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Transcriptional interference caused by *GCN4* overexpression reveals multiple interactions mediating transcriptional activation

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Abstract Overproduction of Gcn4p in yeast cells resulted in the inhibition of transcription from promoters controlled by the GAL4 or dA:dT elements. We have demonstrated that this effect is mediated through the activation domain of Gcn4p and that the function of the transcriptional activator at the affected promoter is impaired. The inhibitory effect of Gcn4p on these promoters persisted in yeast strains disrupted for the *ADA2* and/or *GCN5* genes, whose products are required for only part of the transcriptional activation capacity of Gcn4p and other activators, but was alleviated by overexpression of yTFIIB. These results support the hypothesis that general transcription factors become unavailable at certain promoters when an activator is overexpressed and strongly imply the existence of an Ada2p/Gcn5p-independent pathway of communication between acidic activators and the basic transcription machinery. In a genetic screen, we have isolated a mutation which neutralises the squelching effects of Gcn4p. This *AFR1-1* (*a*ctivation *f*unction *r*educed) mutation is dominant, it affects the transcriptional activation properties of a number of activators and results in lethality when combined with a *gcn5* disruption. Our results suggest that the *AFR1* gene product is involved in the mediation of transcriptional activation.

Key words Transcriptional squelching · Adaptors · TFIIB · *GCN5* · *ADA2*

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Introduction

Transcriptional activator proteins enhance the expression of genes by allowing for a higher frequency of initiation events at the transcription initiation site. This is accomplished even though activators may bind DNA at great distances from that site. The question that arises is how these proteins exert their effect on the basic transcription machinery. A number of mechanisms have been proposed, many of which invoke a common frame of protein-protein interactions between activators bound at upstream activation sequences (UASs) and general transcription factors located near the initiation site, usually on the TATA box (reviewed in Tjian and Maniatis 1994). These interactions eventually facilitate the ordered assembly of a productive preinitiation transcription complex (Lin and Green 1991; Choy and Green 1993; LaMarco 1994).

In support of this notion, evidence has accumulated suggesting interference of one transcriptional activator with the effects of another, both in vitro and in vivo (Kelleher et. al. 1990). Such an interplay between activators has been proposed to account for the reduction in the transcription levels of unrelated genes caused by overexpression of *GAL4* (Gill and Ptashne 1988), *GAL4-VP16* and *GAL4-HAP4* (Triezenberg et al. 1988; Berger et al. 1992), in yeast. In all cases, the interference effect of the activator was correlated with its propensity for activation, thereby indicating a close link between the two qualitatively different phenomena. Moreover, overproduction of these acidic activators resulted in pronounced depression of the growth rate of yeast cells. This indicates a more general effect on transcription which might be attributable to squelching of general transcription factors by an activator, either directly or through protein bridges. The direct interaction model is supported by data from studies indicating contact between the VP16 activation domain and TBP, TFIIB or both (Stringer et al. 1990; Ingles et al. 1991; Roberts et al. 1993; Kim TK et al. 1994). However, experimental evidence exists sug-

gesting that the interaction between the activator and the basic factors is mediated or at least stabilised by non-DNA-binding proteins (Berger et al. 1990; Kelleher et al. 1990; Flanagan et al. 1991). The simulation of transcriptional activation in vitro requires the presence of a multiprotein complex, which has been proposed to mediate the function of the activator (Laurent and Carlson 1992; Thompson et al. 1993; Cairns et al. 1994; Kim YJ et al. 1994).

Genetic screens designed to identify genes encoding such proteins have led to the isolation of the *ADA2* and *ADA3* genes; they encode proteins with properties that resemble those for a putative mediator of a transcription activator (Berger et al. 1992; Brandl et al. 1993; Pina et al. 1993). Loss-of-function mutations in these genes render yeast cells immune to otherwise toxic levels of GAL4-VP16 and reduce the activation potential of a set of transcriptional activators. In a previous report, we have identified Gcn5p as a molecule that potentiates the activity of several transcription factors (Georgakopoulos and Thireos 1992). Further genetic analysis demonstrated that Ada2p and Ada3p function in the same pathway to signal the binding of an activator at the UAS to the basic transcription machinery (Pina et al. 1993). Additional evidence indicates that Gcn5p physically interacts with Ada2p, in the context of a multimeric protein complex which serves as a mediator of transcriptional activation (Georgakopoulos et al., in press).

In this study we demonstrate that interference with transcription of unrelated genes caused by excessive amounts of Gcn4p is not relieved in the absence of Ada2p or Gcn5p or both, but is alleviated by overexpression of *yTFIIB*. We thus surmise the existence of additional interactions, through which Gcn4p modulates a specific step in the assembly of the preinitiation transcription complex, involving TFIIB. To substantiate this hypothesis, we have isolated a mutant strain that exhibits reduced activation by several transcription factors and is insensitive to Gcn4p overproduction. Loss of *GCN5* function in this mutant background is lethal, a fact indicating that, in this background, the affected gene product operates in a second transcriptional activation pathway.

