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An inhibitor domain in Sp3 regulates its glutamine-rich activation domains

Jörg Dennig, Miguel Beato and Guntram Suske[1]

Institut für Molekularbiologie und Tumorforschung, Philipps-Universität Marburg, Emil-Mannkopff-Straße 2, D-35037 Marburg, Germany

[1]Corresponding author

Sp3 is a ubiquitously expressed human transcription factor closely related to Sp1 and Sp4. All three proteins contain a highly conserved DNA binding domain and two glutamine-rich regions, suggesting that they possess similar activation functions. In our previous experiments, however, Sp3 failed to activate transcription. Instead, it repressed Sp1-mediated transcriptional activation, suggesting that it is an inhibitory member of this family of regulatory factors. Here we show that Sp3 can also act as a positive regulator of transcription. The glutamine-rich domains on their own have a strong activation function and interact with the TATA box binding protein (TBP)-associated factor dTAFII110. However, in full-length Sp3 as well as in Gal4–Sp3 fusion proteins, both activation domains are silenced by an inhibitory domain located between the second glutamine-rich region and the DNA binding domain. The inhibitory domain completely suppressed transcriptional activation when fused to a heterologous glutamine-rich domain but only moderately suppressed transcription when linked to an acidic activation domain. Site-directed mutagenesis identified a stretch of highly charged amino acid residues essential for inhibitor function. Substitution of the amino acid triplet KEE by alanine residues within this region changed the almost transcriptionally inactive Sp3 into a strong activator. Our results suggest that the transcriptional activity of Sp3 might be regulated *in vivo* by relief of inhibition.

Keywords: inhibitory domain/repression/Sp1/Sp3/transcription factor

Introduction

Sp3 [originally called SPR-2 (Hagen *et al.*, 1992)], together with Sp1 and Sp4, forms a small family of human transcription factors with very similar structural features. All three proteins contain a highly conserved DNA binding domain close to the C-terminus, and two less conserved glutamine- and serine/threonine-rich amino acid stretches in the N-terminal part of the molecule. Consistent with the high conservation of the DNA binding zinc finger region, all three proteins recognize the classical GC box (GGGCGGG) and related motifs with very similar affinity. In contrast to Sp4, which is expressed predominantly in the brain, Sp3 and Sp1 are ubiquitous proteins (Hagen *et al.*, 1992 and our unpublished results) localized exclusively in the cell nucleus (Birnbaum *et al.*, 1995).

The high degree of structural conservation between Sp1, Sp3 and Sp4 suggested that they exert similar activation functions. Functional analyses, however, demonstrated that Sp3 and Sp4 are not simply functional equivalents of Sp1. Although Sp4 acts as a positive regulator by stimulating transcription from Sp1-responsive promoters, unlike Sp1 it cannot activate synergistically through adjacent binding sites (Hagen *et al.*, 1995). Sp3 failed to activate Sp1-responsive promoter constructs. Instead, it repressed Sp1-mediated transcriptional activation, suggesting that Sp3 is an inhibitory member of this family of regulatory factors (Majello *et al.*, 1994; Hagen *et al.*, 1995). This conclusion was supported further by the finding that a fusion protein consisting of the yeast Gal4 DNA binding domain and the N-terminal part of Sp3 containing both glutamine-rich domains did not activate Gal4-responsive promoters (Majello *et al.*, 1994; Hagen *et al.*, 1995; Figure 1). The intriguing finding that Sp1 and Sp3 can exert opposite transcriptional regulation prompted us to analyze further the transcriptional properties of the Sp3 protein.

Here, we have explored the Sp3 protein in detail by mutational analysis performing co-transfection experiments in mammalian cells and in insect cells, which lack endogenous Sp factors. Our studies demonstrate that both glutamine-rich regions of Sp3 are strong activation domains similar to those present in Sp1 and Sp4. However, these activation modules are silenced by the presence of an inhibitory domain located between the second glutamine-rich region and the zinc fingers. The inhibitory domain can also suppress activation completely when linked to a glutamine-rich activation domain of Sp1 but only moderately when linked to the acidic activation domain of VP16. Detailed characterization of the inhibitor sequence highlighted a motif of highly charged residues important for inhibitor function. Point mutations within this region relieved inhibitor function and changed Sp3 from an almost transcriptionally inactive molecule into a strong activator. We show further that Sp3 can interact functionally with dTAFII110 and that the inhibitory domain does not interfere with dTAFII110 interaction in a superactivation assay.

Results

Sp3 has the potential to activate transcription: both glutamine-rich domains of Sp3 exert activation function

Our previous functional analysis of Sp3 in direct comparison with Sp1 has revealed that it does not exert an activation function (Hagen *et al.*, 1994, 1995) although

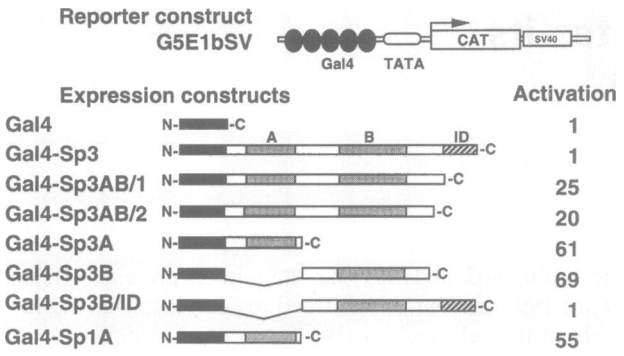

Fig. 1. Identification of activation and silencing modules in Sp3. Ishikawa cells were transfected with 2 μg of G5E1bSV along with 2 μg of expression plasmids for Gal4, Gal4–Sp3, Gal4–Sp3AB/1, Gal4–Sp3AB/2, Gal4–Sp3A, Gal4–Sp3B, Gal4–Sp3B/ID and Gal4–Sp1A as indicated. The cells were subsequently lysed and assayed for CAT activities. The fold activation shown for each Gal4–Sp3 construct is expressed relative to the CAT activity obtained with the Gal4 DNA binding domain (Gal4), which has been given the arbitrary value of 1. The mean value of at least two independent transfections are displayed.

