jun transformed cells (reflecting retroviral transcription of v-jun), the rate of transcription of the endogenous c-jun gene was significantly reduced (Fig. 1c). Moreover, when a plasmid reporter construct containing the bacterial CAT gene under the control of the mouse c-jun gene promoter (pJC6; Fig. 2b; Ref. 15) was transfected into normal and v-Jun-transformed cell cultures, ~10-fold lower levels of transcription were observed in the transformed cell background compared to normal cells (Fig. 2b). This was not a consequence of differential transfection efficiency because other gene promoters such as the murine sarcoma virus long terminal repeat-CAT constructs were transfected into normal (33) or v-Jun-transformed (33) avian fibroblasts, and the levels of CAT activity were determined. Columns, averages of five independently transfected dishes; bars, SD.

The Proximal TRE in the c-jun Promoter Is Differentially Active in Normal and v-Jun-transformed Cells. A number of potential transcription factor-binding sites have been identified in the c-jun promoter, and two of these in particular, a TRE-like motif at position −72 relative to the transcription start site (the proximal junTRE) and an adjacent consensus binding site for factors of the MEF2 family (Fig. 2a), have been implicated in c-jun regulation in a variety of cell types and situations (4, 5, 15–16). These elements are absolutely
conserved in the human, mouse, rat, and chicken c-jun
genes (Fig. 2a), although an additional TRE-like site at po-
tion −190 that has been identified in mammals (the distal
junTRE) is not conserved in the avian promoter and does not
bind AP-1 in vitro (data not shown).

To determine whether the proximal junTRE or MEF2 motifs
contributed to the differential activity of the c-jun promoter,
mutant derivatives with multiple nucleotide substitutions in
either the junTRE site, which abolishes binding of AP-1, or
MEF2 site, which abolishes binding of MEF2 proteins (15),4
were transfected into normal and v-Jun-transformed cells.

As shown in Fig. 2b, this analysis revealed that the pro-
ximal junTRE was essential for efficient basal expression in
normal cells because mutations that eliminated this motif
(pJTX) led to an ~80% reduction in activity. In contrast,
mutations in the adjacent MEF2 site (pJSX) led to a modest
but highly reproducible increase in transcriptional activity.
Because the proximal junTRE and MEF2 sites are extremely
closely juxtaposed (Fig. 2b), we suspect that this may reflect
competition between junTRE-specific and MEF2-specific
factors of differing transcriptional potency for binding to the
composite element in vivo. Thus, elimination of the MEF2 site
may allow unhindered binding of more potent activators to
the adjacent junTRE.

A very different pattern was observed when these pro-
moter constructs were transfected into v-Jun-transformed
cells. In this case, the lower basal level of pJC6 transcription
was not further diminished by elimination of the junTRE
(pJTX), indicating that in this cell background, this element
mediates little if any detectable transcriptional activity. Con-
sistent with this interpretation, mutation of the MEF2 site
(pJSX) did not increase activity.

These results suggest that a deficit of transcriptional ac-
tivity mediated by the proximal junTRE is the major factor
underlying the reduced basal expression of the c-jun pro-
moter in v-Jun-transformed cells. Other mechanisms may
also contribute, however, because c-jun promoter activity is
somewhat lower in the v-Jun-transformed cell background
than can be explained by this deficit alone (Fig. 2b; compare
pJC6 in transformed cells with pJTX in normal). This ad-
tiional minor component is presumably attributable to a re-
duction in the activity of the second TRE-like site at position
−190 in the murine c-jun promoter or of some other func-
tional element contained within the pJC6 reporter (4, 5, 17).
We have not pursued this, however, because additional ex-
periments described below also demonstrate that the prox-
imal junTRE is the primary target of v-Jun.

v-Jun Interacts with the c-jun Proximal TRE in Vitro.

To identify the molecular basis for the reduction in junTRE-
specific transcriptional activity, we first investigated DNA-
binding activities specific for this element in normal and
v-Jun-transformed cells. The junTRE differs from a canonical
consensus TRE (such as that from the collagenase gene
promoter, cofTRE) in that the TGA half-sites are separated by
two nucleotides, a spacing more typical of consensus CREs
(Ref. 17; Fig. 2a). We, therefore, compared the complexes

that formed on the junTRE with those formed on the cofTRE
and the consensus CRE from the fibronectin gene promoter
(fibroCRE) in extracts prepared from either normal or v-Jun-
transformed fibroblasts.

