the spindle also supports a general role for the APC in regulating the mitotic apparatus (*7*).

Our findings also broaden the scope of cellular processes under APC control. In addition to controlling the abundance of mitotic cyclins, the APC regulates sister chromatid cohesion, the cellular DNA content, and the function of the mitotic spindle (*5*, *10*–*12*). The APC proteolytic system may therefore be a global cell-cycle regulator much like the cyclin-dependent kinases.

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25. All strains used in this report are isogenic or congenic to W303a. Cells were arrested in G_1 with α-factor, either 5 μM for *BAR1* strains or 60 nM for *bar1* strains. A *MAT**a** BAR1* W303a strain was used for Northern (RNA) analysis. Probes for Northern analysis are: an antisense riboprobe spanning base pairs 1077 to1739 of the *ASE1* coding sequence, a DNA probe from bases 1 to 1085 of *ACT1*, and a DNA probe containing the entire coding sequence of *CLB2*.
26. A fully functional myc-tagged *ASE1* construct (*13*) was placed under the control of the *GAL1* promoter on a 2μ vector and introduced into a *MAT**a** bar1 ase1*Δ strain. ^{35}S-labeling and measurement of protein stability was done as described [D. Kornitzer, B. Raboy, R. G. Kulka, G. R. Fink, *EMBO J.* **13**, 6021 (1994)]. Ase1 levels were quantitated with a phosphorimager and normalized to the zero time point. Experiments using Ase1 polyclonal antibodies demonstrated that the myc epitope did not alter the half-life of Ase1 or Ase1-db (*19*).
27. Methods are as described (*13*). Cultures for pulse-chase analysis and flow cytometry were split before labeling, and the sample for flow cytometry was grown in parallel in identical medium containing unlabeled methionine.
28. The half-life of Ase1 in cycling cells at 36°C is somewhat shorter than at 30°C (compare Fig. 1 B with Fig. 2A).
29. The Ase1-db mutant was constructed by site-directed mutagenesis using an oligonucleotide of sequence 5′-CATGCAGTAAAACCAGCTCAGCTGGCTCCTA-TCCCGCTGGCTAAAGTCGACACTAAG-3′.

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Combinatorial Control Required for the Specificity of Yeast MAPK Signaling

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In yeast, an overlapping set of mitogen-activated protein kinase (MAPK) signaling components controls mating, haploid invasion, and pseudohyphal development. Paradoxically, a single downstream transcription factor, Ste12, is necessary for the execution of these distinct programs. Developmental specificity was found to require a transcription factor of the TEA/ATTS family, Tec1, which cooperates with Ste12 during filamentous and invasive growth. Purified derivatives of Ste12 and Tec1 bind cooperatively to enhancer elements called filamentation and invasion response elements (FREs), which program transcription that is specifically responsive to the MAPK signaling components required for filamentous growth. An FRE in the *TEC1* promoter functions in a positive feedback loop required for pseudohyphal development.

Because common signaling components such as the MAPK cascade respond to a large number of different stimuli, it is not clear how specific signals are produced. In *Saccharomyces cerevisiae*, elements of the MAPK pathway required for the mating pheromone response are also required for haploid invasive growth and diploid pseudohyphal development. These shared factors include Ste20, Ste11, Ste7, and Ste12 (*1*, *2*). The first three act in sequence and are homologs of the mammalian kinases PAK (p21-activated kinase), MEKK (MAP kinase kinase kinase), and MEK (MAP kinase kinase), respectively (*3*). The transcription factor Ste12 is a terminal component of these signaling cascades, functioning downstream of the kinases to drive either sexual differentiation or filamentous and invasive growth (*3*). In mammalian cells, myriad stimuli activate MAPK pathways, yet only a handful of target transcription factors have been identified (*4*). Therefore, we addressed the question of how a common target of MAPK signaling pathways, Ste12, can direct more than one distinct developmental program.

