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Srb/mediator proteins interact functionally and physically with transcriptional repressor Sfl1

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Srb/mediator proteins that are associated with RNA polymerase II holoenzyme have been implicated in transcriptional repression in *Saccharomyces cerevisiae*. We show here that the defect in repression of *SUC2* caused by mutation of *SRB8*, *SRB9*, *SRB11*, *SIN4* or *ROX3* is suppressed by increased dosage of the *SFL1* gene, and the genetic behavior of the *sfl1Δ* mutation provides further evidence for a functional relationship. Sfl1 acts on *SUC2* through a repression site located immediately 5′ to the TATA box, and Sfl1 binds this DNA sequence *in vitro*. Moreover, LexA–Sfl1 represses transcription of a reporter, and repression is reduced in an *srb9* mutant. Finally, we show that Sfl1 co-immunoprecipitates from cell extracts with Srb9, Srb11, Sin4 and Rox3. We propose that Sfl1, when bound to its site, interacts with Srb/mediator proteins to inhibit transcription by RNA polymerase II holoenzyme.

Keywords: mediator/*Saccharomyces cerevisiae*/Srb/*SUC2*/transcriptional repression

Introduction

Transcriptional regulation requires the interactions of specific regulatory proteins with components of the transcription machinery. Recent work has indicated that RNA polymerase II holoenzyme forms play an important role in transcriptional regulatory mechanisms (for a review, see Greenblatt, 1997). In particular, the Srb/mediator proteins that are associated with the holoenzyme have been implicated in both transcriptional activation and repression.

The *SRB* genes were identified in *Saccharomyces cerevisiae* by Young and colleagues as suppressors of a C-terminal heptapeptide repeat domain (CTD) truncation of RNA polymerase II (Nonet and Young, 1989; Hengartner *et al*., 1995; Liao *et al*., 1995), and the Srb proteins were found associated with an RNA polymerase II holoenzyme that responds to transcriptional activators (Thompson *et al*., 1993; Koleske and Young, 1994; Hengartner *et al*., 1995). A holoenzyme form containing a mediator that confers responsiveness to activators was identified independently by Kornberg and colleagues (Kim *et al*., 1994). The mediator is associated with the CTD and also stimulates basal transcription and phosphorylation of the CTD. The mediator comprises Srb2, Srb4, Srb5, Srb6, Srb7, Gal11, Sin4, Rgr1, Rox3, Hrs1 and Med proteins, but not Srb8, Srb9, Srb10 or Srb11 (Kim *et al*., 1994; Li *et al*., 1995; Gustafsson *et al*., 1997; Myers *et al*., 1998). RNA polymerase II holoenzyme complexes have also been isolated from mammalian cells and found to contain Srb homologs, including Srb7 and cyclin C/cdk8 (Ossipow *et al*., 1995; Chao *et al*., 1996; Maldonado *et al*., 1996; Pan *et al*., 1997; Neish *et al*., 1998), which is a homolog of the Srb10/Srb11 kinase (Surosky *et al*., 1994; Kuchin *et al*., 1995; Liao *et al*., 1995).

Genetic analysis has revealed that some Srb/mediator proteins have roles in transcriptional repression. Mutations in *SRB8*, *SRB9*, *SRB10*, *SRB11*, *SIN4*, *ROX3*, *GAL11*, *RGR1* and *HRS1* affect the negative regulation of a diverse set of promoters and have been isolated in many different mutant searches (Sternberg *et al*., 1987; Sakai *et al*., 1990; Rosenblum-Vos *et al*., 1991; Chen *et al*., 1993; Covitz *et al*., 1994; Stillman *et al*., 1994; Surosky *et al*., 1994; Balciunas and Ronne, 1995; Kuchin *et al*., 1995; Wahi and Johnson, 1995; Song *et al*., 1996; Piruat *et al*., 1997; for review, see Carlson, 1997). The mechanism by which these genes affect repression remains unclear, but evidence suggests a role in the response to DNA-binding repressors. The mutations *sin4* and *rgr1* relieve repression of reporters by Rme1, a repressor of meiotic gene expression (Covitz *et al*., 1994; Shimizu *et al*., 1997); *sin4* and *srb10* relieve repression by α2-Mcm1 (Chen *et al*., 1993; Wahi and Johnson, 1995); and *srb10* and *srb11* reduce repression by Mig1 (Kuchin and Carlson, 1998). α2-Mcm1 and Mig1 function in concert with the Ssn6(Cyc8)–Tup1 corepressor (Keleher *et al*., 1992; Treitel and Carlson, 1995; Tzamarias and Struhl, 1995), and *SRB10* and *SRB11* are required for repression of reporters by LexA fusions to Ssn6 and Tup1 (Kuchin and Carlson, 1998). These data implicate Srb/mediator proteins in the response to Ssn6–Tup1; however, evidence indicates that Ssn6–Tup1 also represses transcription by mechanisms involving chromatin (Cooper *et al*., 1994; Roth, 1995; Edmondson *et al*., 1996). No direct physical interaction between Ssn6–Tup1 and Srb/mediator proteins has been reported.

Our laboratory has focused on the role of Srb/mediator proteins in glucose repression of *SUC2* transcription. We previously identified alleles of *SRB8*, *SRB9*, *SRB10*, *SRB11*, *SIN4* and *ROX3* as *ssn* (suppressor of *snf1*) mutations that affect *SUC2* repression (Carlson *et al*., 1984; Kuchin *et al*., 1995; Song *et al*., 1996); for simplicity, we will refer to these six collectively as *srb/ssn* mutations. Repression of *SUC2* requires Ssn6–Tup1, which is recruited to upstream sites by Mig1 and a second DNA-binding protein, Mig2 (Schultz and Carlson, 1987; Nehlin and Ronne, 1990; Williams *et al*., 1991; Treitel and Carlson, 1995; Tzamarias and Struhl, 1995; Lutfiyya and Johnston, 1996). The *srb/ssn* mutations synergize strongly with *mig1* to relieve glucose repression of *SUC2* (Vallier and Carlson, 1994).