Materials and methods

Strains and media

All yeast strains used in this study were derivatives of S288C cured for the *GAL2* deficiency. Their precise genotypes are as follows. *MAT***a** *ura3-52 leu2-2*; *MAT***a** *AFR1-1 ura3-52 leu2-2*; *MAT***a** *ura3-52 leu2-2 trp1-Δ63*; *MAT***a** *ada2Δ*; *ura3-52 leu2-2 trp1-Δ63*, in which the *ADA2* gene has been inactivated by insertion of an 1.1 kb *Hin*dIII DNA fragment containing the *URA3* gene at an *Afl*II site within the *ADA2* ORF. *MAT***a** *gcn5Δ ura3-52 leu2-2 trp1-Δ63*, in which a genomic region between *Bam*HI and *Eco*RI of *GCN5* has been replaced by the same 1.1 kb *Hin*dIII DNA fragment, and *MAT***a** *ada2Δ gcn5Δ ura3-52 leu2-2 trp1-Δ63. MAT***a** *AFR1-1 gcn5Δ ura3-52 leu2-2* [pRS315-*GCN5*] was constructed as follows. A *MAT***a** *AFR1-1 ura3-52 leu2-2* haploid strain was initially transformed with a plasmid bearing the *GCN5* complementation unit on an 1.8 kb *Xho*I-*Pst*I DNA fragment and a *LEU2* marker (pRS315-*GCN5*). The resulting strain was transformed with a *Mlu*I-*Xba*I *GCN5* DNA fragment in which the region between the *Bam*HI and *Eco*RI sites of *GCN5* had been replaced by the 1.1 kb *Hin*dIII DNA fragment, containing the *URA3* gene. Transformants were selected that could not simultaneously loose uracil and leucine prototrophy which is indicative of integration of *URA3* in the genome. Correct disruption of *GCN5* was confirmed by southern blotting analysis and the pRS315-*GCN5* plasmid recovered from such transformants was shown to be intact.

Minimal media contained yeast nitrogen base (Difco) and 2% glucose, or 2%galactose, depending on whether *GAL4* should be active or not. For obligatory aerobic growth glucose was substituted by 3% dl-lactate.

Reporter plasmids

Construction of *ΔORFGCN4-LacZ* has been described elsewhere (Tzamarias et al. 1986). The *LexAop-LacZ* reporter was obtained from the laboratory of R. Brent as well as the *UASg GAL1-LacZ* reporter. The *DED1-LacZ* plasmid was constructed by inserting the 7.3 kb *Sca*I-*Sal*I DNA fragment from *ΔORFGCN4-LacZ* which contains the *GCN4-LacZ* gene from the transcription initiation site of *GCN4* to the end of *LacZ*, into the *Hin*dIII site of Ycp88, which is located just downstream of the promoter of *DED1* (Struhl 1985). The *GCRE HIS3-LacZ* fusion contains the GCR element between the unique *Bam*HI and *Eco*RI sites in the promoter of *HIS3-LacZ* which was modified to include only the regulated TATA element 18 bp downstream. This plasmid was obtained from D. Tzamarias The *UAS2CYC1-LacZ* reporter gene on YEp24 was a gift of L. Guarente.

Construction of *LexA* fusions

All *LexA* fusions were based on plasmid pAS64F3 which was a gift from D. Tzamarias. This contains 202 amino acids from LexA, sufficient for dimerization and binding, fused to a polylinker in which in-frame insertions can be constructed. The strong *ADH1* promoter drives expression of the fusion genes. The *SSN6/CYC8* terminator follows the polylinker, which, at its 3′ end, contains stop codons in all three reading frames (Tzamarias and Struhl 1994). *LexA-GCN4* was constructed by inserting a 0.2 kb *Bam*HI-*Kpn*I fragment containing the activation domain of *GCN4* (amino acids 54–152, Hope and Struhl 1986) into the *Sma*I site of pAS64F3. *LexA-VP16* was constructed by inserting a 0.4 kb (aminoacids 413–490) *Bgl*II-*Bam*HI fragment from pSJT1193 crf3 (K.Struhl), which includes the VP16 activation domain, into the *Sma*I site of pAS64F3. *LexA-GAL4* was provided by R. Brent (amino acids 768–881 of the *GAL4* activation domain, plasmid clone pHS17-4). Construction of *ΔORFGCN4* has been described elsewhere (Tzamarias et al. 1986). *ADH1ΔORFGCN4* was constructed by inserting a 1.2 kb *Bst*EII-*Dde*I fragment containing only the *GCN4* coding ORF, into the *Hin*dIII site of pDB20 (Fikes et al. 1990). *ADH1TFIIB* was constructed by inserting a 1.1 kb *Nde*I/*Eco*RI fragment from plasmid KSIIB, containing the yTFIIB coding ORF and provided by D. Tzamarias, into the *Hin*dIII site of pDB20.

