The c-Jun δ-Domain Inhibits Neuroendocrine Promoter Activity in a DNA Sequence- and Pituitary-specific Manner*

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The transcription and transformation activity of c-Jun is governed by a 27-amino acid regulatory motif, labeled the δ-domain, which is deleted in v-Jun. We have previously shown that c-Jun is a potent inhibitor of the rat prolactin (rPRL) promoter activity induced by either oncogenic Ras or phorbol esters. Here, we have characterized the structural and cell-specific requirements for this c-Jun inhibitory response, and we show that this c-Jun inhibitory response mapped to the rPRL footprint II repressor site, was pituitary-specific and characterized the structural and cell-specific requirements for this δ-domain. Moreover, alteration of any one of these features (e.g., cis-element, trans-factor, or cell-specific background) switched c-Jun to a transcriptional activator of the rPRL promoter. In HeLa nonpituitary cells, c-Jun alone activated the rPRL promoter via the most proximal GHF-1/Pit-1 binding site, footprint I, and synergized with GHF-1. Finally, recombining GHF-1 interacted directly with c-Jun but not c-Fos proteins. These data provide important fundamental insights into the molecular mechanisms by which the c-Jun δ-domain functions as a modulatory switch and further imply that the functional role of c-Jun is dictated by cell-specific influences and the δ-domain motif.

c-Jun is a member of the BZip family of transcription factors, which are characterized by a basic DNA binding domain and a leucine zipper protein dimerization domain (1, 2). c-Jun was first described as the cellular homologue of the v-jun oncogene, and subsequently, many other BZip family members have been identified, including several J-un-like and Fos-like proteins (3–5). c-Jun and c-Fos can combine to form homo- and heterodimers, with J-un-J homodimers and J-un-F heterodimers (also referred to as an AP-1 complex), binding with high affinity to the consensus DNA binding site, TGACCTA, whereas Fos-Fos homodimers are unable to stably bind to this sequence (1, 6–8). Once bound to DNA, their effects are mediated through amino-terminal transactivation domains (9). In the cell, c-Jun serves as a downstream target for several signaling pathways, including 12-O-tetradecanoyl phorbol 13-acetate (TPA), a phorbol ester that activates protein kinase C (3, 10) and oncogenic Ha-Ras (11, 12). TPA mediates its transcriptional effects through a TPA-responsive element, which is equivalent to the AP-1 site noted above (3, 4, 13, 14). Stimulation of Ha-Ras leads to activation of J un kinase, resulting in the phosphorylation of amino-terminal serines 63 and 73 in the transcription activation domain of c-Jun, thereby enhancing its transcription potency (15–18). Also present in the amino terminus of c-Jun, but deleted in v-Jun, is a region known as the δ-domain (19). This 27-amino acid region is important for cell-specific modulatory effects on transcription and transformation potency of c-Jun versus v-Jun, thought to be mediated via protein-protein interactions (9, 20–23). One such δ-domain-specific interaction is with J un kinase, which binds to the amino-terminal half of the δ-domain spanning amino acids 34–45 and phosphorylates c-Jun un but not v-Jun (9, 18, 24–26).

Additionally, although c-Jun typically activates gene transcription, examples have accumulated documenting that it can also inhibit gene expression. A direct mode of inhibitory action was shown in the c-fos gene, where the AP-1 complex binds to the c-fos promoter to down-regulate c-fos gene expression (27). Alternatively, the AP-1 complex has been shown to bind to a TPA-responsive element that overlaps a critical retinoic acid response element/vitamin D response element required for osteocalcin promoter activity, and thus sterically interferes with retinoic acid receptor/vitamin D receptor binding (28). In certain cases, c-Jun heterodimerizes with other activators, either via its leucine zipper motif, e.g., with the glucocorticoid receptor (29), or via its amino-terminal domain, e.g., with MyoD (30). Indeed, the ability of c-Jun to inhibit the muscle-specific creatine kinase gene (30), cardiac-specific atrial natriuretic factor gene (31), or the liver-specific α-fetoprotein gene (32) was mapped to the amino-terminal domain of c-Jun. In this respect, it is striking that in each case whereby c-Jun inhibits a highly specialized, tissue-specific gene, it does so via its amino-terminal domain, usually requiring just the first 87 amino acids, including the δ-domain.

