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An inhibitor domain in c-Fos regulates activation domains containing the HOB1 motif

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The c-Fos protein has three activation modules at its C-terminus, two of which contain motifs (HOB1 and HOB2) which are also present in the activation domains of c-Jun. Here we show the existence of two additional activation modules at the N-terminus of c-Fos, one of which contains a second HOB1 motif (HOB1-N). The N-terminus also contains an inhibitor domain (ID1) which silences HOB1 activity. GAL4 fusion experiments showed that ID1 can specifically silence HOB1-containing activation domains from c-Fos or c-Jun when linked *in cis*, but will not affect other distinct activation domains. The c-Fos related protein, FosB, also contains an inhibitor domain. Mutagenic and deletion analyses identify an inhibitor motif (IM1) conserved between c-Fos and FosB, which is required for inhibitor function. Mutagenesis of IM1 enhances the ability of c-Fos to activate an AP1 bearing promoter. Finally, squelching experiments suggest that c-Fos ID1 binds a limiting protein involved in inhibition. These results demonstrate the existence of a new class of inhibitor domain within transcriptional activators, which acts in a sequence specific manner to inhibit a subset of activation domains.

Key words: Fos/HOB1/transcription activation

Introduction

The Fos–Jun (AP1) family of transcription factors plays an important role in mediating the cell's response to proliferative signals. Very soon after mitogenic stimulation, the levels of Fos and Jun protein rise and post-translational modification increases the potency of the Fos–Jun complex. The net effect is an increase in the transcription of AP1 site bearing promoters (Curran and Franza, 1988).

The prototypes of the AP1 family, c-Fos and c-Jun, contain a bZIP DNA binding domain (DBD; for review see Kouzarides and Ziff, 1989a). This allows them to form a heterodimer which can bind with high affinity to an AP1 site (Halazonetis *et al.*, 1988; Kouzarides and Ziff, 1988, 1989b; Nakabeppu *et al.*, 1988; Sassone-Corsi *et al.*, 1988; Gentz *et al.*, 1989; Schuermann *et al.*, 1989; Turner and Tjian, 1989). Both c-Fos and c-Jun contribute to the transcriptional activation capacity of the heterodimer (Angel *et al.*, 1989). They each contain multiple activation domains (Angel *et al.*, 1989; Bohmann and Tjian, 1989; Baichwal and Tjian, 1990; Abate *et al.*, 1991; Sutherland *et al.*, 1992) some of which have structural and functional similarities (Sutherland *et al.*, 1992).

The c-Jun protein has at least two activation domains A1 and A2 (Bohmann and Tjian, 1989; Baichwal and Tjian, 1990). The A1 activation domain at the N-terminus of c-Jun contributes the highest activation capacity. The activity of this domain is regulated by extracellular signals, such as UV and tyrosine kinases such as Src and Ha-Ras (Binétruy *et al.*, 1991; Smeal *et al.*, 1991, 1992; Hibi *et al.*, 1993). These agents increase c-Jun activity by inducing the phosphorylation of two Ser residues, S_{63} and S_{73} within A1. Phosphorylation of these sites can be mediated by MAP kinase related proteins *in vitro* (Pulverer *et al.*, 1991; Kyriakis *et al.*, 1994) or a MAP kinase related protein, JNK (Jun N-terminal kinase), *in vivo* (Dérijard *et al.*, 1994). Mutagenesis of S_{63} and S_{73} shows that they are essential for the activity of this c-Jun activation domain (Pulverer *et al.*, 1991). Sequences at and around S_{73} of c-Jun are part of the HOB1 motif shared by an activation domain in c-Fos (Sutherland *et al.*, 1992).

Directly N-terminal to S_{63} and S_{73} lies a sequence (δ region) required for the binding of the kinase (JNK) which phosphorylates these two sites (Dérijard *et al.*, 1994). The δ region is deleted in the v-Jun protein (Adler *et al.*, 1992) and as a consequence S_{63} and S_{73} are not phosphorylated in v-Jun. In addition, using squelching experiments, the δ region has been implicated in binding a cell-specific inhibitor which can silence the activity of c-Jun but not v-Jun (Baichwal and Tjian, 1990).

The c-Fos protein has a number of domains which activate or regulate transcription (Abate *et al.*, 1991; Sutherland *et al.*, 1992; Jooss *et al.*, 1994). Detailed characterization of the C-terminal activation domains has revealed a modular structure (Sutherland *et al.*, 1992). Three co-operating activation modules exist in this region which individually show little independent activity but which can activate transcription synergistically when combined. Two of these modules contain motifs (HOB1 and HOB2) which are present in the A1 activation domain of c-Jun. The HOB-containing regions of c-Jun A1 are interchangeable with those of c-Fos in terms of cooperativity in transcriptional activation. A third module in the c-Fos C-terminus contains a TBP binding motif (TBM) which mediates interaction with TBP both *in vitro* and *in vivo* and allows interaction with the TFIID complex (Metz *et al.*, 1994). The TBP binding activity of TBM is augmented by activation modules containing HOB1 and HOB2, which may explain, at least partly, the co-operativity between different c-Fos activation modules.