Other methods

Ethylmethane sulfonate (EMS)-induced mutagenesis was applied as follows. wild-type haploid yeast cells were transformed with a plasmid carrying *ΔORFGCN4* along with the *DED1-LacZ* reporter. Slow-growing transformants were recovered and mutagenised according to Sherman et al. (1986) to about 10% survival. After plating the cells on minimal plates, fast growing colonies were isolated and cured for the *ΔORFGCN4*-bearing plasmid. To verify the genomic origin of the phenotype, the *GCN4* overexpressing plasmid was reintroduced into these cells and they were checked for maintenance of the fast growth phenotype. By assaying *DED1* expression in the presence or absence of *ΔORFGCN4*,

only mutants that were insensitive to varying levels of *GCN4* expression, were selected.

Transformations were carried out with the lithium acetate method (Rothstein 1991). β-Galactosidase assays and plasmid recovery from yeast were performed as described (Thireos et al. 1984; Sikorski and Hieter 1989).

Results

Gcn4p overexpression interferes with the transcriptional activation from promoters bearing *GAL4* and dA:dT elements

A *GCN4* gene derivative deleted for the small open reading frames (ORFs) located in its 5 untranslated region (*ΔORFGCN4*) expresses relatively high levels of Gcn4p (Tzamarias et al. 1986). We have observed that strains bearing this derivative grow poorly on minimal media (Fig. 3A). These phenotypes were further enhanced by placing *ΔORFGCN4* under the control of the strong *ADH1* promoter. In order to determine the basis for this effect, we first examined the consequences of Gcn4p overexpression for the expression of *LacZ* reporter genes regulated by various promoter elements. Increased *GCN4* expression in the presence of galactose as carbon source (i.e. *GAL1* derepressing conditions) resulted in a severe reduction of the transcriptional activation of a *GAL1-LacZ* reporter gene normally induced by GAL4. Similarly, expression of the *DED1-LacZ* reporter gene, driven by a dA:dT element (Struhl 1985), was also impaired to about the same extent, while no significant decrease in the expression of *ΔORFGCN4-LacZ* was evident (Fig. 1). As expected, the expression of a *LacZ* reporter gene driven by the Gcn4p DNA binding element (GCRE) was increased when Gcn4p was overproduced. We concluded that high levels of Gcn4p interfere with the transcriptional regulation at least through GAL4 and dA:dT elements.

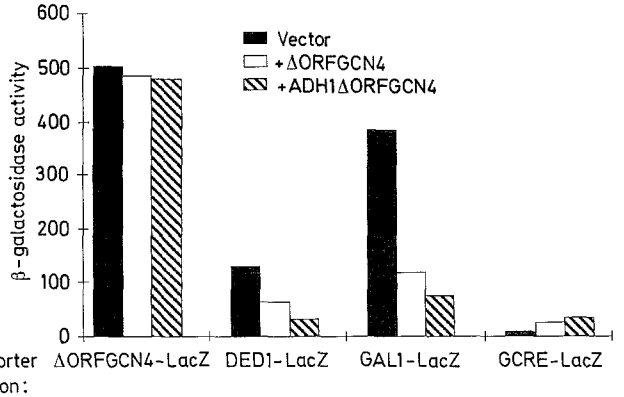

Fig. 1 Graphic representation of the effects of increased Gcn4p amounts on the expression of the indicated reporter genes. Different levels of intracellular Gcn4p production were achieved by introducing into the cells either the plasmid vector (*grey columns*) or the centromeric plasmid bearing the *ΔORFGCN4* derivative (*open columns*). For even higher levels of *GCN4* expression, a multicopy plasmid carrying the *ΔORFGCN4* derivative under the control of the strong *ADH1* promoter was used (*hatched columns*). Gcn4p production was indirectly monitored by using a reporter gene driven by a single GCN4 DNA element (*GCRE-LacZ*). Cells were grown in minimal medium supplemented with all amino acids. Values are in Miller units and represent the average of three independent experiments with less than 10% deviation

The activation domain of Gcn4p inhibits the activation function of Gal4p but not of VP16

We sought to characterise further the Gcn4p mediated inhibition by identifying the protein domain(s) responsible for this effect. With this objective, we fused the activation domain of Gcn4p to LexA and overexpressed this artificial transcriptional activator in yeast cells. Such overexpression also resulted in a slow growth phenotype (data not shown). As shown in Fig. 2A, increased expression of this chimaeric activator resulted in high levels of transcription from a promoter bearing six LexA binding