One of the first indications that the δ-domain serves a regulatory function was noted in cell-free transcription studies. Recombinant c-Jun proteins containing the δ-domain weakly activated an AP-1-driven promoter in J un/Fos-depleted HeLa cell extracts, whereas v-Jun or amino-terminal truncated c-Jun proteins, devoid of the δ-domain, were very active in this in vitro transcription assay (21). Importantly, all of these recombinant J un proteins bound equally well to the TPA-responsive DNA element. Using in vivo gene transfer methods, it was shown that in all cell lines tested, a c-Jun fusion protein with the DNA binding domain of E2, a transcription factor from bovine papilloma virus, activated the appropriate promoter-reporter plasmid (9). Deletion of the δ-domain resulted in an enhancement of J un’s activation effect in HeLa cells (9). However, not all tested cells revealed this differential effect of c-Jun versus v-Jun. For example, the transcriptional activity of c-Jun
and v-j un were the same in REF, SL2, and F9 cells, whereas c-j un was shown to be a better transactivator than v-j un in CEF and HepG2 cells (9, 20, 22). While the notion that the δ-domain operates as a critical negative regulatory domain stems from the observation that simply removing the δ-domain from c-j un results in its oncogenic activation, it has become increasingly clear that transformation and transcription potency of j un proteins are not directly correlated and may even be inversely related (22). Despite this important progress in elucidating the structural features of j un proteins, the precise rules by which the δ-domain functions remain unknown.

The prolactin and growth hormone (GH) genes are two ancestrally related genes whose expression is restricted to the lactotroph and somatotroph cells of the anterior pituitary, respectively (33, 34). Both the ontogeny of these pituitary cells and the expression of these two pituitary-specific genes are regulated by the POU homeodomain transcription factor, GHF-1/Pit-1 (33, 35, 36). Significant insights into basal and hormone-activated PRL and GH gene expression have been provided by GH 4 rat pituitary tumor cells, which are a clonal cell line that maintains cell type-specific functions and hormonal responses (33, 34, 37–39). Previous experiments in this system have demonstrated that c-j un does not function as a downstream target for either oncogenic V12 Ras- or TPA-mediated activation of the rPRL promoter, but instead c-j un inhibits both of these signal transduction pathways (40, 41). Yet this inhibitory effect of c-j un on the V12 Ras- and TPA-mediated activation of the rPRL promoter is promoter-specific and not GH4 cell-specific, since we demonstrated that c-j un enhances V12 Ras stimulation of the AP-1-dependent −73ColCAT promoter-reporter construct (40). These results suggest that c-j un is capable of serving multiple functions within these cells and that some of these functions are promoter-specific. Thus, the rPRL promoter and GH4 pituitary cells provide an important model system in which to elucidate the molecular mechanisms by which c-j un mediates promoter- and cell-specific effects. The goal of the studies presented here was to dissect the mechanism of c-j un inhibition of the rPRL promoter in GH4 neuroendocrine cells. Using transient transfection studies we showed that c-j un selectively inhibits basal rPRL promoter via the amino-terminal c-j un δ-domain and that this inhibition required the rPRL promoter FP II repressor-binding site and pituitary-specific influences. Moreover, eliminating any one of these elements switched c-j un function on the rPRL promoter to an activator. These data provide critical and novel insights into the regulatory functions of the c-j un δ-domain and further imply that the precise functional role of c-j un is dictated by the potential interaction of cell-specific factors with the δ-domain motif.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The pituitary promoter-luciferase constructs, pAΓFLuc-425, pAΓGHLuc, and pAΓhULuc-1760 have been described (39, 42, 43). The −25 to −189, −125, and −36 rPRL promoter deletions were prepared in pSG7PRL by S′exonuclease digestion, shotgun intoSalI/HindIIICUTpAΓLuc, and verified by dideoxy sequencing, and they will be described in detail elsewhere. The site-specific mutations in FP I (pAΓI1Luc), FP II (pAΓI2Luc), and FP II together with a loop-out deletion of the −112 to −80 basal transcription element (BTE) region (pAΓII1Luc) were constructed in the −425 to +73 rPRL promoter as described previously (39, 43). The FP I/FP II double mutant (pAΓI2Luc) was constructed in the −425 to +73 rPRL promoter as described previously (45). Plasmids pRSVc-Jun, pRSVv-Jun, and pRSVGH-1 were generously provided by M. Karin (University of California, San Diego, CA) (3, 46, 47). The plasmid pRSVβ-globin was kindly provided by Drs. Tim Reu edhuber and J ohn D. Baxter (University of California, San Francisco, CA). The plasmid pc-fosTKluc contains two copies of the c-fos enhancer spanning −357 to −276 fused to the TK promoter to −200 to +70 and has been previously described (48). The plasmid pCMVLuc was kindly provided by Dr. Mike Smith (University of Colorado Health Sciences Center, Denver, CO). Plasmids pRSVluc-400 and pAΓSV40luc have been previously described (45, 49). Plasmids pGEM4 and pGEM7 were both obtained from Promega Corp. (Madison, WI).