Ste12 binds cooperatively to pheromone response elements (PREs) of the consensus sequence TGAAACA (*5*, *6*), and two or more of these elements are necessary and sufficient to program pheromone-responsive transcription (*7*). Because Ste12 can act alone during mating, we thought that there might exist a pathway-specific transcription factor that retargets Ste12 during filamentation and invasion through cooperative DNA binding (combinatorial control). The expression of the reporter gene *FG(TyA)::lacZ* depends specifically on the MAPK signaling components that promote filamentous and invasive growth (*8*). Transcription of *FG(TyA)::lacZ* is driven by a fragment of the retrotransposon *Ty1*, whose

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expression requires the *TEC1* gene (9). *TEC1* is also necessary for pseudohyphal and haploid invasive growth (10), which raises the possibility that Tec1 functions within the filamentous signaling pathway. Tec1 contains the conserved TEA or ATTS DNA binding domain, which is shared by several eukaryotic transcription factors, including human TEF-1 and *Aspergillus* abaA (11). TEF-1 binds to the sequence CATTCC, whereas abaA binds to the sequences CATTCC and CATTCT (12). We term these conserved elements TEA/ATTS consensus sequences (TCS). The *Ty1* fragment in *FG(TyA)::lacZ* contains a binding site for Ste12 (a PRE) and an adjacent presumptive binding site for Tec1 (a TCS); the two sites are separated by 14 base pairs (bp). We call this composite DNA element (PRE plus TCS) a filamentation and invasion response element (FRE). This region of *Ty1* binds to a Ste12-containing complex in crude extracts (6). Thus, Ste12 and Tec1 might cooperate to promote pathway-specific transcription of *FG(TyA)::lacZ*.

To test this, we placed the 27-bp *Ty1* FRE upstream of an enhancerless *CYC1::lacZ* reporter gene to create *FRE(Ty1):: lacZ* (13). This construct was expressed constitutively in haploid cells grown in rich medium, which is permissive for invasive growth (Fig. 1A). Mutation of either the PRE (TGAAACG to ACTTACG) or the TCS (CATTCT to CAAACT) reduced expression of the reporter (Fig. 1A). Mutations in elements of the pheromone response pathway that are required for haploid invasion and diploid pseudohyphal development also reduced expression; the *ste20* mutant showed a moderate reduction and *ste11*, *ste7*, and *ste12* mutants exhibited stronger defects (Fig. 1A). Disruption of the *TEC1* gene also reduced FRE activity (Fig. 1A). In contrast, mutations in components of the pheromone response pathway that are not required for filamentation and invasion, including Ste2 (the α-pheromone receptor), Ste4 (the β subunit of the receptor-coupled guanine nucleotide binding protein), Ste5 (a protein that tethers components of the MAPK cascade), and Kss1 or Fus3 (the redundant MAPKs required for mating), did not reduce FRE activity.

To determine whether the FRE is responsive to signals for pseudohyphal development in diploid cells, we introduced *FRE(Ty1)::lacZ* into this cell type. Activity of the FRE was one-thirteenth of that in haploid cells (Fig. 1B). Much of this reduction was overcome by activation of the pathway with the hypermorphic allele *STE11-4*. The activation was abrogated by mutation of the PRE or TCS or by mutation of the downstream signaling components *STE7* and *STE12*. Mutation of *TEC1* also blocked the activation of the reporter, which supports a downstream role (relative to Ste11) for Tec1.

We identified elements similar to the *Ty1* FRE in several genes required for filamentation and invasion (14). One exists in the *TEC1* promoter itself, raising the possibility that *TEC1* participates in an autoregulatory loop. In the *TEC1* FRE, the orientation of the PRE and TCS is reversed compared to that of the *Ty1* FRE, and the spacing between the elements is reduced from 14 to 4 bp.