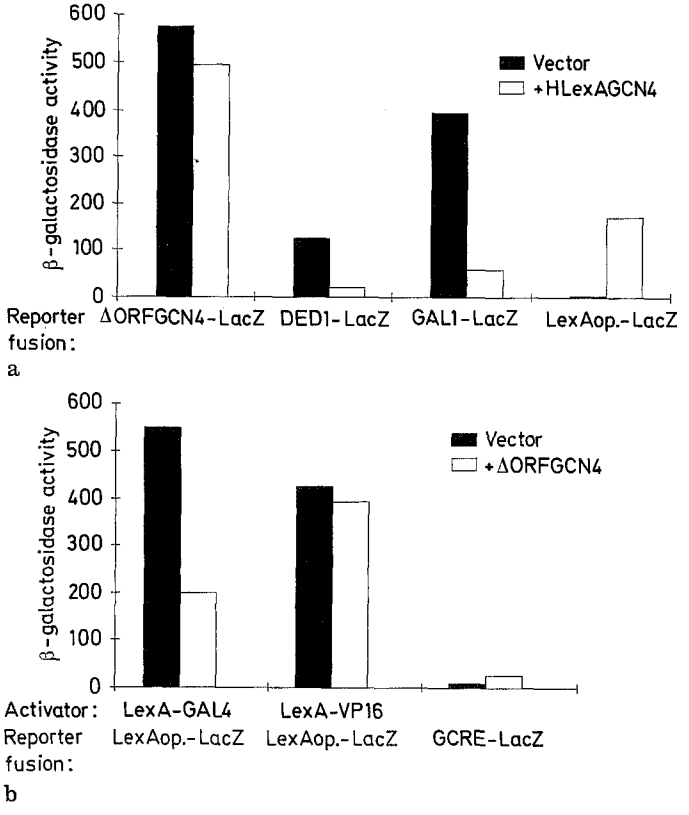

Fig. 2 **A** Graphic representation of the effects of the overexpression of a chimaeric activator consisting of the Gcn4p activation domain, fused to the bacterial LexA binding domain, on the expression of the indicated reporter genes. Yeast cells were either transformed with the plasmid vector (*grey columns*) or with a multicopy plasmid bearing the *LexAGCN4* fusion (*open columns*). Genetic backgrounds and growth conditions were as in Fig. 1. **B** Gcn4p overexpression impairs the function of the Gal4p activation domain but not the function of the VP16 activation domain. Wild-type yeast cells bearing the indicated reporter fusions and chimaeric activators were transformed with either the plasmid vector alone (*grey columns*) or the plasmid bearing the *ΔORFGCN4* derivative (*open columns*). The level of Gcn4p production was monitored as in Fig. 1