Plasmid DNAs were purified either by alkaline-SDS extraction followed by cesium chloride density gradient centrifugation (50) or according to the Qiagen Mega protocol (Qiagen Inc., Chatsworth, CA). Plasmids were quantitated by both absorbance at 260 nm and by coelectrophoresis with DNA standards on agarose electrophoresis (50). No significant difference was observed in transfection results using plasmids prepared by the two different purification methods.

Cell Culture, Electroporation, and Luciferase Assay—GH4T2 rat pituitary tumor cells and HeLa human cervical carcinoma cells were grown in 5% CO2 at 37°C in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% fetal calf serum (HyClone, Logan, UT) and 50 μg/ml of penicillin and streptomycin (Life Technologies). Cells were transfected via electroporation, keeping the total amount of DNA constant with pGEM7 or pGEM4 DNA, and pRSVβ-globin was used to control for nonspecific effects of RSV expression vectors (37). Electroporations were performed in triplicate for each condition within a single experiment, and experiments were repeated using different preparations of each construct. Cells were harvested after transfection unless otherwise stated, cell extracts were prepared, and luciferase assays were performed as described previously (37). Luciferase light units of the control value were set to 1, and the data was expressed as -fold stimulation relative to control. All data was expressed as the mean ± S.E. for replicated experiments. Since c-j un expression modulated the activity of each of the viral promoters typically used to drive a β-galactosidase reporter, these β-galactosidase vectors could not be used as internal controls for transfection efficiency (see Fig. 2). Previously, we have found that by repeating the various transfections multiple times and applying statistical analysis to the resultant data, we are able to achieve consistency of agreement that is equal to or better than using an internal control reporter vector (37–39, 42, 43).

Western Blot Analysis—GH4 cells transiently transfected with the rPRL reporter-reporter and various effector plasmids were harvested with phosphate-buffered saline with 3 mM EDTA. GH4 cells were lysed by sonication using five 10-s pulses on ice in 300 μl of lysis buffer containing 20 mM HEPES, pH 7.9, 0.42 M KCl, 15 mM MgCl2, 25% glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.15 mM spermine, 0.5 mM Triton X-100 and 0.45 mg/ml Boehringer Mannheim protease inhibitor set. After sonication, cell extracts were centrifuged at 10,000 × g for 5 min at 4°C. HeLa cells transiently transfected with the rPRL promoter reporter and various effector plasmids were harvested with phosphate-buffered saline containing 3 mM EDTA. Cells were lysed with three sequential freeze-thaw cycles in 100 mM potassium phosphate, pH 7.8, and 1 mM dithiothreitol. Vortexing ensured cell lysis. The cells were spun down at 10,000 × g for 5 min at 4°C to pellet unlysed cells and cell debris. The protein concentration of the supernatant was determined using the Bio-Rad protein assay. Equal amounts of total cellular protein (100 μg) were resolved on an SDS-10% polyacrylamide gel and transferred to nitrocellulose in 192 mM glycine, 25 mM Tris, 10% methanol at 100 mA for 16 min. Membranes were blocked overnight with 7.5% nonfat dried milk in 20 mM Tris-Cl (pH 7.4), 150 mM NaCl, and 0.2% Tween 20.