As described previously (10), the predominant complexes
that form on the cofTRE in normal and v-Jun-transformed
cells consist of either p39 c-Jun or p65 v-Jun in association
with a variety of Fos-related partners proteins (Fig. 3a). A
superficially similar pattern was observed using the junTRE
(Fig. 3a); however, in this case, addition of antibodies specific
for c-Jun or Fos-related proteins (Pan-Fos) revealed that the
predominant electrophoretic species that formed on this el-
ement in normal cells (Fig. 3a, X) was heterogeneous and
contained a significant proportion of non-c-Jun/-Fos-related
complexes (Fig. 3b). Essentially identical results were ob-
tained with the consensus fibroCRE probe (data not shown).

JunD is the other Jun family protein thought to be ex-
pressed in CEFS (10), and although a small of JunD was
present in complex X, as judged by the effect of a JunD-
specific antibody (Fig. 3c), a significant amount of binding
activity persisted in the presence of both c-Jun- and JunD-
specific antisera. These non-Jun/-Fos related components
exhibited CREB/ATF-like DNA-binding specificity because
they could be effectively competed by the consensus fibro-
CRE but not by the consensus cofTRE (Fig. 3d and data not
shown). In view of this specificity, it seems likely that these
complexes correspond to avian CREB/ATF homologues.
Thus, in normal fibroblasts, both Jun/Fra complexes and
CREB/ATF-like factors interact with the junTRE, whereas
only Jun/Fra complexes bind to the consensus cofTRE.

A more complex pattern consisting of two electrophoretici-

ly distinct complexes formed on the junTRE probe in
v-Jun-transformed cells (Fig. 3a, Y and Z). The more abun-
dant and slowly migrating of these complexes (Y) was unique
to the transformed cell extracts, suggesting that it contained
the gag-linked M, 65,000 v-Jun protein. This was confirmed
by antibody addition experiments, which showed that this
complex was reactive with both anti-c-Jun (Fig. 3b) and
anti-gag antisera (data not shown). It was also evident that
v-Jun was associated with Fos-related partners in complex Y
because it was completely disrupted by addition of Pan-Fos
antisera (Fig. 3b). In contrast, the minor and more rapidly
migrating complex Z was devoid of c-Jun- or Fos-related
components (Fig. 3b) and, as in normal cells, could be ef-
fectively competed by the fibroCRE but not by the cofTRE
competitor (Fig. 3d). These properties suggested that com-
plex Z most likely corresponded to the same CREB/ATF-like
factors that interact with the junTRE in normal cells. Curi-
ously, we saw no evidence for JunD binding to the junTRE
in v-Jun-transformed cells (Fig. 3c), although whether this is
because expression of the endogenous junD gene, like c-jun,
is extinguished remains to be established.

We conclude that the deficit in transcriptional activity me-
diated by the proximal junTRE in v-Jun-transformed cells is
associated with a change in binding activities specific for this
element in vitro; whereas c-Jun/Fra and CREB/ATF-like
complexes mainly interact with this element in normal cells,
v-Jun/Fra complexes predominate in v-Jun-transformed
cells.

4 S. Hussain, unpublished results.
Fig. 3. Analysis of junTRE-specific DNA-binding complexes in normal and v-Jun-transformed cells. a, comparison of complexes formed on oligonucleotides containing the junTRE (right) with the consensus coilTRE (left) in normal and v-Jun-transformed cell extracts. The positions of the previously characterized (10) coilTRE-specific c-Jun/Fra and v-Jun/Fra complexes are indicated. Right, complexes designated X, Y, and Z are indicated. b and c, replicate DNA-binding reactions prepared using extracts from normal or v-Jun-transformed cells were incubated with the indicated antisera prior to the addition of the junTRE oligonucleotide probe. d, replicate binding reactions prepared using normal or v-Jun-transformed cell extracts were incubated with a 50-fold excess of the indicated cold competitor DNAs prior to the addition of the junTRE oligonucleotide probe. In addition to the consensus coilTRE and fibroCRE, oligonucleotides spanning the junTRE alone, MEF2 site alone, or both sites together were used as competitors (Fig. 2a).