We placed the 17-bp *TEC1* FRE upstream of *CYC1::lacZ* to yield *FRE-*

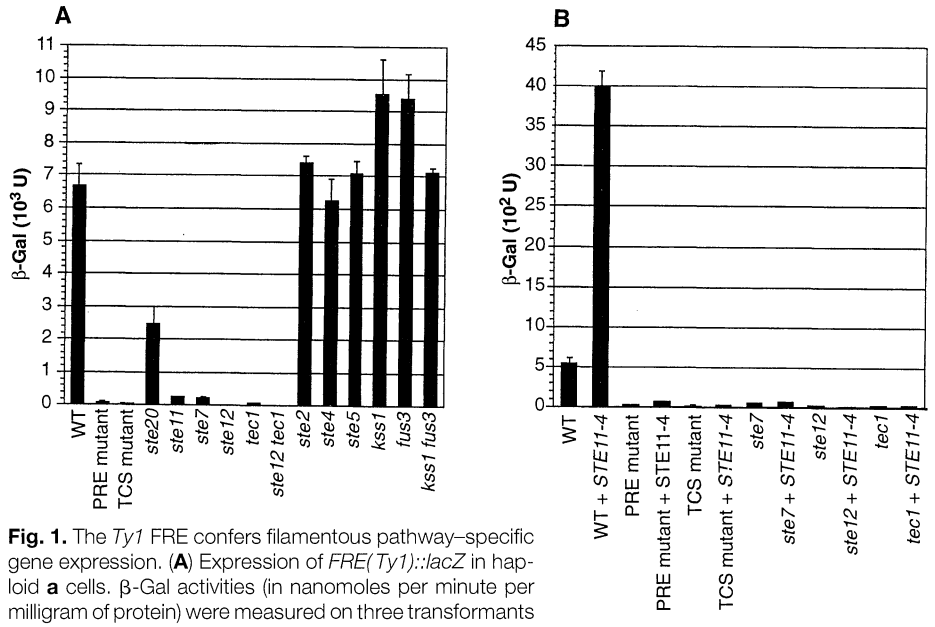

Fig. 1. The *Ty1* FRE confers filamentous pathway–specific gene expression. (**A**) Expression of *FRE(Ty1)::lacZ* in haploid **a** cells. β-Gal activities (in nanomoles per minute per milligram of protein) were measured on three transformants (13). The relevant genotypes are indicated at the bottom of the graph. Strains used in this study were of the Σ1278b background (23). PRE mutant and TCS mutant indicate reporter mutants assayed in the wild-type (WT) haploid strain. Error bars indicate standard deviations. (**B**) Expression of *FRE(Ty1)::lacZ* in **a**/α diploid cells. Expression was measured in homozygous diploids of the indicated genotypes that carried either a vector plasmid or a plasmid encoding the dominant activated allele *STE11-4* (13).

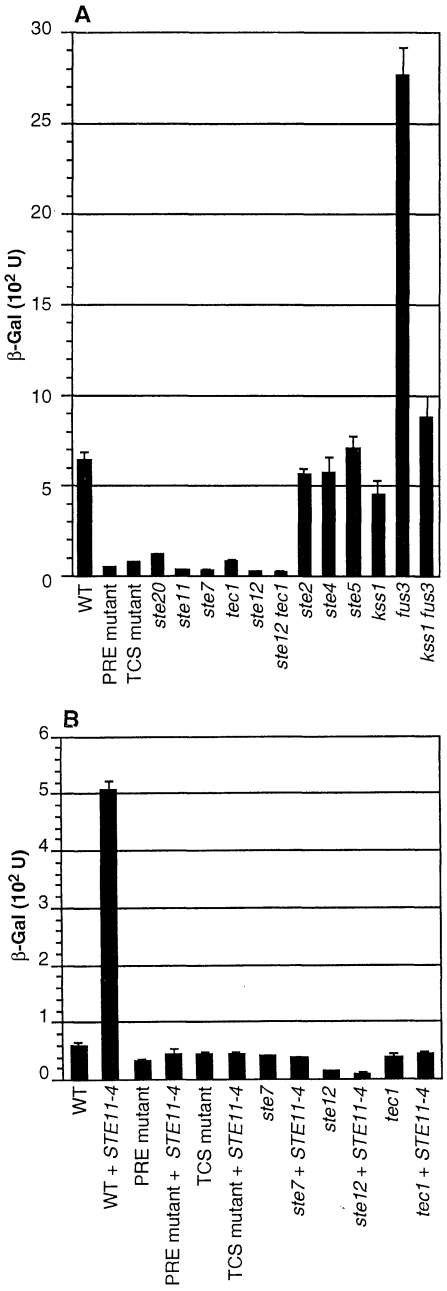

Fig. 2. The *TEC1* FRE confers filamentous pathway–specific gene expression. (**A**) Expression of *FRE(TEC1)::lacZ* in haploid **a** cells. (**B**) Expression of *FRE(TEC1)::lacZ* in **a**/α diploid cells.

(*TEC1*)::*lacZ* (*15*). Like *FRE(Ty1)::lacZ*, this construct was expressed in haploid cells in a PRE- and TCS-dependent manner (Fig. 2A; the PRE mutant is TGAAACA to ACTTACA and the TCS mutant is CATTCC to CAAACC). Mutation of *STE20*, *STE11*, *STE7*, *STE12*, and *TEC1* reduced expression of *FRE(TEC1)::lacZ*, whereas mutation of components specific to the pheromone-responsive MAPK pathway did not. In the *fus3* mutant, expression was increased fourfold, whereas the *kss1* mutant resulted in reduced expression (71% of the wild type; Fig. 2A). The *kss1 fus3* double mutant exhibited expression that was slightly greater than that of wild-type cells. This pattern mimics the effects of these mutations on haploid invasive growth: *fus3* mutants are hyperinvasive, *kss1* strains exhibit a weak invasion defect, and the double mutants invade approximately as well as does the wild type (*2*). Expression of *FRE(Ty1)::lacZ* was not modulated in this way, so the specific arrangement of the PRE and TCS in the FRE might be important for the appropriate response to Fus3 and Kss1.

