

A Jun-binding protein related to a putative tumor suppressor

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ABSTRACT A λ gt11 cDNA library of chicken embryo fibroblasts was screened with biotinylated Jun protein to identify Jun-binding clones. Eight such clones were isolated; one contains a gene referred to as *jif-1* that is homologous to the putative tumor suppressor gene *QM*. *jif-1* codes for a protein of 25 kDa that binds to the leucine zipper of viral and cellular Jun. The Jif-1 protein also binds to itself. Jif-1 does not contain a leucine zipper, and it does not bind to the 12-*O*-tetradecanoylphorbol 13-acetate response element DNA sequence. Complex formation of Jif-1 with Jun inhibits DNA binding and reduces transactivation by Jun. Addition of Fos protein to Jun–Jif-1 complexes restores DNA-binding activity. These observations suggest that Jif-1 is a negative regulator of Jun.

Jun is a member of the transcription factor complex AP-1. It binds to DNA either as a homodimer or heterodimer with Jun B, Jun D, or any member of the Fos family of proteins (1). These dimerizations are mediated by a heptad repeat of leucine residues that interacts with a similar leucine repeat in the partner molecule, forming a coiled-coil structure referred to as the leucine zipper (2). Jun appears to occupy a nodal point in transcriptional regulation in the cell because it also interacts with unrelated transcription factors. It can form dimers with cAMP response element binding proteins and then bind to cAMP response element sequences (3–5). It can interact with helix–loop–helix proteins such as MyoD (6, 7), and it can affect transcriptional regulation by steroid and retinoic acid receptors (8–10).

Jun is also the target of cellular regulatory proteins. A negative regulator has been postulated to interact with the δ domain of Jun (11–13), whereas a different inhibitory protein, IP-1, has been found to interfere with DNA binding of Fos/Jun, acting through the leucine zipper (14, 15). Another cellular protein targets a highly conserved cysteine in the basic domain in a redox control mechanism that regulates Jun–DNA binding (16, 17). Jun is also the substrate of several protein kinases (18–27).

Despite these numerous protein–protein interactions involving Jun, the interplay of Jun with the cellular transcriptional machinery and the control of Jun activation potential are only incompletely understood. Other still unknown proteins probably bind to and regulate Jun. Here, we report on the search for such additional Jun-interacting factors (Jifs) and describe Jif-1,‡ which exhibits properties of a Jun regulatory protein.

MATERIALS AND METHODS

Probe Preparation and Library Screening. The chicken Jun protein was expressed in *Escherichia coli* and purified by nickel chromatography (28). The purified protein was biotinylated as described by Bayer and Wilchek (29). The λ gt11 library of chicken embryo fibroblast (CEF) cDNA (30) was plated in the Y1090 bacterial strain. Soon after the plaques

were visible, nitrocellulose filters (Amersham; Hybond-C extra) impregnated with isopropyl β -D-thiogalactopyranoside (10 mM) were placed over the plaques and incubated overnight at 30°C. The filters were then rinsed of bacterial debris with wash buffer (10 mM Tris, pH 7.5/15 mM NaCl/0.1% Tween 20) and blocked with 5% nonfat dry milk in incubation buffer (20 mM Hepes, pH 7.2/50 mM NaCl/0.1% Nonidet P-40/5 mM dithiothreitol) for 1 hr at 4°C. Biotinylated Jun protein was premixed as a complex with avidin-alkaline phosphatase (Vector Laboratories; 0.9 milliunits) and added to 5% nonfat dry milk in incubation buffer at a final concentration of 100 ng/ml (\approx 3 nM) before incubation with the filter at 4°C with gentle shaking for 5 hr. The filters were washed quickly three times with wash buffer containing 0.2% Triton X-100 at 4°C, followed by four more washes at room temperature for no more than 20 min. The filters were then incubated in substrate solution [100 mM Tris, pH 9.5/100 mM NaCl/5 mM MgCl₂/nitroblue tetrazolium (0.33 mg/ml)/bromochloroindolyl phosphate (0.165 mg/ml)] until color development (1–2 hr).

