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A Common Intermediary Factor (p52/54) Recognizing "Acidic Blob"-Type Domains Is Required for Transcriptional Activation by the Jun Proteins

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The ability of the c-Jun protein, the main component of the transcription factor AP1, to interact directly or indirectly with the RNA polymerase II-initiation complex to activate transcription was investigated by in vivo transcription interference ("squelching") experiments. Coexpression of a Jun mutant lacking its DNA binding domain strongly represses the activity of wild-type c-Jun. Repression depends on the presence of the transactivation domains (TADs), suggesting that a limiting factor interacting with the TADs is essential to link Jun and the components of the transcriptional machinery. The activity of this intermediary factor(s) is restricted to TADs characterized by an abundance of negatively charged amino acids, as demonstrated by the abilities of the TADs of JunB, GAL4, and VP16 to repress c-Jun activity. Depending on the presence of the TADs of Jun, we found physical interaction between Jun and a cluster of three proteins with molecular masses of 52, 53, and 54 kDa (p52/54). Association between Jun and p52/54 is strongly reduced in the presence of VP16, suggesting that the two proteins compete for binding to p52/54. Transcription factors containing a different type of TAD (e.g., GHF1, estrogen receptor, or serum response factor) fail to inhibit Jun activity, suggesting that these proteins act through a different mechanism. We consider the requirement of Jun to interact with p52/54 utilized by other transcription factors a new mechanism in the regulation of transcription of Jun-dependent target genes.

Regulation of transcription is a function of both basal transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and yet-uncharacterized associated proteins) and enhancer-binding proteins that direct the activity of the RNA polymerase II-initiation complex in response to extracellular signals (for reviews, see references 26 and 29). Enhancer factors were initially proposed to interact directly with the RNA polymerase II-initiation complex (28). A growing body of evidence, however, suggests the requirement for bridging factors, also known as coactivators, that link the activities of the transactivation domains (TADs) of the enhancer factors to the initiation complex. In vitro, purified TATA-binding protein (TBP) is incapable of restoring transactivation in a cell extract that lacks TFIID activity but depends on TBP-associated proteins (14, 16, 21, 30). In vivo, a transcriptional interference ("squelching") system in which the transactivation function of a given transcription factor is affected in the presence of a truncated form of a second transcription factor that lacks its DNA binding domain was established (6, 7, 8, 17, 33, 38), suggesting the existence of limiting factors required for transactivation that do not bind directly to DNA but interact with the TADs of the transcription factor to provide the link to the components of the basal transcription machinery.

Protein sequence analysis of various transcription factors revealed several different classes of TADs, all of which are capable of mediating transcriptional activation (for a review, see reference 26). So far, the requirement for a bridging factor interacting directly or indirectly with TBP to activate transcription has been demonstrated for only some of the transcription factors, including Sp1, NTF-1, and CTF (16, 37).

In the present study, we have investigated whether c-Jun, the major component of the transcription factor AP1, which mediates gene regulation in response to growth factors, cytokines, tumor promoters, carcinogens, and overexpression of certain oncogenes (for reviews, see references 5 and 42), requires an intermediary protein for transcriptional activation and, if so, which type. The TADs of c-Jun as well as those of its viral counterpart, v-Jun, are encoded by three short domains in the N-terminal halves of these proteins, which can be severed from the DNA binding domain of Jun and fused to a heterologous DNA binding domain to generate a transcriptional activator of new sequence specificity (6). Both in mammalian cells and in the yeast system, the functions of the TADs depend on the presence of acidic amino acids (6, 36), suggesting that the mechanisms by which Jun affects the activity of the basal transcription machinery have been conserved among eukaryotes.

First evidence for the requirement of a limiting factor in c-Jun-specific transactivation was provided by in vivo competition experiments using a c-Jun mutant that lacks its DNA binding domain (6). Here, we show that diminution by this mutant is dose dependent and relies strictly on the presence of the TADs in the mutant protein, suggesting physical interaction between the TADs and the bridging factor. By immunoprecipitation, we identified a cluster of proteins with molecular masses of 52, 53, and 54 kDa (p52/54) that interacts with the TADs of Jun. Interaction is inhibited by expression of the TAD of VP16 of herpes simplex virus, which is characterized by an abundance of negatively charged amino acids. In line with the interpretation that p52/54 interacts specifically with the negatively charged TAD of Jun, other transcription factors characterized by an

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"acidic blob"-type TAD, such as JunB and GAL4, interfere with the transactivation function of Jun. In contrast, transcription factors containing a different type of TAD, such as GHF1 and the estrogen receptor, have no effect on Jun activity, suggesting that these proteins either utilize a different type of bridging factor or interact directly with TBP or TBP-associated factors (TAFs). The strict requirement of Jun to interact with p52/54 identified this protein(s) as a novel player in the regulation of Jun-dependent target genes.