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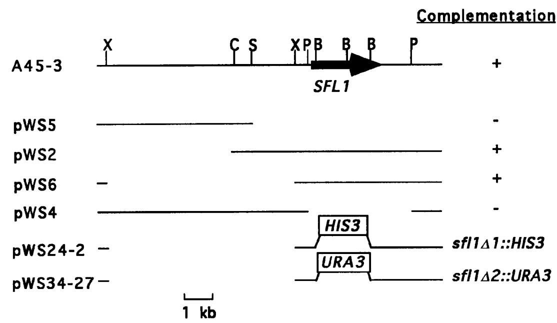


Fig. 1. Restriction maps of *SFL1* plasmids. Only the inserted yeast DNA is shown. Plasmids were tested for complementation of the flocculent phenotype of strain MCY3304 (*srb9 mig1 snf1*). Clones A45-3 and pWS6 were also tested for complementation of the *SUC2* repression defect, and the invertase activities in glucose-grown transformants were 4.4 and 16 U, respectively, compared with 53 U in cells carrying the vector YCp50. The invertase activity in a *mig1 snf1* mutant was 4 U (Song *et al.*, 1996). Restriction sites: B, *Bsp*120I; C, *Cla*I; P, *Pvu*II; S, *Sal*I; X, *Xho*I.

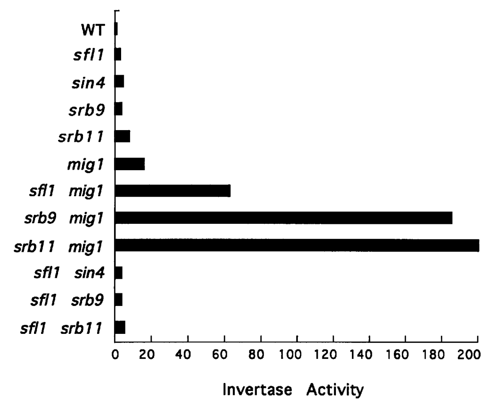


Fig. 2. Genetic interactions between *sfl1Δ* and the mutations *mig1Δ srb9Δ*, *srb11Δ* and *sin4Δ*. Bars represent the invertase activity in cells of the indicated genotype grown in SC + 3% glucose. Values are averages of two to four assays. Standard errors were <10%. All strains have the S288C genetic background, and the alleles were *sfl1Δ1::HIS3*, *srb9/ssn2Δ1::URA3*, *srb11/ssn8Δ2::LEU2*, *ssn8Δ2::HIS3* and *sin4Δ::TRP1*. Wild type (WT) was FY250.

In this work, we have identified a new mechanism for repression of *SUC2* that directly involves Srb/mediator proteins. We recovered the *SFL1* gene as a suppressor of *srb9*. We show that the Sfl1 protein functions as a repressor, binds to a repression site near the *SUC2* TATA sequence, and interacts functionally and physically with Srb/mediator proteins.

Results

Increased dosage of the SFL1 gene suppresses srb/ssn mutations

While cloning the *SRB9* gene (Song *et al.*, 1996), we recovered a low-copy suppressor of the *srb9* mutation. Our cloning strategy took advantage of the flocculent phenotype conferred by *srb9* and the synergy between *srb9* and *mig1* in relieving glucose repression of *SUC2* (see Materials and methods). We transformed an *srb9 mig1* strain with a library in a centromere vector and recovered clone A45-3, which suppressed both phenotypes (Figure 1). Subcloning and sequencing identified the gene as *SFL1*, which encodes a 767-amino-acid protein with homology (residues 65–142 and 182–205) to the conserved DNA-binding domain of heat-shock transcription factors (Fujita *et al.*, 1989).

To test whether *SFL1* suppresses defects associated with other *srb/ssn* mutations, we used pWS6 (Figure 1) to transform strains carrying each of the mutations *srb8*, *srb10*, *srb11*, *sin4* and *rox3* in a *mig1* mutant background. pWS6 partially suppressed the flocculent phenotypes of all the mutants and, except in the case of *srb10*, their defects in glucose repression of *SUC2* (Table I). A likely explanation for the lack of suppression of *srb10* is that Sfl1 is unstable in this mutant background; tagged Sfl1 proteins were smaller than full size in the *srb10* mutant (data not shown). Suppression was not dependent on the presence of *mig1*, as pWS6 also suppressed the *SUC2* repression defect caused by a single *srb11* mutation.

Disruption of SFL1 confers phenotypes similar to those of srb/ssn mutations

To disrupt the *SFL1* gene, we introduced deleted alleles (Figure 1) into wild-type haploid strains; the gene is not essential for viability (Fujita *et al.*, 1989). The *sfl1Δ* mutation caused flocculence and a slight defect in glucose repression of *SUC2*, and synergized with *mig1Δ* to relieve glucose repression (Figure 2). The *sfl1Δ* mutation also weakly suppressed the growth defect of a *snf1Δ* mutant on sucrose (data not shown) and can thus be categorized as an *ssn* suppressor. No temperature sensitivity, cold sensitivity, or defect in mating, sporulation or derepression of *SUC2* was observed.

We also examined genetic interactions between *sfl1* and *srb/ssn* mutations. *sfl1Δ* did not synergize with *srb9Δ*, *srb11Δ* or *sin4Δ* to release repression of *SUC2* (Figure 2), whereas each of these mutations showed synergy with *mig1* (Figure 2; Vallier and Carlson, 1994). In crosses of the *sfl1Δ1* mutant to *srb/ssn* mutants, we observed partial non-complementation between *sfl1Δ1* and *srb8 (ssn5-4)* for the flocculent phenotype.

The similar mutant phenotypes, genetic interactions of the mutations and dosage suppression together provide

Table I. Suppression of *srb/ssn* mutations by low-copy *SFL1*

Relevant genotype	Invertase activity	
	YCp50	pWS6
srb8 mig1 snf1	52	17
srb9 mig1 snf1	37	16
srb10 mig1 snf1	49	48
srb11 mig1 snf1	56	19
sin4 mig1 snf1	82	16
rox3 mig1 snf1	130	54
mig1 snf1	5.4	5.4
srb11	13	5.2
Wild type	2.4	2.6

Strains (see Table III) were transformed with the vector YCp50 or pWS6, carrying *SFL1*. Transformants were grown in SC +2.5% glucose and assayed for secreted invertase activity. Values are in most cases averages for assays of three transformants, and standard errors were <20%. The presence of a *snf1* allele reduces invertase activity to some extent even in glucose-grown cultures. *srb snf1*, *sin4 snf1* and *rox3 snf1* double mutants produce no more than 2 U of activity (Vallier and Carlson, 1994).