Here we show that the c-Fos protein contains an

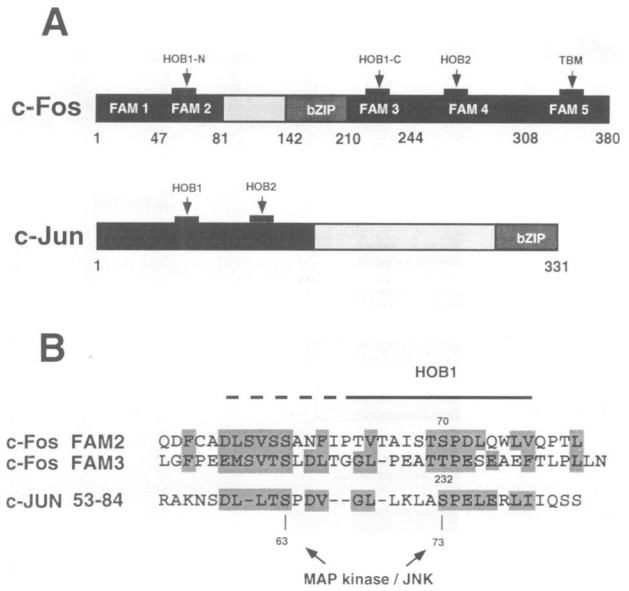

Fig. 1. Activation domains and conserved motifs within the c-Fos and c-Jun proteins. (**A**) The c-Fos protein contains five activation modules, FAM1 to FAM5. Two of these, FAM1 and FAM2, are described here whereas FAM3, 4 and 5 have been described previously (Sutherland *et al.*, 1992). Two of these modules, FAM2 and FAM3, have sequence similarity which includes the previously defined HOB1 motif (designated HOB1-N and HOB1-C respectively). The HOB1 motif, along with a second motif HOB2, is conserved in c-Jun (Sutherland *et al.*, 1992). The TBM in FAM5 mediates binding to TBP (Metz *et al.*, 1994). The bZIP DNA binding domain in c-Fos and c-Jun is indicated. Residues 81–142 in c-Fos contain the inhibitor activity described in this paper. (**B**) Alignment of residues in FAM2, FAM3 and c-Jun 53–84. The position of the previously defined HOB1 motif is indicated by a solid line. The N-terminal extension of HOB1, revealed by this new alignment, is indicated by a broken line. The residues in c-Jun (S63 and S73) which are phosphorylated by MAP kinase and JNK are indicated. The S/T residues in FAM2 and FAM3 which correspond to c-Jun S63 and S73 in this alignment are indicated.

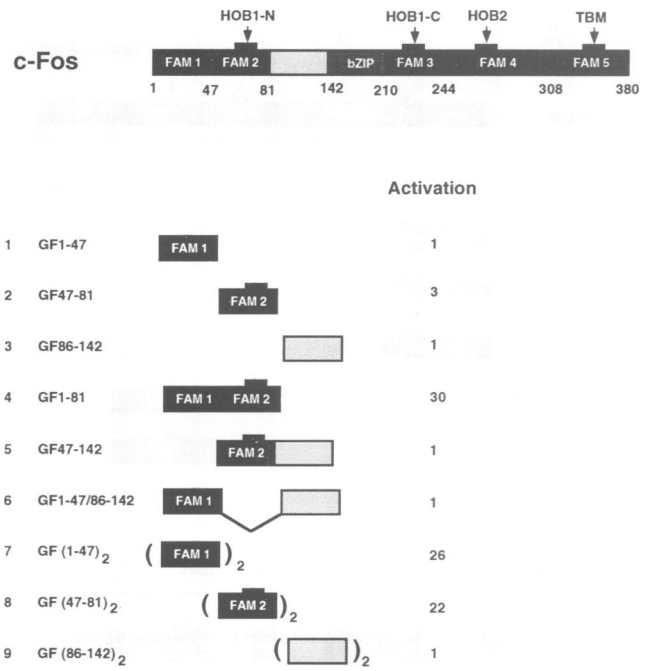

Fig. 2. Identification of activation modules at the N-terminus of c-Fos. Three regions of c-Fos 1–47 (FAM1), 47–81 (FAM2) and 81–142 were linked to the GAL4 DNA binding domain, either individually (lanes 1–3), in combination (lanes 4–6) or duplicated (7–9). These chimeras expressed from an SV40 bearing promoter (1 μg) were co-transfected with a target reporter containing GAL4 sites linked to the CAT gene (pUAS10CAT 4 μg) into 1BR cells. Extracts from these cells were used for CAT assays which were quantified using a PhosphorImager. The activity shown for each c-Fos domain represents the fold induction above that of the GAL4 DBD.

inhibitory domain (ID1) at its N-terminus which acts to silence activation modules containing the HOB1 motif. ID1 can suppress activation when linked *in cis* to two HOB1-containing activation domains from c-Fos and one from c-Jun, but will not silence a heterologous activation domain from CREB. Detailed characterization of the inhibitor sequence highlights a motif containing basic residues which is important for inhibition. Mutagenesis of this motif activates the potency of the c-Fos protein.

Results

c-Fos has modular activation domains at its N-terminus

Our deletion analysis of GAL4−Fos fusions has revealed the presence of activation domains at both the C-terminus and the N-terminus of the c-Fos protein (Sutherland *et al.*, 1992). Extensive analysis of the C-terminal domains revealed the presence of three Fos activation modules (FAMs) at 210–244, 244–308 and 308–380, which we now refer to as FAM3, FAM4 and FAM5 respectively (Figure 1A). FAM3 and FAM4 contain the previously identified HOB1 and HOB2 motifs (which are also present in c-Jun); FAM5 contains the newly identified TBM (Metz *et al.*, 1994).

Close analysis of the N-terminus of c-Fos reveals a stretch of 35 amino acids (FAM2, Figure 1B) with 20% identity and 43% homology to the FAM3 domain of c-Fos. The similarity between FAM2 and FAM3 also extends to c-Jun (Figure 1B). It encompasses sequences at and around S_{63} and S_{73} which are phosphorylated by the MAP kinase/JNK family of proteins (Pulverer *et al.*, 1991; Dérijard *et al.*, 1994; Kyriakis *et al.*, 1994). This alignment reveals that sequences N-terminal to the originally defined HOB1 are conserved between FAM2, FAM3 and c-Jun (dashed line, Figure 1B). This raises the possibility that HOB1 is more extensive than was first recognized and may span the length of these activation modules.