The membranes were then probed with a rabbit polyclonal c-j un antibody directed against amino acids 95–105 (Santa Cruz Biotech; Santa Cruz, CA), a rabbit antibody, GHF-1 antibody (Amersham, Arlington Heights, IL), amino acids 214–230 (BabCO; Richmond, CA), or a mouse monoclonal actin antibody, clone C4 (Boehringer Mannheim) in a 1:1000 dilution in blocking buffer with 1% dried milk. The membranes were extensively washed and developed with a 1:5000 dilution of goat anti-rabbit or goat anti-mouse antibodies linked to horseradish peroxidase (Life Technologies, Inc.) as a luminescence kit from American Quanta Biosciences Inc. Between probes with different antibodies, the nitrocellulose membranes were stripped by incubation at 50°C for 30 min in a solution of 0.7% β-mercaptoethanol, 2% (w/v) SDS, 62.5 mM Tris, pH 6.8. After stripping, the membranes were blocked overnight, and the membrane was then reprobed as described above.

In Vitro Protein Binding Studies—The protein AGF/H1 fusion (pa/ GHF-1) vector was constructed by filling in, with Klenow polymerase, the NcoI to NotI fragment of the GHF-1 clone, SK-9 (47), in which the ATG codon of GHF-1 was modified to an NcoI site and the NotI site is
downstream of the stop codon. The blunt-ended fragment was inserted into the SmaI site of the pA vector, Rit 32 (a modification of the Rit 2 vector) (51). The amino-terminal start codon of GHF-1 is thus fused, in frame, to the carboxyl tail of protein A. The pA and pA/GHF-1 vectors were transformed into N4830-1 bacteria (Pharmacia Biotech Inc.) grown in 500 ml of Luria broth (LB) at 30°C until the A600 = 0.6 and induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside and increasing the temperature to 42°C by the addition of 500 ml of LB at 55°C; the N4830-1 bacteria used to grow the pA and pA/GHF-1 constructs contain a temperature-sensitive λ cl repressor which, with the Lac repressor, regulates the Rit32 promoter. The pA- and pA/GHF-1-expressing cells were collected 45 min after induction, since pA/GHF-1 expression was quite toxic to the cells.

Bacterial cell pellets were resuspended with 7–8 ml of TST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 5 μg/ml each of the protease inhibitors antipain, chymostatin, leupeptin, and pepstatin A) and lysed by sonication, and the debris was pelleted for 20 min at 15,000 rpm in an SS34 rotor. The pA- and pA/GHF-1-containing lysates were then passed over 0.5 ml of IgG-agarose (Sigma) columns, washed twice with 5 ml of TST and then twice with 5 ml of buffer A (25 mM Hepes, pH 7.9, 80 mM KCl, 6 mM MgCl2, 10% glycerol, and protease inhibitors listed above). The beads were then resuspended with 1.5 ml of buffer A and stored at 4°C. Efficient production of either fusion protein was assessed by boiling approximately 10 μl of packed beads in SDS sample loading buffer, loading onto an SDS-10% polyacrylamide gel and staining with Coomassie Blue.

Reticulocyte lysates (Promega) were programmed with RNA transcribed by T7 polymerase, resulting in either human c-Jun, rat c-Fos, or rat GHF-1 protein labeled with [35S]Met (DuPont NEN). Efficiency of labeling was assessed by electrophoresing 1 μl of each sample on an SDS protein gel followed by autoradiography. Roughly equivalent amounts of each radiolabeled protein (2–5 μl of each programmed reticulocyte lysate) were incubated with about 5 μl of packed pA or pA/GHF-1 beads in 100 μl of buffer A containing 0.05% Nonidet P-40 and 200 μg/ml ethidium bromide (52) for 1.5 h at 4°C with gentle rocking in an Eppendorf tube. Unprogrammed reticulocyte lysate was added to each incubation so that all incubations were done with the same amount of lysate. Following incubation, the beads were pelleted, the supernatant removed, and the pellet was washed six additional times each with 170 μl of buffer A, 0.1% Nonidet P-40. To each pellet or input sample was added 15 μl of SDS gel loading buffer. The samples were boiled and loaded onto an SDS 3% stacking, 10% resolving polyacrylamide gel, the signal was enhanced with “Amplify” (Amersham), and the gel was dried and visualized by autoradiography.