Cloning and Sequencing. Inserts of clones selected from the λ gt11 library of CEF cDNA were amplified by PCR using forward and reverse primers located upstream and downstream, respectively, of the unique *Eco*RI site of the *lacZ* gene in λ (Promega). Sequences of PCR products were determined by automated sequencing (Applied Biosystems) using the upstream λ primer. The cloned insert was sequenced by using a combination of automated and manual protocols described by the manufacturer (Sequenase; United States Biochemical). PCR products were cloned into pCRII (Invitrogen) using the manufacturer's recommended procedure.

Plasmid Construction. Plasmids VJ-0, VJ-9, VJ-8, VJ-4, VJ-1, CJ-1, and CJ-4, used for *in vitro* transcription and translation, were described (31). The transcription and translation vector for *jif-1*, pCRII-ATGJIF, was synthesized by PCR amplification of the cloned phage insert using oligonucleotides that fused a terminal *Hind*III site and the first five codons of the human *QM* gene to the 5' coding sequence of *jif-1* and a phage T7 oligonucleotide (reverse primer) complementary to sequences in pCRII and located downstream of the *jif-1* termination codon. The resulting PCR product was digested with *Hind*III/*Eco*RI and then cloned into *Hind*III/*Eco*RI sites in pCRII. pRC/RSV-JIF was constructed by cloning the *Hind*III/*Xba* I insert of pCRII-ATGJIF into the *Hind*III/*Xba* I site of pRC/RSV (Invitrogen). The bacterial

Abbreviations: Jif, Jun-interacting factor; RSV, Rous sarcoma virus; GST, glutathione S-transferase; CEF, chicken embryo fibroblasts; TRE, 12-*O*-tetradecanoylphorbol 13-acetate response element [the target DNA sequence of the AP-1 transcription factor (ATGACT-CAT)]; CAT, chloramphenicol acetyltransferase.

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‡The Jif-1 sequence has been deposited in the GenBank data base (accession no. L13234).

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expression vector of chicken c-Jun, pDSH6JUN, was constructed by PCR amplification of CJ1, using oligonucleotides that placed a *Sph* I site and six histidine codons immediately upstream of the *jun* coding sequence at residue 5 and a *Bam*HI site after the termination codon. The resultant PCR product was digested with *Sph* I/*Bam*HI, and the fragment was cloned into the *Sph* I/*Bam*HI sites of pDS56 (32). The GST-Jif-1 and GST-cJun fusion proteins were made from pGEX1Jif-1 and pGEX1cJun-1, respectively, which contain the glutathione *S*-transferase (GST) coding sequence fused, respectively, to the 5' end of the *jif-1* and *jun* coding sequences in-frame with GST in the *E. coli* expression vector pGEX1.

Binding Assays. *In vitro*-synthesized mRNA transcripts were translated in the presence of 25 μ Ci (1 Ci = 37 GBq) of [³⁵S]methionine (1200 Ci/mmol) by using rabbit reticulocyte lysate following the manufacturer's recommendations (Promega). The lysates were examined by SDS/PAGE, followed by fluorography. The GST-Jif and GST-Jun fusion proteins were expressed in *E. coli* strain TG-1. Bacterial lysates were prepared by sonication, and GST fusion proteins were analyzed by SDS/PAGE and Coomassie staining. Equal amounts of fusion proteins or GST control were incubated with glutathione-agarose at 4°C. The agarose pellets were rinsed and aliquoted. Translated ³⁵S-labeled proteins were incubated with glutathione-bound fusion protein (typically containing 2 μ g of fusion protein as judged by SDS/PAGE and Coomassie staining) for 30 min at 4°C. Unbound proteins were removed by washing four times in phosphate-buffered saline with 1% Triton X-100. The agarose pellet was resuspended in sample buffer, and the boiled extract was analyzed by SDS/PAGE and fluorography.