MATERIALS AND METHODS

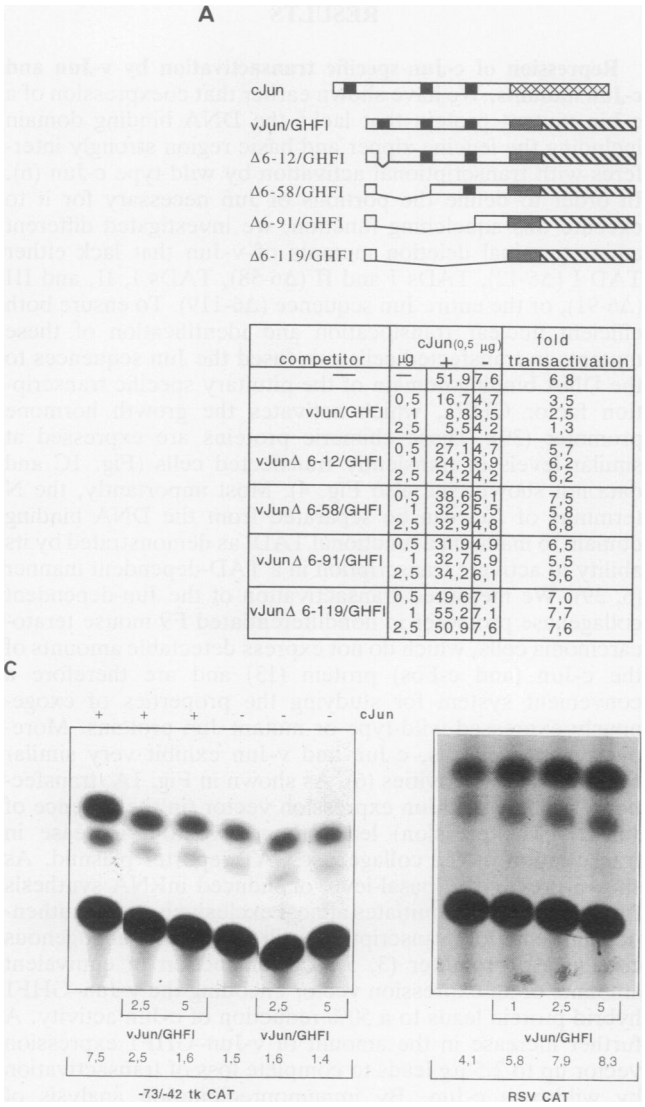
Cells and transfections. F9 embryonal carcinoma stem cells were grown in F12-Dulbecco's modified Eagle medium (1:1) supplemented with 10% fetal calf serum and 10^{-4} M β-mercaptoethanol. Each 100-mm-diameter culture dish was incubated with a calcium phosphate-DNA coprecipitate (1, 3) containing the different expression and reporter plasmids in various amounts, as indicated in the figure legends. Coprecipitates were removed after 7 h. Sixteen hours later, the cells were harvested and chloramphenicol acetyltransferase (CAT) activity was determined as described previously (1, 3).

Immunoprecipitation. For immunoprecipitations, cell proteins were labeled for 2 h with L-[^{35}S]methionine (final concentration, 200 μCi/ml) in methionine-free RPMI medium. Cells were harvested in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 10 mM Tris·HCl [pH 8.0], and 0.1 mM phenylmethylsulfonyl fluoride). Nuclear extracts were prepared as described previously (11). Lysates were cleared by treatment with preimmune serum followed by immunoprecipitation with the GHF1-specific antiserum as previously described (9, 11).

Plasmid constructions. RSV-vJunΔ6-12/GHF1, RSV-vJunΔ6-58/GHF1, and RSV-vJunΔ6-91/GHF1 were constructed by exchanging the 0.4-kb *Hin*dIII-*Nco*I fragment of RSV-vJun/GHF1 (6) with the corresponding fragment from RSV-Δ6-12, RSV-Δ6-58, or RSV-Δ6-91 (6), respectively. The remaining part of the POU domain containing the region recognized by the GHF1 antiserum (10) was inserted by exchanging the *Bgl*II-*Asp*718 fragments of the Jun-GHF1 deletion mutants with the corresponding fragment from RSV-Jun-G126 (39). RSV-GHF1/SRF was constructed as follows. A 1-kb *Bgl*II-*Hin*cII fragment of the complete serum response factor (SRF) cDNA (27) encoding amino acids 245 through 508 and 3′ untranslated sequences was inserted into the *Xho*I-filled-in, *Bgl*II-digested vector RSV-vJunΔ6-91/GHF1 (6) to generate RSVΔG. The complete DNA binding domain of GHF1 (amino acids 126 through 291) was amplified by a polymerase chain reaction using RSV-cJun/GHF1 and two primers which are identical with nucleotides 599 through 623 of c-Jun (5′-CCCGACGTGGGGCTGCTCAAG CTG-3′) and 873 through 851 (underlined) of GHF1 (5′-GGG ATCCGTCTCTCTGCACTCAAGATGCTCCTTTG-3′). The amplified product was digested with *Bam*HI and *Bgl*II and cloned into the *Bgl*II site of RSVΔG between the Rous sarcoma virus (RSV) long terminal repeat (LTR) and SRF sequences. SV-vJun, pUC-SV, pUC-RSV, RSV-cJun, RSV-cJun/GHF1, RSV-JunB-GHF1, RSV-GHF1, −73/+63 collagenase promoter-CAT (−73/+63 CollCAT), and −73/−42 tk-CAT are described elsewhere (1–4, 13). The expression vectors encoding ER, ER-GAL4 and GAL4-VP16, and ERE-tkCAT were described previously (20, 22, 33, 38, 43).