RESULTS

C-Jun Selectively Inhibits the rPRL Promoter in a Doseresponsive Fashion—Since c-Jun has previously been shown to inhibit basal rPRL promoter activity (41), we first tested whether this effect was dose-responsive on the rPRL promoter in GH4 cells. As shown in Fig. 1A, c-Jun produced a dose-dependent inhibition of the −425-base pair rPRL promoter reaching 58% inhibition at a dose of 20 μg of pRSV-c-jun. Although the inhibitory effect of c-Jun is modest, it is very reproducible and statistically significant (p < 0.05). Also, similar doses of pRSV-c-jun inhibited a −2.5 kilobase pair rPRL promoter construct, containing the distal enhancer (data not shown). The maximal dose of pRSV-c-jun, 20 μg, was then used in all further studies. The inhibition of the −425 rPRL promoter by c-Jun reached 50% by 12 h post-transfection and remained constant from 12 to 24 h post-transfection, with maximal inhibition reaching 77% at 36 h post-transfection (data not shown). In order to ensure that the transiently transfected c-Jun expression vector resulted in detectable levels of c-Jun protein in GH4 cells, we performed Western blot analysis. As shown in Fig. 1B, control (lane 1) and mock-transfected (lane 2) GH4 cells did not appear to have detectable levels endogenous c-Jun, whereas transfection of pRSV-c-jun resulted in readily detectable levels of c-Jun protein (lane 3). The same blot was reprobed with an anti-GHF-1 antibody in order to verify that the proteins in the cellular extract loaded in lanes 1–3 were equivalent and intact. The results show that the levels of endogenous GHF-1 protein were equivalent (Fig. 1B, lanes 4–6). Also, c-Jun expression had no effect on GHF-1 protein levels, indicating that the negative effect of c-Jun on the rPRL promoter was not due to a decrease in endogenous GHF-1 protein levels.

To determine whether the inhibition of the rPRL promoter was promoter-specific, the effect of c-Jun was examined on a variety of pituitary and nonpituitary promoters in GH4 cells. As shown in Fig. 2, c-Jun inhibited the −425 rPRL promoter and the −2.5 kilobase pair rPRL promoter 54 and 34%, respectively. Likewise, the pituitary-specific growth hormone promoter was also inhibited by c-Jun to 53% of its basal activity. However, c-Jun was not a general inhibitor of pituitary promoters as evidenced by its 1.7-fold stimulation of the human α-globin promoter.

FIG. 1. C-jun inhibits the rPRL promoter in a dose-dependent fashion. A, c-Jun dose response. Exponentially growing GH4 rat pituitary tumor cells were transiently transfected by electroporation in triplicate with 3 μg of pA/rPRLuc-425 and with or without 2, 5, 10, 15, or 20 μg of pRSV-c-jun. An equal amount of pRSV-β-globin was added to control for nonspecific promoter effects as well as to maintain total DNA equivalent in all transfections as described under “Experimental Procedures.” The -fold basal rPRL promoter activity was calculated by dividing the mean total light units in the presence of pRSV-c-jun by the mean total light units in the presence of pRSV-β-globin alone. Different plasmid preparations were used for separate experiments. Data are expressed as the mean -fold ± S.E. for three transfections done in triplicate, and specific comparisons of the means were conducted by Student’s t test. The results for DNA doses of 5 and 20 were significant to p < 0.05, and 15 and 10 were significant to p < 0.01. B, c-Jun Western blot. Equal amounts (100 μg) of cell protein from transfected GH4 cells were electrophoresed on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose. The membrane was probed with c-Jun/ AP-1 rabbit polyclonal antibody and detected using Amersham ECL with a 5-min exposure. The membrane was stripped and reprobed with GHF-1 rabbit polyclonal antibody and detected using Amersham ECL with a 30-min exposure. Lanes 1 and 4, wild-type GH4 cells untransfected; lanes 2 and 5, GH4 cells transfected with empty pRSV-β-globin vector; lanes 3 and 6, GH4 cells transfected with pRSV-c-jun. Arrows point to size standards of 46 kDa (ovalbumin) and 30 kDa (carbonic anhydrase).
Fig. 2. c-jun differentially modulates various pituitary and viral promoters in GH4 pituitary cells. GH4 cells were transfected with 5 μg of pA3Rluc-425, p2SbfRluc, pGRHluc, pAhuc-1760, p-cfosTKluc, pCMVluc, pSV40luc, pA3RSVluc, or pA3luc with either 20 μg of pRSV-jun or pRSV-c-jun. Fold activation was determined relative to the basal activity of each individual promoter construct. Data are expressed as mean fold ± S.E. of eight transfections for pA3Rluc-425, six transfections for p2SbfRluc, four transfections for pGRHluc, three transfections for pAhuc-1760, two transfections for p-cfosTKluc, five transfections for pCMVluc, five transfections for pSV40luc, three transfections of pSRVluc, and three transfections for pA3luc. Each experiment consisted of transfections performed in triplicate. The inhibitory effects of c-jun on both the −425 and the −2.5 kb Rluc promoters were significant to p < 0.01, whereas the effect of c-jun on the parental pA3luc vector was not statistically significant compared with untreated.