Mobility Shift Assays. Gel retardation assays were performed as follows: 0.5 μ g of purified Jun protein was mixed with either 1 μ g of glutathione-agarose-purified GST or GST-Jif-1 in 20 mM Tris, pH 7.4/50 mM KCl/3 mM MgCl₂/1 mM EDTA/2 mM dithiothreitol/bovine serum albumin (2 mg/ml) and incubated with 1 μ g of poly(dI-dC) and radiolabeled oligonucleotide containing the collagenase 12-*O*-tetradecanoylphorbol 13-acetate response element (TRE) (\approx 20,000 cpm/58 pmol). After a 20-min incubation at room temperature, the protein-DNA complexes were resolved from unbound oligonucleotides on low-ionic-strength polyacrylamide gels in 0.25 \times Tris/borate/EDTA.

Transactivation Assay. Transactivation was measured by chloramphenicol acetyltransferase (CAT) assay as described (33), using the pRC/RSV-Jun and pRC/RSV-Jif-1 expression plasmids and the Col-CAT (34), RSV-CAT, and -154 GRP78-CAT (35) reporter plasmids.

RESULTS

Isolation of a Jif. Approximately 0.5×10^6 plaques were screened with biotinylated Jun protein, yielding eight Jun-binding clones. The inserts of these clones were amplified by PCR. Partial DNA sequences were used to search for homologous sequences in the GenBank data base; no homologies were found except for one clone referred to as Jif-1. This clone contains a gene that is closely related to a gene, *QM*, preferentially expressed in Wilm's tumor cell lines that were made nontumorigenic by transfer of a human der(11)t(X;11)(q26-qter;pter-q23) chromosome (36). *QM* is thought to be involved in the maintenance of the nontumorigenic phenotype of the Wilm's tumor revertants. Because of this interesting homology, we characterized this Jif clone first and describe some of its properties in this communication. Comparison of the predicted amino acid sequence of the Jif-1 protein to that of *QM* suggested that the isolated Jif-1 clone represents a partial cDNA; five amino-terminal residues appear to be missing (Fig. 1). The total length of the poly-

Jif-1	----P-RCYR YCKNKYPYKPS RFCRGVDPDK IRIFDLGRKK AKVDFEPLCG	45
QM	MGRRPARCVR YCKNKYPYKPS RFCRGVDPDAK IRIFDLGRKK AKVDFEPLCG	50
Jif-1	HMVSDEYEQL SSEALEAARI CANKYMKVSK GKDGPHIRVR LHPFHVIRIN	95
QM	HMVSDEYEQL SSEALEAARI CANKYMKVSK GKDGPHIRVR LHPFHVIRIN	100
Jif-1	KMLSCAGADR LQTGMRGAFG KPQGTVARVH MGQVIMSIRT KAQNKEHVVE	145
QM	KMLSCAGADR LQTGMRGAFG KPQGTVARVH IGQVIMSIRT KLQNKEHVIE	150
Jif-1	ALRRAKFKFP GRQKIHISKK WGFTKFNADA FEEMVAQKRL IPDGCGVKYV	195
QM	ALRRAKFKFP GRQKIHISKK WGFTKFNADA FEDMVAEKRL IPDGCGVKYI	200
Jif-1	PGRGPLDRWR ALHAA	210
QM	PSRGPLDKWR ALHS-	214

Fig. 1. Alignment of the predicted amino acid sequences of Jif-1 and QM. Mismatched residues are marked with asterisks.

peptide including the missing residues is 215 amino acids. The amino acid homology to QM is 92%. Thirteen amino acid residues are substituted but are conserved with the exception of residues 26 (alanine to proline), 128 (methionine to isoleucine), 177 (glutamic acid to glutamine), and 199 (glycine to serine) in Jif-1. The stop codon results in a different carboxyl-terminal residue. Computer analysis of the amino acid sequence suggests that the protein is primarily basic, with predominantly α -helical structure, containing potential myristoylation and protein kinase C phosphorylation sites (data not shown). The Jif-1 protein does not contain heptad repeats of leucine residues or of similar hydrophobic amino acids, and it does not show any structural resemblance to known transcription factor motifs (37).