RESULTS

Repression of c-Jun-specific transactivation by v-Jun and c-Jun mutants. We have shown earlier that coexpression of a c-Jun mutant protein that lacks the DNA binding domain including the leucine zipper and basic region strongly interferes with transcriptional activation by wild-type c-Jun (6). In order to define the portions of Jun necessary for it to execute this squelching function, we investigated different amino-terminal deletion mutants of v-Jun that lack either TAD I (Δ6-12), TADs I and II (Δ6-58), TADs I, II, and III (Δ6-91), or the entire Jun sequence (Δ6-119). To ensure both efficient nuclear translocation and identification of these mutants in transfected cells, we fused the Jun sequences to the DNA binding domain of the pituitary specific transcription factor GHF1, which activates the growth hormone promoter (39). These chimeric proteins are expressed at similar levels in transiently transfected cells (Fig. 1C and data not shown; see also Fig. 4). Most importantly, the N terminus of Jun can be separated from the DNA binding domain to maintain a functional TAD, as demonstrated by its ability to activate transcription in a TAD-dependent manner (6, 39). We measured transactivation of the Jun-dependent collagenase promoter in nondifferentiated F9 mouse teratocarcinoma cells, which do not express detectable amounts of the c-Jun (and c-Fos) protein (13) and are therefore a convenient system for studying the properties of exogenously expressed wild-type or mutant Jun proteins. Moreover, in these cells, c-Jun and v-Jun exhibit very similar transactivation activities (6). As shown in Fig. 1A, transfection of 500 ng of c-Jun expression vector (in the absence of Jun-GHF1 expression) leads to a sevenfold increase in transcription of the collagenase-CAT reporter plasmid. As shown previously, basal-level or induced mRNA synthesis from this construct initiates almost exclusively at the authentic start site of transcription utilized in the endogenous collagenase promoter (3, 5). Cotransfection of equivalent amounts of an expression vector encoding the v-Jun–GHF1 hybrid protein leads to a 50% reduction of c-Jun activity. A further increase in the amount of v-Jun–GHF1 expression vector up to 2.5 μg leads to complete loss of transactivation by wild-type c-Jun. By immunoprecipitation analysis of radioactively labeled F9 cells transfected with the expression vectors encoding wild-type c-Jun or v-Jun–GHF1 in a 1:3 ratio, the amount of v-Jun–GHF1 protein was found to exceed the level of c-Jun protein by about five- to sixfold (Fig. 1B). Deletion of TAD I (Δ6-12), however, almost completely eliminates squelching of c-Jun-specific transcriptional activation. Deletion of TADs I and II (Δ6-58) renders the Jun-GHF1 chimeric protein incapable of repressing wild-type c-Jun activity. Very similar results were obtained by using a v-Jun–GHF1 hybrid in which TADs I, II, and III had been deleted (Δ6-91; Fig. 1A). These data show that the inhibitory effect of the v-Jun–GHF1 hybrid cannot be attributed to the GHF1 sequences but proved entirely dependent on the TADs of the Jun portion of the chimeric protein. In agreement with these results, we found that a truncated v-Jun–GHF1 hybrid protein that lacks part of the DNA binding domain of GHF1 (without affecting nuclear translocation) represses c-Jun-specific transactivation in a manner similar to that of wild-type v-Jun–GHF1 (data not shown). The TAD-dependent squelching activity of the Jun-GHF1 mutants is consistent with our previous results on the mapping of the TADs in v-Jun, showing that deletion of TAD I strongly reduces Jun-specific transactivation while deletion of TADs I and II completely obliterates c-Jun activity (6).



competitor	μg	cJun(0,5 μg) +	-	fold transactivation
—	—	51,9	7,6	6,8
vJun/GHFI	0,5	16,7	4,7	3,5
	1	8,8	3,5	2,5
	2,5	5,5	4,2	1,3
vJunΔ 6-12/GHFI	0,5	27,1	4,7	5,7
	1	24,3	3,9	6,2
	2,5	24,2	4,2	6,2
vJunΔ 6-58/GHFI	0,5	38,6	5,1	7,5
	1	32,2	5,5	5,8
	2,5	32,9	4,8	6,8
vJunΔ 6-91/GHFI	0,5	31,9	4,9	6,5
	1	32,7	5,9	5,5
	2,5	34,2	6,1	5,6
vJunΔ 6-119/GHFI	0,5	49,6	7,1	7,0
	1	55,2	7,1	7,7
	2,5	50,9	7,6	7,6