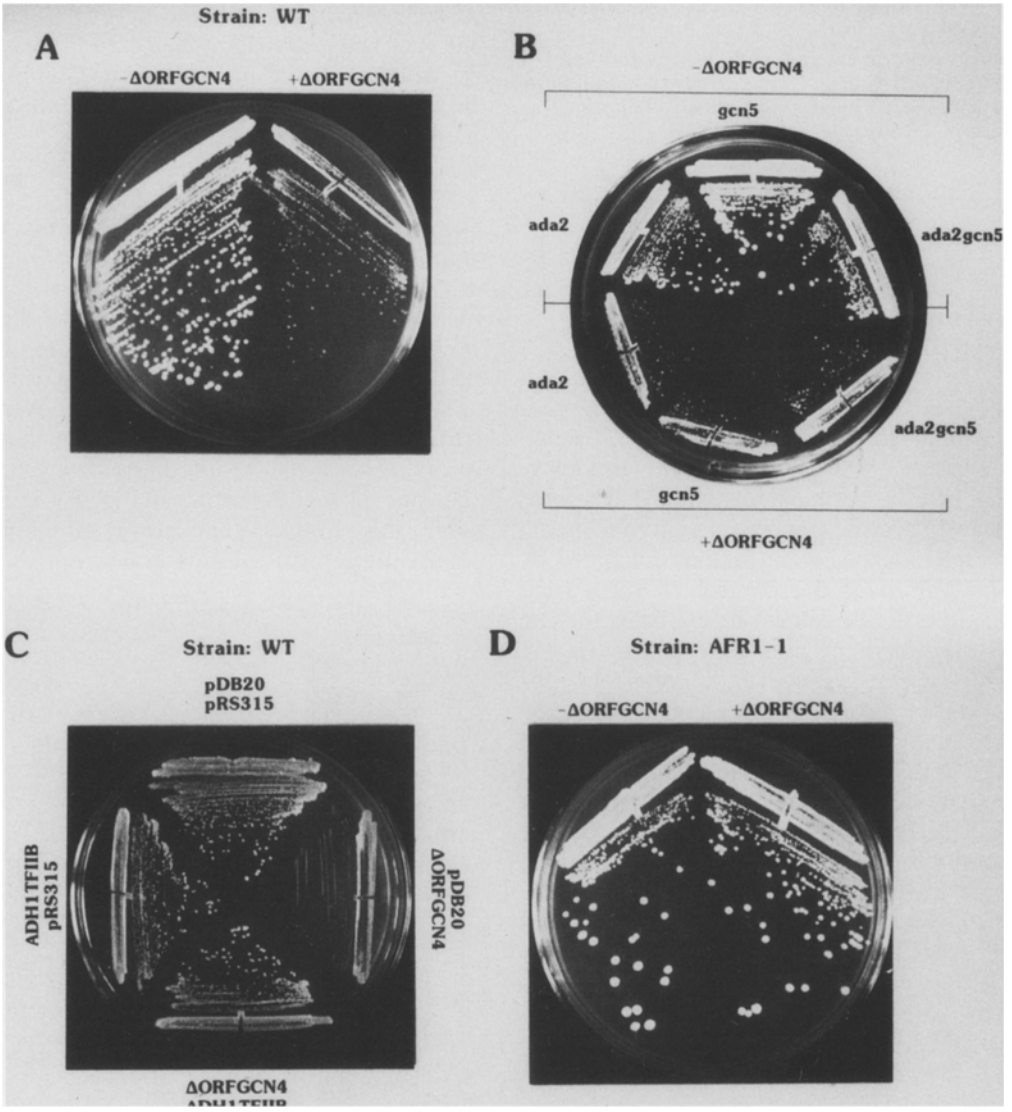

Fig. 3 Plate phenotypes induced by Gcn4p overproduction in various genetic backgrounds. Growth was monitored at 30°C after 3 (**B** and **D**) or 4 days (**A** and **D**) of incubation. **A** Gcn4p imposes a growth handicap on wild-type yeast cells when overproduced. Cells transformed with a *ΔORFGCN4* derivative-bearing plasmid (*+ΔORFGCN4*) grow poorly as compared with cells transformed with the vector alone (*-ΔORFGCN4*). **B** The absence of Ada2p or Gcn5p or both does not alleviate the toxicity of Gcn4p overproduction. The indicated yeast strains were either transformed with the plasmid vector alone (*-ΔORFGCN4*, upper level) or with the Gcn4p overproducing plasmid (*+ΔORFGCN4*, lower level). **C** Overexpression of the yTFIIB completely alleviates the toxic effects of Gcn4p overproduction. Plasmids bearing the indicated gene derivatives were introduced into a wild-type strain. Cotransformation with a yTFIIB overexpressing plasmid (*ΔORFGCN4, ADH1TFIIB*) reversed the slow growth phenotype of a wild-type strain bearing the *ΔORFGCN4* derivative (*ΔORFGCN4*/pDB20). No effect on growth was manifested upon overexpression of yTFIIB alone (*ADH1TFIIB*/pRS315 vs *pDB20*/pRS315). **D** The *AFR1-1* mutant strain is insensitive to toxic levels of Gcn4p. No inhibition of growth is imposed by the introduction of the *ΔORFGCN4* derivative-bearing plasmid into an *AFR1-1* mutant strain (*+ΔORFGCN4*), as compared with the untransformed cells (*-ΔORFGCN4*). The *AFR1-1* strain does not exhibit any growth defect on minimal media at 30°C as compared to the wild-type parental strain (*-ΔORFGCN4* of **A** vs *-ΔORFGCN4* of **D**)

sites but in a reduction in transcription from the *GAL1* and the *DED1* promoters. We concluded that the observed transcriptional inhibition was not the consequence of an indirect effect of Gcn4p overexpression but rather a true squelching effect caused by the Gcn4p activation domain.

In order to determine the target of the Gcn4p squelching effect, we utilised a fusion between the LexA binding and the Gal4p activation domain, along with a *LacZ* reporter gene driven solely by six *LexA* operators. Transactivation of this gene by LexA-GAL4 was monitored under low and high *GCN4* expression levels. Substantial reduction of β-Galactosidase activity was observed when *GCN4* was overexpressed (Fig. 2B), indicating that the activation function of Gal4p was impaired by Gcn4p. The capacity of the VP16 activation domain to stimulate transcription of the same reporter was also examined by employing a LexA-VP16 fusion. In contrast to the impairment of Gal4p-mediated activation, we did not observe any significant effect on VP16 function, as shown in Fig. 2B. We concluded that, under conditions of *GCN4*

Table 1 Inhibition of transcriptional activation by Gcn4p overproduction in wild-type, *ada2*, *gcn5* and *ada2 gcn5* yeast strains. β-Galactosidase activity was measured in the yeast strains transformed with the indicated fusion genes, either together with the *ΔORFGCN4*-bearing plasmid (*+ΔORFGCN4*) or without it (*−ΔOFGCN4*). In the last three fusions a reporter gene driven by six *LexA* operators was employed to monitor the capacity for activation by the indicated chimaeric activators, under conditions of low and high *GCN4* expression (− or *+ΔORFGCN4* respectively). Values are in Miller units and represent the averages of three independent measurements showing<10% deviation