The presence of the HOB1 motif at the N-terminus of c-Fos prompted us to characterize the activation functions of this region. We were particularly interested in whether the new HOB1-containing region had modular activity like that of HOB1-C. Guided by the alignment of the HOB1-containing sequences shown in Figure 1B, we linked regions of the c-Fos N-terminus onto the GAL4 DBD and asked whether they had the characteristics of activation modules. Figure 2 shows that the N-terminus of c-Fos contains two activation modules, FAM1 and FAM2. These two domains do not activate transcription efficiently when they are independently linked to the GAL4 DBD (lanes 1 and 2) but can do so very efficiently and co-operatively when combined with each other (lane 4). In addition, each of these domains can activate co-operatively when duplicated (lanes 7 and 8). In contrast, sequences between FAM2 and the bZIP domain (81–142)

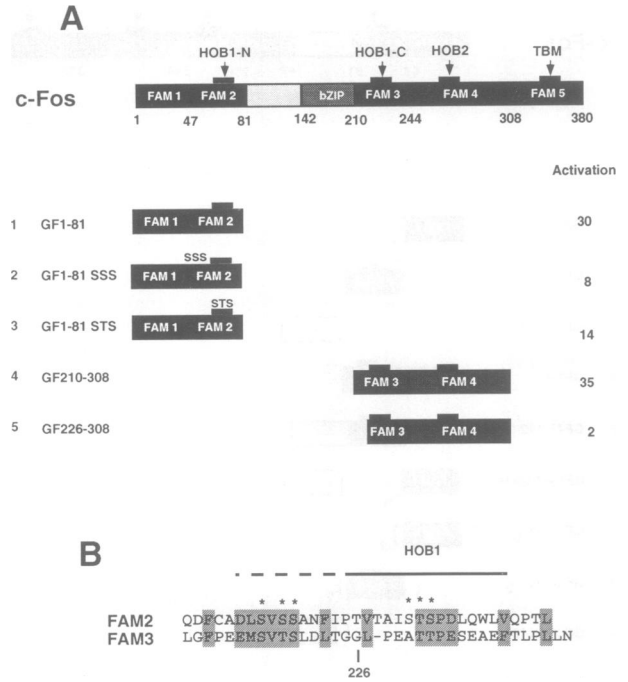

Fig. 3. Sequences N-terminal to HOB1 contribute to the activity of the HOB1-containing modules. (**A**) Domains of c-Fos carrying mutations or deletions within FAM2 or FAM3 were cloned in frame with the GAL4 DBD and assayed for activity on a GAL4 bearing promoter as described in Figure 2. (**B**) Alignment of FAM2 and FAM3 showing the residues mutated or deleted in (A).

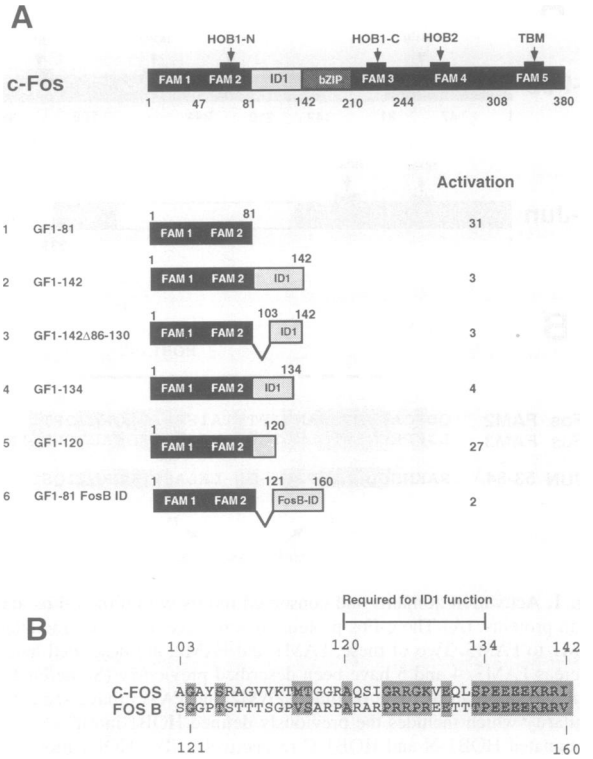

Fig. 4. c-Fos has an inhibitor domain (ID1). (**A**) Domains of the c-Fos protein (or FosB) linked to the GAL4 DBD were introduced into 1BR cells along with a GAL4 site bearing promoter linked to CAT as described in Figure 2. The activity shown represents fold induction relative to the GAL4 DBD. (**B**) Alignment of c-Fos and FosB sequences which mediate inhibition of transcriptional activation. Conserved residues are shaded. The region required for inhibitor function in c-Fos, according to the deletion analysis in (A), is shown.

show none of these characteristics. This region will not activate transcription on its own (lane 3), will not co-operate with FAM1 or FAM 2 (lanes 5 and 6) and will not activate transcription when duplicated (lane 9). These results, along with those reported previously (Sutherland *et al.*, 1992), indicate that c-Fos has five activation modules (FAM1–5). These are defined as domains which have very low activity as GAL4 fusions but which can co-operatively activate transcription when combined with another module or when duplicated.

The alignment in Figure 1 suggests that sequences N-terminal to the originally defined HOB1 motif are contributing to the activity of FAM2 and FAM3. The results in Figure 3 support this interpretation. Deleting these N-terminal sequences in FAM3 (but still retaining HOB1) abolishes co-operativity with FAM4 (Figure 3, lanes 4 and 5). The function of FAM3 is also affected by a point mutation in HOB1 (Sutherland *et al.*, 1992), indicating that the conserved residues throughout the FAM3 domain are required for its activity. The same is true for FAM2: mutagenesis of the HOB1 motif (STS) or mutagenesis of a conserved set of Ser residues (SSS) at the N-terminus of FAM2 affects transcriptional activation (lanes 1, 2 and 3). These results confirm that conserved residues N-terminal to HOB1 contribute to the activity of the HOB1-containing domain of c-Fos. The same is true for c-Jun since mutagenesis of either S_{63} or S_{73} affects activity (Pulverer *et al.*, 1991).

c-Fos has an inhibitor domain

During the course of these experiments we uncovered the presence of a domain (86–142) which acts to inhibit transcriptional activation (Figure 4). The effect of this domain is evident when comparing the activity of c-Fos residues 1–81 (containing FAM1 and FAM2) with that of 1–142 (lanes 1 and 2). Inclusion of the inhibitor domain (ID1, 81–142) reduces the activity of the FAM1/FAM2 combination 10-fold. The effect of inhibition is independent of distance since deleting 22 residues from the N-terminus of ID1 does not affect its inhibitor function (lane 3). Deletions introduced from the C-terminus of ID1 indicate that a region between residues 120 and 134 is required for inhibitor function (lanes 4 and 5).