\[ \text{Relative Promoter Activity} \]

\begin{align*}
\text{-425 Rluc} & : 2.5 \text{kb Rluc} \\
\text{pGRHluc} & : pAhuc-1760 \\
p-cfosTKluc & : pCMVluc \\
pSV40luc & : pA3RSVluc \\
pA3luc & : \text{pA3luc}
\end{align*}

\text{c-jun Inhibition Maps to the FP II Region of the rPRL Promoter—In order to address the question of mechanism, we sought to determine which region of the rPRL promoter was important for mediating this c-jun inhibition. In the rPRL promoter, footprints (FPs) I, III, and IV bind GHF-1/Pit-1, a pituitary-specific POU-homeodomain transactivator (36, 53). FP II binds a ubiquitous repressor denoted as F2F, and the −117 to −80 BTE binds a ubiquitous basal transcription-activating factor, both of which have yet to be characterized (43). Using a series of 5′ deletions and site-specific mutations of the rPRL promoter immunizing on these various regulatory dis-acting sites (shown in Fig. 3A), we show that c-jun equally inhibited rPRL promoter constructs with 5′ end points of −425, −255, and −189 (Fig. 3B). However, the effect of c-jun switched to an activating response with rPRL promoter 5′ end points of −125, −54, and −36 (Fig. 3B). Of note, statistical analysis (Student’s t test) revealed that the effects of c-jun are statistically significant to p < 0.05 for the −425 and −125 end points and significant to p < 0.01 for the −255 and −189 end points, whereas the effects of c-jun on the −54, −36, and promoterless constructs were not statistically significant. These data indicate that DNA sequences between −189 and −125 were responsible for the inhibitory effect of c-jun, while sequences between −125 and −54 were responsible for c-jun’s activating effect. Moreover, neither the negative nor positive effects of c-jun were due to a cryptic AP-1 site in the pA3luc background vector, since c-jun had no effect on the pA3luc promoterless vector (Figs. 2 and 3B).

Within the −189 to −125 region of the rPRL promoter lie both FP III and FP II. In order to determine which of these two footprints was important for c-jun action, we used a panel of site-specific mutants that featured a Sall linker sequence substituted for specific bases within the various footprints and a
The activating effect of c-Jun on the intact rPRL promoter in HeLa cells. As shown in Fig. 3A, repression of the rPRL promoter maps to the FP II region. The site-specific rPRL promoter mutants include 1) the Δ1 site-specific mutant indicated by the open rectangle in the FP I region to yield pA3Δ1luc; 2) the Δ2 site-specific mutant indicated by the black rectangle in the FP II region to yield pA3Δ2luc; 3) the Δ2,D site-specific deletion mutant containing a 32-base pair deletion of the -112 to -80 BTE region in the Δ2 background to yield pA3Δ2,Dluc; and 4) the Δ1,3 site-specific mutant indicated by the open rectangles in both the FP I and FP III regions to yield pA3Δ1,3luc. B, c-Jun repression of the rPRL promoter maps between -189 and -125 base pairs. The indicated rPRL promoter deletions (5 µg) were transiently transfected into GH4 cells with either 20 µg of pRSVβ-globin or pRSVc-Jun. -Fold activation was determined relative to the basal activity of each individual promoter construct. Data are expressed as mean -fold for four transfections done in triplicate. Statistical analysis (student t test) reveal that the effects of c-Jun are significant to p < 0.05 for the -425 and -125 end points and to p < 0.01 for the -255 and -189 end points. Additionally, the effects of c-Jun on the -54, -36, and promoterless constructs were not statistically significant. C, c-Jun repression of the rPRL promoter maps to the FP II region. The indicated rPRL site-specific mutants (5 µg) were transiently transfected into GH4 cells with either 20 µg of pRSVβ-globin or pRSVc-Jun. -Fold activation was determined relative to the basal activity of each individual promoter construct. Data are expressed as mean -fold ± S.E. for three transfections done in triplicate.

