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Yeast SUB1 is a suppressor of TFIIB mutations and has homology to the human co-activator PC4

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Activation of transcription in eukaryotes depends upon the interplay between transcriptional activators and general transcription factors. While direct contacts between activators and general factors have been demonstrated *in vitro*, an additional class of proteins, termed co-activators, is also required for transcriptional activation. Here we describe a yeast protein, SUB1, that was isolated as a suppressor of the cold-sensitive TFIIB R78H mutant. The N-terminal third of SUB1 is highly similar to the mammalian co-activator PC4. We show that increased expression of SUB1 suppresses two alleles of TFIIB (E62G, R78H) specifically and that the deletion of *SUB1* is lethal in combination with these same two alleles. We show that SUB1 binds to TFIIB *in vitro* and that it specifically inhibits the formation of TBP–TFIIB–promoter complexes. Furthermore we show that increasing the copy number of *SUB1* stimulates transcriptional activation *in vivo*. Based on our results and recent observations of others, we propose that SUB1 plays a role in the release of TFIIB from the transcription complex during transcription initiation.

Keywords: co-activator/genetics/SUB1/TFIIB/transcription

Introduction

Co-activators play an important role in activator-dependent stimulation of transcription. They were first identified as factors required for activation *in vitro* (Berger *et al.*, 1990; Kelleher *et al.*, 1990; Pugh and Tjian, 1990), and include members of the TFIID complex—the TBP-associated factors (TAFs; reviewed in Goodrich and Tjian, 1994), the CREB binding protein (CBP; Chrivia *et al.*, 1993; Alberts *et al.*, 1994; Kwok *et al.*, 1994), a CBP-related factor that binds to E1A (p300; Eckner *et al.*, 1994) and BCL-3 which associates with NF-κB p50 homodimer–DNA complexes (Fujita *et al.*, 1993). Another mammalian co-activator, PC4, was found to bind to transcriptional activators as well as to a promoter-bound TFIIA–TFIID complex and may function as an adaptor bridging the interaction between activators and basal transcription factors (Ge and Roeder, 1994; Kretzschmar *et al.*, 1994).

Genetic studies in yeast have led to the identification of several co-activators which may function by diverse mechanisms. The SWI–SNF complex (Peterson and Herskowitz, 1992; Cairns *et al.*, 1994; Peterson *et al.*, 1994) appears to function by antagonizing repression mediated by nucleosomes. Suppressors of *swi* mutations reside in components of chromatin (Hirschhorn *et al.*, 1992; Winston and Carlson, 1992) and the SWI–SNF complex was found to stimulate activator binding to nucleosomal DNA *in vitro* (Côté *et al.*, 1994). Another yeast co-activator, the ADA complex, binds to the activation domain of VP16 *in vitro* and is required for the full activity of certain yeast activators *in vivo* (Berger *et al.*, 1992; Piña *et al.*, 1993; Marcus *et al.*, 1994; Silverman *et al.*, 1994; Horiuchi *et al.*, 1995). The SRB proteins associate with the C-terminal domain of the large subunit of RNA polymerase II and are functional components of the holoenzyme complex (Kim *et al.*, 1994; Koleske and Young, 1994). Finally, mutations in *GAL1* and *SUG1* were isolated as suppressors of weak activation domains (Gann *et al.*, 1992; Swaffield *et al.*, 1995). Both proteins interact with activation domains and were found to be members of the RNA polymerase II holoenzyme (Kim *et al.*, 1994; Koleske and Young, 1994).

The recent isolation of yeast TAFs (Poon and Weil, 1993; Reese *et al.*, 1994), a mammalian SWI–SNF complex (Imbalzano *et al.*, 1994) and the human homolog of the yeast co-activator SUG1 (Lee *et al.*, 1995) indicates that co-activator function might be highly conserved between lower and higher eukaryotes.

The basal transcription factor IIB has been implicated as one of the key targets for transcriptional activators. First, acidic activation domains bind to TFIIB and facilitate its recruitment to a TBP–promoter complex *in vitro* (Lin and Green, 1991; Lin *et al.*, 1991). Second, mutations in human TFIIB have been found which eliminate activated transcription *in vitro* while basal transcription remains unaffected (Roberts *et al.*, 1993). Third, recent evidence suggests that upon the interaction with an activation domain, TFIIB undergoes a conformational change which may be important in the activation process (Roberts and Green, 1994). Finally, the interaction of TFIIB with a component of the TFIID complex has been reported (Goodrich *et al.*, 1993).

In this report, we initiate a genetic study of yeast TFIIB. We have isolated a panel of temperature- and cold-sensitive mutations in *SUA7*, the gene encoding TFIIB (Pinto *et al.*, 1992). We were able to identify a gene, *SUB1*, which, when overexpressed, suppresses the cold sensitivity of the TFIIB E62G and R78H mutants. Interestingly, the N-terminal domain of SUB1 bears strong homology to the human co-activator PC4. We show that the function of both TFIIB mutants absolutely depends on the presence of SUB1 *in vivo*. Additional evidence suggests that SUB1 specifically interacts with TFIIB *in vitro* and