Table II. Overexpression of GAD–Sfl1 and HA_3–Sfl1 allows *SUC2* expression in glucose-grown cells

Overexpressed protein	Invertase activity			
	Wild type		*sfl1Δ*	
	R	D	R	D
GAD–Sfl1	74	260	83	390
GAD	1.6	120	2.2	120
Sfl1	1.1	69	1.4	76
None (vector)	1.1	110	1.4	130
HA_3–Sfl1	14	ND	ND	ND
Sfl1–HA_4	1.1	ND	ND	ND
HA_3	1.2	ND	ND	ND

Strains were FY250 and its derivative MCY3802. Plasmids were pWS35, pACTII, pWS42, pSK37, pWS96, pWS94 and pWS93. Cultures were grown in SC + 3 % glucose (R, repressed) and shifted to SC + 0.05% glucose for 3 h (D, derepressed). Values are averages for assays of two to four transformants. Standard errors were <10%. ND, not determined.

strong genetic evidence for a functional connection between Sfl1 and the Srb/mediator proteins.

DNA-bound LexA–Sfl1 represses transcription

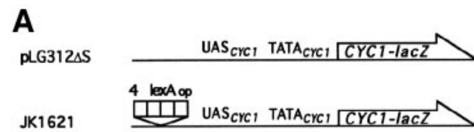
The genetic evidence suggested that Sfl1 functions in transcriptional repression of *SUC2*. In addition, overexpression of Sfl1 from the *ADH1* promoter reduced derepression of *SUC2* by 40% relative to the vector control (Table II). We therefore assayed LexA–Sfl1, containing the LexA DNA-binding domain fused to Sfl, for the ability to repress transcription of a *CYC1–lacZ* reporter with *lexA* operators 5′ to the UAS. LexA–Sfl1 repressed the expression of this reporter 29-fold in glucose-grown cells (Figure 3B). In raffinose-grown cells, no significant repression was detected (Figure 3B) and the LexA–Sfl1 protein was undetectable (Figure 3C).

To determine whether repression by Sfl1 requires Srb9, we assayed an isogenic *srb9Δ* mutant. Repression by LexA–Sfl1 was reduced by a factor of 4 (from 29- to 7.6-fold); immunoblot analysis showed that the level of LexA–Sfl1 protein was not reduced (Figure 3C).

We also examined the dependence on Ssn6–Tup1, which is required for repression of *SUC2*. Repression by LexA–Sfl1 was abolished in *ssn6Δ* and *tup1Δ* mutants (1.3- and 1.6-fold repression, respectively; Figure 3B and data not shown); however, immunoblot analysis of the *ssn6Δ* strain showed that the level of LexA–Sfl1 protein was ~4-fold lower than that in wild type (Figure 3C). The loss of repression and the instability of Sfl1 in the absence of Ssn6 suggest a functional connection.

The ability of Sfl1 to repress transcription distinguishes Sfl1 from the Srb/mediator proteins. LexA fusions to Srb9, Srb10 and Srb11 do not repress this reporter (data not shown). Conversely, DNA-bound LexA fusions to Srb9, Srb11, Sin4, Rox3 and Gal11 activate transcription of reporters (Himmelfarb *et al*., 1990; Jiang and Stillman, 1992; Kuchin *et al*., 1995; Song *et al*., 1996), whereas LexA–Sfl1 does not function as an activator in such assays (data not shown).

A



B

Relevant Genotype	Expressed Proteins	Repressed lexAop 0	Repressed lexAop 4	Repressed Fold	Derepressed lexAop 0	Derepressed lexAop 4	Derepressed Fold
WT	LexA	230	120	1.9X	500	230	2.2X
	LexA-Sfl1	160	5.7	29 X	670	250	2.7X
srb9Δ	LexA	270	120	2.2X			
	LexA-Sfl1	180	24	7.6X			
ssn6Δ	LexA	43	29	1.5X			
	LexA-Sfl1	38	29	1.3X			
WT	LexA +GAD	280	260	1.1X			
	LexA-Sfl1+GAD	97	11	8.8X			
	LexA +GAD-Sfl1	160	64	2.4X			
	LexA-Sfl1+GAD-Sfl1	140	43	3.3X			

C

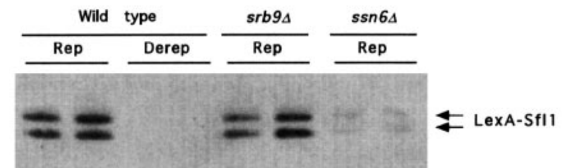


Fig. 3. LexA–Sfl1 represses transcription. (**A**) Target plasmids. JK1621 (Keleher *et al*., 1992) is derived from pLG312ΔS (Guarente and Hoar, 1984). (**B**) Repression of target gene expression by the indicated LexA protein in wild-type (WT) and mutant strains. Strains were MCY3647, MCY3817 and MCY1974. Expression plasmids were pSH2-1, pWS41, pACTII and pWS35. Transformants were grown in SC + 4% glucose (repressed), or SC + 2% raffinose + 0.05% glucose (derepressed). β-galactosidase activity was assayed in permeabilized cells and expressed in Miller units. Values are average for three transformants. In each case, the fold repression was derived by comparing the β-galactosidase activity obtained for the target with four *lexA* operators with the activity for the target with no *lexA* operator. Standard errors were <10%. (**C**) The level of LexA–Sfl1 was monitored by immunoblotting using anti-LexA antibody. Cultures of two independent transformants were collected by centrifugation, and the pellet was resuspended in sample buffer containing 5 mM EDTA and immediately boiled for 5 min. The supernatant was collected after centrifugation and the equivalent of 3 ml of culture at OD_{600} = 0.5 was used for each lane.

GAD–Sfl1 acts through the element for response to Sfl1 (ERS) site immediately 5′ to the SUC2 TATA box

The presence of a putative DNA-binding domain in the Sfl1 protein suggested that Sfl1 contributes to repression of *SUC2* by binding to the promoter. We reasoned that a Gal4 activation domain (GAD) fusion to Sfl1 might function as a transcriptional activator and thereby facilitate localization of the Sfl1 recognition site. Expression of GAD–Sfl1 from the *ADH1* promoter strongly activated *SUC2* expression in glucose-grown cells (Table II) and also caused flocculence and slow growth. These effects of GAD–Sfl1 were also detected in an *sfl1Δ* mutant, indicating that the native Sfl1 protein is not required. In control experiments, expression of the unfused Sfl1 from the *ADH1* promoter did not cause these phenotypes (Table II).