To directly determine whether c-Jun and GHF-1 physically interact, we performed in vitro binding assays whereby the ability of either recombinant protein A alone or a protein A-GHF-1 fusion protein prebound to IgG beads to pull down radiolabeled c-Fos, c-Jun, or GHF-1 proteins was assessed (Fig. 7). Twenty percent of the radiolabeled c-Fos, human c-Jun, and rat GHF-1 is shown in lanes 1, 4, and 7, respectively. Rat c-Fos showed no interaction with either the protein A alone or protein A-GHF-1 beads (lanes 2-3). By contrast, c-Jun showed a low level of interaction with the protein A beads (lane 5), whereas there was significant binding of c-Jun to protein A-GHF-1 (lane 6).

Since GHF-1 is known to homodimerize (36), we showed that radiolabeled GHF-1 interacted with the protein A-GHF-1 beads, as a positive control for the binding assay (lane 9) but that labeled GHF-1 binds to protein A beads only minimally.
The inability of labeled c-Fos to bind to protein A- 
GHF-1 beads indicates that there is specificity to the GHF-1- c-Jun interaction.

**DISCUSSION**

Although our understanding of c-Jun function has pro-
gressed quite rapidly, some of the original observations relat-
ing to the differential and cell-specific effects of c-Jun versus 
v-Jun in transcription and transformation assays have re-
mained unexplained. In this paper, we show that c-Jun inhibition of rPRL promoter basal activity requires the c-Jun \( \delta \)-domain, the FP II site, and the pituitary-specific cell type. Alteration of any one of these features switches the inhibitory effect of Jun to that of an activator. Moreover, since the putative DNA binding sites for both c-Jun and v-Jun proteins are the same, the striking differences in their effects on the rPRL promoter strongly suggest that c-Jun and v-Jun mediate their differential effects upon the same promoter in the same cell type due to a mechanism independent of DNA binding specificities. Taken together, our data are most consistent with the model that the structural difference between these two Jun isoforms, specifically the \( \delta \)-domain, dictates a second level of transcription control by governing factor-factor interactions. The information gained provides critical mechanistic insights into the molecular code by which Jun proteins regulate gene expression.

The absence of the \( \delta \)-domain in v-Jun renders this protein unable to bind or to be phosphorylated by Jun kinases (JNKs), yet v-Jun is typically a much more potent oncogene, raising the interesting possibility that phosphorylation is required primarily to "inactivate" the \( \delta \)-domain in c-Jun (25, 55). From these data, it is tempting to speculate that catalytically inactive JNK, which binds tightly to the \( \delta \)-domain, functions as the putative repressor, possibly by masking the amino-terminal
tobindaputativepituitary-specificrepressorviathe
produce no effect, as reported previously (9, 21). If c-Jun were
activity rather than to mediate a partial activation or simply
transactivation domain and/or influencing c-Jun's ubiquitina-
and degradation, and that JNK activation causes its re-
lease from c-Jun (18, 24–26). Nevertheless, several lines of
evidence argue against this hypothesis: 1) recent studies have
demonstrated that only a small fraction (5–10%) of c-Jun in quiescent
GH4 cells. Additionally, the rPRL promoter does not
contain a canonical AP-1 site (41). With regard to mechanism,
if c-Jun were to titrate a putative cell-specific co-activator, then
this co-activator would have to display specificity for the rPRL
and rGH genes, since c-Jun inhibits both promoters (Fig. 2) and
v-Jun activates both promoters (Fig. 4 and data not shown). In
this respect, we initially surmised that c-Jun might be inter-
fering with the function of Pit-1/GHF-1 by binding to this
factor, thereby inhibiting both rPRL and rGH promoter activ-
ities. Surprisingly, our data show that c-Jun does interact with
Pit-1/GHF-1 directly (Fig. 7) but that such an interaction re-
sults in activation of the rPRL promoter in a HeLa cell recon-
stitution assay (Fig. 5) via the most proximal Pit-1/GHF-1
binding site (Fig. 6). By contrast, mapping of the cis-acting
element mediating c-Jun's inhibitory response co-localized the
J/NK responsive element to the FP II site, previously identified
as a binding site for the putative repressor, F2F (43). Since F2F
(43) and the c-Jun inhibitor both require an intact FP II site,
the formal possibility remains that these two proteins might
belong to the same family of transcription factors. Neverthe-
less, it is highly unlikely that they will be the same factor.
Indeed, there are several lines of evidence that show that the
F2F repressor functions and is expressed in a variety of non-
pituitary cell lines, including HeLa and Rat 2 cells (43),
whereas the putative target of c-Jun functions in a GH4 pitui-
itary- and FP II-specific manner (Fig. 3 and 5). It is the appar-
tent absence of the c-Jun inhibitor in HeLa nonpituitary cells
that allows c-Jun to switch function and become an activator of
the rPRL promoter. These data imply that the interaction
between the c-Jun and the pituitary-specific inhibitor is
dominant, and abrogation of this interaction is required in
GH4 pituitary cells in order to unmask the recessive and activ-
ating effects of c-Jun, which are mediated by FP I and GHF-1.