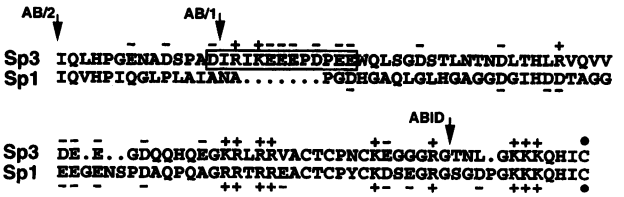

Fig. 2. Amino acid sequence alignment of the Sp3 inhibitory domain with the corresponding region of Sp1. The filled circles depict the first cysteine of the Sp1 and Sp3 zinc finger region. Positively (+) and negatively (–) charged amino acids are indicated. The arrows indicate the deletion endpoints of the Gal4 constructs Gal4–Sp3 (ABID), Gal4–Sp3AB/1 (AB/1) and Gal4–Sp3AB/2 (AB/2) shown in Figure 1. The highly charged amino acid stretch present in Sp3 but not in Sp1 is boxed.

both glutamine-rich regions share similarity with the two activation domains of Sp1, which are characterized by an array of glutamine residues interspersed by hydrophobic amino acids (Gill *et al.*, 1994). Close inspection of the glutamine-rich regions of Sp3 revealed a similar array of glutamine and bulky hydrophobic residues, suggesting that these domains may have the potential to activate transcription.

To test this idea, we fused both glutamine-rich regions of Sp3 (from here on referred to as domains A and B) independently to the DNA binding domain of the yeast transcription factor Gal4 (Gal–Sp3A and Gal–Sp3B in Figure 1) and performed transfection experiments in the mammalian cell line Ishikawa using a Gal4-responsive promoter construct as the reporter gene [G5E1bSV in Figure 1 (Hagen *et al.*, 1995)]. These experiments revealed that both glutamine-rich domains of Sp3 can stimulate transcription as efficiently as a corresponding activation domain of Sp1 (Gal4–Sp1A, Figure 1). This finding was surprising, since the construct Gal4–Sp3 containing the N-terminal part of Sp3 encoding both glutamine-rich regions (amino acids 1–484 according to Kingsley and Winoto, 1992) fused to the Gal4 DNA binding domain did not activate transcription from the G5E1bSV reporter construct. However, an analogous Gal4–Sp1 fusion protein stimulated transcription efficiently (Hagen *et al.*, 1995).

The activation domains of Sp3 are silenced by an inhibitory domain

To uncover the molecular basis for the inactivity of the complete N-terminal region of Sp3, we constructed and tested deletion mutants of Gal4–Sp3 (Gal4–Sp3AB/1 and Gal4–Sp3AB/2 in Figure 1) by removing 64 and 79 amino acids, respectively, from the C-terminal end of Gal4–Sp3. Both constructs activated transcription of G5E1bSV by >20-fold. Electrophoretic mobility shift assay (EMSA) experiments revealed that all three proteins are expressed at similar levels after transfection (see Figure 3D). It should be noted that the short expression constructs bearing only a single glutamine-rich region (Gal4–Sp3A and Gal4–Sp3B) were expressed at higher levels compared with the long constructs (Gal4–Sp3, Gal4–Sp3AB/1 and Gal4–Sp3AB/2) containing both glutamine-rich domains (data not shown), thus explaining the stronger activation potential of these constructs (compare Gal4–Sp3A and Gal4–Sp3B with Gal4–Sp3AB/1 and Gal4–Sp3AB/2 in Figure 1).

Our results suggest that the activation domains of Sp3 are silenced by an inhibitory domain located between the second glutamine-rich region and the DNA binding domain. This interpretation was supported by another Gal4–Sp3 mutant (Gal4–Sp3B/ID). Gal4–Sp3B/ID contains the glutamine-rich region B of Sp3 in conjunction with the 79 amino acids located between region B and the first zinc finger of the DNA binding domain. This construct did not stimulate CAT transcription. The inhibitory domain of Sp3 is thus able to inhibit activation function when fused to a single Sp3 glutamine-rich activation domain.

Point mutations in the inhibitory domain of Sp3 relieve inhibitor function

An alignment of the amino acid sequence of the inhibitory domain of Sp3 with the corresponding sequences of Sp1 and Sp4 revealed a highly charged region of 13 amino acids [DIRIKEEEPDPEE (amino acids 419–431 according to Kingsley and Winoto, 1992)] present in Sp3 but not in Sp1 (Figure 2) and Sp4. To evaluate the role of this region for inhibitor function, we generated a small deletion in Gal4–Sp3 (SD in Figure 3A) and tested the activation property of this mutant with the G5E1bSV reporter construct (Figure 3B). Introduction of the small deletion turned inactive Gal4–Sp3 into an active form, showing that the presence of these 13 amino acids is required for inhibitor function.

To delineate further the amino acids necessary for inhibitor function, we replaced several charged residues within this region by alanine residues (Figure 3A). In addition, an adjacent serine residue was mutated to an alanine or an aspartic acid residue, respectively. All mutants were expressed at similar levels (Figure 3D and data not shown). Substitution of the single arginine (R421) and the serine residue (S416) as well as substitution of the triplet PEE (amino acids 429–431) by alanines did not impair the activity of Gal4–Sp3 (Figure 3B). However, substitution of the KEE triplet (amino acids 423–425) by alanines completely relieved the inhibitor function of the domain. Thus, the integrity of the triplet KEE is required to silence the activation domains in Gal4–Sp3. These data