As with *FRE(Ty1)::lacZ*, expression of *FRE(TEC1)::lacZ* in diploid cells was lower than that in haploid cells (Fig. 2A). Again, expression was increased by *STE11-4* (eightfold), and this induction was blocked by mutation of the PRE and TCS or by mutation of the downstream signaling components *STE7*, *STE12*, or *TEC1*.

To establish the relevance of the *TEC1* FRE to the transcriptional and biological activities of the full-length *TEC1* promoter, we mutated the PRE and TCS, either together or individually, in the native promoter (*16*) and examined the effects of these mutations on the activation of a *TEC1::lacZ* fusion gene in diploid cells (*17*). Activation of the MAPK pathway with *STE11-4* produced a fourfold increase in expression of *TEC1::lacZ* (Fig. 3A). This increase was largely blocked by the PRE-TCS double mutant as well as by the PRE and TCS single mutants. We also introduced a high-copy plasmid containing the *TEC1* gene into cells harboring

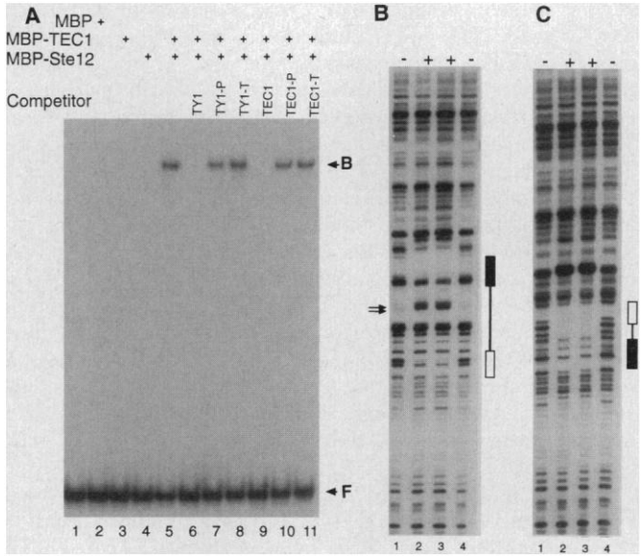

Fig. 4. Cooperative binding of Ste12 and Tec1 derivatives to FREs. (**A**) Native gel analysis of protein-FRE complexes. An autoradiogram of a nondenaturing acrylamide gel is shown. B, bound; F, free. ^{32}P-labeled *Ty1* FRE was applied to the gel after incubation with the indicated components (*20*). Approximate protein concentrations were as follows: MBP, 5×10^{-9} M; MBP-Tec1, 6×10^{-10} M; and MBP-Ste12, 6×10^{-10} M. ^{32}P-labeled *Ty1* FRE concentration was approximately 3×10^{-10} M. Unlabeled competitor DNAs were added as indicated and are labeled as follows: TY1, wild-type *Ty1* FRE; TY1-P, PRE mutant; TY1-T, TCS mutant; TEC1, wild-type *TEC1* FRE; TEC1-P, PRE mutant; and TEC1-T, TCS mutant. (**B**) Deoxyribonuclease I footprint analysis of protein binding to the *Ty1* FRE. Shown is an autoradiogram of a denaturing polyacrylamide gel (8%). Reactions contained a ^{32}P–end-labeled probe derived from the *FRE(Ty1)::lacZ* construct (*21*). Lanes 1 and 4 correspond to reactions lacking MBP-Tec1 and MBP-Ste12; lanes 2 and 3 correspond to reactions containing both proteins. The positions of the TCS (open box) and of the PRE (solid box) are indicated. Arrows indicate a pair of hypersensitive sites induced between the PRE and TCS. (**C**) Deoxyribonuclease I footprint analysis of protein binding to the *TEC1* FRE. Analysis was performed as in (B), except that the probe was derived from *FRE(TEC1)::lacZ*.