Based on the results presented here, we have formulated a
model, presented in Fig. 8, which incorporates all of these
features. In this model, we propose that c-Jun stabilizes
the binding of a pituitary cell-specific repressor protein to FP II, by
a protein-protein interaction mechanism that would require
the Δ-domain of c-Jun. Thus, deletion of the Δ-domain, elimina-
tion of the pituitary-specific inhibitor, or site-specific mutation
of FP II, would nullify this inhibitory effect and

![Model for c-Jun repression of rPRL promoter activity in GH4 cells.](image)

**Fig. 8.** Model for c-Jun repression of rPRL promoter activity in GH4 cells. c-Jun stabilizes the binding of a pituitary cell-specific repressor protein to FP II by a protein-protein interaction mechanism that requires the Δ-domain of c-Jun. Thus, deletion of the Δ-domain, elimination of the pituitary-specific inhibitor, or site-specific mutation of FP II would nullify this inhibitory effect. In HeLa nonpituitary cells, the putative repressor is represented by the black oval, and JNK cooperative activator is represented by the gray rectangle.
unmask the recessive activating effect of the GHF-1 of c-Jun un complex acting via FP I. Consistent with this model is the observation that both c-Jun and v-Jun functionally interact with GHF-1 to cooperatively activate the rPRL promoter via FP I (Figs. 5A and 6) in HeLa nonpituitary cells devoid of the pituitary-specific repressor. These results indicate that a region distinct from the α-domain of c-Jun interacts with GHF-1, since both c-Jun and v-Jun synergize with GHF-1 (Fig. 5A). Nevertheless, the α-domain does appear to contribute to the GHF-1-c-Jun interaction, since c-Jun cooperates more efficiently with GHF-1 than does v-Jun (Fig. 5A). However, if c-Jun is able to interact with GHF-1 to stimulate rPRL promoter activity, why is this interaction recessive to the c-Jun-pituitary-specific pituitary-specific repressor. Thus, if c-Jun enhances the inhibitory effect of the FP II site basal activity, despite intact GHF-1 binding sites (43, 44). The recessive interaction between c-Jun and the pituitary-specific FP II-binding factor should dominate over the c-Jun-GHF-1 response. Finally, implicit in this model is that DNA binding of either c-Jun or v-Jun to the rPRL promoter is not necessary, but instead c-Jun and v-Jun would mediate their effects through protein-protein interactions with other factors whose presence is dictated by the developmental state of the cell. This notion would be consistent with the lack of a canonical AP-1 site in the rPRL promoter.

Although the α-domain definitely affects c-Jun un activity, the cumulated data indicate that its function is much more complicated than a simple interaction of the α-domain with a putative cell-specific repressor, as initially postulated (9, 23, 48). In this respect, the ability of c-Jun to induce differentiation in F9 teratocarcinoma cells (11) and to inhibit the basal activity of highly specialized promoters (30–32), suggest that the putative effects of the α-domain may be governed by multiple regulatory influences, including the differentiated state of the cell, environmental cues, signaling events, repressors, co-activators, and ubiquitination machinery. Indeed, as if to verify this point, several reports have shown that the c-Jun un amino-terminal transactivation domain, including the α-domain, functionally (and in some cases physically) interacts with 1) certain transcription factors, such as MyoD, myogenin, steroid receptors, and STAT3β, to either repress or activate transcription of specific target genes, and 2) JNK (18, 24, 29–32, 57). These data suggest that the α-domain may contain several functional faces, one interacting with JNK and a separate face that might interact with other proteins, some of which may be cell-specific.

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