Sequences within the ID1 are conserved between c-Fos and other Fos related proteins. We therefore tested whether one of these, FosB, has an inhibitor domain in a position similar to that of c-Fos. We chose to analyse FosB because its sequence is the most divergent of the Fos-related proteins in this region and would therefore give us clues about the identity of the residues required for inhibition, if FosB retained inhibitor function. Figure 4, lane 6, shows that FosB sequences 121–160 (equivalent to c-Fos residues 103–142) inhibit the activity of the FAM1/FAM2 combination to the same extent as the c-Fos ID1.

Alignment of the c-Fos and FosB sequences reveals a number of conserved residues (Figure 4B). In particular, within the region required for ID function (120–130 in c-Fos) there is a conserved block of residues with the consensus G/P, R, R, G/P, K/R (IM1). In order to identify residues required for inhibitor function, we mutated this

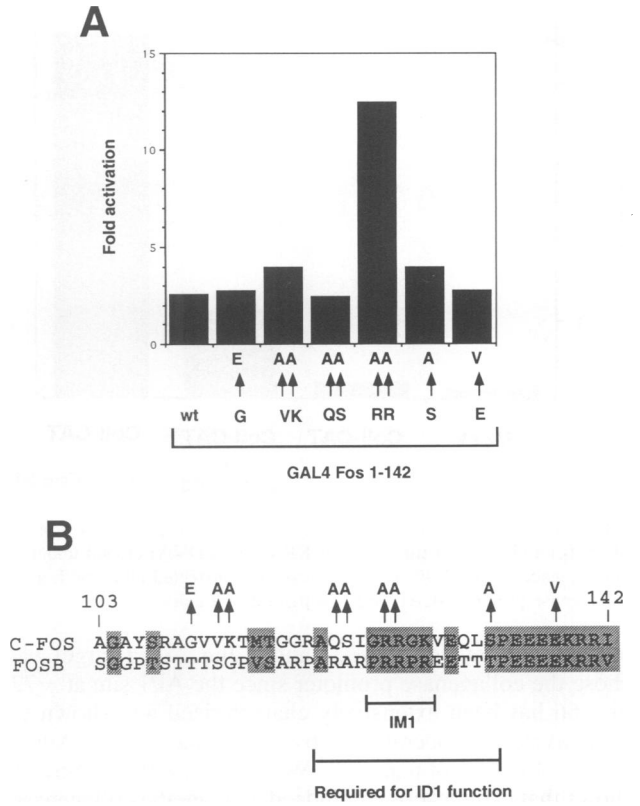

Fig. 5. Point mutations in ID1 relieve inhibitor function. (**A**) Point mutations were introduced into GAL4–Fos 1–142 within the region of the inhibitor domain. The CAT activity of these mutants relative to the GAL4–Fos 1–142 (WT) is shown. The activity represents fold induction above that of the GAL4 DBD. Transfection into 1BR cells was as described in Figure 2. (**B**) Positions of point mutations within the inhibitor domain. Residues conserved between the inhibitor region of c-Fos and FosB are shaded. The region required for ID1 function, according to the deletion analysis in Figure 4, is shown. The inhibitor motif, IM1, is indicated.

conserved motif as well as other residues in this region. Figure 5 shows that changing the two Arg residues within IM1 (RR to AA) severely impairs the ability of ID1 to inhibit activation, and unmasks the activity inherent in residues 1–142. In contrast, mutating a separate, conserved residue (S to A) or other non-conserved residues (AS to AA and VK to AA) had very little effect. These data are perfectly compatible with the results from the deletion analysis in Figure 4 and support the idea that the conserved motif centred around sequences 120–130 is part of the sequence mediating the inhibitor effect.

The v-Fos protein, carried by the transforming retroviruses FBR and FBJ, contains point mutations in the region defined here as ID1 (for review see Müller, 1986). One of these (E to V) has been shown to increase the immortalizing functions of c-Fos (Jenuwein and Müller, 1987). We therefore examined the possibility that these mutations change the capacity of this region to inhibit transcriptional activation. We find that two point mutations, E to V and G to E, present within v-Fos in FBR and FBJ respectively, do not affect inhibitor function (Figure 5). Since these mutations fall outside the region required for ID function, as identified by deletion analysis, they support the conclusion that the sequences essential for inhibitor function lie within residues 120–134 of c-Fos.

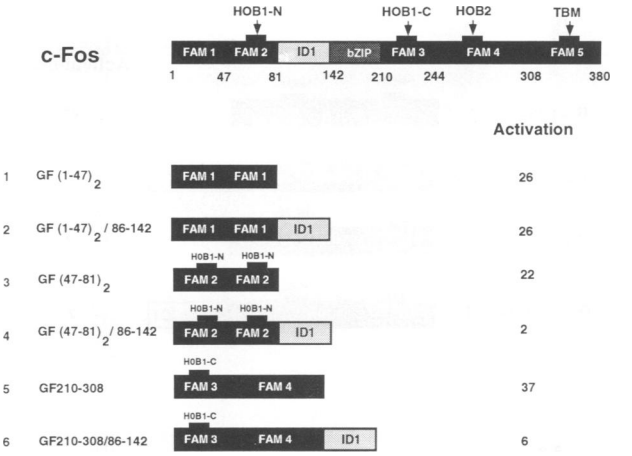

Fig. 6. The ID1 silences a subset of c-Fos activation modules which contain the HOB1 motif. Active GAL4–Fos chimeras which do [GF(47–81)$_2$ and GF210–308] or do not [GF(1–47)$_2$] contain a HOB1 motif were fused to ID1 (86–142) and assayed for CAT activity following co-transfection with pUAS10CAT into 1BR cells as described in Figure 2. The CAT activity shown is relative to the GAL4 DBD.