Jif-1 Binds to Jun. To verify binding of Jif-1 to Jun, Jif-1 was expressed as a GST fusion protein, allowed to react with *in*

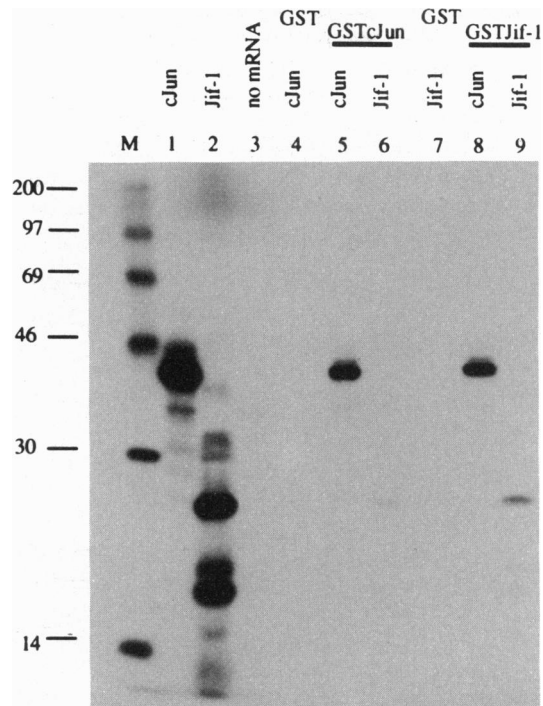


Fig. 2. Binding of Jif-1 to Jun. Lane M, molecular size markers (in kDa). Lanes 1-3, 1 μ l of *in vitro*-translated ³⁵S-labeled proteins c-Jun and Jif-1 and no mRNA, respectively. The major Jif-1 band migrates at 25 kDa; smaller bands at 19 kDa were not seen in all experiments and may be due to internal initiation. Lane 4, Jun protein fails to bind to GST; lane 5, Jun bound by GST-Jun; lane 6, Jif-1 bound by GST-Jun; lane 7, Jif-1 fails to bind to GST; lane 8, Jun bound by GST-Jif-1; lane 9, Jif-1 bound by GST-Jif-1.

in vitro-translated, [³⁵S]methionine-labeled Jun and with control proteins, and then precipitated with glutathione-agarose. Proteins in the precipitate were separated by SDS/polyacrylamide gel electrophoresis and identified by autoradiography (Fig. 2). Jif-1 was found to bind cellular and viral Jun (Fig. 3C). A weak interaction was found between Jif-1 and JunD, but no interaction was found with Fos (data not shown). Conversely, a GST-Jun fusion protein precipitated *in vitro*-translated Jif-1 (Fig. 2). Neither Jif-1 nor Jun was bound by GST. The binding reactions were resistant to the presence of ethidium bromide at 200 μ g/ml used as an indicator of DNA-independent protein association (38). This result suggests a direct protein-protein interaction between Jif-1 and Jun rather than indirect complex formation, where both proteins could bind to DNA. To test whether Jif-1 could interact with itself, Jif-1 was translated *in vitro* in an expression vector that fused the first five codons of QM to the 5' portion of the *jif-1* coding sequence. The expressed Jif-1

protein also bound GST-Jif-1 (Fig. 2), suggesting that Jif-1 can form multimeric complexes.