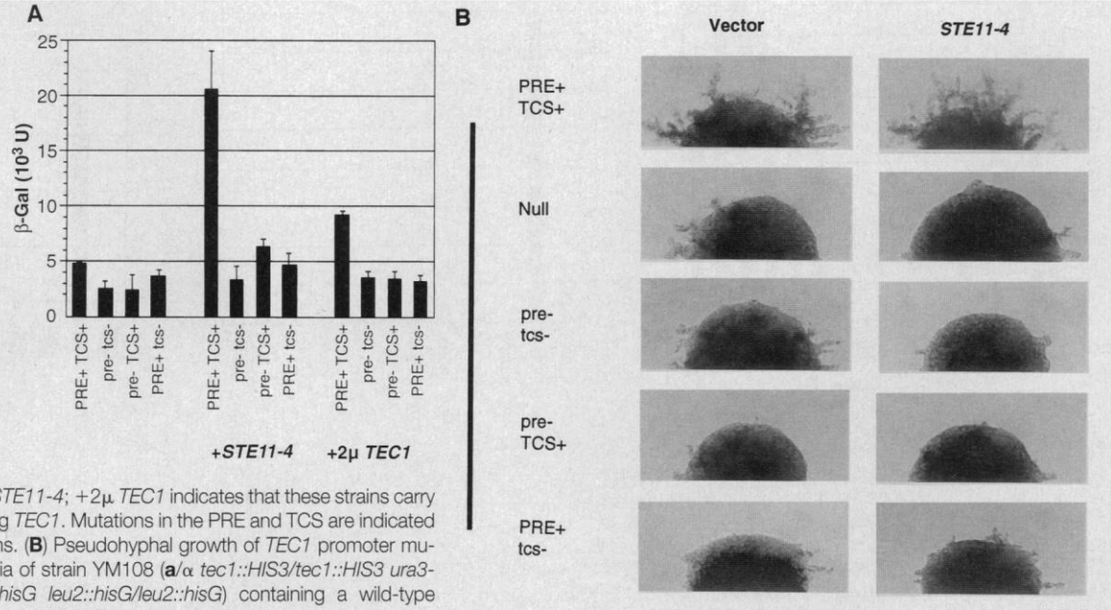

Fig. 3. Requirement of the *TEC1* FRE for signal-responsive transcription and pseudohyphal development. (**A**) Expression of *TEC1* promoter mutants in wild-type diploid cells. Expression of *TEC1::lacZ* promoter fusions carrying point mutations in the FRE was measured on three transformants (*17*). Solid bars show the average activity in nanomoles per minute per milligram of protein, and error bars show the standard deviation. *+STE11-4* indicates that these strains contain a plasmid encoding *STE11-4*; *+2μ TEC1* indicates that these strains carry a high-copy plasmid encoding *TEC1*. Mutations in the PRE and TCS are indicated by the lowercase designations. (**B**) Pseudohyphal growth of *TEC1* promoter mutants. Growth on SLAD media of strain YM108 (**a**/α *tec1::HIS3/tec1::HIS3 ura3-52/ura3-52 his3::hisG/his3::hisG leu2::hisG/leu2::hisG*) containing a wild-type *TEC1* allele integrated at *URA3* (wild type) was compared to that of cells transformed with the empty vector (null) or *tec1* alleles containing mutations in the FRE. The strains contained a plasmid encoding *STE11-4* on a *LEU2, CEN* vector (right column) or a *LEU2, CEN* control vector that lacked an insert (left column). Plates were incubated for 5 days at 30°C.

the reporter. Overexpression of *TEC1* increased expression of *TEC1::lacZ* approximately twofold. This effect was blocked by mutations in the FRE, which supports the positive feedback model.

To ascertain the importance of the *TEC1* FRE to pseudohyphal development, a single copy of the native *TEC1* gene with wild-type or mutant promoter sequences was introduced into a homozygous *tec1/tec1* mutant diploid by integrative transformation (*18*). The wild-type promoter allele complemented the pseudohyphal growth defect of the null allele (Fig. 3B). The PRE-TCS double mutant blocked filament production, as did the PRE and TCS single mutants. In the presence of the wild-type allele, *STE11-4* enhanced filamentation, whereas the activation of filamentation was blocked by the *tec1* null allele. The PRE and TCS mutants also blocked the induction of filamentation by *STE11-4*.