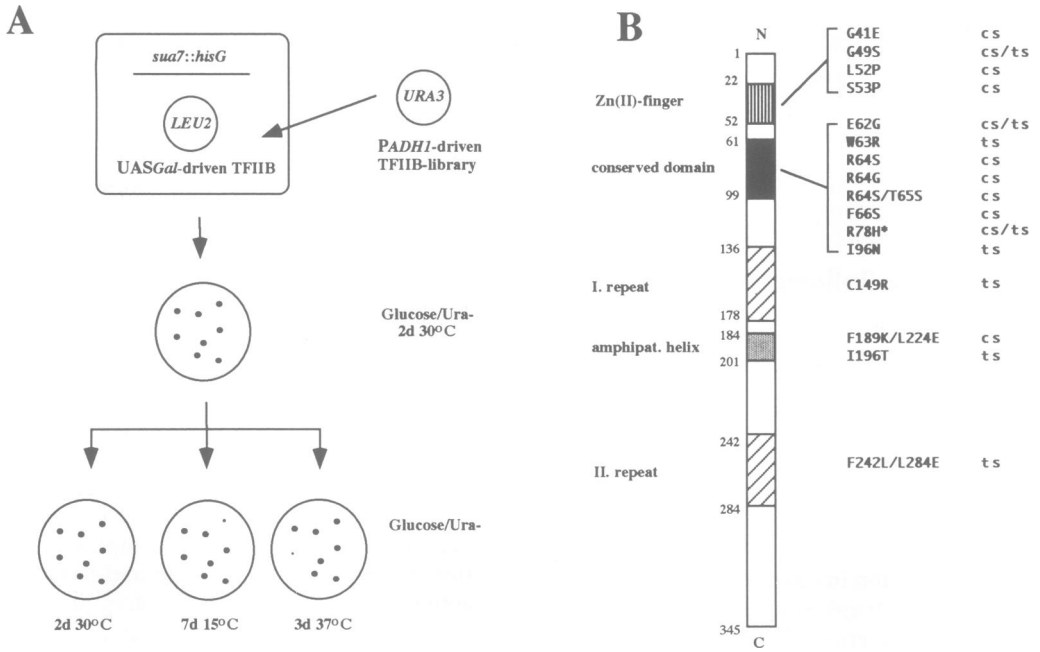


Fig. 1. Generation and isolation of temperature-sensitive (ts) and cold-sensitive (cs) TFIIB mutants. (**A**) Schematic of the isolation. The yeast strain RKY16 which carries a deletion of chromosomal *SUA7* and a plasmid with *SUA7* under the control of a galactose-dependent promoter (Materials and methods) was transformed with a library of mutagenized *SUA7* genes under control of the constitutive *ADH1* promoter (Materials and methods). Transformants were selected on SD medium supplemented with casamino acids (0.1%), adenine (0.01%) and glucose (2%) at 30°C. Under these conditions, TFIIB is only expressed from the *ADH1* promoter-driven *SUA7* genes of the library plasmids. After 2 days, the transformants were replica plated onto the same medium and incubated at 30, 15 and 37°C. Ts and cs mutants were identified by their inability to grow at 37 and 15°C respectively. (**B**) Ts and cs mutations in TFIIB. The coding regions of 16 *SUA7* alleles were sequenced and, where necessary, multiple mutations were separated by recombination with the wild-type allele. Recombinant *SUA7* genes were transferred to plasmid pRK151 (Materials and methods), sequenced and subsequently tested for their ts and cs phenotype in RKY16. For the double mutants shown, the mutations have not been separated from each other. One mutant (*) has been mapped indirectly by showing that secondary mutations are phenotypically silent.

that this interaction strongly inhibits the formation of TBP–TFIIB–promoter complexes. The implications of these findings for mechanisms of transcriptional activation are discussed.

Results

Isolation of temperature- and cold-sensitive TFIIB mutants

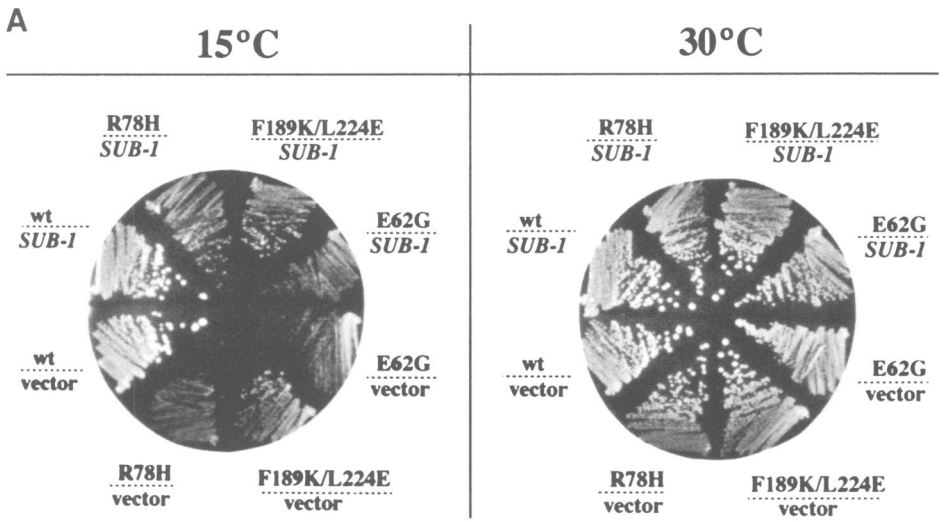
A collection of TFIIB mutants was generated by PCR mutagenesis of the *SUA7* gene. The mutant alleles, expressed from the constitutive *ADH1* promoter on an *ARS-CEN* or 2μm plasmid, were introduced into strain RKY16, in which TFIIB expression is under the control of a galactose-dependent promoter (Materials and methods). Temperature- and cold-sensitive mutants were identified on glucose medium as outlined in Figure 1A. Strikingly, most mutations clustered in two N-terminal domains of TFIIB: the putative Zn(II) finger domain and the conserved domain which is the most highly conserved region between yeast and human TFIIB (Figure 1B).