Jif-1 Interacts with the Jun Leucine Zipper. The region of the Jun molecule that binds to Jif-1 was determined by expressing a set of Jun deletion mutants (Fig. 3A) and testing the interactions of these proteins with GST-Jif-1 (Fig. 3B and C). Jif-1 failed to bind to all carboxyl-terminal deletions of Jun that removed the leucine zipper (Fig. 3B), suggesting that an intact leucine zipper is required for the formation of Jun-Jif-1 complexes.

Jif-1 Inhibits Jun DNA Binding and Transactivation. Bacterially expressed Jun protein binds to its consensus DNA target, the TRE oligonucleotide, and this binding can be demonstrated in a gel retardation assay (39). When Jun is complexed with GST-Jif-1, however, binding to the TRE is suppressed (Fig. 4). GST-Jif-1 alone does not bind the TRE oligonucleotide. Since Jif-1 binds to the region containing the heptad repeat of Jun, it appeared possible that it could be

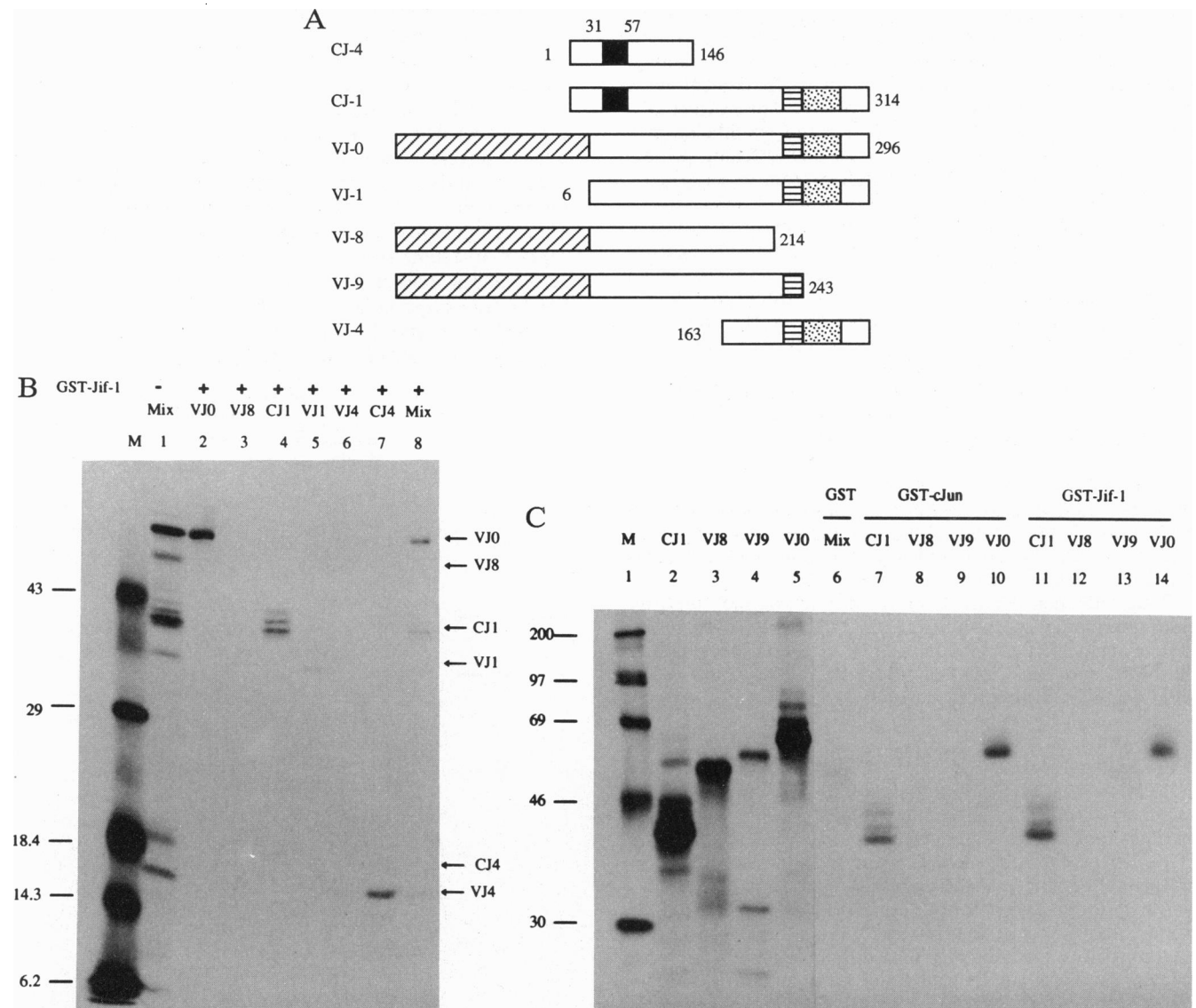


FIG. 3. Mapping the binding site of Jif-1 on Jun. (A) Schematic diagram of Jun deletion mutants. Hatched boxes represent viral Gag sequences; the remainder are Jun sequences. Black boxes, δ region; striped boxes, basic region; stippled boxes, leucine zipper. (B) Jif-1 binds to the basic region-leucine