Strain	β-Galactosidase activity							
	Wild type		*ada2*		*gcn5*		*ada2 gcn5*	
	ΔORFGCN4		*ΔORFGCN4*		*ΔORFGCN4*		*ΔORFGCN4*	
	−	+	−	+	−	+	−	+
Fusion								
ΔORFGCN4-LacZ	308.6	282.2	291.1	269.3	250.1	232.3	294.6	282.3
DED1-LacZ	215.6	98.7	88.7	42.2	65.0	41.2	94.2	38.9
GAL1-LacZ	351.9	186.3	203.4	69.1	170.3	72.8	163.7	71.3
GCRE-LacZ	6.3	17.4	3.9	7.3	2.6	14.1	3.5	7.8
LexAop-LacZ/LexA-GAL4	451.4	263.8	110.6	63.7	98.3	42.5	101.6	61.9
LexAop-LacZ/LexA-VP16	395.3	352.6	173.4	68.7	212.3	108.7	162.2	55.6
LexAop-LacZ/LexA-GCN4	110.4	34.2	48.3	12.1	44.0	14.8	37.4	12.1

overexpression, the function of only some acidic activation domains is affected.

Inhibition by Gcn4p is alleviated by overexpression of yTFIIB

Squelching by potent activators has been proposed to reflect titration of a basic transcription factor, thereby affecting expression of a broad set of genes unrelated to the activator. A crucial initial step in the assembly of a productive preinitiation complex, aided by an activator, is the recruitment of TFIIB to the TATA-associated factors (Choy and Green 1993). If the growth defect manifested upon overexpression of an activator results from depletion of TFIIB, this inhibition should be relieved by co-overexpressing TFIIB. In accordance with this assumption, the introduction of a multicopy plasmid bearing the yeast TFIIB coding ORF under the control of the *ADH1* promoter, completely reversed the detrimental effect of *ΔORFGCN4* on the growth of yeast cells (Fig. 3C). This result strongly suggested that TFIIB, is the titrated molecule involved in the process of Gcn4p mediated inhibition.

Inhibition by Gcn4p is not relieved in the absence of *ADA2*, or *GCN5* or both

Although direct contacts of transcriptional activation domains with TFIIB have been demonstrated biochemically (Choy and Green 1993), recent evidence supports the notion of a requirement for mediator molecules that physically connect transcriptional activators with basic factors (Cairns et al. 1994; Y.J. Kim et al. 1994). The *ADA2* and *GCN5* gene products are required for the transcriptional activating function of Gcn4p, Gal4p and VP16 (Berger et al. 1992; Georgakopoulos and Thireos 1992) and define one pathway for such transcriptional mediation. Does this pathway mediate the inhibition effect exerted by Gcn4p? To answer this question, we repeated the analysis described in the previous sections using strains in which *ADA2*, *GCN5* or both had been inactivated. A first observation was that Gcn4p overproduction could still impose a growth handicap on *ada2*, *gcn5* and *ada2 gcn5* strains (shown in Fig. 3B). Secondly, although the expression of the *GAL1*- and *DED1*-driven reporters was reduced in these strains, Gcn4p overexpression effected a further reduction in their expression (Table 1). Taken together, these results suggested that the squelching effect of Gcn4p on Gal4p- and dA:dT-dependent activators was not mediated by Ada2p or Gcn5p. Interestingly, in strains deleted for *ADA2*, *GCN5* or both, VP16-mediated transcriptional activation was rendered vulnerable to Gcn4p overdose. (see Table 1). The functional implications of this effect will be discussed.

The *AFR1-1* mutation completely alleviates *GCN4* mediated squelching

In the context of the proposed mechanism that accounts for inhibition phenomena exerted by potent activators (Ptashne 1988), Gcn4p could sequester essential transcription factors, such as TFIIB, via linker proteins other than Ada2p and Gcn5p. With the aim of isolating such gene products we employed a genetic approach. Following EMS mutagenesis, we selected for mutants resistant to levels of Gcn4p which were otherwise toxic for growth. Approximately 10^7 cells were mutagenised and 16 resistant mutants were recovered. Of those, 14 were plasmid-linked and two were genomic. This methodology yielded one recessive and one unlinked dominant mutation, named *AFR1-1* (Fig. 3D) for *a*ctivation *f*unction

Table 2 Transcriptional inhibition by high levels of *GCN4* expression in a *AFR1-1* background. The analysis presented in Table 1 was repeated for the *AFR1-1* mutant strain. The absolute values in this Table may be compared to the respective values of the strains in Table 1 as the assays were done simultaneously and the genetic backgrounds were otherwise isogenic. The respective values of a wild-type strain under low *GCN4* expression (*−ΔORFGCN4*) are shown in parentheses for comparison. Values are in Miller units and represent the averages of three independent experiments showing<10% deviation