Cloning of SUB1

We wanted to identify genes whose products, when overexpressed, would suppress the cold sensitivity of mutations in the N-terminal domains of TFIIB. For this purpose, we used derivatives of strain RKY16 carrying *ARS-CEN* plasmids with *SUA7* alleles expressed from the *ADH1* promoter. These strains were transformed with a library in which yeast cDNA inserts were expressed from a galactose-inducible promoter. Transformants were screened for enhanced growth on galactose medium at 15°C. From all strains, library plasmids with cDNA inserts encoding TFIIB were isolated multiple times. Only one mutant, R78H, gave rise to another cDNA that suppresses cold sensitivity. This cDNA was used to isolate the corresponding gene from a yeast genomic DNA library. As shown in Figure 2A, the high copy plasmid carrying a genomic DNA fragment bearing the suppressor gene partially suppresses the cold-sensitive phenotype of the TFIIB R78H mutant, but fails to suppress other cold-sensitive TFIIB mutants, such as E62G and F189K/L224E (Figure 2A) or G41E and G49S (not shown). Interestingly, when SUB1 was expressed from the initially isolated cDNA clone under the control of a strong galactose-dependent promoter, the cold sensitivity of the TFIIB E62G mutant is also partially suppressed (not shown).

Suppression did not result from an increase in expression of the mutant TFIIB protein, since overexpression of SUB1 does not affect steady-state levels of TFIIB (Figure 3B), and overexpression of the TFIIB mutants does not alleviate cold sensitivity (not shown).

The sequence of both the cDNA and the genomic clone revealed an open reading frame encoding a predicted protein of 292 amino acids (Figure 2B). We named the protein SUB1 for its ability to suppress TFIIB mutations. Strikingly, its N-terminal third shows strong similarity to a 73 residue region encompassing the majority of the 127 amino acid mammalian co-activator PC4 (48% identity, 70% similarity, Figure 2C). This portion of PC4 possesses sequences essential for co-activator function as well as



B

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SUB-1:   1 MSYYNRYRNK RRSDNGGGNL SNSNNNNGGM PSGLSASDAI FDLGKNKRVT VRQFRNINLI DIREYYLDSS  70
        71 TGEMKPGKKG ISLTEDLYDE LLKHRLNIDE ALRRLGSKRP KTKMVRLLSD DEYEDDNNND STNNDKDKNG 140
       141 KDKNSPKKRR EDKSKASNES HDLEPRSKKE KPAPPTLLPH EENIQNAERE ANATLIIPGQ AGRKQQEERK 210
       211 QKEKEEAEEA KAKAVAEQEK EAKEKEKIAE PEPEPVPTLQ AKKEDIVSNI NESKDANSSD EEFAQSLEAE 280
       281 MNKAEDDISE EEZ
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C

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SUB-1:   32 SGLSASDAIFDLGKNKRVTVRQFRNINLIDIREYYLDSSTGEMKPGKKGISLTEDLYDELLKHRLNIDEALRRL 105
             S  S  D +F +GK + V+VR F+   LIDIREY++D   GEMKPG+KGISL  + + +L +   +ID+A+R+L
PC4:     55 SSSSRDDNMFQIGKMRYVSVRDFKGKVLIDIREYWMDPE-GEMKPGRKGISLNPEQWSQLKEQISDIDDAVRKL 127
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Fig. 2. SUB1: suppression of TFIIB R78H, amino acid sequence and homology to human co-activator PC4. (**A**) Expression of *SUB1* from a multicopy plasmid suppresses the cold sensitivity of the TFIIB mutant R78H. Yeast strain RKY16 and its derivatives expressing the indicated TFIIB mutants under control of the *ADH1* promoter of plasmid pRK151 were transformed with a vector carrying either the 7 kb genomic DNA fragment with the *SUB1* gene (*SUB1*) (Materials and methods) or no insert (vector). Transformants were tested for growth on SD medium supplemented with amino acids (0.05 g/l each, no leucine), adenine (0.01%) and glucose (2%) at the restrictive (15°C) and permissive (30°C) temperature. (**B**) Amino acid sequence of SUB1. (**C**) Homology of SUB1 and human co-activator PC4. Sequence alignment was performed at the NCBI using the BLAST network service. Identical amino acids as well as conservative changes (+) are indicated.

single-stranded DNA binding activity (Ge and Roeder, 1994; Kretzschmar *et al.*, 1994).

SUB1 affects activation in vivo

Evidence that SUB1 functions in transcriptional activation was obtained from two *in vivo* experiments. First, the effect of overexpression of SUB1 on the activity of various cellular activators was tested in *lacZ* reporter assays. SUB1 expressed from the *ADH1* promoter on a high copy plasmid stimulates the activity of the cellular activators GCN4 (Hope and Struhl, 1986) or HAP4 (Forsburg and Guarente, 1989) (Figure 3A). This effect is specific, since the activity of a promoter under control of the GAL4 activator (Ma and Ptashne, 1987) is not affected (Figure 3A). As mentioned above, overexpression of SUB1 does not alter steady-state levels of TFIIB (Figure 3B).

Second, the entire *SUB1* coding sequence was deleted from the chromosome of strain BWG1-7A. Deleting *SUB1* is not lethal, does not affect growth on medium containing galactose or lactate as carbon source (not shown) and exerts only small effects on several reporters tested (Figure 3A). However, deleting *SUB1* results in inositol auxotrophy (Figure 4A), a phenotype also observed in SWI, SNF, SRB and RNA polymerase II CTD mutants (Peterson and Herskowitz, 1992; Thompson *et al.*, 1993; Cairns *et al.*, 1994; Peterson *et al.*, 1994).

SUB1 is essential for the function of TFIIB E62G and R78H in vivo

Since the deletion of *SUB1* exerts only modest effects in a strain expressing wild-type TFIIB, we examined possible synthetic lethality between the deletion and mutant alleles of TFIIB. Such lethality would be a further indication of an interaction between SUB1 and TFIIB *in vivo*.