zipper domain of Jun. Lane M, molecular size markers (in kDa). Lane 1, mixture (Mix) of 1 μ l of each of the *in vitro*-translated proteins, CJ-1, CJ-4, VJ-0, VJ-8, VJ-4, and VJ-1. Lanes 2-8, proteins bound by GST-Jif-1. Lane 2, VJ-0; lane 3, VJ-8; lane 4, CJ-1; lane 5, VJ-1; lane 6, CJ-4; lane 7, VJ-4; lane 8, CJ-1, CJ-4, VJ-0, VJ-8, VJ-4, and VJ-1. (C) Binding of *in vitro*-translated proteins to GST-Jif-1 in the presence of ethidium bromide at 200 μ g/ml (38). Lane 1, molecular size markers (in kDa); lanes 2-5, *in vitro* translation products; lane 6, a mixture of VJ-0, VJ-8, VJ-9, and CJ-1 fails to bind to GST; lanes 7-10, proteins bound by GST-Jun; lanes 11-14, proteins bound by GST-Jif-1. Lanes 2, 7, and 11, CJ-1; lanes 3, 8, and 12, VJ-9; lanes 4, 9, and 13, VJ-8; lanes 5, 10, and 14, VJ-0.

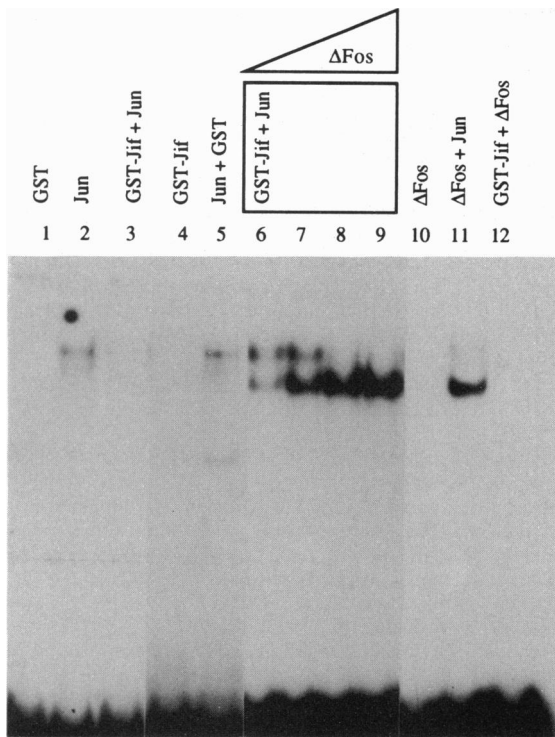


FIG. 4. Effect of Jif-1 on Jun-DNA binding. Binding of bacterially expressed Jun and GST-Jif-1 to TRE was examined by mobility shift assay. The proteins present in the binding reaction are indicated at the top of the figure. Δ Fos is a truncated Fos protein. Increasing amounts (50–400 ng) of Δ Fos protein were added to the Jun/Jif-1 mixture (lanes 6–9), and 200 ng was added to Jun alone (lane 11).