Repression of c-jun Promoter Activity by v-Jun in Vivo.
To determine what effect v-Jun might have on c-jun promoter activity in vivo, we transfected the pJC6 and mutant derivative reporter plasmids into normal cells together with expression vectors encoding either v-Jun or c-Jun. As shown in Fig. 4a, expression of exogenous v-Jun repressed transcription of pJC6 by ~10-fold compared to control in normal cells, whereas c-Jun stimulated an increase in promoter activity of ~2-fold. Both repression by v-Jun and activation by c-Jun were dependent on the proximal junTRE because neither c-Jun nor v-Jun had any effect on the expression of the pJTX or pJSSTX reporters, both of which lack this element. In contrast, pJSX, which lacks the adjacent MEF2-binding site, was activated by c-Jun and repressed by v-Jun as effectively as wild-type pJC6. Interestingly, the effect of v-Jun could be mimicked by a dominant-negative mutant of c-Jun (Tam67; Ref. 19) that lacks the NH2-terminal activation domain (data not shown), indicating that, in this context, v-Jun lacks significant transactivation capacity.

Ectopic expression of c-Jun and v-Jun had quite different effects on c-jun promoter activity in the v-Jun-transformed cell background (Fig. 4b). Here, the much lower level of basal transcription could not be further depressed by v-Jun; however, expression of c-Jun stimulated c-jun promoter activity by a greater factor than that in normal cells (~10-fold compared to 2-fold). As in normal cells, stimulation by c-Jun was dependent on the proximal junTRE because pJTX and pJSSTX were completely nonresponsive, whereas pJSX was indistinguishable from wild-type pJC6 (Fig. 4b).

Thus, whereas exogenous v-Jun represses the c-jun promoter in normal cells, the reintroduction of c-Jun can substantially rescue the transcriptional deficit evident in v-Jun-transformed cells, and both of these effects are mediated through the proximal junTRE. Taken together with the DNA-binding analysis described above, these results strongly suggest that down-regulation of c-jun transcription in v-Jun-transformed cells is attributable, in large part, to the interaction of v-Jun with this element in vivo.

The c-jun Promoter is Refractory to Induction via the SAPK/JNK Pathway in v-Jun-Transformed Cells. In many cell types, c-jun transcription can be induced by external signals that activate one or more mitogen-activated protein kinase pathways, and a number of studies have identified the junTRE and adjacent MEF2 sites as potential targets for such signals (4, 5, 15, 17, 18). In particular, it has been proposed that agonists of the SAPK/JNK pathway, such as UV light, induce c-jun expression by directly phosphorylating and, thereby, stimulating the transcriptional activity of het-
erodimer complexes of c-Jun and ATF-2 bound to the proximal and distal junTRE motifs (4). Because a large body of evidence suggests that the v-Jun oncoprotein is uncoupled from SAPK/JNK-mediated phosphorylation in vivo as a result of the S deletion (10, 20–22), it was of interest to determine whether regulation of c-jun transcription by this mechanism was disturbed in v-Jun-transformed cells.

Initial attempts to introduce chemical or radiant agonists of SAPK/JNK, such as UV, cycloheximide, or anisomycin (23), into the reporter assay were complicated by severe effects on cell viability and/or protein synthesis.4 As an alternative means of manipulating SAPK/JNK activity, we cotransfected expression vectors encoding MEKK, a potent catalytic activator of the SAPK/JNK pathway (24), or alternatively, a catalytically inactive mutant of the SAPK/JNK upstream activator SEK-1 (SEK-1 A/L; Ref. 25), together with the pJC6 and mutant derivative reporters.