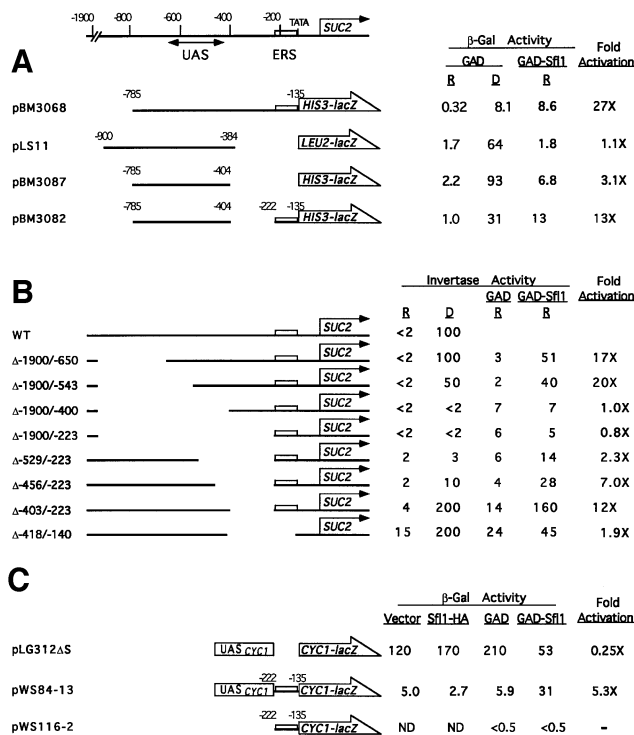


Fig. 4. GAD–Sfl1 interferes with transcriptional repression through the ERS site 5′ to the *SUC2* TATA box. The upstream region of *SUC2* is drawn to scale at the top. In each case, the fold activation was derived by comparing the activity under glucose-repressing conditions (R) of transformants expressing GAD–Sfl1 with that of transformants expressing GAD. (**A**) Effects of GAD–Sfl1 on the expression of *lacZ* reporters under the control of *SUC2* sequences. Strain MCY3824, which carries an integrated copy of pLS11, a *LEU2–lacZ* reporter driven by the upstream region of *SUC2* (Sarokin and Carlson, 1985), was transformed with pWS35 (GAD–Sfl1) or vector pACTII (GAD). Strain MCY3647 was co-transformed with pBM3068, pBM3082 or pBM3087, which carry *HIS3–lacZ* reporters driven by *SUC2* upstream sequences (Ozcan *et al*., 1997), and either pWS35 or pACTII. Transformants were grown in SC + 4% glucose (R, repressed) and shifted to SC + 0.05% glucose for 3 h (D, derepressed). β-galactosidase activity was assayed in permeabilized cells and expressed in Miller units. (**B**) Effects of GAD–Sfl1 on the expression of invertase from a series of deletions at the genomic *SUC2* locus. Values for invertase activity of these deletion mutants are taken from Sarokin and Carlson (1984). Mutants were transformed with either pWS53 (GAD–Sfl1) or vector pWS52 (GAD), grown in SC + 4% glucose and assayed for invertase activity. (**C**) Effects of GAD–Sfl1 and Sfl1–HA on the expression of *CYC1–lacZ* reporters containing the ERS sequence. Transformants of strain MCY3647 were grown in SC + 4% glucose and assayed as in (A). Expression plasmids were pWS64, its parent vector pSK37, pWS35 and pACTII. Reporters were pLG312ΔS (Guarente and Hoar, 1984) and the indicated derivatives. Values in this figure are averages for two to four transformants; standard errors were <10%.

To localize the site of Sfl1 function, we first showed that in glucose-grown cells GAD–Sfl1 activates a *lacZ* reporter containing the entire *SUC2* upstream region and the *HIS3* TATA sequence (pBM3068; Figure 4A). We then tested the effect of GAD–Sfl1 on expression of reporters with the *SUC2* UAS and the *HIS3* or *LEU2* TATA sequence; *SUC2* sequences between –650 and –418 are required for wild-type levels of *SUC2* derepression and are sufficient to confer glucose-regulated expression to a heterologous promoter (Sarokin and Carlson, 1984; Sarokin and Carlson, 1986). GAD–Sfl1 did not significantly affect expression of either reporter (pLS11 and pBM3087; Figure 4A), indicating that the *SUC2* UAS does not mediate the effect of GAD–Sfl1. The region critical for the action of GAD–Sfl1 was identified by comparison of pBM3082 and pBM3087, which differ only by the presence of the *SUC2* sequence from –222 to –135 (Figure 4A).

The importance of this region was confirmed by analysis of upstream deletions in the genomic *SUC2* locus. Activation by GAD–Sfl1 required the sequence from –222 to –140; GAD–Sfl1 activated expression of the Δ–403/–223 deletion but had no significant effect on the Δ–418/–140 locus (Figure 4B). The sequence from –222 to –135 is termed ERS. The ERS is located immediately 5′ to the *SUC2* TATA box at –133.

Evidence that GAD–Sfl1 interferes with repression of SUC2

Analysis of the *SUC2* deletions also revealed that activation by GAD–Sfl1 requires the function of the *SUC2* UAS. The level of activation by GAD–Sfl1 in glucose-grown cells correlated with the integrity of the UAS, as monitored by *SUC2* expression in derepressed cells (Figure 4B). These findings indicated that the activation of *SUC2* by GAD–Sfl1 does not reflect simple transcriptional activation by the GAD sequence. An alternative possibility was that GAD–Sfl1 acts as a dominant-negative factor to disrupt a repression mechanism involving the native Sfl1 and its recognition site. The finding that GAD–Sfl1 confers flocculence, a phenotype characteristic of *sfl1Δ*, also supported this view.

To test this idea, we first determined whether GAD–Sfl1 interferes with repression by LexA–Sfl1. In the presence of GAD–Sfl1, LexA–Sfl1 did not repress much more effectively than $LexA_{87}$, whereas in the control with GAD, LexA–Sfl1 repressed 8-fold better than $LexA_{87}$ (Figure 3B). Secondly, we showed that the GAD moiety is not specifically required for the observed effects; overexpression of HA_3–Sfl1, with an N-terminal triple hemagglutinin (HA) epitope, also activated expression of *SUC2* in glucose-grown cells (Table II) and conferred flocculence. Neither Sfl1–HA_4 (tagged at the C terminus), Sfl1–HA nor $LexA_{87}$–Sfl1 caused either phenotype. Together, these findings strongly suggest that certain N-terminal modified derivatives of Sfl1, when overexpressed, function to relieve Sfl1-mediated repression.