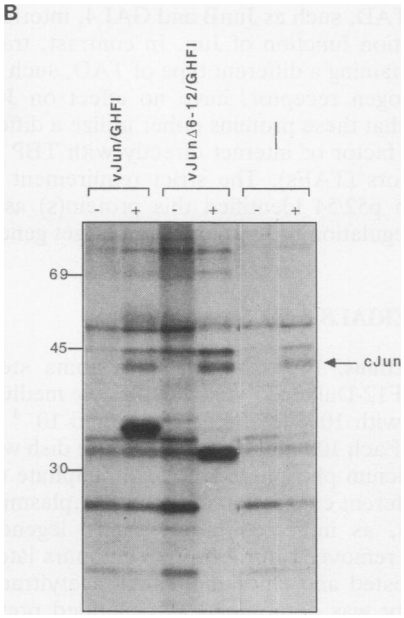


FIG. 1. Transcriptional interference properties of the TADs of v-Jun. (A) Nondifferentiated F9 cells were transfected with 5 μg of the AP1-dependent −73/+63 CollCAT reporter plasmid and increasing amounts of v-Jun–GHF1 expression vectors containing or lacking parts of the TADs of v-Jun in the presence (+) or absence (−) of 0.5 μg of an expression vector encoding wild-type c-Jun. The total amount of expression vector was kept at 10 μg by adding the appropriate amount of the control vector pUC-RSV lacking any Jun or GHF1 sequences. Values are percents conversion of [^{14}C]chloramphenicol to the acetylated form (averages of at least three independent experiments which did not deviate by more than 20%). Fold transactivation was determined by comparing the expression of the reporter plasmid in the presence and absence of the c-Jun expression vector. The organization of c-Jun and the chimeric Jun-GHF1 proteins is shown at the top. Solid boxes, TADs of c-Jun and v-Jun; crosshatched box, DNA binding domain of c-Jun; hatched boxes (homeobox domain) and shaded boxes (POU domain), the DNA binding domain of GHF1. (B) F9 cells were transfected with 15 μg of pUC-RSV (—), RSV-vJun/GHF1, or RSV-vJunΔ6-12/GHF1 in the presence (+) or absence (−) of 5 μg of RSV-cJun. After transfection, the cells were labeled for 2 h with [^{35}S]methionine, and then nuclear extracts were prepared. The exogenously expressed proteins translocated to the nucleus were purified by immunoprecipitation with a polyclonal antibody raised against the complete Jun protein. Molecular masses in kilodaltons are shown on the left. (C) Nondifferentiated F9 cells were transfected with 2 μg of either −73/−42 collagenase-tkCAT (plus 0.5 μg of RSV-cJun) or RSV-CAT (without RSV-cJun) in the presence of increasing amounts of RSV-vJun/GHF1. The total amount of expression vectors (10 μg) was kept constant by using pUC-RSV. Values referring to the rate of conversion of [^{14}C]chloramphenicol are averages of four independent experiments which did not deviate by more than 10%.

Coexpression of the various Jun-GHF1 mutants that squelch or do not squelch Jun-specific transactivation reduces neither the synthesis nor the nuclear translocation of wild-type Jun, as demonstrated by immunoprecipitation of exogenously expressed proteins from nuclear extracts of transiently transfected F9 cells (Fig. 1B).

Complete squelching is not restricted to Jun interacting with the collagenase promoter but is also observed in the context of the Jun binding site fused to the thymidine kinase (TK) promoter of herpes simplex virus (Fig. 1C). Most importantly, expression of the Jun-GHF1 hybrid proteins does not affect basal-level expression of the −72/−42 tk-CAT construct, whose activity is driven by one CTF and two Sp1 sites in the TK promoter. In F9 cells, both CTF and Sp1 activities are present, as found by in vivo footprinting analysis of the endogenous c-Jun promoter carrying binding sites for both CTF and Sp1 (18). In addition, expression of a CAT reporter gene driven by the highly active LTR of RSV is not reduced but is even slightly enhanced (up to twofold) by Jun-GHF1 (Fig. 1C).

Squelching is not specific for v-Jun sequences but is also exerted by the N-terminal half of c-Jun linked to the DNA binding domain of GHF1 (Fig. 2). We also observed significant reductions of the basal-level activity of the −73/+63 collCAT construct by all GHF1 hybrid proteins that contain v-Jun sequences but not c-Jun sequences (Fig. 1A and 2). The reason for these differences between c-Jun and v-Jun is unknown but can be attributed to the residual 33 amino acids of v-Jun present in vJunΔ6-91/GHF1 (Fig. 1A; compare Δ6-91 and Δ6-119). It is possible that these sequences, when fused to the GHF1 DNA binding domain, force the formation of a secondary structure that interferes nonspecifically with the functions of other proteins binding to the collage-