displaced from this site by another protein that binds the same domain of Jun (e.g., Fos). Fos was therefore added to the GST-Jif-1-Jun complex, and increasing amounts of Fos restored Jun binding to the TRE in the form of Jun-Fos heterodimers (Fig. 4).

Expression of Jif-1 in CEF using the Rc/RSV vector together with the cotransfected collagenase promoter-CAT

construct resulted in repression of the endogenous AP-1 activity of CEF (Fig. 5A). When Rc/RSV-cJun was cotransfected with Rc/RSV-Jif-1, TRE-dependent CAT activity was also repressed by Jif-1 (Fig. 5B). This repression was not seen with reporter constructs lacking an AP-1 binding site such as the Rous sarcoma virus (RSV) long terminal repeat or the GRP78 promoters (Fig. 5A).

DISCUSSION

Jif-1 appears to be a regulator of Jun. It binds specifically to the Jun protein. The binding is a direct protein-protein interaction as suggested by the fact that it takes place in the presence of ethidium bromide. Ethidium bromide would interfere with indirect complex formation mediated by Jun and Jif-1 both binding to contaminating DNA that may be present in the preparations. Binding of Jif-1 to other transcription factors (WT-1 and VP-16) has been tested, and no binding was detected, suggesting that Jif-1 binds specifically to members of the Jun family (unpublished results).

The target of Jif-1 in Jun is the region containing the leucine heptad repeat. It is not known whether Jif-1 binds to Jun monomers or dimers, and it is not known whether Jif-1, which forms multimers with itself, binds to Jun in monomeric or multimeric form. As would be expected from an interaction with the leucine heptad repeat in Jun, Jif-1 can compete with Fos, which binds to the same target domain. This competition between Jif-1 and Fos suggests an interaction with Jun monomers. Jif-1 does not contain a leucine zipper. However, computer analysis of the Jif-1 sequence reveals two small noncontiguous areas with permissivity for participating in a coiled-coil structure (unpublished data). Because Jif-1 not only inhibits DNA binding by Jun but also interferes with transcriptional activation, it could affect the expression of genes that are controlled by Jun. It could also change the affinity of Jun for specific DNA target sequences and thus alter the spectrum of genes affected by Jun.

Jif-1 is not identical with one of the previously described Jun regulators. Unlike inhibitory protein 1 (IP-1) (14, 15), which has a higher reported molecular weight than Jif-1, Jif-1 associates only with Jun and not with the Fos-Jun complex. The association with the leucine zipper also distinguishes