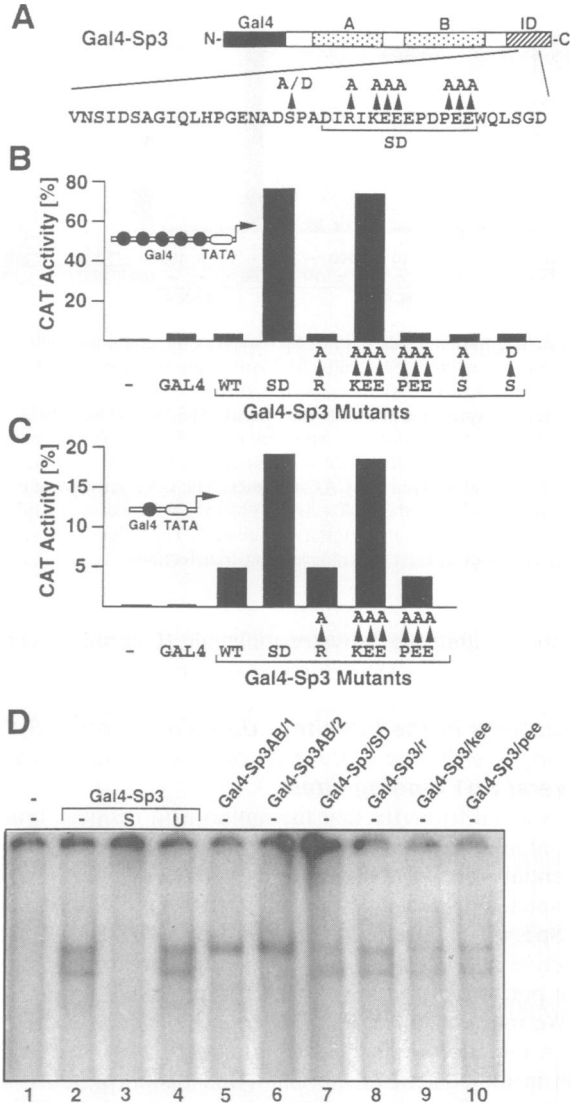

Fig. 3. Point mutations in the inhibitory domain of Sp3 relieve inhibitor function. (**A**) Point mutations (S to A, S to D, R to A, KEE to AAA and PEE to AAA) or a small deletion (SD) were introduced into Gal4–Sp3 within the region of the inhibitory domain as indicated. (**B**) and (**C**) Transfections into Ishikawa cells were performed as described in Figure 1 with G5E1bSV (B) and G1E1bSV (C) as reporter constructs. The CAT activity of the Gal4–Sp3 mutants is shown. (**D**) Transient expression of Gal4–Sp fusion proteins in Ishikawa cells. Gel retardation assays were performed with crude nuclear extracts from Ishikawa cells transfected with 8 μg of expression plasmids for Gal4–Sp3 (lanes 2, 3 and 4), Gal4–Sp3AB/1 (lane 5), Gal4–Sp3AB/2 (lane 6), Gal4–Sp3/SD (lane 7), Gal4–Sp3/r (lane 8), Gal4–Sp3/kee (lane 9), Gal4–Sp3/pee (lane 10) or mock DNA (pUC8 plasmid) (lane 1). All reactions contained 0.2 ng of labeled Gal4 oligonucleotide and 2.5 μg of protein extract. In lanes 3 and 4, a 100-fold molar excess of unlabeled Gal4 oligonucleotide (S) or non-specific oligonucleotide (U) was included in the binding reaction.

are compatible with the results obtained from the deletion analysis shown in Figure 1 and support the idea that the highly charged region of 13 amino acids is at least part of the domain mediating inhibitor function.

In the course of studying the activation properties of Gal4–Sp3 mutants, we also generated a reporter construct that contains only a single Gal4 binding site fused to the E1bTATA box (G1E1bSV in Figure 3C). Surprisingly,

Table I. Point mutations in the inhibitory domain of Sp3 relieve inhibitor function in CV-1, COS-1 and NIH 3T3 cells

Activator	Relative CAT activity		
	CV-1	COS-1	NIH 3T3
–	0.1	0.2	0.1
Gal4	1	1	1
Gal4–Sp3	0.4	1.5	1.8
Gal4–Sp3/kee	3.7	6.0	17
Gal4–Sp3/r	0.6	1.1	1.4

wild-type Gal4–Sp3 already activated transcription of this reporter construct (Figure 3C). However, deletion of the 13 charged amino acids or substitution of the KEE triplet by AAA residues, but not the substitution of the PEE by AAA residues, further enhanced CAT gene expression from this reporter (4-fold). The repression potency of the inhibitory domain of Sp3 thus appears to be much less pronounced on a promoter containing only a single binding site (see also below).

The transfection experiments described so far have been performed with Ishikawa cells, a cell line derived from a human endometrial carcinoma. To investigate whether the inhibitory effect of the ID domain of Sp3 could also be seen in other mammalian cell lines or whether it acts in a cell type-specific manner, we tested the transcriptional activity of Gal4–Sp3 and its derivatives Gal4–Sp3/kee and Gal4–Sp3/r in CV-1, COS-1 and NIH 3T3 cells. In all three cell lines Gal4–Sp3 was inactive, and the KEE/AAA mutation (in Gal4–Sp3/kee), but not the R/A mutation (in Gal4–Sp3/r), relieved the inhibitor function of the inhibitory domain of Sp3 (Table I). Thus, it appears likely that the inhibitory domain functions in many if not all cell types.

The inhibitory domain of Sp3 silences heterologous glutamine-rich activation domains

Given that the inhibitory domain of Sp3 silences the activity of the Sp3 activation domains, we tried to establish whether the inhibition was specific for the activity of these two domains or whether heterologous activation domains are also influenced by the presence of this sequence. The inhibitory domain was linked to the glutamine-rich activation domain A of the transcription factor Sp1 (Gal4–Sp1A/IDwt) and to the strong acidic activation domain of the viral protein VP16 (Gal4–VP16/IDwt), respectively. Figure 4 shows that the presence of the inhibitory domain completely abolished the activity of the glutamine-rich activation domain A of Sp1. To demonstrate that the inhibitory effect is specific for the wild-type inhibitory domain, we introduced the KEE to AAA mutation, which abolished inhibitor function in the Gal4–Sp3 construct, into the inhibitory domain (Gal4–Sp1A/IDkee in Figure 4). This mutated domain did not impair the activation capacity of the Sp1 activation domain (Figure 4), demonstrating that it is not the C-terminal extension of Gal4–Sp1A *per se* but rather the nature of the protein sequence which is responsible for inhibition. Fusion of the inhibitory domain to the VP16 acidic domain reduced the activation capacity of the Gal4–VP16 fusion protein only moderately (3.8-fold). However, this effect seems to be specific since the KEE to AAA mutation also relieved

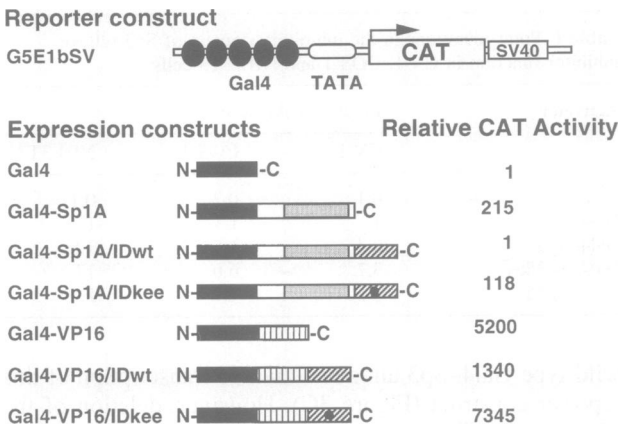

Fig. 4. The Sp3 inhibitory domain specifically inhibits glutamine-rich activation domains. Fusion proteins consisting of the Gal4 DNA binding domain linked to the glutamine-rich activation domain A of Sp1 or the acidic activation domain of VP16 were coupled to the wild-type inhibitory domain (Gal4–Sp1A/IDwt and Gal4–VP16/IDwt) or the inhibitory domain containing the KEE to AAA mutation (Gal4–Sp1A/IDkee and Gal4–VP16/IDkee). These constructs (2 μg) were co-transfected with a Gal4-bearing promoter (G5E1bSV, 2 μg) into Ishikawa cells. The relative CAT activity shown for each chimeric Gal4 construct is expressed relative to the CAT activity obtained with the Gal4 DNA binding domain (Gal4), which has been given the arbitrary value of 1.

inhibitor function in this construct. These results suggest that the inhibitory domain of Sp3 has specificity and can inhibit glutamine-rich activation domains much more efficiently than acidic activation domains.