The inhibitor domain silences HOB1-containing activation domains

Given that ID1 can inhibit the activity of the FAM1/FAM2 combination, we next tried to establish whether the inhibition was specific for the activity of FAM1 or FAM2. The inhibitor domain was linked to an active GAL4 fusion containing either a duplicated FAM1 or a duplicated FAM2 domain. Figure 6 shows that ID1 does not affect the activity of duplicated FAM1 but severely inhibits the activity of the duplicated FAM2 (lanes 1–4). These results suggest that the inhibitor domain will only suppress activity of certain activation domains and that this inhibition is not due to a general effect on the DNA binding ability of GAL4 fusions. Since FAM2 contains a HOB1 motif (HOB1-N) we asked whether a domain containing HOB1-C is also inhibited. Figure 6 shows that GF210–308 containing HOB1-C is indeed inhibited by ID1 (lanes 5 and 6).

These results suggest that ID1 can inhibit a subset of c-Fos activation modules which contain the HOB1 motif. Since c-Jun also contains HOB1, we next asked whether the HOB1-containing activation domain of c-Jun could be inhibited by the c-Fos ID1. Figure 7 shows that indeed this domain of c-Jun is inhibited but that the equivalent region of v-Jun is not (lanes 1–4). The v-Jun protein has sustained a deletion of 27 residues N-terminal to HOB1. This region contains the binding site for JNK which binds to c-Jun and phosphorylates the HOB1 motif at S_{63} and S_{73} (Dérijard *et al.*, 1994). In v-Jun, phosphorylation of these residues is prevented by this deletion (Adler *et al.*, 1992). Consequently v-Jun has an inactive HOB1 motif but can still activate transcription, presumably via a separate activation domain. The inability of ID1 to repress v-Jun activity is therefore consistent with its role as a HOB1 domain specific inhibitor.

Since HOB1 requires phosphorylation for its activity, we asked whether ID1 would inhibit other phosphorylation dependent activation domains, such as that of CREB. The kinase inducible domain (KID) of CREB can activate transcription as a GAL4 fusion but only in response to

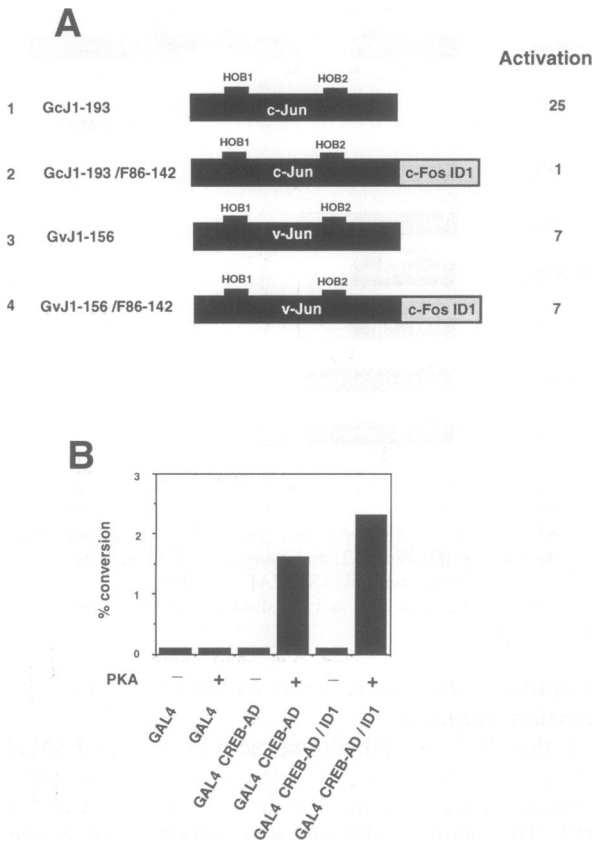

Fig. 7. The c-Fos ID1 can specifically inhibit heterologous activation domains containing the HOB1 motif. (**A**) A GAL4 chimera of the N-terminal activation domain of c-Jun (GcJ1–193) or the equivalent region of v-Jun (GvJ1–156) was linked to the c-Fos ID1 (86–142). These chimeras (1 μg) were co-transfected with a GAL4 bearing promoter (G5-E1bCAT, 4 μg) into 1 BR cells. The CAT activity is relative to the GAL4 DBD. (**B**) A GAL4 chimera containing a duplicated KID from CREB was linked to the c-Fos ID1 and assayed for CAT activity. The GAL4 chimeras (1 μg) were co-transfected along with G5-E1b-CAT (4 μg) into F9 cells in the presence or absence of a PKA expressing plasmid (1 μg).

phosphorylation by protein kinase A (PKA) (Gonzalez *et al.*, 1991). We find that this PKA inducibility is increased to relatively high levels (16-fold) when the KID is duplicated (Figure 7B). Fusing the c-Fos ID1 onto the CREB KID does not inhibit its PKA responsive activation capacity, supporting the idea that ID1 has specificity and can inhibit only a subset of activation domains.