As shown in Fig. 5a, cotransfection of MEKK stimulated c-jun transcription in normal cells, whereas the inhibitory SEK-1 A/L mutant induced a dramatic inhibition of basal promoter activity. As with the effects of c-Jun and v-Jun, stimulation by MEKK and inhibition by SEK-1 A/L was me-
Fig. 5. Effect of activators or inhibitors of the SAPK/JNK pathway on c-jun promoter activity in normal and v-Jun-transformed cells. a, expression plasmids encoding the constitutively active SEK-1 activator MEKK or a catalytically inactive form of SEK-1 (SEK-1 A/L) were cotransfected together with the indicated c-jun-CAT reporter constructs into either normal (3) or v-Jun-transformed (8) fibroblasts, and the relative levels of CAT activity were determined. Columns, averages of five independently transfected dishes; bars, SD. b, effect of exposure to UV on p39 c-Jun protein expression in normal and v-Jun-transformed fibroblasts. Cultures were irradiated with UV light at a fluence of 40 J/m², and after 3 h, cell extracts were prepared and analyzed by Western blotting using anti-Jun antisemur (730; Ref. 10). The positions of the p39 c-Jun and p65 v-Jun proteins are indicated.

mediated through the proximal junTRE because neither catalytic regulator had any effect on pJTX or pJSTX, whereas pJSX was equally responsive (Fig. 5a).

Remarkably, neither MEKK or SEK-1 A/L had any significant positive or negative effect on the activity of the wild-type c-jun promoter (pJC6) or of the TRE-deficient pJTX and pJSTX reporters in the v-Jun-transformed cell background. A small increase in transcription response to MEKK was noted with the MEF-deficient pJSX reporter; however, in marked contrast to wild-type pJC6, SEK-1 A/L did not reduce pJSX basal activity. We conclude that, in v-Jun-transformed cells, the c-jun promoter is rendered relatively insensitive to induced fluctuations in SAPK/JNK activity that, in normal cells, stimulate or inhibit promoter activity primarily through the proximal junTRE.

To confirm that these findings could be extended to the endogenous c-jun gene, we exposed normal and v-Jun-transformed fibroblasts with UV light, a potent agonist of JNK and inducer of c-jun expression (2). As shown in Fig. 5b, although UV induced a significant accumulation of p39 c-Jun protein in normal fibroblasts, there was no corresponding induction of p39 c-Jun in v-Jun-transformed cells, indicating that, as predicted, the endogenous c-jun gene is nonresponsive to JNK activation. Interestingly, c-jun mRNA and protein expression are also refractory to induction by serum growth factors in v-Jun-transformed cells (data not shown; Ref. 10).

Discussion

A number of groups have noted that expression of endogenous p39 c-Jun expression is specifically extinguished in avian fibroblasts transformed by v-Jun (8–10); however, the molecular basis of this negative autoregulatory phenomenon has not been elucidated. Here, we show that down-regulation of c-jun mRNA levels in v-Jun-transformed cells is associated with a decrease in the rate of c-jun transcription and that this, in turn, is largely attributable to a deficit of transcriptional activity mediated by a specific element in the c-jun promoter, the proximal junTRE. We also find that this deficit correlates with a change in DNA-binding activities specific for this element in vitro; whereas c-Jun/Fra and CREB/ATF-like factors predominantly interact with the junTRE in normal cells, v-Jun/Fra complexes predominate in v-Jun-transformed cells. Finally, we show that expression of exogenous v-Jun represses c-jun promoter activity in normal cells, whereas expression of exogenous c-Jun can rescue the transcriptional deficit evident in v-Jun-transformed cells, and that both of these effects are mediated specifically through the proximal junTRE.

On the basis of these observations, we propose that repression of c-jun transcription in v-Jun-transformed cells results primarily from the interaction of v-Jun with the proximal junTRE in competition with, or at the expense of, the c-Jun/Fra and CREB/ATF-like factors that normally bind to this element in untransformed cells. The salient features of this model are summarized schematically in Fig. 6, in which we assume that v-Jun acts primarily as a heterodimer in association with Fra partners as seen in vitro, although clearly we cannot formally rule out a role for v-Jun homodimers or other v-Jun-containing complexes in vivo. Indeed, the ability of exogenous v-Jun to suppress the c-jun promoter in transfection experiments suggests that v-Jun homodimers are capable of interacting with this element in vivo. In addition, other, quantitatively less significant mechanisms may also contribute to repression of c-jun transcription in v-Jun-transformed cells; however, these remain to be defined.