The inhibitory domain of Sp3 silences the glutamine-rich activation domains in SL2 cells

In transiently transfected *Drosophila* Schneider SL2 cells lacking Sp1-like activity, Sp3 was almost inactive on a promoter bearing two GC boxes (Hagen *et al.*, 1994). To see whether the glutamine-rich domains of Sp3 can exert principally an activation function in SL2 cells and whether the activation would be dependent on the absence of the inhibitory domain also in this insect cell line, we constructed and tested several internal deletion mutants of Sp3 (Figure 5). As reporter constructs we used the plasmids BCAT-2 and pSV2CAT. BCAT-2 contains two Sp1 binding sites fused to the E1bTATA box (Pascal and Tjian, 1991). In pSV2CAT, the CAT gene is driven by the SV40 promoter that contains six GC boxes (Gorman *et al.*, 1982). In the presence of the inhibitory domain, almost no activation was observed with Sp3 expression constructs that contain either of the activation domains A or B (Sp3ΔB and Sp3ΔA in Figure 5). However, when the glutamine-rich domain A was fused adjacent to the DNA binding domain of Sp3 thereby removing the inhibitory domain (Sp3ΔBID), with 100 ng of expression plasmid we obtained a 33-fold and 15-fold activation of BCAT-2 and pSV2CAT, respectively. These results show that at least the glutamine-rich domain A of Sp3 can exert an activation function in SL2 cells and that the inhibitory domain is able to silence this activity. When comparing these results with the previous results obtained with Gal4–Sp3 fusion proteins (see above), these experiments further show that the inhibitory domain functions independently of the nature of the DNA binding domain (Gal4 versus Sp3 DNA binding domain) and independently of its

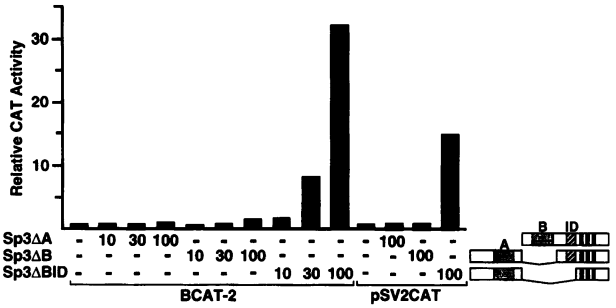

Fig. 5. The inhibitory domain of Sp3 silences the glutamine-rich activation domains in SL2 cells. SL2 cells were transfected with 8 μg of BCAT-2 or pSV2CAT along with variable amounts (10, 30 or 100 ng) of expression plasmids for Sp3ΔA, Sp3ΔB and Sp3ΔBID, respectively, as indicated. The structure of the Sp3ΔA, Sp3ΔB and Sp3ΔBID proteins is illustrated schematically. The cells subsequently were lysed and assayed for CAT activities. The CAT activities are expressed relative to the CAT activity obtained with the vector (pPac), which has been given the arbitrary value of 1. The values represent mean values of at least two independent transfections.

position within the activator molecule (C-terminal versus N-terminal to the DNA binding domain).

Mutations in the inhibitory domain of native Sp3 strongly enhance activation of promoters bearing several Sp1 binding sites

We wanted to verify that the amino acid residues crucial for inhibitor function in Gal4–Sp3 fusion proteins are also essential for silencing the activation potential of the complete Sp3 molecule. Mutations in the inhibitory domain of Sp3 which changed Gal4–Sp3 from a transcriptionally inactive into an active protein should increase the activation potency of the full-length Sp3 protein.

We introduced the small deletion (SD) and the KEE to AAA mutation (Figure 6A), which both alleviated inhibition in the context of the Gal4–Sp3 fusion proteins, into full-length Sp3. As a control, we introduced the PEE to AAA mutation that had no effect on Gal4–Sp3 activation. Band-shift analysis showed that all four proteins were expressed at comparable levels after transfection (Figure 6B). As a target for Sp3 and its mutants, we used, in addition to the SV40 promoter–CAT plasmid (pSV2CAT) and BCAT-2, BCAT-1, which contains only a single Sp1 binding site fused to the E1bTATA box (Pascal and Tjian, 1991). Constant amounts of the reporter plasmids along with variable amounts of Sp3 expression vectors were transfected into SL2 cells and the CAT activity of the resulting cell extracts was plotted as a function of the amount of DNA used to transfect the cells (Figure 6C–E). Wild-type Sp3 already slightly activated CAT gene expression from pSV2CAT in a dose-dependent manner. However, when we used the Sp3 mutants containing either the 13 residue deletion or the KEE to AAA mutation, transcription from the SV40 promoter occurred up to 10-fold more efficiently (Figure 6C). In contrast, the Sp3/pee mutant behaved like the wild-type protein. The effect of the KEE to AAA and SD mutations was even more pronounced when we used BCAT-2 as reporter. On BCAT-2, wild-type Sp3 and the Sp3/pee mutant were almost inactive, whereas the mutant proteins Sp3/kee and Sp3/SD activated transcription from BCAT-2 up to 1000-fold (Figure 6D). The effect of the Sp3 mutants on the