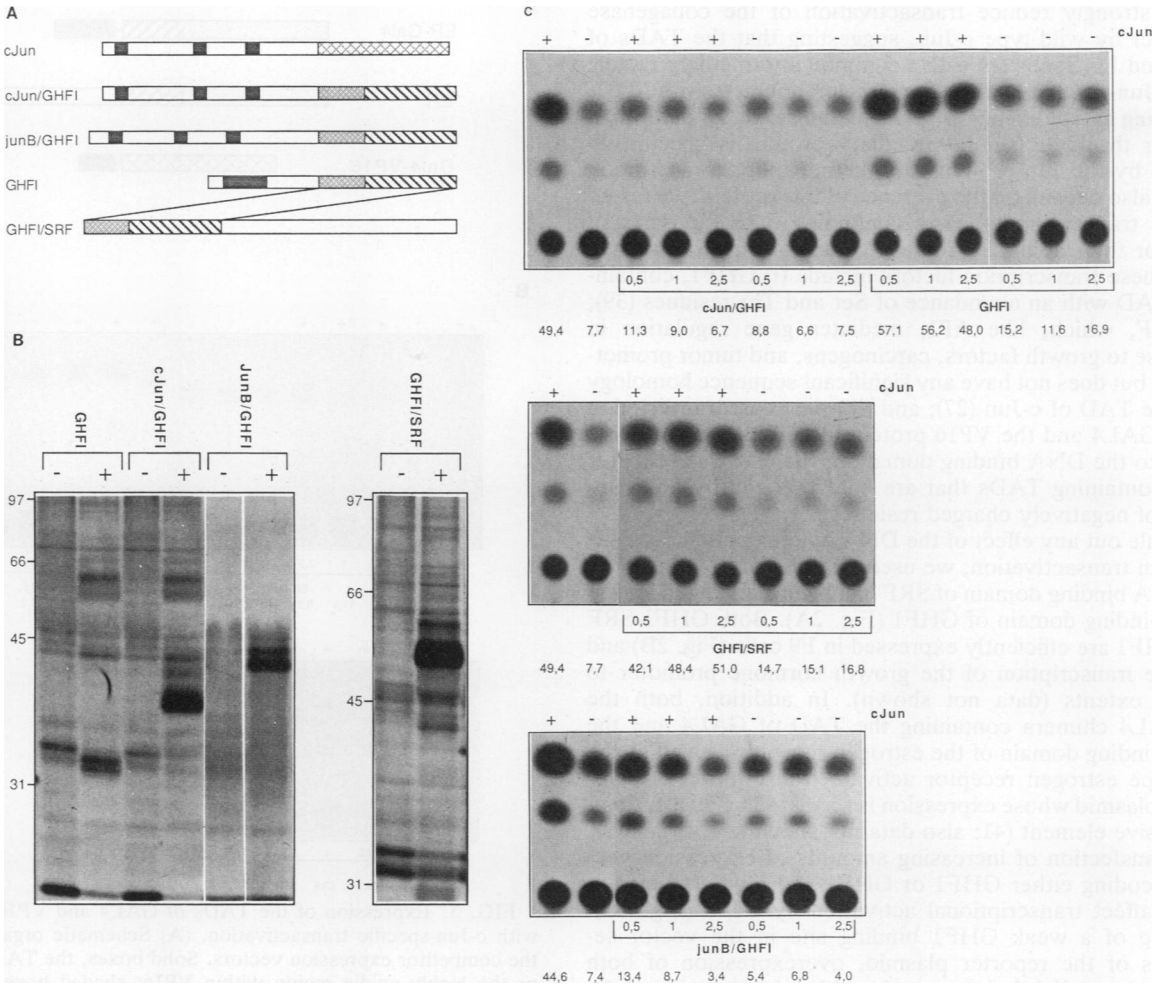


FIG. 2. Selective inhibition of c-Jun-specific transactivation by other transcription factors. (A) Structures of the products encoded by the various RSV-driven expression vectors. Solid boxes, TADs of c-Jun, JunB, and GHF1 (amino acid residues 1 through 73); crosshatched box, DNA binding domain of c-Jun; hatched boxes (homeobox domain) and shaded boxes (POU domain), DNA binding domain of GHF1. (B) Detection of GHF1, c-Jun–GHF1, JunB-GHF1, and GHF1-SRF in transiently transfected F9 cells by immunoprecipitation. F9 cells were transfected with 15 μg of the various expression vectors. After the DNA precipitate was removed, the cells were labeled for 2 h with [^{35}S]methionine. Expression of the products containing GHF1 sequences was determined by radioimmunoprecipitation with (+) and without (−) a GHF1-specific antipeptide antibody as described in Materials and Methods. The predicted sizes of the different exogenously expressed proteins are 33 and 34 kDa (GHF1 [9, 10]), 40 kDa (c-Jun–GHF1), 43 kDa (JunB-GHF1), and 47 kDa (GHF1-SRF). (C) Selective inhibition of c-Jun-specific transactivation by other transcription factors. Nondifferentiated F9 cells were transfected with 2.5 μg of the AP1-dependent −73/+63 CollCAT reporter and increasing amounts (0.5, 1, and 2.5 μg) of expression vectors encoding the proteins indicated at the bottom of each panel in the presence (+) or absence (−) of RSV-cJun, and CAT activity was determined. The result of one typical assay is shown. Percents conversion of [^{14}C]chloramphenicol to the acetylated form (averages of at least three independent experiments which did not deviate by more than 15%) are shown at the very bottom of each panel.

nase promoter, e.g., the factor binding to the region between positions −60 and −35 (box I [4]).

In summary, these results demonstrate that the TADs of c-Jun and v-Jun compete for the same limiting component required for transactivation. It is unlikely that this component is TBP itself or another general component of the initiation complex (e.g., TFIIA or TFIIB) because expression of c-Jun–GHF1 affects neither basal-level expression of the collagenase promoter or the TK promoter nor the high level of transcriptional activity of the LTR from RSV (Fig. 1B) or the simian virus 40 enhancer (data not shown). Our results suggest rather that the limiting factor(s) is a component that links the TADs of Jun and components of the basal transcription machinery. This factor either interacts directly with TBP or associates with another protein, e.g., a specific type of TAF (16), to link Jun and TBP.

c-Jun and JunB compete for interaction with a common coactivator. To determine whether the identified intermediary protein(s) is also utilized by other members of the Jun protein family, we examined the squelching capacity of JunB, whose N terminus shows high degrees of similarity with c-Jun and v-Jun (32). The JunB-GHF1 protein is efficiently expressed in F9 cells (Fig. 2B) and stimulates transcription from the growth hormone promoter in a manner similar to that of c-Jun–GHF1 (13). As shown in Fig. 2, increasing amounts of an expression vector encoding JunB-

GHF1 strongly reduce transactivation of the collagenase promoter by wild-type c-Jun, suggesting that the TADs of c-Jun and JunB interact with a common intermediary factor.

The Jun-specific intermediary factor interacts with TADs containing an abundance of acidic amino acids. To determine whether the identified intermediary protein is specifically utilized by the Jun proteins or whether other transcription factors also depend on the presence of this protein, we tested various transcription factors containing different types of TAD for their ability to repress c-Jun-specific transactivation. These transcription factors include (i) GHF1, containing a TAD with an abundance of Ser and Thr residues (39); (ii) SRF, which, like AP1, mediates gene regulation in response to growth factors, carcinogens, and tumor promoters (12) but does not have any significant sequence homology with the TAD of c-Jun (27); and (iii) the yeast transcription factor GAL4 and the VP16 protein of herpes simplex virus (fused to the DNA binding domain of the estrogen receptor [43]), containing TADs that are characterized by an abundance of negatively charged residues (24, 41).