Mutating ID1 elevates c-Fos activation of an AP1 bearing promoter

We next wanted to verify that the ID1 domain represses activity in the context of the full length c-Fos protein. Since ID1 inhibits a subset of c-Fos activation domains, we argued that alleviating this inhibition, by mutating ID1, should increase the overall potency of the c-Fos protein since additional activation modules should now be available. We therefore introduced into c-Fos the RR to AA mutation within IM1, which alleviates inhibition in the context of GAL4 Fos 1–142 (Figure 5). We then compared the transactivating capacity of this c-Fos RR mutant with that of the full length c-Fos protein. These experiments were carried out in F9 cells where the basal level of AP1 bearing promoters is low as a result of low

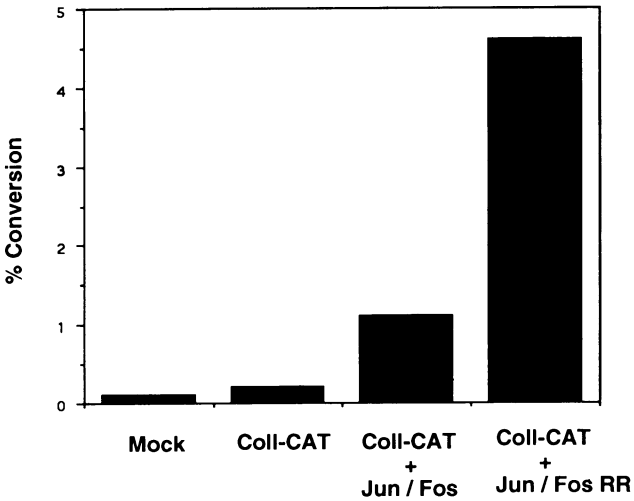

Fig. 8. Mutagenesis of ID1 elevates c-Fos activation capacity. c-Jun (0.15 μg) and c-Fos (1 μg) or c-Fos RR (1 μg) cDNAs cloned under the influence of an SV40 promoter were co-transfected along with a collagenase promoter–CAT target (4 μg) into F9 cells.

endogenous levels of c-Fos and c-Jun. As a target we chose the collagenase promoter since the AP1 site at −72 to −66 has been extensively characterized and shown to be activated co-operatively by c-Jun and c-Fos (Angel *et al.*, 1987; Gutman and Wasylyk, 1990). Figure 8 shows that c-Jun/c-Fos can indeed activate the collagenase promoter, as previously reported. When we use the c-Fos RR mutant under the same conditions we find that c-Jun/c-Fos RR can activate transcription more efficiently than c-Jun/c-Fos, as expected if the RR mutation has allowed additional activation domains to be available.

ID1 binds a limiting protein involved in inhibition

Finally, we wished to examine the mechanism by which ID1 could bring about inhibition. Characterization of other transcription factors such as c-Jun and PHO4 (Baichwal and Tjian, 1990; Jayaraman *et al.*, 1994) suggests the existence of proteins which can mediate inhibition. To establish whether ID1 can bind such a protein we used 'squelching' experiments which have previously been successful in analysing the c-Jun inhibitor. We linked c-Fos residues 86–142 (ID1) to a nuclear localization sequence and asked whether excess free ID1 could alleviate the inhibitory effect of ID1 present within GAL4–Fos 1–142. Such 'derepression' would come about if ID1 was able to compete for the binding of an 'inhibitor' protein bound to GAL4–Fos 1–142. Figure 9 shows that increasing concentrations of c-Fos 86–142 elevate the activation capacity of GAL4–Fos 1–142 (lanes 5–8). This effect is not due to the increased concentration of expression plasmid (lanes 9–11). Furthermore, the squelching activity is specifically directed against Fos residues 1–142 and not the GAL4 DBD since c-Fos 86–142 does not affect the low but detectable activation capacity of GAL4 1–147 (lanes 1–4). These results suggest that c-Fos 1–142 binds a protein involved in inhibition which can be sequestered away when excess ID1 is provided *in trans*.

Discussion

The c-Fos protein possesses a number of activation modules (FAMs) which activate transcription co-