ERS site mediates repression by Sfl1

To test directly whether the *SUC2* ERS confers repression, we inserted the ERS between the UAS and TATA sequence of *CYC1–lacZ* in pLG312ΔS (pWS84-13; Figure 4C). Insertion of the ERS sequence reduced expression of β-galactosidase 24-fold (from 120 to 5.0 U), and overexpression of Sfl1–HA increased the repression to 64-fold (from 170 to 2.7 U). In contrast, GAD–Sfl1 alleviated repression by ERS; repression was 36-fold in the presence of GAD (from 210 to 5.9 U) and only 1.7-fold in the presence of GAD–Sfl1 (from 53 to 31 U). As observed for other reporters containing ERS, the effect of GAD–Sfl1 was also apparent as activation of expression of pWS84-13 (5.3-fold relative to GAD). In control experiments, GAD–Sfl1 did not activate a reporter containing the ERS 5′ to the *CYC1* core promoter (pWS116-2), confirming that the effects of GAD–Sfl1 on pWS84-13

are not due to transcriptional activation by GAD–Sfl1 bound to ERS.

Analysis of the *SUC2* deletions provided further evidence that ERS is a repression site. The deletion Δ–418/–140 partially relieves glucose repression of *SUC2*, allowing invertase expression in glucose-grown cells (15 U; Figure 4B). Moreover, the Δ–418/–140 mutation exhibits synergy with *mig1Δ*; repressed invertase activity in the double mutant was 93 U. In this respect, the deletion behaves similarly to *sfl1Δ* and *srb/ssn* mutations (see Figure 2).

Sfl1 binds the SUC2 ERS

Genetic evidence that Sfl1 functions via the *SUC2* ERS suggested that Sfl1 binds to this site. We therefore tested whether immobilized HA-tagged Sfl1 protein from yeast protein extracts can specifically retain ^{32}P-labeled ERS DNA. Extracts were prepared from glucose-grown cells expressing Sfl1–HA, Sfl1–HA_4 or HA_3-Sfl1, and were incubated with monoclonal anti-HA antibody. Immune complexes were immobilized onto rProtein A–Sepharose beads and assayed by a DNA-binding reaction for ability to retain a ^{32}P-labeled ERS fragment. All three HA-tagged Sfl1 proteins bound labeled ERS fragment (Figure 5A, lanes 4–6). Control experiments showed that binding requires antibody (lane 3) and HA-tagged Sfl1; no retention was observed in experiments with HA_3, Sfl1 or HA_3–Srb10 protein (lanes 1, 2 and 7). Competition experiments showed that this binding was ERS-specific (Figure 5B). Binding was effectively competed by addition of unlabeled ERS fragment (lanes 3–6) but not by a 50-fold excess of an unrelated 88 bp fragment [non-specific (NS)] with identical ends and similar G/C content (Figure 5B, lane 7). Moreover, labeled NS fragment was not retained by Sfl1–HA_4 in a binding assay (lane 8). Thus, Sfl1 binds specifically to the ERS sequence *in vitro*. It is possible that other proteins co-purify with Sfl1 and contribute to this binding.

Sfl1 co-immunoprecipitates with Srb9, Srb11, Sin4 and Rox3

The genetic interactions between *SFL1* and *SRB/SSN* alleles, together with the binding of Sfl1 to a site adjacent to the TATA sequence, suggested the possibility of direct interaction between Sfl1 and Srb/mediator proteins that are associated with RNA polymerase II holoenzyme. To test for physical interaction, we carried out co-immunoprecipitation experiments. Extracts were prepared from glucose-grown cells expressing Sfl1–HA_4 and a LexA fusion to Srb9, Srb11, Sin4 or Rox3. Sfl1–HA_4 was immunoprecipitated with monoclonal HA antibody, and the precipitates were analyzed by immunoblotting with LexA antibody. All four LexA fusion proteins co-immunoprecipitated with Sfl1–HA_4 (Figure 6A–C). In control experiments, very little or no LexA fusion protein was precipitated when an untagged Sfl1 protein was expressed; moreover, the control protein LexA–Snf6 did not co-immunoprecipitate with Sfl1–HA_4 (although LexA–Snf6 was weakly detected after long exposure). Nor did we detect any co-precipitation of Sfl1–HA_4 and LexA–Srb9 if an unrelated mouse monoclonal antibody or no antibody was used (data not shown).

We further tested for co-immunoprecipitation of LexA–Sfl1 with HA_3–Srb9, HA_3–Srb11 and HA_3–Sin4. Extracts were prepared from cells expressing each pair of proteins, monoclonal HA antibody was used to immunoprecipitate the HA-tagged protein, and the precipitates were analyzed by immunoblotting with LexA antibody. LexA–Sfl1 co-immunoprecipitated with all three HA-tagged proteins, but did not precipitate when only the triple HA tag was expressed (Figure 6D and E).

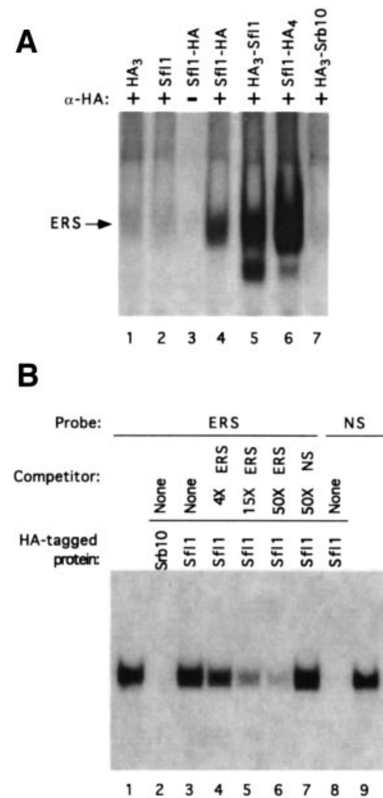


Fig. 5. Sfl1 binds the ERS sequence. Protein extracts were prepared from glucose-grown strain MCY3806 (*sfl1Δ1::HIS3*) expressing the indicated proteins from plasmids pWS93, pWS42, pWS64, pWS94, pWS96 and pSK84 (expresses HA_3–Srb10 from vector pWS93; gift of S.Kuchin). HA-tagged proteins were immunoprecipitated with anti(α)-HA and then were assayed for their ability to retain ^{32}P-labeled ERS sequence in a DNA-binding reaction, as described in Materials and methods. (**A**) Anti-HA antibody was added to the binding reactions, except for lane 3, in which no antibody was added. (**B**) Probes were ^{32}P-labeled ERS sequence (lanes 1–7) and the non-specific NS sequence (see Materials and methods; lanes 8 and 9); free probes are shown in lanes 1 and 9. DNA-binding reactions were carried out using protein extracts prepared from MCY3806 expressing HA_3–Srb10 (lane 2) or Sfl1–HA_4 (lanes 3–8). Unlabeled ERS DNA (specific competitor) was added to the binding reaction in 4×, 15× or 50× molar excess relative to the labeled probe (lanes 4–6); unlabeled NS DNA (non-specific competitor) was added in 50× excess (lane 7). Autoradiograms are shown.