We used strains RKY42 and RKY42Δ*sub1* (Materials and methods), which are deleted for the chromosomal copy of *SUA7* (TFIIB) and express wild-type TFIIB from plasmid pRK161 (*ARS-CEN, URA3*, P_{ADH1}). Both strains were transformed with either a control vector pRK151 (*ARS-CEN, LEU2*, P_{ADH1}) or with derivatives thereof carrying one of several *SUA7* allelles (Materials and methods). Transformants were tested for their ability to grow on medium containing 5-fluoroorotic acid, which selects for loss of the *URA3* plasmid expressing wild-type TFIIB. Survival therefore depends on the function of the TFIIB protein expressed from the pRK151 plasmid. As shown in Figure 4B, the deletion of *SUB1* from cells expressing the TFIIB mutants E62G or R78H, but not other TFIIB mutants, results in synthetic lethality. Interestingly, these are the same two TFIIB mutants whose cold sensitivity is suppressed by overexpression of SUB1. This synthetic lethality is not due to an alteration in TFIIB expression in the Δ*sub1* strain (Figure 3B).

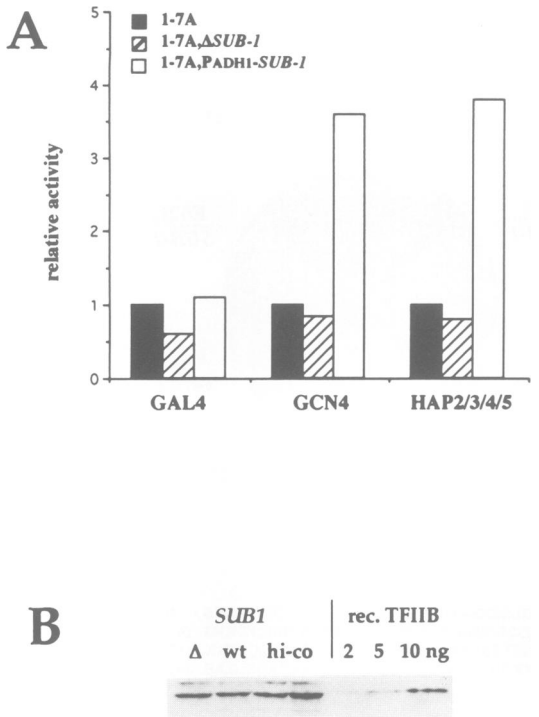

Fig. 3. Overexpression of SUB1 stimulates transcriptional activation *in vivo*. (**A**) Relative activities of the transcriptional activators GAL4, GCN4 and the HAP2/3/4/5 complex in the strain BWG1-7A, BWG1-7A/Δ*sub1* and BWG1-7A/ P_{ADH1}-*SUB1* (overexpressing SUB1 from the *ADH1* promoter on a high copy number plasmid). Promoter activities were measured using β-galactosidase assays with reporter plasmids carrying the *lacZ* gene under control of the *CYC1* TATA boxes and UASs for the activators listed above. The standard error in the assays was usually <20% and never >30% of the mean. Activities are given relative to those obtained for the wild-type strain BWG1-7A. (**B**) Expression of TFIIB in cells carrying zero (Δ), one (wt) or multiple copies (hi-co) of *SUB1*. The Western blot analysis was performed as described in Materials and methods. As a control, various amounts of purified, recombinant TFIIB were included. The recombinant protein carries a fusion of six histidines at its N-terminus and therefore migrates more slowly in the gel than native TFIIB.

SUB1 specifically interacts with TFIIB in vitro

The allele-specific suppression of TFIIB mutants by overexpression of SUB1 and the observed synthetic lethality between the same mutants and the *sub1* deletion suggest that SUB1 and TFIIB interact directly. To address this possibility, far-Western experiments were performed. Recombinant TFIIB, TBP and dihydrofolate reductase (DHFR) were subjected to denaturing gel electrophoresis, transferred to a nitrocellulose membrane, renatured and subsequently analyzed for their ability to interact with *in vitro* translated, [^{35}S]methionine-labeled SUB1 (Materials and methods). In these assays, SUB1 interacts strongly with TFIIB, very weakly with TBP and not at all with DHFR (Figure 5A). The smaller product visible in the lane with TFIIB represents a C-terminal fragment of the protein that clearly interacts with both Gal4-VP16 and SUB1. In control experiments performed under identical conditions, *in vitro* translated luciferase does not interact with any of the recombinant proteins, whereas *in vitro* translated GAL4-VP16 specifically interacts with TBP and TFIIB as expected.

SUB1 specifically inhibits the formation of TBP–TFIIB–promoter complexes

In order to confirm the results of the far-Western experiments described above, recombinant SUB1 purified from *Escherichia coli* was analyzed in electrophoretic mobility shift experiments. We wanted to demonstrate that the interaction between SUB1 and TFIIB would specifically alter the mobility of TBP–TFIIB–promoter complexes but not that of TBP–TFIIA–promoter complexes in native polyacrylamide gels. However, we observed that the addition of SUB1 strongly inhibits the formation of TBP–TFIIB–promoter complexes, whereas the formation of TBP–TFIIA–promoter complexes is not affected (Figure 5C). This inhibition was observed at equimolar amounts of SUB1 and TFIIB. As a further demonstration that this inhibition is specific, we were able to show that it is reversed almost completely by increasing the TFIIB concentration 5-fold (Figure 5C). We also found that SUB1 inhibited TFIIB binding in a reaction containing TBP, TFIIB and TFIIA (not shown).