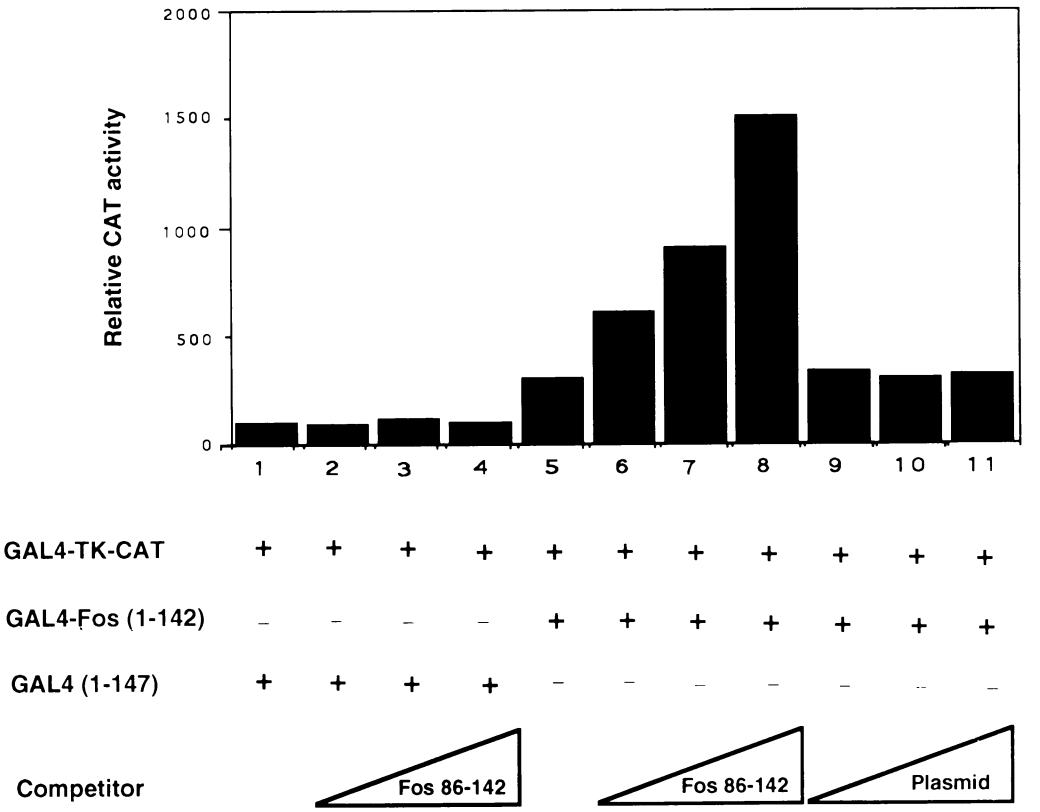

Fig. 9. ID1 binds a limiting protein in squelching experiments. pHKGAL4-Fos 1–142 or pHKGAL4 1–147 (1 μg) was co-transfected along with 4 μg of a GAL4 site bearing TK-CAT reporter within a total DNA concentration of 20 μg made up with salmon sperm (SS) DNA. Where indicated, 5, 8 and 10 μg of competitor DNA were included in place of SS DNA. The plasmid competitor was pHK, which contains the SV40 promoter; the c-Fos 86–142 sequences were expressed from the pHKnt plasmid which has an in frame nuclear localization sequence from nucleoplasmin. The results represent the CAT activity relative to GAL4 (1–147), lane 1, which was given a value of 100.

operatively with other modules. Here we identify two FAMs at the c-Fos N-terminus which (along with the FAMs identified previously) bring the total to five. These modules have very little activity when assayed independently, but when in combination with a second module, they activate transcription synergistically. Several lines of evidence support the notion that these FAMs are units, rather than parts of a larger activation domain. First, these domains can activate transcription efficiently when duplicated (Figure 2). Secondly, mutagenesis and deletion analysis (Figure 3) shows that residues spanning the length of FAM2 and FAM3 are required for their activity. Finally, more circumstantial evidence comes from the exon structure of the c-*fos* gene: the first exon (1–47) corresponds precisely to FAM1, whereas the second exon (48–131) corresponds to FAM2 plus the FAM2-specific inhibitor domain, ID1. Thus the boundary between FAM1 and FAM2, which we originally defined purely on the basis of homology between FAM2 and FAM3, we now recognize to be a natural boundary between exons.

The modularity of activation domains is not unique to c-Fos. A number of other transcription factors have modules whose activity is evident only after duplication (Emami and Carey, 1992; Seipel *et al.*, 1992; Chi and Carey, 1993). What is perhaps surprising about c-Fos is the number of activation modules it possesses. Collectively these modules span almost the entire protein. Indeed, the only domain (outside the bZIP) which shows no transcriptional activation capacity (86–142) has been defined an as inhibitor domain. The complexity of the c-Fos protein probably reflects its multi-faceted nature. A multiplicity of activation modules will allow for more diversity if a particular combination of modules is necessary to activate certain promoters and not others. An example of this redundancy has already been described for FAM5. This module contains a motif (TBM) which is necessary for the activation of a TATA-box bearing promoter but not for a promoter containing only an Inr sequence (Metz *et al.*, 1994).

Why c-Fos has evolved to have two highly homologous activation modules containing the HOB1 motif is unclear. If these two modules (FAM2 and FAM3) function in a similar way, it may be an indication that the potency of this particular domain has to be alleviated beyond a threshold level. An alternative, although not mutually exclusive, explanation is that FAM2 and FAM3 function in a similar way but are regulated differently. These two modules have S/T residues conserved at positions analogous to S_{63} and S_{73} in c-Jun and are therefore likely candidates for regulation by phosphorylation. The kinase responsible may be related to JNK, considering the similarity in sequence surrounding these Ser residues (Figure 1). However, the enzymes phosphorylating FAM2 and FAM3 may be activated by different signalling cascades resulting in the differential use of FAM2 and FAM3.

The HOB1-N containing module identified here (FAM2) overlaps with a region recently shown to be essential for

v-Fos induced transformation (Jooss *et al.*, 1994). This report, which appeared when our work here was in progress, shows that a region between residues 60 and 84 of c-Fos is required for both transcriptional activation and transformation. Consistent with these results we find that mutagenesis of sequences in this region of HOB1-N (STS, Figure 3) affects transcriptional activation. In contrast to the requirement of HOB1-N for v-Fos induced transformation, the HOB1-C motif is not required for this process. This region has suffered a deletion in v-Fos which would abolish the activation capacity of HOB1-C. This may be an indication that HOB1-N and HOB1-C have distinct roles. If they are required for the activation of a distinct set of promoters, then the promoters activated by HOB1-N may be those required to mediate the transformation process.

We have identified a domain, ID1, that can specifically inhibit the activation potential of domains containing the HOB1 motif. The c-Fos ID1 can inhibit HOB1-N and HOB1-C containing activation domains of c-Fos as well as the HOB1-containing activation domain of c-Jun. Regulatory regions, capable of influencing Fos–Jun activity *in vitro*, have been described (Abate *et al.*, 1991). One of these maps in the region of c-Fos ID1. Removal of this domain elevates the activity of the Fos–Jun complex *in vitro*. These results are consistent with the elevation of activity seen on the Fos–Jun complex *in vivo*, following mutagenesis of IM1 (Figure 8). In a recent report Jooss *et al.* (1994) failed to detect ID1 activity when analysing domains at the c-Fos N-terminus. We do not know the reason for this but it could be due to the different conditions used in their experiments. These include different cells (NIH-3T3), different endpoints of c-Fos domains and a different DBD (lex A).

Little is known about sequences which mediate transcription inhibition *in cis* and which are present within transcriptional activators. Inhibitor domains have been described in c-Jun (Baichwal and Tjian, 1990), c-myb (Dubendorff *et al.*, 1992), C/EBP (Nerlov and Ziff, 1994) and the yeast activator PHO4 (Jayaraman *et al.*, 1994; Kaffman *et al.*, 1994). Perhaps the least characterized sequences are those present in c-Jun, where two independent regions (δ and ε) are required to inhibit the A1 activation domain. This bipartite, negative regulator of activation appears to function by binding a tissue specific inhibitor protein. We have no evidence that this cJun inhibitor is related in sequence or function to the inhibitor domain of c-Fos. Firstly, there is no sequence similarity between the δ/ε regions and ID1, and secondly, the c-Jun inhibitor can repress a heterologous activation domain, whereas ID1 cannot.