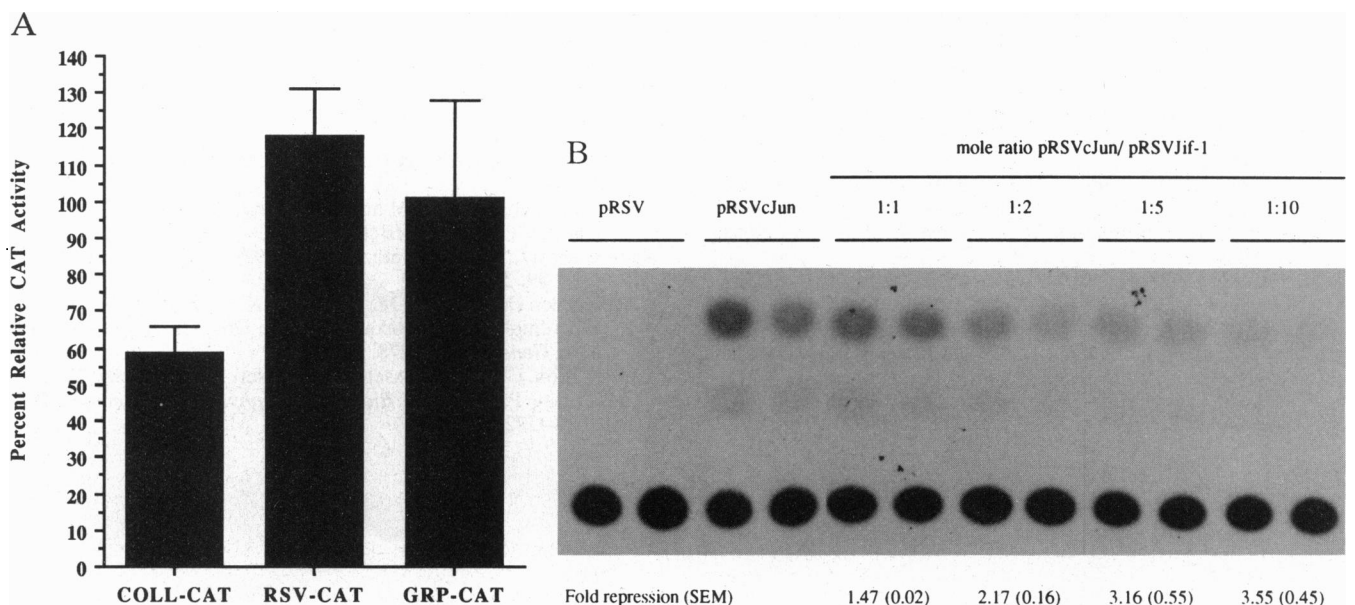


FIG. 5. Transactivation assay. (A) Effect of Jif-1 on the basal activity of COLL-CAT, RSV-CAT, and -154 GRP78-CAT reporter plasmids. Bars represent the residual CAT activity with Jif-1 added at a 1:2.5 mole ratio of reporter to Jif-1 expression construct. (B) Repression of c-Jun transactivation by Jif-1 using 2 μ g of RSV-c-Jun is shown with a representative autoradiograph. Constructs and ratios used are indicated above the figure. The fold repression by Jif-1 with SEM from at least three independent experiments with duplicate samples is shown below the figure.

Jif-1 from the factor that regulates Jun transactivation potential by interacting with the δ domain (11–13). Jif-1 shows no homology to Ref-1, the DNA repair enzyme involved in the redox control that targets the cysteine-248 residue of chicken c-Jun (residue 272 of rat c-Jun) (16, 17, 40). Recently Jun-binding proteins have been isolated by interaction cloning in yeast (41). Like Jif-1, they bind to the Jun leucine zipper, forming coiled-coil structures. The amino acid sequences of these proteins, JZA-3, JZA-20, and mTR67, show no homology to Jif-1 or the other Jif clones.

Jif-1 is closely related to the product of the *QM* gene. It may be the chicken homolog of *QM*. However, *QM* belongs to a multigene family, and Jif-1 could also be the homolog of another member of this family. The human *QM* has now been mapped to the X chromosome (42). A recent report found *QM* sequences fused to a laminin receptor mRNA in a human mammary tumor cell line (43).

QM has some characteristics of a tumor suppressor gene. It is expressed in nontumorigenic revertants of Wilm's tumor cells and may be involved in the maintenance of this nontumorigenic phenotype. Preliminary observations in our laboratory suggest that Jif-1 can inhibit Jun-induced transformation. Because of these potential antioncogenic effects of Jif-1, the condition of this gene in human tumors needs to be examined. Much remains to be learned about the normal role of Jif-1 in the control of cell growth and possibly of development and differentiation. Gene disruption experiments, the generation of Jif-1 transgenic animals, and tests for other protein-protein interactions involving Jif-1 will offer important clues.

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