Discussion

Previous studies have shown that Srb/mediator proteins contribute to transcriptional repression of *SUC2*. Here we present genetic and biochemical evidence that the Sfl1 protein is functionally related to Srb/mediator proteins and that Sfl1 represses transcription of *SUC2* via the ERS site 5′ to the TATA sequence.

Several lines of evidence support the view that Sfl1 is functionally related to Srb/mediator proteins. First, we

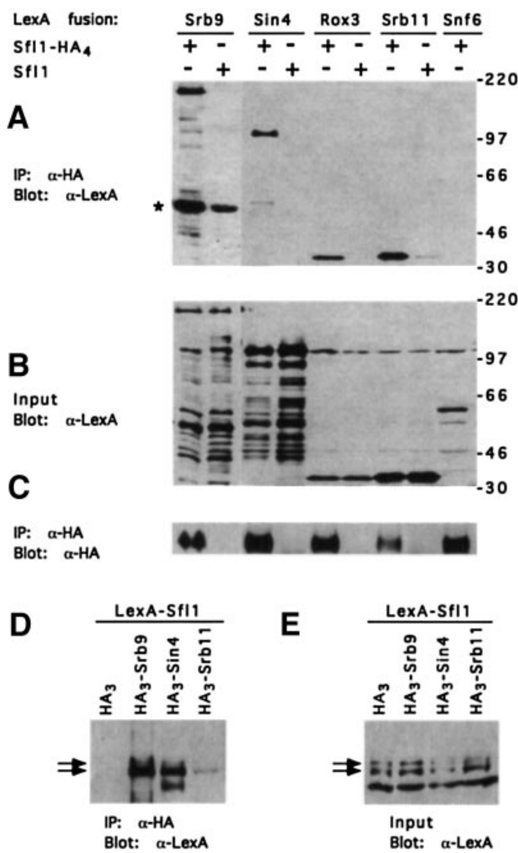


Fig. 6. Srb9, Srb11, Sin4 and Rox3 co-immunoprecipitate with Sfl1. Protein extracts (250 μg, except 500 μg for LexA–Snf6 control) were prepared from a glucose-grown wild-type strain FY250 expressing the indicated fusion proteins. (**A**) HA-tagged proteins were immunoprecipitated (IP) with α-HA, separated by 7% SDS–PAGE and immunoblotted with α-LexA. The LexA–Srb9 lanes are from an independent experiment; the predominant degradation product of LexA–Srb9, marked by an asterisk, co-migrates with a cross-reacting IgG band that is visible in the second lane and was also visible in the LexA–Snf6 control lane for this experiment (not shown). (**B**) Immunoblot analysis of the input proteins (25 μg, except for 50 μg for the LexA–Snf6 control). (**C**) The immunoblot shown in (A) was reprobed with α-HA to confirm the precipitation of Sfl1–HA_4. (**D**) HA-tagged proteins were immunoprecipitated, separated by SDS–PAGE and immunoblotted with α-LexA. The positions of LexA–Sfl1 polypeptides are marked by arrows. The immunoblot shown here was reprobed with α-HA to confirm the precipitation of HA_3–Srb9, HA_3–Sin4 and HA_3–Srb11 (not shown). (**E**) Immunoblot analysis of the input LexA–Sfl1 protein (25 μg) used in (D). Proteins were expressed from (A–C) pWS94, pWS42, pWS54, pWS125, pIT220 (LexA–Rox3; Song *et al*., 1996), pSK32 (LexA-Srb11; Kuchin *et al*., 1995) and pLexA–SNF6 (Laurent and Carlson, 1992) and (D and E) pWS93, pWS121, pWS98 and pSK86 (expresses HA_3–Srb11 from vector pWS93; gift of S.Kuchin). Positions of the size standards (kDa) are marked.

recovered the *SFL1* gene as a low-copy suppressor of *srb9* and showed that it also suppresses *srb8*, *srb10*, *srb11*, *sin4* and *rox3* mutations for flocculence and/or *SUC2* regulation. Secondly, the *sfl1Δ* deletion resembles *srb/ssn* mutations in causing similar phenotypes and showing synergy with *mig1* for release of glucose repression of *SUC2*. In contrast, *sfl1Δ* shows no synergy with *srb9*, *srb11* or *sin4*, consistent with a related function. Thirdly, transcriptional repression of a reporter by DNA-bound LexA–Sfl1 was partly dependent on Srb9. Finally, Sfl1 co-immunoprecipitated with tagged Srb9, Srb11, Sin4 and Rox3 proteins. These findings indicate that Sfl1 interacts with complexes containing Srb/mediator proteins. Sfl1 has not been reported as an integral component of such complexes.

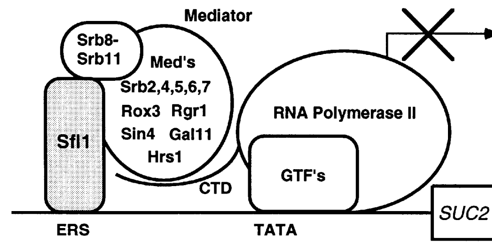


Fig. 7. Model for interaction of Sfl1 with Srb/mediator proteins at the *SUC2* promoter. The Sfl1 protein is shown bound to the ERS site, where it interacts with Srb/mediator proteins to repress transcription. Possible mechanisms are discussed in the text. Other proteins may bind to the ERS with Sfl1. The RNA polymerase II holoenzyme is depicted with the mediator associated with its CTD. The relationship of Srb8–Srb11 to the mediator is controversial (Hengartner *et al*., 1995; Myers *et al*., 1998), so these proteins are shown as a separate, associated complex. Other general transcription factors (GTFs) are not represented in detail. Not drawn to scale.