Discussion

We have created a collection of mutant *sua7* alleles encoding temperature- and cold-sensitive forms of yeast TFIIB. Interestingly, many of these amino acid changes are located in the Zn(II) finger and in the conserved domain at the N-terminus of the protein (Figure 1B). We used a GAL1-dependent yeast cDNA expression library (Liu *et al.*, 1992) to isolate suppressors of the cold sensitivity of these N-terminal mutations. One cDNA specifically suppresses the cold sensitivity of two TFIIB mutants (E62G and R78H). This cDNA was used to clone the corresponding gene, *SUB1*, which encodes a novel yeast protein with high homology to the human co-activator PC4.

Chromosomal deletions of *SUB1* result in inositol auxotrophy, a phenotype also associated with mutations in a number of other transcription factors including members of the SWI–SNF and SRB complex. Deletion of *SUB1* has relatively small effects on the reporters tested, suggesting that other mechanisms are sufficient to stimulate transcription from these promoters. The inositol auxotrophy of the strain lacking *SUB1* may highlight a restricted subset of yeast genes that normally require this factor.

We have demonstrated that the deletion of *SUB1* is synthetically lethal with the same TFIIB mutants that require high levels of *SUB1* for the suppression of their cold sensitivity (Figure 4B). These allele-specific genetic interactions strongly support the existence of a functional SUB1–TFIIB interaction *in vivo*. A role for SUB1 in transcriptional activation is also indicated by our findings that overexpression of SUB1 significantly augments the activities of activators, such as GCN4 and HAP4 (Figure 3A), *in vivo*.

Two different biochemical assays provided evidence that SUB1 directly contacts TFIIB *in vitro*. The results of the far-Western experiments revealed a specific interaction between SUB1 and both full-length TFIIB and its C-terminal fragment (Figure 5A). The C-terminus of TFIIB has been shown previously to be sufficient for TBP binding. We discuss below a model of how SUB1 binding to the C-terminus of TFIIB can suppress specifically two

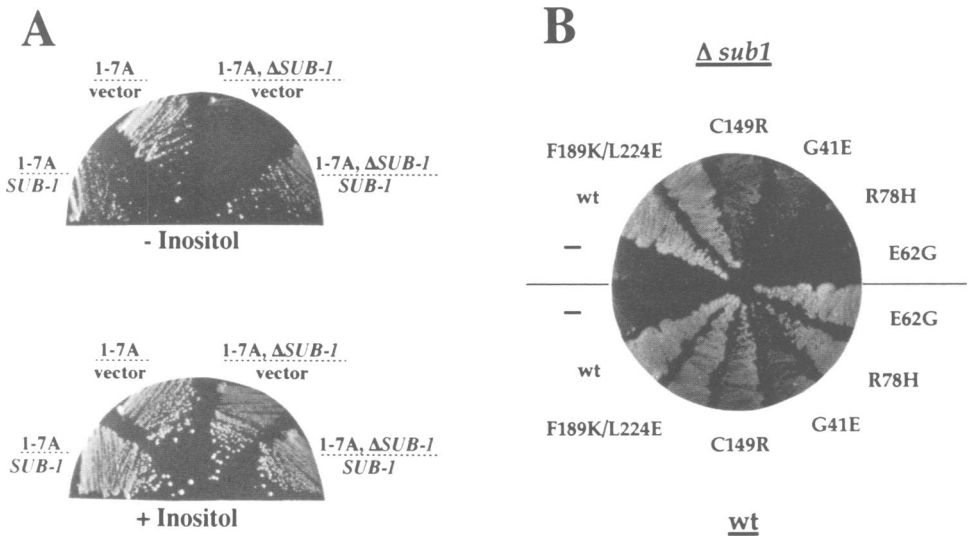

Fig. 4. Deletion of *SUB1* causes inositol auxotrophy in wild-type cells and synthetic lethality in cells expressing the TFIIB mutants E62G or R78H. (**A**) A derivative of yeast strain BWG1-7A lacking *SUB1* fails to grow on inositol starvation medium. The strains BWG1-7A and BWG1-7A/Δ*sub1* (Materials and methods) carrying either a control plasmid pRS316 (vector) or a plasmid carrying the genomic DNA fragment with the *SUB1* gene (*SUB1*) (Materials and methods) were first grown on SD medium supplemented with casamino acids (0.1%), adenine (0.01%) and glucose (2%). Single colonies were picked, streaked on inositol starvation medium and incubated for 3 days (+ inositol) and 5 days (– inositol) respectively at 30°C. Inositol starvation medium was prepared as previously described (Sherman *et al.*, 1986) and was supplemented with amino acids (0.05 g/l each), uracil (0.01%), adenine (0.01%) and glucose (2%). Where indicated, inositol was added to a concentration of 200 μM. (**B**) The deletion of *SUB1* is synthetically lethal with TFIIB E62G or R78H. Strains RKY42 and RKY42/Δ*sub1* (Materials and methods) were transformed with plasmid pRK151 (–) or derivatives encoding one of the indicated TFIIB mutants. Transformants were first selected on omission medium lacking leucine and uracil and subsequently were restreaked on medium containing 5-fluoroorotic acid as shown. Plates were incubated for 5 days at 30°C.