Strain	β-Galactosidase activity	
	AFR1-1	
	− ΔORFGCN4	*+ ΔORFGCN4*
Fusion		
ΔORFGCN4-LacZ	311.3 (308.6)	298.4
DED1-LacZ	58.1 (215.6)	59.4
GAL1-LacZ	93.6 (351.9)	88.4
GCRE-LacZ	3.1 (6.3)	6.9
LexAop-LacZ/LexA-GAL4	89.7 (451.4)	79.6
LexAop-LacZ/LexA-VP16	415.6 (395.3)	410.3
LexAop-LacZ/LexA-GCN4	58.7 (110.4)	55.6

Table 3 Transcriptional activation of a *UAS2(CYC1)-LacZ* reporter gene under strictly aerobic conditions in wild-type and *AFR1-1* backgrounds. Lactate was used to impose strictly aerobic growth. β-Galactosidase activity was measured after growth of the indicated strains for 15 h at 30° C in minimal-glucose (*Glucose*) or after transfer for 5 h in minimal-lactate medium following 10 h of growth in minimal-glucose medium (*Lactate*). Values are in Miller units and represent the averages of three independent measurements with<10% deviation

Strain	β-Galactosidase activity			
	Wild type		*AFR1-1*	
	Glucose	Lactate	Glucose	Lactate
Fusion				
UAS2(CYC1)-LacZ	5.2	40.4	2.4	14.6

reduced, since it affected the activity of several transcription factors (see below). *AFR1-1* strains exhibited additional phenotypes: inability to grow under amino acid starvation conditions, severe growth defect when grown in galactose and inability to grow in non-fermentable carbon sources. The AFR1-1 strain was dominant for all these phenotypes which co-segregated after three rounds of mating with the parental *wild-type* strain, and random spore analysis indicated the presence of a single mutation. The growth properties were in concert with the fact that transcriptional activation through the *GCRE* and *GAL1* promoters was reduced in this strain (Table 2). In addition, the transcriptional activation of a *CYC1-LacZ* reporter driven solely by UAS2 was also reduced in an *AFR1-1* strain (Table 3). UAS2 is the the target element of the Hap2p/Hap3p/Hap4p heterotrimeric activator involved in the regulation of genes coding for respiratory functions (Forsburg and Guarente 1989).

In order to define the impaired function of transcription factors in *AFR1* mutant strains we employed *LexA* fusions of the activation domains of Gcn4p, Gal4p and VP16. With the exception of LexA-VP16, which could still function to normal levels, the other two fusions lost half of their activity in the *AFR1* mutant background (Table 2). The important fact was that, unlike the situation with the *ada2* and *gcn5* strains, high levels of Gcn4p in an *AFR1-1* strain did not any further affect the transcription of the *GAL1* and *DED1* reporters nor the activity of the LexA-GAL4 fusion (Table 2). We concluded that the *AFR1* gene product potentiates the transcriptional activating function of a number of regulators and defines at least one factor that mediates the squelching properties of Gcn4p.

An *AFR1-1 gcn5* double mutant strain is not viable

Based on the phenotypes of the *AFR1-1* mutation the corresponding gene product might define a function analogous to that attributed to Gcn5p and Ada2p: linking activators with the basic transcriptional machinery. The question that arises is whether the *AFR1* gene product operates within, or in a parallel to the Ada2p/Gcn5p pathway. To answer this question we investigated the impact of a *GCN5* deletion in a haploid *AFR1-1* strain. Initially, a *GCN5* complementing plasmid was introduced into an *AFR1* mutant strain. In this strain we disrupted the chromosomally located copy of the *GCN5* gene, as described in Materials and methods. In such disrupted strains, repeated efforts to segregate the plasmid-borne *GCN5* gene away were unsuccessful, while it could be readily lost from non-disrupted transformants. Moreover, viability of the disrupted transformants under non-selective conditions for the plasmid was reduced as compared to wild-type transformants. These results strongly suggested that an *AFR1-1 gcn5* double mutant strain is not viable. This synthetic lethality indicated that Afr1p functions in parallel to the Ada2p/Gcn5p pathway and that blockage of both pathways impairs transcription in a way detrimental for survival.

Discussion

Overexpression of Gcn4p has a toxic effect on cell growth. In this study we have demonstrated that this Gcn4p-mediated toxicity correlates with transcriptional interference. We have examined the effects of Gcn4p overproduction on the transcription of two other genes, *GAL1* and *DED1*, which are regulated by different transcriptional activators. Our analysis demonstrated that Gcn4p can interfere with the function of the Gal4p and the dA:dT factors, reducing their activation potential. Finally, we have mapped this interference to the activation domain of the Gcn4p. Therefore, Gcn4p can be added to the set of potent acidic transcription activators, such as Gal4p, GAL4-VP16 and GAL4-HAP4, which

have been shown to impose inhibition on the expression of non-target genes and retard growth upon overexpression (Gill and Ptashne 1988; Triezenberg et al. 1988; Berger et al. 1992).