The genetic effects of *sfl1Δ* on *SUC2* expression, together with the ability of LexA–Sfl1 to repress a reporter, implicate Sfl1 in transcriptional repression of *SUC2*. We mapped the sequence that mediates Sfl1 function to the ERS 5′ to the *SUC2* TATA box. Several lines of genetic evidence indicate that the ERS is a site for repression by Sfl1. Deletion of the ERS partially relieves glucose repression of *SUC2*, and this deletion, like *sfl1Δ*, acts synergistically with *mig1Δ*. Moreover, insertion of the ERS between the UAS and the TATA sequence confers repression to a *CYC1–lacZ* fusion, and this repression is relieved by the overexpression of GAD–Sfl1. The sequence homology of Sfl1 to the DNA-binding domains of heat-shock transcription factors suggested that Sfl1 binds to the *SUC2* promoter, and we showed that HA-tagged Sfl1, when purified from cell extracts, specifically binds the ERS DNA sequence *in vitro*. These studies support a model in which Sfl1 binds to the ERS, perhaps in conjunction with other DNA-binding proteins, and functions to repress *SUC2* transcription. This Sfl1-dependent repression is complementary to other repression mechanisms that involve Mig1, Mig2 and the Ssn6–Tup1 complex. The regulation of Sfl1 function by the glucose signal remains to be examined; both LexA–Sfl1 and Sfl1–HA_4 are difficult to detect in extracts from glucose-limited cells (W.Song, unpublished results), which may reflect the operation of a regulatory mechanism.

Why does overexpression of GAD–Sfl1 or HA_3–Sfl1 dramatically relieve repression of *SUC2*, whereas loss of Sfl1 causes only a minor effect? A possible explanation is that one or more proteins function redundantly with Sfl1, and overexpression of GAD–Sfl1 or HA_3–Sfl1 has a dominant-negative effect on their function. The yeast genome includes four genes encoding proteins with similar DNA-binding domains: *HSF1*, *MGA1*, *SKN7* and YJR147w. Alternatively, it is possible that GAD–Sfl1 and HA_3–Sfl1 relieve repression by interacting with Srb/mediator proteins and interfering with a general repression mechanism.

We have shown that Sfl1 binds to a repression site near the *SUC2* TATA sequence, that Sfl1 contributes to

Table III. List of *S.cerevisiae* strains

Strain[a]	Genotype
MCY1974	*MAT*α *ssn6Δ9 ade2-101 his3Δ200 lys2-801 ura3-52 trp1Δ1*
MCY3304	*MAT*α *srb9/ssn2-4 mig1Δ2::LEU2 snf1 ade2-101 his4-539 ura3-52*
MCY3309	*MAT*α *srb10/ssn3-1 mig1Δ2::LEU2 snf1 ade2-101 his4-539 ura3-52*
MCY3312	*MAT***a** *sin4/ssn4-1 mig1Δ2::LEU2 snf1 ade2-101 his4-539 lys2-801 ura3-52*
MCY3316	*MAT*α *srb8/ssn5-4 mig1Δ2::LEU2 snf1 ura3-52 his4-539*
MCY3319	*MAT***a** *rox3/ssn7-1 mig1Δ2::LEU2 snf1 ura3-52 his4-539*
MCY3322	*MAT***a** *srb11/ssn8-1 mig1Δ2::LEU2 snf1 ura3-52 his4-539*
MCY3337	*MAT*α *mig1Δ2::LEU2 snf1 ade2-101 his4-539 lys2-801 ura3-52*
MCY3644	*MCY3647 srb11/ssn8Δ2::LEU2*
MCY3647	*MAT*α *his3Δ200 leu2-3,112 lys2-801 ura3-52*
MCY3802	FY250 *sfl1Δ1::HIS3*
MCY3806	MCY3647 *sfl1Δ1::HIS3*
MCY3817	MCY3647 *srb9Δ::hisG*
MCY3824	*MAT***a** *his3Δ200 leu2-3,112 ura3-52::SUC2-LEU2-lacZ::URA3*
FY250[b]	*MAT*α *his3Δ200 ura3-52 leu2Δ1 trp1Δ63*

[a]All strains are derived from S288C. Current *srb*/*ssn* names are followed by original allele designations (Carlson *et al*., 1984; Vallier and Carlson, 1994; Kuchin *et al*., 1995; Song *et al*., 1996). Alleles were *snf1Δ3* or *snf1-28*.
[b]FY250 was provided by F.Winston.

transcriptional repression of *SUC2*, and that Sfl1 interacts functionally and physically with Srb/mediator proteins. We propose that Sfl1, when bound to its site, interacts with Srb/mediator proteins to repress transcription (Figure 7). It is unlikely that Sfl1 serves primarily to recruit Srb/mediator proteins to the promoter because previous studies have implicated such recruitment in transcriptional activation (Barberis *et al*., 1995; Farrell *et al*., 1996); rather, the interaction of Sfl1 with these proteins must have a specific inhibitory effect. Many steps in the transcription process are possible targets for repression (Johnson, 1995; Hanna-Rose and Hansen, 1996), and a variety of mechanisms can be envisioned. Sfl1 may play an active role in a mechanism by which certain Srb/mediator proteins inhibit transcription; for example, Sfl1 may modulate the activity of the Srb10–Srb11 kinase, which has a role in CTD phosphorylation (Liao *et al*., 1995). Alternatively, the physical interaction of Srb/mediator proteins with DNA-bound Sfl1 may block interactions with other proteins or restrict conformational changes in the holoenzyme, thereby hindering a step in the transcription process such as assembly of a functional complex, initiation or promoter clearance. Another model is that Sfl1 binds tightly to Srb/mediator proteins and simply restrains RNA polymerase II holoenzyme from leaving the promoter.

Materials and methods

Strains, plasmids and genetic methods

S.cerevisiae strains are listed in Table III. Standard methods for yeast genetic analysis and transformation were followed (Rose *et al*., 1990). Selective synthetic complete (SC) medium was used to maintain selection for plasmids. Plasmids are listed in Table IV. pWS35, 41, 42, 53 and 96 were constructed with a *Bam*HI PCR fragment produced from template pWS6. For pWS64 and pWS94, a *Bam*HI PCR product encoding Sfl1 with an added C-terminal HA sequence was used. pWS93 is a derivative of pSH2-1 in which the $LexA_{87}$ coding sequence between the *Hin*dIII and *Eco*RI sites has been replaced by a *Bgl*II site followed by a sequence encoding HA_3; in addition, the *HIS3* marker was replaced with *URA3*. pWS98 and pWS125 contain a *Bam*HI fragment encoding Sin4 (Song *et al*., 1996) cloned into the *Bam*HI site of pWS93 and pSH2-1, respectively. pWS121 was made by cloning a *Bam*HI–*Sal*I fragment containing *SRB9* into the cognate sites of pWS93. pWS54 has been described previously as pLexA–SSN2 (Song *et al*., 1996). The *Escherichia coli* strain used was XL1-Blue.