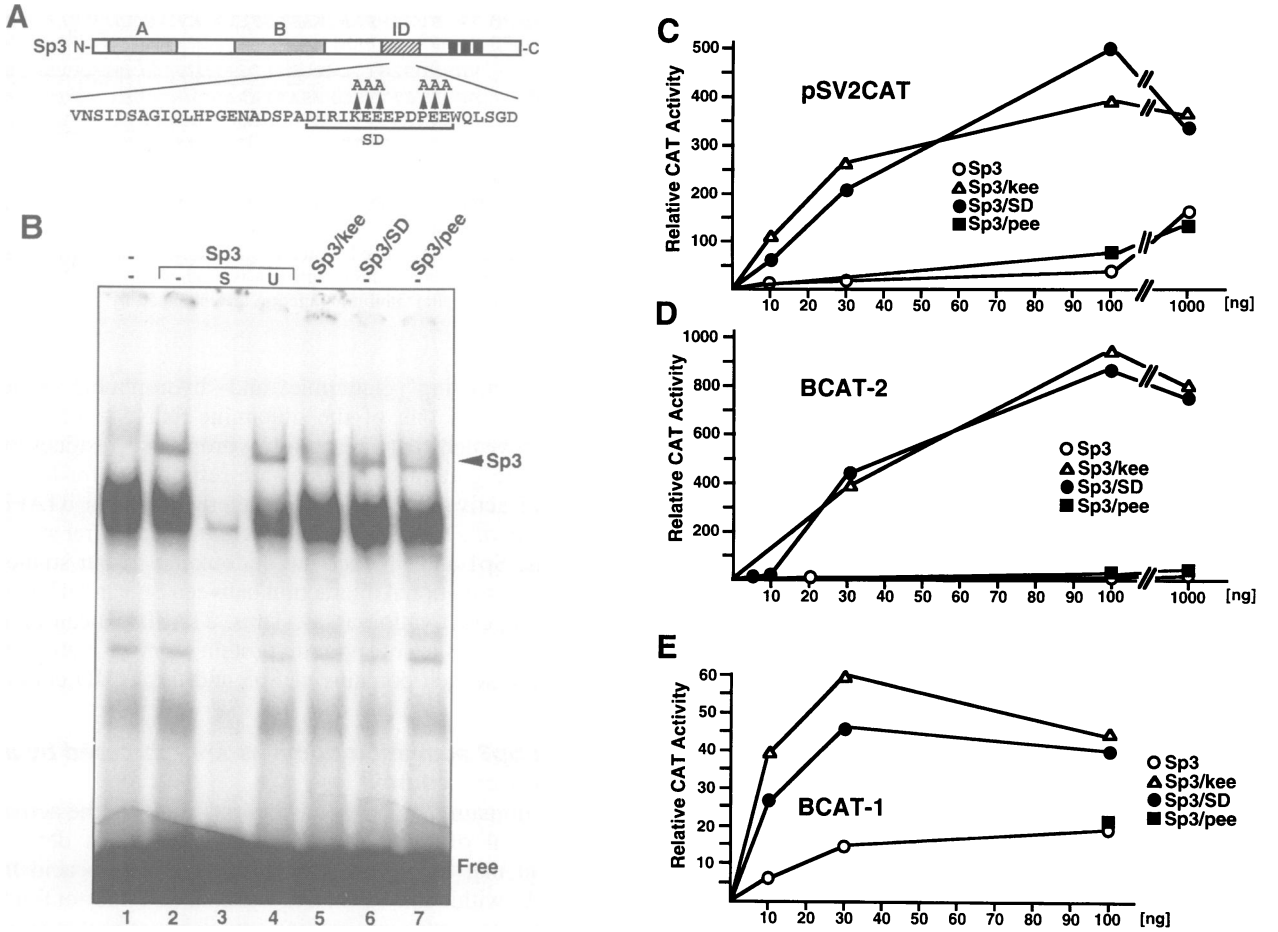

Fig. 6. Point mutations in the inhibitory domain strongly enhance Sp3 activation capacity. (**A**) The small deletion (SD) and the point mutations KEE to AAA and PEE to AAA were introduced into the intact Sp3 molecule within the region of the inhibitory domain as indicated. (**B**) Transient expression of Sp3 mutant proteins in SL2 cells. Gel retardation assays were performed with crude nuclear extracts from SL2 cells transfected with 8 μg of expression plasmids for Sp3 (lanes 2, 3 and 4), Sp3/kee (lane 5), Sp3/SD (lane 6), Sp3/pee (lane 7) or mock DNA (pPac plasmid) (lane 1). All reactions contained 0.2 ng of labeled GT oligonucleotide and 2.5 μg of protein extract. In lanes 3 and 4, a 100-fold molar excess of unlabeled GT oligonucleotide (S) or non-specific oligonucleotide (U) was included in the binding reaction. (**C**) SL2 cells were transfected with 8 μg of pSV2CAT along with variable amounts of expression plasmids for Sp3 and the appropriate Sp3 mutants (Sp3/kee, Sp3/SD and Sp3/pee). The CAT values are expressed relative to the CAT activity obtained with the vector (pPac), which has been given the arbitrary value of 1. The values represent mean values of at least two independent transfections. (**D**) and (**E**) SL2 cells were transfected with the same expression plasmids as in (B) along with 8 μg of BCAT-2 (D) or BCAT-1 (E) as reporter plasmids.

promoter with a single GC box (BCAT-1), however, was much less pronounced. Similarly to the results obtained with Gal4–Sp3 on a reporter construct containing a single Gal4 binding site, transcription from BCAT-1 was already activated by wild-type Sp3 (Figure 6E). Nevertheless, the Sp3/kee and the Sp3/SD but not the Sp3/pee mutant further enhanced CAT gene expression up to 4-fold.

Taken together, our transfection experiments demonstrate that the inhibitory domain of Sp3 is active in the context of the full-length Sp3 molecule. The previously observed inactivity of full-length Sp3 on the GC box-bearing promoter BCAT-2 in SL2 cells (Hagen *et al.*, 1994) thus was at least partially due to the pronounced silencing effect of the inhibitory domain on this promoter construct.

The inhibitory domain of Sp3 does not interfere with dTAFII110 interaction in a superactivation assay

Sp1 binds and requires dTAFII110 for activation *in vitro* (Hoey *et al.*, 1993; Gill *et al.*, 1994). It is assumed, therefore, that dTAFII110 functions as a co-activator by serving as a site of protein–protein contacts between Sp1 and TFIID. Consequently, we asked whether Sp3 could also interact functionally with dTAFII110 and whether the inhibitory domain would interfere with this interaction.

A Gal4–dTAFII110 fusion construct [Gal4–dTAFII110-(N308) in Hoey *et al.*, 1993] and the Gal4 DNA binding sites containing reporter construct G5E1b–CAT were transfected into SL2 cells along with DNA binding-deficient mutants of Sp3 containing either the wild-type inhibitory domain (Sp3ΔDBD) or the mutated inhibitory domain (Sp3/SDΔDBD), respectively (Figure 7). Co-transfection of the Sp3ΔDBD expression construct led to a strong increase in transcription from G5E1b–CAT (up to 10-fold). This superactivation is dependent on the dTAFII110 sequences since Sp3ΔDBD was unable to stimulate transcription when co-transfected with the Gal4 DNA binding domain alone. Superactivation of Gal4–dTAFII110(N308) by a DNA binding-deficient Sp3 construct is similar when the inhibitory domain of Sp3 is mutated. Thus, the inhibitory domain present in Sp3ΔDBD does not interfere