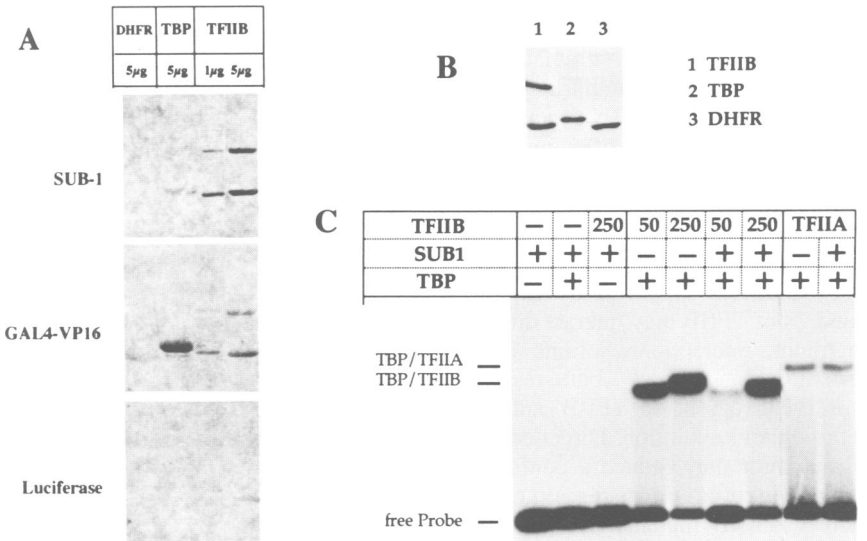

Fig. 5. SUB1 directly and specifically interacts with TFIIB *in vitro*. (**A**) Far-Western analysis of SUB1 interaction with TFIIB, TBP and DHFR. *In vitro* translated [^{35}S]methionine-labeled SUB1 (Materials and methods) interacts with immobilized recombinant TFIIB and TBP (Materials and methods) but fails to interact with recombinant DHFR. In identical control experiments, *in vitro* translated GAL4-VP16 interacts with TFIIB and TBP whereas *in vitro* translated luciferase (Materials and methods) fails to interact with any of the immobilized proteins. The lower band in the lanes with TFIIB is a C-terminal proteolytic fragment of the recombinant protein. (**B**) A Coomassie Blue-stained SDS–polyacrylamide gel showing 2 μg each of the recombinant proteins used in the *in vitro* experiments. (**C**) Electrophoretic mobility shift analysis of the SUB1 interaction with TBP–TFIIB and TBP–TFIIA complexes at the TATA box of the adenvirus major late promoter. The assay was performed as described in Materials and methods. The reactions contained 50 ng of SUB1, 10 ng of TBP and 4 U of TFIIA where indicated (+) and either 50 or 250 ng of TFIIB as shown. The position of the free probe and of the TBP–TFIIB and TBP–TFIIA complexes is indicated. Added singly, none of the proteins was able to form a stable protein–DNA complex (not shown).

mutations in the N-terminus of the protein. Interestingly, recombinant SUB1 inhibits the formation of TBP–TFIIB but not TBP–TFIIA gel shift complexes. This inhibition, which occurs at equimolar amounts of both proteins, is completely reversed by the addition of excess TFIIB (Figure 5C). We propose that the observed inhibition of the TBP–TFIIB–promoter complexes by SUB1 is the result of the mutually exclusive binding of SUB1 and TBP to the C-terminal domain of TFIIB.

While it has been proposed that the mammalian co-

activator PC4 bridges interactions between activation domains and an assembled TFIID–TFIIA complex (Ge and Roeder, 1994; Kretzschmar *et al.*, 1994), a major target of SUB1 is TFIIB. The general factor TFIIB has been suggested as a key target for transcriptional activation domains (Lin and Green, 1991). Interestingly, TFIIB seems to undergo a conformational change in the activation process. The direct interaction between an acidic activation domain and the C-terminal domain of TFIIB disrupts an intramolecular interaction between the N- and C-terminal domains of TFIIB, enabling it to adopt an active conformation. This conformational change seems to be necessary to promote the recruitment of other general transcription factors into the pre-initiation complex (Roberts and Green, 1994). It is interesting to note that a glutamine-rich activator was found to contact the N-terminal domain of TFIIB directly (Colgan *et al.*, 1995). This suggests that the interaction of activators with distinct regions of TFIIB may induce a similar conformational change in the protein.

Although the exact nature of the activator-induced conformational change in TFIIB is unclear, recent genetic data suggest that the glutamic acid and arginine residues at positions 62 and 78 of TFIIB are bonded to one another through ionic interaction (Pinto *et al.*, 1994). This intramolecular interaction could play an important role in the observed conformational change in TFIIB.

Consistent with this hypothesis, our TFIIB E62G and R78H mutants, neither of which could form the proposed salt bridge, exhibit a number of identical phenotypes; cold sensitivity, suppression of the slow growth of cells lacking the transcriptional adaptor ADA2, and a stimulation of transactivation *in vivo* (not shown). Most importantly, the *SUB1* gene dosage specifically affects the function of these two TFIIB mutants *in vivo*. Cells expressing either mutant are not viable in the absence of *SUB1* and require an increased *SUB1* copy number at 15°C.

Based on the above considerations, we propose the following model for SUB1 function. As previously suggested (Pinto *et al.*, 1994), and as supported by our own findings, residues 62 and 78 of TFIIB may interact directly with each other through ionic interactions, holding TFIIB in an inactive conformation. Interestingly, both residues are in the most conserved region of TFIIB and are absolutely conserved throughout evolution. Direct contact with a transcriptional activator may induce a conformational change in TFIIB that involves the disruption of this salt bridge. Consequently, TFIIB mutants like the ones described above might more easily adopt the active conformation and could thereby exhibit an increased affinity for their targets in the pre-initiation complex. Importantly, the reverse reaction, namely the disruption of the interaction between TFIIB and these factors during the process of transcription initiation, would be negatively affected by the E62G and R78H mutations.