To rule out any effect of the DNA binding domain of SRF on c-Jun transactivation, we used a hybrid protein in which the DNA binding domain of SRF had been exchanged for the DNA binding domain of GHF1 (Fig. 2A). Both GHF1-SRF and GHF1 are efficiently expressed in F9 cells (Fig. 2B) and activate transcription of the growth hormone promoter to similar extents (data not shown). In addition, both the ER-GAL4 chimera containing the TAD of GAL4 and the DNA binding domain of the estrogen receptor as well as the wild-type estrogen receptor activate transcription of a reporter plasmid whose expression is regulated by an estrogen-responsive element (41; also data not shown).

Cotransfection of increasing amounts of expression vectors encoding either GHF1 or GHF1-SRF does not significantly affect transcriptional activation by c-Jun (Fig. 2C). Because of a weak GHF1 binding site in the vector sequences of the reporter plasmid, overexpression of both proteins even slightly increases basal-level expression of the collagenase promoter. These results strongly suggest that neither GHF1 nor SRF interacts with the Jun-specific bridging factor or that each interacts with it only with a very low affinity.

In contrast to GHF1 and GHF1-SRF, coexpression of ER-GAL4 inhibits c-Jun-specific transactivation in a dose-dependent manner while basal-level expression of the reporter plasmid in the absence of c-Jun is not significantly affected (Fig. 3B). This effect is specific for the GAL4 TAD because overexpression of the wild-type estrogen receptor has no effect. In a manner similar to that of GAL4, the highly acidic portion of VP16 (fused to the DNA binding domain of GAL4) rapidly decreases c-Jun-specific activation of the collagenase promoter to the level observed in the absence of c-Jun expression (Fig. 3B).

These results strongly suggest that the TADs of Jun, GAL4, and VP16 interact with a common mammalian factor(s) required for transcriptional activation that can be characterized by its property to recognize TADs containing acidic amino acid residues. It also suggests that the transactivation function of the Jun proteins depends on a single type of intermediary protein (or class of proteins) that recognizes acidic domains whose inactivation leads to a complete loss of the transactivation activity of Jun.

p52/54 interacts with the TAD of Jun. While the experiments described above can give only indirect evidence for the existence of one protein or multiple proteins interacting with the TAD of Jun, we tried to demonstrate physical

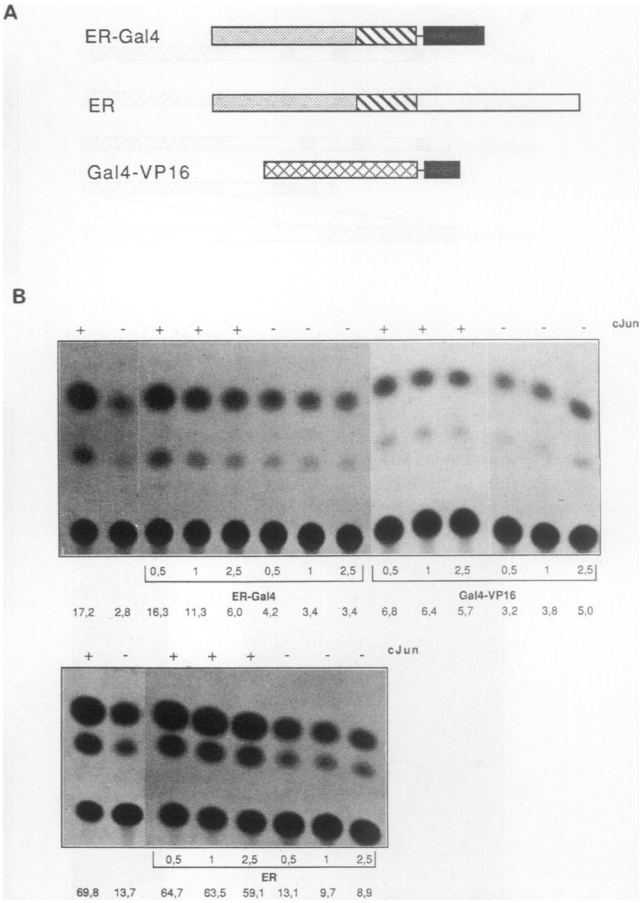


FIG. 3. Expression of the TADs of GAL4 and VP16 interferes with c-Jun-specific transactivation. (A) Schematic organization of the competitor expression vectors. Solid boxes, the TAD of GAL4 or the highly acidic region within VP16; shaded boxes, the A/B region of ER containing TAF-1; open box, the E/F region of ER containing TAF-2; hatched boxes, DNA binding domain of ER; crosshatched box, DNA binding domain of GAL4. (B) Repression of c-Jun activity by GAL4 and VP16. F9 cells were transfected with 5 μg of the −73/+63 CollCAT reporter and increasing amounts of competitor expression vectors in the presence (+) or absence (−) of 500 ng of SV-Jun, and CAT activity was analyzed 16 h later. Because the expression of ER, ER-GAL4, and GAL4-VP16 is under the control of the simian virus 40 promoter-enhancer, pUC-SV was used instead of pUC-RSV to keep a constant amount of expression vector (10 μg). The result of a typical assay is shown. Percents conversion of [^{14}C]chloramphenicol to the acetylated form (averages of three independent experiments which did not deviate by more than 20%) are shown at the bottom.