Inhibitor sequences from a yeast protein PHO4 also appear to be bipartite. Domains spanning either side of the activation domain are required for inhibition. The protein which interacts with this domain and mediates inhibition is PHO80 (Jayaraman *et al.*, 1994). This protein has extensive similarity to yeast cyclins and, along with its cyclin dependent kinase partner PHO85, phosphorylates PHO4 (Dubendorff *et al.*, 1992). We have not detected significant sequence similarity between the inhibitor sequences of PHO4 and c-Fos ID1. The c-myb inhibitor can inhibit transcription not only *in cis* but also *in trans* (Dubendorff *et al.*, 1992). We cannot inhibit activity *in trans* when we use the ID1 domain (our unpublished results) and so we believe the c-myb inhibitor is unrelated to ID1.

More is known about the sequence of repressor domains which are found in repressor proteins. These domains can repress transcription efficiently when directed to the promoter via a heterologous DNA binding domain (Cowell and Hurst, 1994; Witzgall *et al.*, 1994). They can be rich in Ala, Glu or Pro residues, as in the Eve and Kr proteins (Licht *et al.*, 1990; Han and Manley, 1993), or they can be positively charged, as identified using a random screen (Saha *et al.*, 1993). Recently a repressor domain (Krüppel-associated box, KRAB) has been identified in a number of zinc finger proteins. The KRAB sequence, which is ~40 residues long and has a predicted α-helical structure, is sufficient to direct repression (Margolin *et al.*, 1994; Witzgall *et al.*, 1994).

The IM1 motif characterized here has basic residues interspersed with helix breakers such as Gly and Pro. Indeed motifs very similar to this are present in repressor domains isolated by a random screening approach (Saha *et al.*, 1993). Our analysis has identified this motif in a number of other transcription factors, although verification of their authenticity remains to be proven experimentally. However, it is worth noting that a plant transcription factor (Menkens and Cashmore, 1994) with characteristics and limited sequence similarity to c-Fos has this motif conserved at precisely the same relative position.

The identification of a domain which specifically inhibits a subset of activation domains in the Fos–Jun complex raises the question of differential usage of activation domains. When are the HOB1 domains active? Is ID1 inhibition alleviated under certain conditions to allow activity of HOB1 motifs? One possibility is that HOB1 activity depends on the context of the promoter. Evidence for this comes from the C/EBP protein whose inhibitory domain is functional on some promoters but not others (Nerlov and Ziff, 1994). This raises the possibility that ID1 function is dependent on the presence or absence of certain transcription factors on a given promoter. This is an attractive model which may explain the co-operativity between different transcription factors. A particular activator may have the capacity to neutralize the effect of the ID1, possibly by physical contact, thereby increasing the activity from the Fos–Jun complex by allowing the HOB1 motifs to function.

How does ID1 mediate such specific inhibition of activation? This domain does not repress basal transcription when linked to the GAL4 DBD and therefore does not have the characteristics of other defined repressor domains (Cowell and Hurst, 1994; Witzgall *et al.*, 1994). One possibility is that ID1 interacts with a sequence common to all HOB1-containing domains and in doing so masks the activating residues. Using an *in vitro* interaction assay (GST-pull down) we have no evidence that a domain containing HOB1 (Fos1–81) can interact with ID1 in solution. However, these experiments may not detect a weak interaction. Indeed such an interaction may be stabilized when the two domains are in a natural situation, namely within the same protein. An alternative (but not exclusive) possibility, favoured by the results shown in Figure 9, is that ID1 binds a protein which is involved in the inhibition process. Once bound to ID1 such a protein

may affect HOB1 activity in one of several ways. First, it could directly contact residues within HOB1 and mask their activation potential; the extensive similarity outside the originally defined HOB1 motif (Figure 1) may reflect a binding site for such a protein. Second, it could affect the phosphorylation and subsequent activation of the HOB1 motif. Third, it could prevent an interaction between HOB1 and a co-activator protein. Further analysis of ID1 and its associated inhibitory activity will shed more light on the mechanism of transcriptional inhibition by this sequence-specific inhibitor domain.

Materials and methods

Recombinant DNA

For the GAL4 fusion experiments, various domains from c-Fos or other transcription factors were cloned into pHKG (Sutherland *et al.*, 1992) using PCR or engineered restriction sites. pHKG has the GAL4 DBD (1–147) under the control of an SV40 promoter. For activation of the collagenase promoter the c-Fos and c-Jun cDNAs were expressed from the pHK vector (described above) which lacks the GAL4-DBD. The target was collagenase sequences from −517 to −42 linked to a TK-CAT reporter. For the squelching experiments c-Fos sequences were cloned into pHKnt which contains the nucleoplasmin NLS (AVKRPAATKKAGQAKKKKLDKEDESS).

Transfections

Approximately 1×10^6 1BR cells [SV40 T antigen-transformed human skin fibroblasts (Mayner *et al.*, 1986)] grown at 37°C (5% CO_2) in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum were transfected with a total of 7.5 μg of DNA using the calcium phosphate co-precipitation technique. The precipitate was washed 6 h after transfection and the cells were harvested 24 h after transfection. Approximately 2×10^7 F9 cells were seeded on 0.1% gelatin coated plates and grown for 24 h in DMEM supplemented with 10% fetal calf serum. Four hours before transfection HEPES was added to 50 mM. Transfection, using a total of 10 μg of DNA, was carried out using the calcium phosphate co-precipitation technique. After 16 h the cells were washed and allowed to grow for a further 24 h in DMEM/10% FCS with 15 mM HEPES.

Extracts from transfected cells were then used for CAT assays and for Western blots. The CAT assays were quantitated by a PhosphorImager. The Western blots were probed with an antibody against the GAL4 DNA binding domain (gift of M.Ptashne) to check the level of protein expressed by each vector. All plasmids expressed proteins at relatively equivalent levels. Each transfection was repeated a minimum of twice.

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