Recent results (Zawel *et al.*, 1995) demonstrate that during the initiation of transcription by RNA polymerase II complexes *in vitro*, TFIID remains associated with the promoter, TFIIB is released from the complex and the other basal factors (at least initially) leave the promoter along with RNA polymerase II. Since TFIIB is released very early, its interactions with proteins remaining at the promoter (e.g. activators, TBP, TAF32) and factors traveling with the transcription complex (e.g. TFIIF, RNAPII) must be disrupted. We suggest that SUB1 is a clearance factor that promotes the release of TFIIB from the promoter by disrupting the interaction between TFIIB and TBP. By this model, the E62G and R78H mutants could depend critically on SUB1 function for their efficient release from TBP during transcription initiation, rendering the *SUB1* gene essential. Accordingly, it would explain why, at low temperatures, where the interaction between TBP and both TFIIB mutants may be stabilized further, higher levels of *SUB1* are required. Our model also explains how SUB1 can bind to the C-terminus of TFIIB yet suppresses mutations in the N-terminus of the protein. Finally, recent experiments indicate that SUB1 can dislodge a pre-assembled TBP–TFIIB–promoter complex (not shown). In this respect, SUB1 is reminiscent of MOT1, a protein that dislodges a TBP–promoter complex (Auble *et al.* 1994). Unlike MOT1, SUB1 does not require ATP for its activity. While our model is consistent with findings reported here and elsewhere, other functions for SUB1, such as those described for its putative mammalian homolog PC4, cannot be excluded.

Proteins that facilitate the disassembly of transcription complexes at promoters may be prevalent and function as key determinants of transcriptional activation *in vivo*.

Materials and methods

Yeast strains

For strain RKY16 (*MATα ura3-52 leu2-3,2-112 his4-519 ade1-100 ade2-101 lys2 sua7::hisG*) the chromosomal copy of *SUA7* was disrupted by *hisG–URA3–hisG* insertion (Alani *et al.*, 1987) between the sequence encoding amino acid 69–324 of TFIIB. Since TFIIB is expressed from the galactose-inducible promoter of plasmid pRK152, strain RKY16 grows on medium with galactose but not with glucose as carbon source.

Strain RKY42 is identical to RKY16 except that TFIIB is expressed from the ADH1 promoter on plasmid pRK161. Strain BWG1-7A has been described before (Guarente and Mason, 1983).

Plasmids

For *SUA7* under *GAL* promoter control, the *Stu*I–*Sma*I fragment of plasmid pLGSD5,*HAP1* Δ*Xho*I (Turcotte and Guarente, 1992) carrying the UAS_{GAL} upstream of the *CYC1* TATA boxes was inserted into the *Sma*I site of pRS315 (Sikorski and Hieter, 1989). This plasmid (pRK152) was cleaved subsequently with *Not*I and ligated to the PCR-amplified *SUA7* coding region flanked by *Not*I sites, resulting in plasmid pRK1521.

Plasmids pRK161 (*ARS-CEN*) and pRK261 (2μm) carrying the *URA3* gene and the *ADH1* promoter were used as recipients for mutagenized *SUA7* DNA. Briefly, the *ADH1* promoter–terminator cassette was moved from pDB20 (Becker *et al.*, 1991) to derivatives of pRS316 (Sikorski and Hieter, 1989) and pRS306,2μm (P.Sugiono, unpublished). The plasmid pRS306,2μm is a derivative of pRS306 (Sikorski and Hieter, 1989) carrying the 2μm element of Yep 352 (Hill *et al.*, 1986).

The *ARS-CEN LEU2* plasmid pRK151 carries the *ADH1* promoter–terminator cassette from pDB20. It was constructed in the background of pRS315 (Sikorski and Hieter, 1989).

PCR mutagenesis and library construction

The coding region of *SUA7* was mutagenized randomly by 'error-prone PCR' using *Taq* DNA polymerase (Stratagene), 1 ng of plasmid pDW5462 (Pinto *et al.*, 1992) as template, 50 pmol of the primers (5′-GGGCC-CAGTGTGTTGGTCATGATGACTAGGGAGAGCATAG-3′ and 5′-GGG-CCCAGTGTGATGGTTATTTCTTTTCAACGCCCGGTAAG-3′), 0.5 mM $MnCl_2$, 0.5 mM of each nucleotide and PCR buffer (Stratagene) for 35 cycles (94°C 1 min, 54°C 1 min, 72°C 2 min). The PCR products were purified, subjected to *Bst*XI cleavage and were cloned into the *Bst*XI site downstream of the *ADH1* promoter of plasmids pRK161 or pRK261. Approximately 3×10^5 *E.coli* transformants were pooled and the plasmid DNA was isolated. More than 90% of the plasmids contained the *SUA7* coding region. Sequence analysis of randomly picked library clones revealed a mutation frequency of ~$2–3\times10^{-2}$ per nucleotide.

Isolation of SUB1

Derivatives of yeast strain RKY16 expressing the TFIIB mutants G41E, G49S, E62G or R78H from plasmid pRK151 were transformed with a library of yeast cDNAs expressed from the *GAL1* promoter (Liu *et al.*, 1992). Library plasmids were isolated from all transformants which showed galactose-dependent suppression of cold sensitivity. The cDNA which suppressed the cold-sensitive phenotype of TFIIB R78H was used to isolate a corresponding clone from a yeast genomic DNA library (2μm, *URA3*, the genomic DNA was derived from strain YPH1 *MATa ura3-52 lys2-801 ade2-101*; P.Heiter, unpublished) by *E.coli* colony hybridization (Sambrook *et al.*, 1989). A plasmid carrying an ~7 kb yeast genomic DNA insert with the *SUB1* coding region and ~4 kb of upstream and 2 kb of downstream sequence was isolated from the library.

Deletion of SUB1

The *SUB1* sequence from 3 bp upstream of the coding region to codon 276 was deleted from the chromosome of BWG1-7A and RKY16 by *hisG–URA3–hisG* insertion (Alani *et al.*, 1987). The deletions were confirmed by Southern blot analysis.