interaction by immunoprecipitation analysis of metabolically labeled F9 cells transiently transfected with the various Jun-GHF1 expression vectors. As shown in Fig. 4, precipitation of the hybrid protein containing the complete N terminus of Jun with either a GHF1-specific antibody (Fig. 4A) or a Jun-specific antibody (Fig. 4B) leads to coprecipitation of three proteins with molecular masses of 52, 53, and 54 kDa (p52/54). In contrast, v-Jun mutants that lack parts of the TADs do not interact with p52/54. Most importantly, the ability of the Jun-GHF1 mutants to interact with p52/54 strictly correlates with squelching of wild-type Jun activity (Fig. 1A), suggesting that p52/54 might be the limiting factor required for c-Jun-specific transactivation. Denaturing the

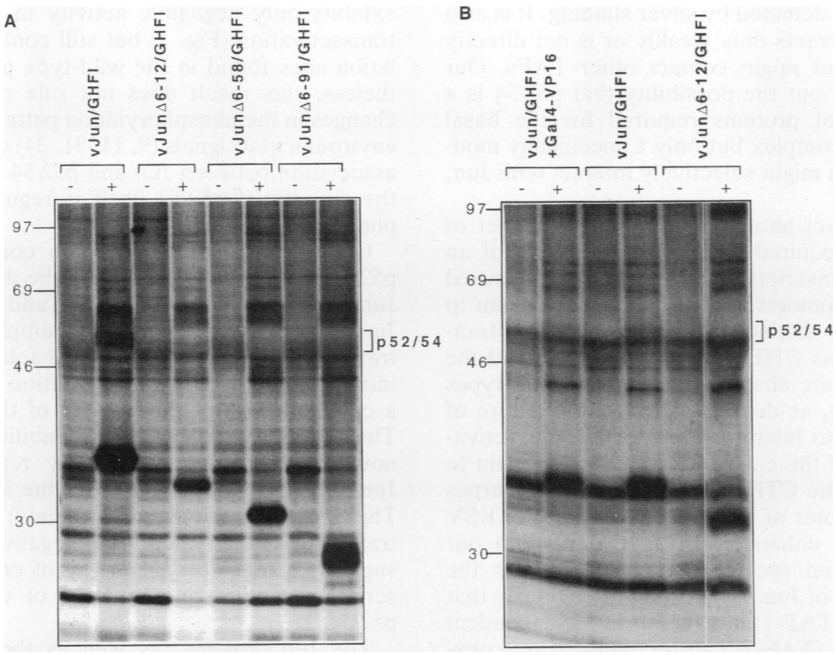


FIG. 4. (A) Coprecipitation of v-Jun–GHF1 and p52/54. Nondifferentiated F9 cells were transfected with 15 μg of expression vectors encoding the various Jun-GHF1 hybrid proteins indicated at the top. Expression was measured by immunoprecipitation of metabolically labeled cells as described in the legend to Fig. 1B, using either preimmune serum (−) or an antibody raised against GHF1 (+). The location of p52/54 is indicated on the right. (B) Expression of GAL4-VP16 inhibits association of Jun and p52/54. F9 cells were transfected with 10 μg of expression vector vJun/GHF1 or vJunΔ6-12/GHF1 together with 5 μg of an expression vector encoding GAL4-VP16 (two left lanes) or the control vector pUC-SV. Expression was determined by immunoprecipitation as described for panel A, using a polyclonal antibody raised against the complete Jun protein.

protein extracts prior to immunoprecipitation leads to a loss of p52/54-Jun interaction (data not shown).

To get further support for the role of p52/54 in mediating Jun activity, we questioned whether coexpression of the TAD of VP16, which efficiently interferes with Jun transactivation (Fig. 3B), would also affect the association of Jun and p52/54. Because of limitations of the transient transfection system with respect to the amounts of transfected DNA, we used 10 and 5 μg of expression vectors encoding v-Jun–GHF1 and the TAD of VP16 (fused to the DNA binding domain of GAL4), respectively. As shown in Fig. 4B, even under these conditions, association of Jun and p52/54 is almost completely lost. By excising the radioactive bands from the gel, association of p52/54 was found to be reduced by almost 80% while the expression of v-Jun–GHF1 was reduced by about 15%.

Although we cannot completely rule out the possibility that expression of GAL4-VP16 inhibits the expression of p52/54, our results favor the model that the TADs of Jun and VP16 compete for binding to p52/54.

DISCUSSION

The existence of bridging proteins that recognize transactivation domains composed of stretches of acidic amino acids, also known as acidic blobs, that may form amphipathic α helices has been suggested on the basis of transcriptional activation by the yeast transcription factor GAL4 and by VP16 from herpes simplex virus in mammalian cells (20, 43). So far, only one example of a mammalian transcription factor, the human glucocorticoid receptor, that interacts with this type of protein in vivo has been described (38). In a study similar to ours, however, GAL4 and VP16 only slightly affected transactivation by the wild-type glucocorticoid receptor, suggesting that the limiting factor recognizing negatively charged TADs is, to a large extent, dispensable for transactivation by the receptor (38).

Our data now show that the Jun protein family, GAL4, and VP16 make use of the same factor or class of factors required for transcriptional activation. By immunoprecipitation analysis, we have identified a cluster of three proteins with molecular masses of 52, 53, and 54 kDa (p52/54) that is associated with the TAD of Jun. There are two lines of evidence that p52/54 and the bridging protein defined by our in vivo competition experiments are identical: (i) the ability of any Jun-GHF1 hybrid to repress the activity of wild-type c-Jun perfectly correlates with the ability to interact with p52/54, and (ii) expression of the TAD of VP16 inhibits both transcriptional activation by Jun and the association of Jun and p52/54. At present, we do not know whether the p52/54 cluster represents different proteins or whether it is the result of alternative splicing or differences in posttranslational modification of a single type of protein.