These so-called squelching effects (Gill and Ptashne 1988) reflect mechanistic aspects of the transcriptional activation process and are consistent with two alternative models. First, any two factors that interfere with each other might employ common cofactor(s) to exert their function, which would be titrated by the one overexpressed. Second, overexpressed activators could somehow trap general transcription factors at their target sites, reducing their availability (Ptashne 1988). Trapping could be achieved by virtue of either direct interaction of the activator with components of the basic transcription machinery or through protein bridges. We have shown that overexpression of TFIIB relieves the growth handicap imposed by elevated amounts of Gcn4p. This observation favours the second model and suggests that Gcn4p either directly or through protein bridges can titrate TFIIB. Thus, a step in the formation of productive preinitiation complexes involving TFIIB is impaired by elevated amounts of Gcn4p.

The titration hypothesis predicts that mutations that disrupt the interaction between the activator and general factors would alleviate the growth handicap (Berger et al. 1990). A genetic scheme designed to isolate genes whose products would facilitate these interactions has proven fruitful in identifying Ada2p and Ada3p as putative adaptor molecules (Berger et al. 1992). A study of the *gcn5* mutation, originally selected to confer a degree of sensitivity to amino acid starvation, also demonstrated that Gcn5p is a candidate pleiotropic mediator of transcriptional activation (Georgakopoulos and Thireos 1992). Genetic analysis argues strongly that Ada2p and Gcn5p interact and define one pathway for transcriptional mediation of a class of acidic activators which includes Gcn4p (Georgakopoulos et al., in press). Similar studies suggest that Ada3p also participates in the same pathway (Pina et al. 1993). As we have demonstrated in this study, Gcn4p still exerts its inhibitory effects in the absence of *ADA2*, *GCN5* or both. This result indicates that the Gcn4p squelching effects are not mediated exclusively by the presumptive Ada2p/Gcn5p/Ada3p mediation pathway. This fact suggests that either these effects are mediated through direct interactions with basic factors or implies the existence of additional components or pathways that mediate or stabilise such interactions.

In order to identify molecules that mediate the Gcn4p squelching effects we utilised a genetic scheme that aimed to isolate of mutations that neutralise these effects. We have isolated a dominant mutation, *AFR1-1*, that relieves the growth handicap that Gcn4p mediates and blocks its interference on the function of other activators. This mutation reduces the activating function of Gcn4p and affects the transcriptional activation capacity of several other transcription factors. These pleiotropic phenotypes are consistent with a co-activator function for the *AFR1* gene product. In contrast to the non-additive effects of *ada2 gcn5* double mutants (Table 1; Georgakopoulos et al., in press), which indicate that the corresponding gene products function in the same pathway, a *GCN5* deletion is lethal in *AFR1-1* strains. This synthetic lethality suggests functional independence of the corresponding gene products and indicates the existence of an additional step for the mediation of transcriptional activation.

Based on observations regarding the reduction in the activity of various activation domains in *AFR1-1* or *ada2* and *gcn5* strains, we speculate that these domains exhibit different affiliations to each pathway in which Afr1p or Ada2p and Gcn5p participate. The Gcn4p, Gal4p and the dA:dT factor-mediated activation depend almost equally on both pathways. By contrast, the VP16-mediated activation is mostly facilitated by the Ada2p/Gcn5p pathway and only a small portion is Afr1p dependent. This is consistent with the observation that growth inhibition by GAL4-VP16 is suspended in the absence of Ada2p and/or Ada3p (Berger et al. 1992; Pina et al. 1993), whereas inhibition by Gcn4p still persists in *ada2 gcn5* strains. This differential contribution of the two presumptive pathways to VP16 activation explains the fact that the squelching effects of Gcn4p on VP16 are evident only in *ada2* strains. In such strains the absence of the major contributor to VP16-mediated activation allows the Gcn4p interference via the other pathway to become evident.

The genetic evidence presented in this paper supports the notion that transcriptional activation in vivo is mediated by a multiplicity of interactions the sum of which determines the extent of transcription initiation events. These interactions may operate in the context of a single physical protein complex such as the one reported to be required for transcriptional activation in vitro (Cairns et al. 1994), or be exerted by different mediator complexes. This notion can further be tested by isolating the *AFR1* gene and biochemically probing the interactions of the corresponding protein. The dominant nature of the *AFR1-1* mutation will be a valuable tool in such studies since it suggests reinforcement of non-productive interactions. This analysis should eventually help to reveal how these interactions modulate the assembly of a productive rather than abortive transcription preinitiation complex.

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