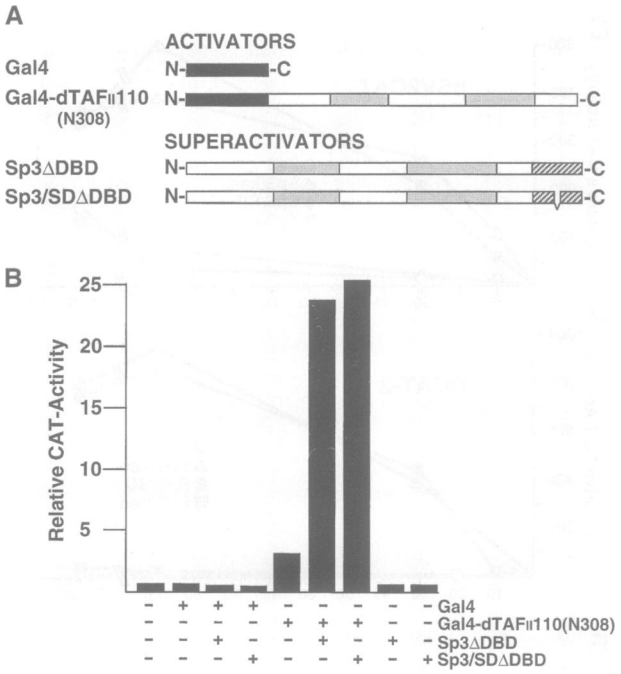

Fig. 7. The inhibitory domain of Sp3 does not interfere with dTAFII110 interaction in a superactivation assay. (**A**) Schematic illustration of the activator plasmids Gal4 and Gal4–dTAFII110(N308) (activators) and the fingerless mutants Sp3ΔDBD and Sp3/SDΔDBD (superactivators). The black and the stippled boxes indicate the DNA binding domain of Gal4 and the glutamine-rich domains of dTAFII110(N308) and Sp3, respectively. The hatched box indicates the Sp3 inhibitory domain. (**B**) Five micrograms of the reporter G5E1b were transfected along with 100 ng of the activators Gal4 or Gal4–dTAFII110(N308) in the absence or presence of 1 μg of the superactivator Sp3ΔDBD or Sp3/SDΔDBD, respectively, as indicated.

with the functional interaction of the glutamine-rich activation domains of Sp3 with dTAFII110. Prevention of an interaction between the activation domains of Sp3 and dTAFII110, thus, very probably does not account for the low activity or inactivity, respectively, of complete Sp3.

Discussion

Sp3 has the potential to activate transcription

In our previous studies, we found that Sp3 did not act as a transcriptional activator like Sp1 or Sp4 (Hagen *et al.*, 1994, 1995; Majello *et al.*, 1994). Instead, Sp3 repressed Sp1- and Sp4-mediated activation and was therefore considered as an inhibitory member of the Sp transcription factor family (Hagen *et al.*, 1994, 1995). In another recent report, however, Sp3 has been described as an activator (Udvadia *et al.*, 1995). The picture of Sp3 that emerges from the present study is much more complex and clarifies this apparent contradiction. The Sp3 protein has the potential to activate transcription. It possesses at least two strong transcriptional activation domains which both, however, can be silenced by an inhibitory domain that is located between the second glutamine-rich domain and the zinc finger region.

Both glutamine-rich activation domains of Sp3 share significant similarity with those described for Sp1 (Pascal and Tjian, 1991; Gill *et al.*, 1994), Sp4 (Hagen *et al.*, 1995) and cAMP response element binding protein (CREB; Gill *et al.*, 1994). These activation domains are characterized

Fig. 8. The inhibitory domain of Sp3 shows similarity with the inhibitory domains of C/EBPβ and c-Fos/FosB. Residues conserved between the inhibitory regions of Sp3, rat C/EBPβ, human C/EBPβ, FosB and c-Fos are boxed. Mutations in the inhibitory domains of Sp3 and c-Fos relieving inhibitor function are indicated by +. Mutations which did not alter inhibitor function are indicated by –.

by alternating glutamine and hydrophobic residues. Mapping of one of the glutamine-rich domains of Sp1 had revealed that the bulky hydrophobic residues rather than the glutamine residues are responsible for transcriptional activation and for the interaction with dTAFII110 (Gill *et al.*, 1994). Consistent with the structural similarity of the Sp1 and Sp3 activation domains, in our studies we found a functional interaction between Sp3 and dTAFII110 in a superactivation assay. Thus, dTAFII110 can function also as a site of protein–protein contact between the glutamine-rich domains of Sp3 and the TFIID complex.

The Sp3 activation domains are repressed by an inhibitory domain

The domain that is responsible for silencing the activation potential of Sp3 has been characterized in detail. We found that the integrity of a charged amino acid triplet (KEE) within this domain is essential for inhibitor function. There is little information available about sequences which mediate transcriptional inhibition in *cis*, although inhibitory domains have been described in several transcription factors including c-Jun (Baichwal and Tjian, 1990; Baichwal *et al.*, 1991), NGFI-A (also called Egr-1, Krox 24 and Zif268) (Gashler *et al.*, 1993; Russo *et al.*, 1993), C/EBP (Nerlov and Ziff, 1994), c-Myb (Dubendorff *et al.*, 1992), B-Myb (Tashiro *et al.*, 1995), ATF-2 (Li and Green, 1996), the yeast activator PHO4 (Jayaraman *et al.*, 1994) and c-Fos and FosB (Brown *et al.*, 1995).

The inhibitory domains of C/EBPβ [RD1 in (Williams *et al.*, 1995)] and c-Fos/FosB (Brown *et al.*, 1995) share some similarity with the inhibitory domain of Sp3 (Figure 8). In c-Fos, residues which are necessary for inhibition of c-Fos activation have been mapped. The only mutation which relieved inhibitor function in Fos was the replacement of two arginine residues by alanine residues. One of these two arginine residues is conserved between c-Fos/FosB and Sp3 (Figure 8). Substitution of the conserved arginine residue in Sp3, however, did not relieve Sp3 inhibitor function. Moreover, the Fos inhibitory domain specifically inhibited heterologous activation domains containing the HOB1 motif but not the activation domain of CREB. A different specificity for a subset of activation domains seems to apply for the Sp3 inhibitory domain. It completely inhibited the glutamine-rich activation domains of Sp3 and Sp1 but the acidic activation domain of VP16 was inhibited to a much smaller extent. Thus, it seems unlikely that the inhibition exerted by the inhibitory domains of c-Fos/FosB and Sp3 are identical, although similar mechanisms might be at work. Further experiments will be necessary to unravel the functional relationship

between the inhibitory domains of Sp3, c-Fos/FosB and C/EBPβ.

How might the inhibitory domain mediate inhibition of activation?