Most recently, at least six protein factors were identified by immunoprecipitation and silver staining to bind directly to the *Drosophila* TBP, and they are expected to confer a coactivator function (16). At present, we do not know whether p52/54 belongs to this class of proteins. In a complex consisting of TBP, p52/54, and the competitor (e.g., Jun-GHF1), TBP might still get to the promoter and support basal transcription while p52/54 is blocked in its ability to respond to promoter-bound Jun. However, none of the TAFs described by Dynlacht et al. (16) has a molecular mass of 52 to 54 kDa. The level of expression of p52/54 might, of

course, be too low to be detected by silver staining. It is also possible that p52/54 interacts only weakly or is not directly associated with TBP but might contact other TAFs. Our results also do not rule out the possibility that p52/54 is a member of the class of proteins required for the basal transcription initiation complex but only a specifically modified form of this protein might selectively interact with Jun, JunB, GAL4, or VP16.

Regardless of the exact nature of p52/54, this cluster of proteins seems to be required during the formation of an activated state of the transcription initiation complex caused by Jun bound to the promoter. This type of mechanism to activate transcription is distinct from those of other transcription factors, such as GHF1, SRF, CTF, Sp1, and the estrogen receptor, that are characterized by different types of TAD (15, 25, 39, 40), as demonstrated by the failure of GHF1 and GHF1-SRF to inhibit c-Jun-specific transactivation and the inability of the c-Jun–GHF1 hybrid protein to repress the activity of the CTF- and Sp1-dependent herpes simplex virus TK promoter or the very potent LTR of RSV or the simian virus 40 enhancer. In agreement with our finding that the estrogen receptor does not repress the transactivation function of Jun, it was shown previously that neither the basal-level (TAF-1) nor the hormone-dependent transactivation function (TAF-2) can be inhibited by coexpression of GAL4 or VP16 (40). In line with our results, neither VP16 nor GAL4, both of which efficiently titrate the c-Jun-specific intermediary protein, represses basal-level expression of the reporter plasmids used in the in vivo competition experiments. Although a direct interaction between VP16 and components of the basal transcription machinery has been observed in vitro (19, 23, 35), our results are consistent with previous studies that did not detect effects of VP16 and GAL4 on TFIID activity in vivo (8, 38, 40). In agreement with our in vivo data, the isolated coactivator required for Sp1 activity is not involved in transcriptional activation by VP16 (37).

The ability of Jun, GAL4, and VP16 to activate transcription in both yeast cells (24, 36, 41) and mammalian cells (for examples, see references 1, 20, and 43) and the transcriptional interference experiments described here suggest that p52/54 or p52/54-related proteins are utilized in both systems and that the structural properties of these proteins, at least at the site of interaction with Jun, GAL4, and VP16, are evolutionarily conserved. Interestingly, Berger and coworkers isolated a yeast protein of 434 amino acids (ADA-2), which is required for transcriptional activation of VP16 in yeasts (7). Therefore, it is tempting to speculate that p52/54 might be a mammalian homolog of ADA-2 from yeasts.

Because Jun regulates transcription in response to extracellular signals (e.g., growth factors or enhanced expression of various oncogenes), an obvious question is whether this is, at least in part, due to differences in the association between Jun and p52/54. Recently, expression of the Ras oncoprotein was shown to augment Jun-specific transactivation which was accompanied by enhanced phosphorylation of Jun on Ser residues that are located within TAD II (9, 34) and which are utilized by mitogen-activated protein kinases, at least in vitro (31). Therefore, squelching of c-Jun-specific transactivation by the Jun-GHF1 hybrid could also be explained by the competition for a specific protein kinase. We consider this explanation unlikely for two reasons. First, neither VP16 nor GAL4, both of which strongly repress c-Jun activity, has been found to be phosphorylated in mammalian cells. Second, and more important, a v-Jun deletion mutant lacking amino acids 6 through 12 (TAD I) exhibits only negligible activity in repressing Jun-specific transactivation (Fig. 1) but still contains all the phosphorylation sites found in the wild-type protein (31, 34). Nevertheless, this result does not rule out the possibility that changes in the phosphorylation pattern of Jun in response to environmental signals (9, 11, 31, 34) could be involved in the association between Jun and p52/54. It is also possible that the activity of p52/54 itself is regulated by protein phosphatases or kinases.

On the basis of our in vivo competition experiments, p52/54 is likely to interact with the different members of the Jun protein family (v-Jun, c-Jun, and JunB [and, most likely, JunD]), representing the first examples of a specific type of transcription factor that depends solely on the activity of an intermediary protein whose titration or inactivation leads to a complete loss of the activity of the transcription factor. The absolute need for the availability of p52/54 provides a novel mechanism to efficiently regulate transcription of Jun-dependent target genes on the level of p52/54 activity. The fact that the same protein(s) is shared by several transcription factors carrying negatively charged TADs also suggests a regulatory pathway of cross talk between transcription factors on the level of competitive binding to p52/54.

The Jun proteins (as well as the members of the Fos protein family) have been proposed to be a key element in the regulation of cell proliferation, differentiation, and transformation (for reviews, see references 5 and 42). Our findings on the requirement of p52/54 greatly expand the knowledge of mechanisms controlling the activities of AP1-dependent target genes during these biological processes.

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