The proposal that v-Jun exerts a negative effect on transcription through a specific TRE element is somewhat heterodox, because it has been widely considered that v-Jun induces cellular transformation by acting as a super-activated form of c-Jun (12, 13). Negative effects of v-Jun on transcription are, however, not unprecedented, particularly
Gene Repression by v-Jun

Fig. 6. Proposed mechanism of down-regulation of endogenous c-Jun proto-oncogene transcription by v-Jun. In normal fibroblasts (a), approximately equal amounts of c-Jun/Fra and CREB/ATF-like DNA-binding complexes are available to interact with the proximal JunTRE and sustain a certain level of basal expression (small amounts of JunD are also present but have been omitted for clarity). After transformation by v-Jun (b), c-Jun expression is lost, and v-Jun/Fra complexes become the predominant JunTRE-binding activity, partially displacing the remaining CREB/ATF-like factors by competition and leading to c-Jun repression. It is assumed, although not yet verified experimentally, that the occupancy of this site in situ is likely to reflect the relative abundance of the complexes detected in vitro.

when the activity of v-Jun has been evaluated in avian fibroblasts, the natural target for oncogenic conversion (14). For example, cotransfection of v-Jun has been shown to repress TRE-dependent transcription in transient transfection assays in this system (Ref. 9; this study), whereas other work has documented an inverse correlation between the relative transforming and transcriptional potency of mutant derivatives of v-Jun (26).

In considering potential explanations for transcriptional inhibition by v-Jun, it is important to stress that, although c-Jun mRNA and protein expression may be said to be repressed in v-Jun-transformed cells (i.e., compared to the levels present in untransformed cells), we do not infer from our observations that v-Jun actively represses transcription at the molecular level. Although we cannot rule this out, the current results are consistent with v-Jun being either transcriptionally inert or a comparatively weak activator that inhibits transcription by competing with and displacing more potent junTRE-specific factors. Why should this be? One possibility is that overexpression causes v-Jun to displace inherently more potent activators, such as the unidentified CREB/ATF-like factors that are clearly potentially capable of interacting with the proximal junTRE in these cells. Alternatively, because phosphorylation mediated by SAPK/JNK is considered to be a major determinant of c-Jun transactivation in vivo (2) and v-Jun is uncoupled from this regulation by the δ deletion (10, 20–22), it is also possible that v-Jun’s transcriptional potential is impaired, even in comparison to its normal counterpart, by a lack of positive regulatory phosphorylation.

Clearly, these possibilities are not mutually exclusive; however, it is noteworthy that the major SAPK/JNK regulatory sites (Ser-63 and Ser-73) in v-Jun have recently been shown to be underphosphorylated by a factor of ~10 compared to the corresponding residues in c-Jun in vivo, indicating that a deficit of positive regulatory phosphorylation similar in scale to the observed reduction in transcriptional activity exists (2). Occupation of the junTRE by the mutant v-Jun oncoprotein could also account for the failure of MEKK to stimulate or SEK-1 A/V to inhibit c-jun promoter activity in v-Jun-transformed cells because regulatory phosphorylation of v-Jun is insensitive to fluctuations in the activity of the SAPK/JNK pathway in vivo (10, 22).

This mechanism could also potentially be reconciled with previous suggestions that fluctuations in the phosphorylation state of DNA bound c-Jun (or ATF-2) underlie the induction and subsequent repression phases of c-jun expression in response to inducing signals (5) because by displacing these signal-regulated activators, v-Jun would effectively mimic the repression phase continually in the absence of any prior inducing signal. It is also conceivable that overexpression of c-Jun could lead to impaired phosphorylation of Ser-63/ Ser-73 in c-Jun-overexpressing cells by saturating SAPK/ JNK activity in vivo. This might explain why overexpression of c-Jun can also down-regulate endogenous c-jun expression (8) and suggests a potential homeostatic mechanism through which the repression phase of c-jun autoregulation might normally be imposed after gene induction. Further work will be required to evaluate these possibilities. It will also be important to determine whether the two point mutations within the v-Jun DNA-binding domain (6) enhance or contribute to gene repression by v-Jun.

To our knowledge, c-jun is the first example of an endogenous cellular gene, the expression of which is repressed by v-Jun in vivo. Although it is well established that v-Jun can also stimulate gene transcription in a variety of cell types and experimental contexts (9, 12, 13, 27), the possibility that repression of cellular genes with growth-inhibitory or pro-apoptotic functions may contribute to oncogenesis by v-Jun clearly merits further investigation.