The number of binding sites in a promoter appears to be one determinant of the strength of inhibition. Most significantly, promoters with a single Gal4 binding site (G1E1bSV in Figure 3) or a single GC box (BCAT-1 in Figure 6) already showed significant activation by Gal4–Sp3 or wild-type Sp3, respectively. Mutations which relieved inhibitor function enhanced the activation potential of Sp3 on these promoters only to a small extent. However, it should be noted that the inhibitory domain does not simply repress synergistic activation since the activation of a promoter with a single Gal4 site or GC box was stronger in comparison with a construct containing five Gal4 sites or two GC boxes. Possibly, the association of two Sp3 molecules bound to adjacent sites results in a conformational change which makes the inhibitory domain more accessible.

Principally, two different mechanisms underlying the repression exerted by the inhibitory domain have to be considered. One possibility would be that the inhibitory domain masks the activation domains by interacting intramolecularly *in cis* with an amino acid sequence present within or close to the activation domain. Such an inhibitory intramolecular interaction has been proposed recently for the inhibitory domain of ATF-2 (Li and Green, 1996). So far, we have no evidence for such an interaction. Moreover, since the inhibitory domain repressed activation independently of its position within an activator molecule (C- or N-terminal of the DNA binding domain) and independently of the context (deletion of various regions of Sp3 did not affect inhibition, and inhibition was observed on a heterologous activation domain), we consider an intramolecular interaction mechanism unlikely.

Other possibilities to explain how the inhibitory domain exerts its function would include intermolecular interactions with (i) an inhibitory protein, (ii) another activator molecule tethered to DNA or (iii) the basal transcription machinery directly. To test whether an inhibitory protein is involved in mediating inhibition of Sp3, we have performed competition experiments by overexpressing the inhibitory domain of Sp3 along with Sp3 or Gal4–Sp3. Similar experiments have been successful in establishing the existence of a protein interacting with the c-Jun and c-Fos inhibitory domains (Brown *et al.*, 1995). Overexpressing the free Sp3 inhibitor domain or complete Sp3, however, did not derepress Sp3 or Gal4–Sp3 activation (our unpublished data). Thus, a protein which can be sequestered by an excess of the inhibitory domain provided in *trans* seems not to be involved in the inhibitory process.

Very recently, it was shown that the complete amino-terminal region of Sp3 tethered to a promoter via a heterologous DNA binding domain silences transcriptional activation of various positive regulators bound to adjacent sites (De Luca *et al.*, 1996). It is very likely that the observed repression is mediated by the inhibitory domain identified and characterized here. This observation would be compatible with the idea that the inhibitory domain of Sp3 interacts with another activator molecule in *trans* or with a component of the general transcription machinery.

Potential interaction partners of Sp3 with the general transcription machinery besides dTAFII110 and its human homolog hTAFII135 are TATA box binding protein (TBP) and hTAFII55, since these proteins are also known to interact with Sp1. In a superactivation assay as well as in an *in vitro* interaction assay (GST pull down assay, our unpublished results), the interaction of Sp3 with dTAFII110 was not influenced by the inhibitory domain of Sp3. Thus, prevention of the dTAFII110 interaction with the glutamine-rich domains of Sp3 seems not to account for the observed inhibition. As an alternative to the prevention of a positive interaction, it is feasible that the inhibitory domain destabilizes the initiation complex by interacting with other components of the basal transcription machinery. Such a mechanism has been shown recently for the *Drosophila* zinc finger protein Krüppel. In the case of Krüppel, an interaction of the dimeric protein with TFIIEβ resulted in transcriptional repression (Sauer *et al.*, 1995). Further analysis of the inhibitory domain and its properties will be necessary to unravel the mechanism of transcriptional inhibition exerted by Sp3.

What might be the physiological function of the inhibitory domain?

The identification of a domain which inhibits the activation potential of Sp3 raises the question of its physiological significance. Is the inhibition alleviated under certain conditions to allow full activation by the glutamine-rich domains? One possibility would be that Sp3 acts as an activator in a subset of cell types, whereas in other cell types its activity is silenced by the inhibitory domain. Such a cell type specificity has been reported for c-Jun. The inhibitory domain of c-Jun is active in HeLa tk-, NIH 3T3 and L-M tk- cells but not in SL2, F9 and HepG2 cells (Baichwal and Tjian, 1990; Baichwal *et al.*, 1991). So far, we have no evidence that the inhibitory domain of Sp3 acts in a cell type-specific manner. It silenced the activation domains in all tested cell lines, including Ishikawa, NIH 3T3, CV-1, COS-1 and SL2 cells. Notably, Sp3 has been reported recently to act as a transcriptional activator in the teratocarcinoma cell line NTera2-D1 but not in HeLa cells (Sjøttem *et al.*, 1996). Thus, it might be possible that in NTera2-D1 cells the inhibitory domain of Sp3 is inactive. This observation also raises the possibility that the 'activity' of the inhibitory domain might be dependent on the presence or absence of certain transcription factors on a given promoter. A particular transcription factor or a set of transcription factors may interact with the inhibitory domain thereby allowing the glutamine-rich domains to function.

A most intriguing possibility would be that the activity of Sp3 could be a target for regulatory events. The presence of strong activation domains and an inhibitory domain offers the possibility that transcriptionally inactive Sp3 could be changed into an active molecule. Regulation of the activity of transcription factors by alleviating inhibition is not unusual and has been demonstrated for several transcription factors including c-Jun (Baichwal *et al.*, 1991) and ATF-2 (Li and Green, 1996). Further investigations will be necessary to understand the functional interplay between the activation and inhibitory domains of Sp3.

Materials and methods

Plasmid constructions

The construction of the reporter plasmid G5E1bSV has been described (Hagen *et al.*, 1995). The plasmid G1E1bSV was generated from G5E1bSV by replacing the *Pst*I–*Bam*HI promoter fragment by a double-stranded oligonucleotide containing a single Gal4 binding site. Expression of Gal4 fusion proteins was driven by the SV40 promoter. The plasmid pSG-Gal4–Sp3 was described previously (Majello *et al.*, 1994). To generate the C-terminal deletion mutants pSG-Gal4–Sp3AB and pSG-Gal4–Sp3A, we removed a 245 bp *Bst*1107I–*Xba*I fragment and a 1.0 kb *Xba*I fragment, respectively, of pSG-Gal4–Sp3. To generate the plasmid pSG-Gal4–Sp3B/ID, we first cloned the appropriate cDNA fragment as 1 kb *Bam*HI–*Xba*I (blunted) from pSG-Gal4–Sp3 (dam$^-$ DNA) into a pPacUbx vector (see *Drosophila* vectors) leading to pPacUSp3B/IDΔDBD. Then, the *Bam*HI (blunted)–*Eco*RI insert of pSG-Gal4–Sp3 was replaced by the *Bam*HI (blunted)–*Eco*RI Sp3B/ID-encoding cDNA fragment from pPacUSp3B/IDΔDBD. The plasmid pSG-Gal4–Sp3B, in which the activation domain B of Sp3 is fused to Gal4 without the inhibitory domain, was generated by removing the 245 bp *Bst*1107I–*Xba*I fragment from pSG-Gal4–Sp3B/ID.