High-copy SUB1

The *SalI–NotI* fragment carrying the entire *SUB1* cDNA was released from the library plasmid, treated with Klenow, ligated to *NotI* linkers, cut with *NotI*, gel-purified and inserted into the *NotI* site of plasmid pRK251 (2μm, *Leu2*, P_{ADH1}). Plasmid pRK251 is a derivative of pRS325 (Sikorski and Hieter, 1989) carrying the *ADH1* expression cassette of pRK161.

Recombinant TBP, TFIIB, SUB1 and DHFR

Six codons for histidine were fused to the 3′-end of the coding regions of *SUA7*, *SUB1* and *SPT15* (Eisenmann *et al.*, 1989; Hahn *et al.*, 1989) by PCR. The recombinant coding regions were then inserted between the *NcoI–SalI* sites of a derivative of the bacterial expression vector pQE-7 (Qiagen) from which the *BsmI–BsmI* fragment had been deleted (N.Silverman, unpublished). The recombinant proteins were expressed in the *E.coli* strain M15, pREP4 (Qiagen) and were purified from lysates of that strain over Ni-NTA columns (Qiagen). Recombinant DHFR–6× His expressed from plasmid pQE19 (Qiagen) was isolated the same way.

Western blots

For Western blotting, cultures were grown to OD_{600} = 1 in omission medium lacking leucine and uracil. Cultures were lysed in a solution containing 1.85 M NaOH and 7% β-mercaptoethanol. The proteins were TCA precipitated, rinsed with 1 M Tris-base and subsequently resuspended in 8 M urea, 0.1 M Na_2HPO_4, 10 mM Tris–HCl pH 8.0. Equal amounts (20–30 μg) of proteins were run on a 12% SDS–polyacrylamide gel and electroblotted to a Immobilon™-P membrane (Millipore). The filters were probed with rabbit antiserum raised against recombinant TFIIB–6× histidine fusion protein. The blots were developed using the Lumi-Phos™ 530 System (Boehringer) as described by the manufacturer. As secondary antibody a 1:8000 dilution of alkaline phosphatase-conjugated goat α-rabbit IgG (Sigma) was used.

In vitro transcription/translation

For *in vitro* transcription/translation of *SUB1*, the *SstI–SalI* fragment carrying the entire *SUB1* cDNA was inserted between the *EcoRI* (filled in with Klenow) and the *XhoI* site of pT7-link (Dalton and Treisman, 1992). *In vitro* transcription/translation was carried out following standard procedures (Promega). The GAL4–VP16 *in vitro* transcription/translation construct was obtained by insertion of the *BamHI* fragment of pJR3 (Berger *et al.*, 1992) into the *BamHI* site of pT7-link and subsequent treatment of the plasmid with *NcoI–AccI*, Klenow and DNA ligase. Luciferase was expressed from the control template provided by Promega.

β-Galactosidase assays

β-Galactosidase assays were performed as described previously (Rose and Botstein, 1983). All strains were transformed with reporter plasmids carrying the *lacZ* gene under control of the *CYC1* TATA boxes and the upstream activation sequences for GAL4 (pLGSD5, Guarente *et al.*, 1982), GCN4 (*HIS14x2*; Hinnebusch *et al.*, 1985) or the Hap2/3/4/5 complex (pLG265-UP1; Forsburg and Guarente, 1989). For the assays, single colonies were grown in SD medium supplemented with casamino acids (0.1%), adenine (0.01%) and glucose (2%) for pLG265-UP1 transformants, SD medium supplemented with amino acids (0.05 g/l each), adenine (0.01%) and raffinose (2%) for pLGSD5 transformants or minimal medium supplemented with leucine, histidine, adenine (0.01% each) and glucose (2%) for *HIS14x2* transformants, to an OD_{600} of 1. In order to induce GAL4 activity, galactose (4%) was added to cultures with cells carrying the pLGSD5 reporter plasmid at least 4 h before harvesting.

Far-Western analysis

One or 5 μg of the recombinant proteins were run on a 12% SDS–polyacrylamide gel and subsequently were electroblotted onto a nitrocellulose membrane (Amersham). The immobilized proteins were first denatured in 6 M guanidinium-hydrochloride for >1 h at room temperature and then subjected to a stepwise renaturation into 20 mM HEPES–KOH pH 7.3, 60 mM KCl, 7.5 mM $MgCl_2$, 2.5 mM dithithreitol (DTT) and 5% glycerol. After blocking the filter with 5% bovine serum albumin (BSA) in the same buffer for >1 h at room temperature, *in vitro* translated [^{35}S]methionine-labeled SUB1 (5 μl of a 12.5 μl standard *in vitro* translation reaction) was added and binding was allowed for at least 5 h at room temperature. Finally, the filter was washed three times with the above buffer for 20 min at room temperature before the experiment was analyzed in a PhosphorImager (Molecular Dynamics).

Electrophoretic mobility shift assays

Reactions (20 μl) contained 10 ng of TBP, 50 ng of SUB1, 50 or 250 ng of TFIIB, 4 U of TFIIA, 25 fmol of end-labeled probe (carrying the adenovirus major late promoter sequences from position −50 to +33) and 100 ng of poly(dGdC). Proteins, probe and competitor were mixed at 4°C in gel shift buffer [20 mM HEPES–KOH, pH 7.3; 60 mM KCl; 7.5 mM $MgCl_2$, 2.5 mM DTT; 5% (v/v) glycerol, 0.1% (v/v) Brij58 and 100 μg/ml BSA] and were incubated for 30 min at 20°C. The reactions were subjected to electrophoresis on native polyacrylamide gels (6%, 37:1) at 4°C and 100–150 V in Tris–glycine buffer (25 mM Tris, 190 mM glycine; pH 8.3).

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