Materials and Methods

Cell Culture and Transfection Assays. Secondary CEFs were cultured in DMEM supplemented with 10% tryptose phosphate broth, 10% newborn calf serum, and 2% chicken serum. v-Jun-transformed CEFs were generated by infecting secondary CEFs with ASV17 (regenerated from pASV17-241; Ref. 14) essentially as described (28). For control purposes, normal CEF cultures were infected with helper virus alone. To activate JNK, cultures were irradiated with UV light using a Stratalinker, as described previously (10).

For functional analyses, 60-mm dishes of normal or v-Jun-transformed CEF were transfected with 5 μg of reporter plasmid using N-[1-(2,3-diacryloyloxypropyl)-N,N,N-trimethylammonium]methy sulfate (Boehringer Mannheim). After 24 h, the cultures were fed with fresh growth medium and incubated for a further 24 h prior to harvesting. Cell extracts were prepared, and CAT activity was assayed as described previously (10). The c-jun promoter-CAT constructs pJC6, pJTX, pJJSX, and pJSTX have been
described previously (15). For cotransfection experiments, 5 µg of reporter plasmid were transfected together with 5 µg of the appropriate control vector or expression plasmid. c-Jun and v-Jun were expressed from pFR5-luc-3 and pFR5-luc-1, respectively (29); MEK5 was expressed from pCMV5 (30); and SEK-1 A-L (25) from pFR5CMV.6

Preparation of WCEs and Gel Retardation Analysis. WCEs were prepared according to the method of Marais et al. (31). Briefly, cultures (90-mm dishes) were washed twice with PBS at 4°C, drained thoroughly, and lysed by addition of 100 µl of buffer E [20 mM HEPES (pH 7.9), 5 mM EDTA, 5 mM EGTA, 5 mM NaF, 0.1 µg/ml okadaic acid, 10% (v/v) glycerol, 1 mM DTT, 0.4 mM KCI, and 0.4% (v/v) Triton X-100]. Extracts were clarified by Eppendorf centrifugation for 30 min at 4°C, and the protein concentration measured using a Comassie protein determination kit (Pierce).

DNA-binding reactions were performed as described previously (10) using 10 µg of WCEs and 0.1–0.5 ng of 32P-labeled double-stranded oligonucleotide probe. Reactions were resolved by electrophoresis on 4% (w/v) 0.5× Tris-borate EDTA acrylamide gels at 4°C, after which the gels were fixed and dried and complexes were visualized by autoradiography. The following double-stranded oligonucleotides were used as probes: c-TRE (32), junTRE (33), and fibroCRE (34). Additional oligonucleotides spanning the c-Jun MEF2 site or this site together with the adjacent junTRE motif (Fig. 2b) were also used as competitors.

Where indicated, a 50% excess of cold competitor DNA or 3 µl of polycyclonal antiserum was added to the reaction and incubated on ice for 15 min prior to the addition of the probe. The c-Jun-specific antisera 948 and 730 have been described previously (10), whereas the Pan-Fos (sc-253) and JunD (sc-74) antisera were obtained from Santa Cruz Biotechnology. Titration experiments indicated that antibody–antigen interactions were saturated for each of the antisera at the concentrations used.

Northern and Run-on Analysis. Total RNA was prepared from 150-mm dishes of normal or ASV17-transformed cells using RNAzol and poly(A)+ mRNA purified using a poly(A)+ -Tract isolation system (Promega). One-µg portions of poly(A)+ were resolved by electrophoresis on 1% agarose-formaldehyde gels, transferred to nitrocellulose, and hybridized with the appropriate 32P-labeled DNA probes. Nuclear run-on transcripts were synthesized using isolated nuclei and hybridized to appropriate DNA fragments cloned in pSP6T19 (10) and immobilized on nitrocellulose essentially as described (35).

Acknowledgments
We thank R. Prywes for generously providing the pJC6 and mutant c-jun-CAT reporter plasmids; N. Jones and R. Treisman for antisera against the ATF-2 and MEF-2 proteins, respectively; I. Morgan and P. Vogt for Jun expression plasmids; D. Bentley for advice on nuclear run-on assays; and C. Bartholomew, I. Morgan, and J. A. Wyke for helpful comments on this manuscript.

References

6 D. A. F. Gillespie, unpublished results.