To generate the small deletion and the point mutations in pSG-Gal4–Sp3, we used the Clontech transformer kit and appropriate oligonucleotides, leading to pSG-Gal4–Sp3/SD, pSG-Gal4–Sp3/kee, pSG-Gal4–Sp3/pee, pSG-Gal4–Sp3/r, pSG-Gal4–Sp3/s-a and pSG-Gal4–Sp3/s-d.

The plasmids pSG-Gal4–Sp1A/IDwt and pSG-Gal4–Sp1A/IDkee, which contain the inhibitory domain of Sp3 fused to the activation domain A of Sp1, were obtained by cloning the appropriate 245 bp *Bst*1107I–*Xba*I fragments from pSG-Gal4–Sp3 and pSG-Gal4–Sp3/kee, respectively, into the *Sal*I (blunted)–*Xba*I-restricted plasmid pSG-Gal4–Sp1A (Southgate and Green, 1991).

The plasmids pSG-Gal4–VP16/IDwt and pSG-Gal4–VP16/IDkee were constructed by replacing the Sp3 sequences of pSG-Gal4–Sp3 or pSG-Gal4–Sp3/kee by an appropriate VP16-encoding PCR fragment generated from Gal4–VP16 (Sadowski *et al.*, 1988).

Sp3 expression in *Drosophila* Schneider cells was driven by the *Drosophila* actin 5C 5′-flanking region and the constructs were generated as follows. To ensure full-length expression of Sp3, we first constructed the plasmid pPacUSp3, which contains the ultrabithorax leader sequence (Courey and Tjian, 1988) 5′ to the Sp3 coding sequence. We cloned a 2.35 kb *Bam*HI–*Xho*I fragment from pGEX-2TK-Sp3 (see below) into the *Bam*HI–*Xho*I sites of pPacUbx which was generated from pPacSp1 (Courey and Tjian, 1988) by removing the Sp1 cDNA with *Bam*HI and *Xho*I. The expression plasmid pPacUSp3ΔA, in which the activation domain A of Sp3 is removed, was obtained by replacing the Sp4 insert of pPacSp4 (Hagen *et al.*, 1995) by a 1.8 kb *Xba*I fragment from pBS-Sp3 (dam$^-$ DNA) via 12mer *Xho*I linkers. The two plasmids pPacUSp3ΔB and pPacUSp3ΔBID were generated by replacing the *Xba*I–*Xho*I insert of pPacUSp3 by appropriate PCR fragments. Expression plasmids for mutated full-length Sp3 containing either a small deletion (pPacUSp3/SD) or point mutations (pPacUSp3/kee and pPacUSp3/pee) in the inhibitory domain were obtained by replacing the wild-type Sp3 cDNA from pPacUSp3 by the mutated Sp3 cDNAs from pGEX-2TK-Sp3 as *Bam*HI–*Xho*I fragments. The corresponding pGEX-2TK-Sp3 mutants were obtained by cloning the Sp3 mutants from pSG-Gal4–Sp3 vectors (see above) into pGEX-2TK-Sp3 as 1.4 kb *BamHI*–*Afl*II fragments.

The expression plasmid for the fingerless Sp3 mutant (pPacUSp3ΔDBD), in which the 168 C-terminal codons of Sp3 are removed, was obtained by cloning a 1.55 kb *Eco*RI (blunted)–*Bam*HI fragment from pSG-Gal4–Sp3 (Majello *et al.*, 1994) into the *Xho*I (blunted)–*Bam*HI site of pPacU obtained from pPacSp4 (Hagen *et al.*, 1995). The expression constructs for human Sp1 and Gal4–dTAFII110(N308) were described previously (Courey and Tjian, 1988; Hoey *et al.*, 1993).

Cell culture, transfections and CAT and luciferase assays

Ishikawa cells were cultured in MEME, CV-1, COS-1 and NIH 3T3 cells in Dulbecco's modified Eagle's medium (DMEM). Ishikawa and NIH 3T3 cells were transfected by the DEAE-dextran method (Cato *et al.*, 1986; Slater *et al.*, 1990) and CV-1 and COS-1 cells by the calcium phosphate method. Every plate (9 cm for Ishikawa and 6 cm for CV-1, COS-1 and NIH 3T3 cells) received 2 μg of reporter plasmid, 2 μg of Gal4–Sp expression plasmid and 2 μg of RSVLuc. Transfected cells were harvested for CAT assays 72 h after transfection. Variations in transfection efficiencies were corrected by determining the luciferase activities (Brasier and Fortin, 1987).

SL2 cells (Schneider, 1972) were maintained in Schneider medium supplemented with 10% fetal calf serum at 25°C. One day prior to transfection, cells were plated onto 6 cm plastic dishes at a density of 4.3×10^6 cells per plate. Cells were transfected by the calcium phosphate method described by DiNocera and Dawid (1983). Every plate received up to 14 μg of DNA including 8 μg of reporter plasmid and 4 μg of the β-galactosidase expression plasmid p97b as internal reference. Variable amounts of expression plasmids were compensated for with the plasmid pPac. The medium was changed 24 h after addition of DNA, and 24 h later the cells were washed twice with phosphate-buffered saline (PBS) and harvested.

For CAT assays, cells were suspended in 250 mM Tris–HCl, pH 7.8 and lysed by three rounds of freezing and thawing. Protein concentrations and reaction times in the CAT assays were adjusted to bring the extent of CAT conversion into a range that is linear with the CAT enzyme concentration. CAT conversion was assayed by thin-layer chromatography, and quantitation of acetylated and non-acetylated forms of [^{14}C]chloramphenicol was performed with an automated Imaging Scanner (United Technologies Packard). The ratio of acetylated to total chloramphenicol was displayed as a percentage of conversion. The β-galactosidase assays were performed according to Hall *et al.* (1983). The values were used to normalize the CAT conversion data for plate to plate variations in transfection efficiency.

Nuclear extracts and gel retardation assays

Nuclear extracts from transfected Ishikawa and SL2 cells were prepared from one 9 cm plate according to Andrews and Faller (1991). Gel retardation assays were performed essentially as described (Fried and Crothers, 1981; Garner and Revzin, 1986) with oligonucleotides containing the Gal4 or the GT box binding sites (Hagen *et al.*, 1995), respectively.

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