Table IV. Expression plasmids constructed for this study

Name	Description
pSK37	pACTII with the GAD sequence deleted
pWS35	GAD–Sfl1; vector pACTII
pWS41	LexA–Sfl1; vector pSH2-1
pWS42	Sfl1; vector pSK37
pWS52	pACTII with *LEU2* marker changed to *URA3*
pWS53	GAD–Sfl1; vector pWS52
pWS54	LexA–Srb9; vector pSH2-1
pWS64	Sfl1–HA; vector pSK37
pWS93	pSH2-1 with LexA replaced by HA_3 sequence and *HIS3* replaced by *URA3*
pWS94	Sfl1–HA_4; vector pWS93
pWS96	HA_3–Sfl1; vector pWS93
pWS98	HA_3–Sin4; vector pWS93
pWS121	HA_3–Srb9; vector pWS93
pWS125	LexA–Sin4; vector pSH2-1

Vectors were pACTII (gift of S.Elledge; Legrain *et al*., 1994) and pSH2-1 (Hanes and Brent, 1989). LexA fusions constructed in pSH2-1 express only the DNA-binding domain of LexA ($LexA_{87}$). LexA fusions and HA-tagged proteins are expressed from the *ADH1* promoter.

Isolation of SFL1 as a suppressor of srb9

A genomic library in the centromere vector YCp50 (Rose *et al*., 1987) was used to transform the *srb9/ssn2-4 mig1* strain MCY3304 (the *snf1* allele is irrelevant for this study). We enriched for non-flocculent transformants by differential sedimentation (Song *et al*., 1996), and plated for single colonies. Non-flocculent colonies were identified and tested for recovery of flocculence after selection on 5-fluoroorotic acid for plasmid loss. Plasmids were isolated by passage through bacteria. When used to retransform MCY3304, clone A45-3 complemented the defect in repression of *SUC2*.

Disruption of chromosomal SFL1 locus

pWS6 was made by deleting the *Xho*I fragment in clone A45-3. pWS17-4 is pWS6 with the *Sma*I–*Nru*I fragment deleted from the YCp50 backbone. The *Bsp*120I fragments (1.6 kb) in pWS17-4 were then replaced with a *Bsp*120I–*Eag*I*HIS3* fragment or a *Sma*I*URA3* fragment, generating pWS24-2 or pWS34-27, respectively. The *Pvu*II fragments from these plasmids were used to disrupt the genomic locus, yielding the alleles *sfl1Δ1::HIS3* and *sfl1Δ2::URA3*.

β-galactosidase and invertase assays

Cultures were grown to mid-log phase. β-galactosidase activity was assayed in permeabilized cells and is expressed in Miller Units (Guarente, 1983). The invertase activity was assayed as described previously (Vallier

and Carlson, 1994) and is expressed as μmol glucose released per min per 100 mg cells (dry weight).

Co-immunoprecipitation assays

Preparation of protein extracts and immunoprecipitation were essentially as described previously (Yang *et al*., 1992). The extraction buffer was 50 mM HEPES pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 5 mM EDTA, 1 mM dithiothreitol, 10% glycerol, containing 2 mM phenylmethylsulfonyl fluoride (PMSF) and Complete protease inhibitor cocktail (Boehringer Mannheim). rProtein A immobilized on Sepharose beads (RepliGen) was added to protein lysates, which were rotated for 20 min and cleared by centrifugation at 12 000 r.p.m. for 10 min. Monoclonal mouse anti-HA antibody (12CA5) was added, and samples were mixed for 30 min and cleared by centrifugation for 5 min at 10 000 r.p.m. The supernatant was mixed with immobilized rProtein A for 1.5 h. The beads were collected by brief centrifugation and washed four times in 1 ml extraction buffer containing 1 mM PMSF by rotating for 10–15 min. The procedure was done at 4°C or on ice. Proteins were separated by SDS–PAGE and blotted. Primary antibodies were anti-LexA (gift of C.Denis) and were detected by enhanced chemiluminescence with ECL reagents (Amersham).

DNA-binding assays

The ERS DNA probe, containing *SUC2* nucleotides –221 to –135, was prepared by PCR with template pRB58 and primers U221T32, 5′-GGAATTCTCGAGCTCTATAGTAAACCATTTGG-3′ and U135B31, 5′-GGAATTCTCGAGTTTCTTTTCAGGAGGAAGG-3′ (added *Xho*I sites are underlined). The NS fragment contains nucleotides 1127–1214 from the *SUC2* coding region, and was prepared by PCR with the same template and primers SUC1127T, 5′-GGAATTCTCGAGTTTATTACAATGTCGATTTGAGCAAC-3′, and SUC1214B, 5′-GGAATTCTCGAGTTAAATATGGTTTGTGTGGTGTTAACAGC-3′. Products were digested with *Xho*I, gel purified and labeled with Klenow fragment (New England Biolabs) to a specific activity of 4–5$\times 10^4$ c.p.m./ng. Protein extracts were prepared from transformants grown in selective SC+4% glucose. The extraction buffer was 50 mM HEPES pH 8.0, 100 mM NaCl, 5 mM EDTA, 10% glycerol, containing 2 mM PMSF and Complete protease inhibitor cocktail (Boehringer Mannheim). Monoclonal anti-HA (0.5 μl per 50 μg of protein extract) was added to the protein extracts and mixed for 30 min. rProtein A immobilized on Sepharose beads was added and mixed for 2 h. Beads were collected by centrifugation at 3000 r.p.m. for 10 s and washed with 1 ml of extraction buffer lacking Complete protein inhibitor cocktail. For each assay, an aliquot of beads (8–10 μl) which had been incubated with 80 μg (Figure 5A) or 60 μg (Figure 5B) of protein was then incubated with ^{32}P-labeled ERS (1 ng) in 50 μl of DNA-binding reaction buffer containing 50 mM HEPES pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mg/ml BSA, 10 μg/ml poly(dI–dC)·poly(dI–dC), and 10% glycerol (Sorger and Nelson, 1989). After mixing for 2 h at 4°C, the beads were collected by centrifugation at 3000 r.p.m. for 10 s. The beads were washed twice in 50 mM HEPES pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM PMSF and 10% glycerol by mixing at 4°C for 15 min. The beads were collected and resuspended in sample buffer (50 μl). After extraction with phenol:chloroform:isopropanol (25:24:1), DNA was subjected to electrophoresis on a 5% native polyacrylamide gel in 89 mM Tris-borate, 2 mM EDTA, pH 8.3. Gels were dried and autoradiographed.

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