We examined the ability of purified derivatives of Ste12 and Tec1 to interact with FREs in vitro. Full-length Ste12 and Tec1 were expressed in *Escherichia coli* as protein fusions with maltose-binding protein (MBP). A FLAG peptide tag was added to the COOH-termini to aid in purification (*19*). Binding of these proteins to the *Ty1* FRE was examined with native gel electrophoresis (Fig. 4B) (*20*). No protein complex was observed upon incubation of a ^{32}P-labeled fragment containing the 27-bp *Ty1* FRE with MBP, MBP-Tec1, or MBP-Ste12 alone. However, incubation of the *Ty1* FRE with both MBP-Tec1 and MBP-Ste12 resulted in the appearance of a complex of lower mobility. The requirement of both proteins for complex formation implies cooperative binding. A 100-fold excess of the wild-type *Ty1* FRE eliminated the formation of the complex, but the same amount of *Ty1* FRE containing mutations in either the PRE or TCS did not. A 100-fold excess of the unlabeled *TEC1* FRE also eliminated binding to the *Ty1* FRE (Fig. 4A, lane 9), whereas *TEC1* FREs containing point mutations in either the PRE or TCS did not compete.

We also performed deoxyribonuclease I (DNase I) protection experiments with end-labeled fragments from the *FRE(Ty1)::lacZ* and *FRE(TEC1)::lacZ* constructs (*21*). Incubation of the *FRE(Ty1)::lacZ* or *FRE(TEC1)::lacZ* probe with MBP-Tec1 and MBP-Ste12 resulted in the protection of specific nucleotides within the PRE and TCS (Fig. 4, B and C). Binding to *FRE(Ty1)::lacZ* resulted in the appearance of a pair of adjacent hypersensitive sites between the TCS and PRE that may be indicative of a distortion in the DNA (Fig. 4B).

Our data demonstrate that the appropriate transcriptional response to overlapping, upstream, MAPK signaling components in yeast requires combinatorial control. Ste12 can act as a homomultimer to promote pheromone-responsive transcription (*7*). During filamentation and invasion, Ste12 acts with a second transcription factor, Tec1, to drive transcription that is specifically responsive to the MAPK pathway that promotes filamentation and invasion. The *TEC1* FRE is necessary for normal pseudohyphal development, which establishes at least one role for Ste12 and Tec1 in the expression of a gene involved in filamentation. Mating pheromone does not activate the MAPK pathway that leads to filamentation and invasion (*8*), yet it activates Ste12. Thus, the mechanism by which Ste12 is switched on by the mating MAPKs, Fus3 and Kss1, must not operate on Ste12-Tec1 complexes. The inhibitory action of Fus3 on FRE-dependent transcription could also play a role in preventing the activation of Ste12-Tec1 during mating. Because Fus3 and Kss1 together are dispensable for filamentation and invasion (although individually they can modulate haploid invasion), there likely exists a MAPK equivalent that specifically activates Ste12-Tec1 complexes.

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13. *FRE(Ty1)::lacZ* was constructed by replacing the Xho I fragment of pLG669-Z (*22*) with a double-stranded oligonucleotide corresponding to the *Ty1* FRE (CATTCTTCTGTTTTGGAAGCTGAAACG) flanked by Xho I ends. The FRE extends from positions +60 to +86 with respect to the *Ty1* δ sequence. β-galactosidase (β-Gal) assays were performed as described (*8*) on extracts of exponentially growing cells in liquid synthetic complete medium lacking uracil for haploid cells and liquid SLAD (synthetic low-ammonia dextrose) media (*23*) for diploid cells.
14. H. D. Madhani and G. R. Fink, unpublished observations.
15. The *FRE(TEC1)::lacZ* construct was made in the same manner as the *FRE(Ty1)::lacZ* construct, except that an oligonucleotide corresponding to the *TEC1* FRE (TGAAACACGCACATTCC) was cloned between the Xho I sites of pLG669-Z. The *TEC1* FRE extends from positions –383 to –399 with respect to the start codon.
16. The Eco RI–Xba I fragment containing the *TEC1* promoter was cloned into YEp356R (2μ, *URA3*), which contains a promoterless *E. coli lacZ* gene. The PRE and TCS mutants were constructed by polymerase chain reaction (PCR) with the use of mutagenic primers and Pfu polymerase.
17. β-Gal assays were performed on cultures grown on SLAD plates for 3 days as described (*8*); for the *TEC1::lacZ* fusion, solid medium gave more reproducible results than liquid medium.
18. The Xba I–Sac I fragment containing the *TEC1* coding sequence and 3′ flank was cloned in pRS306 (*URA3*). Wild-type and mutant promoter fragments from the *TEC1::lacZ* constructs were cloned upstream of the Xba I site.
19. The *TEC1* and *STE12* coding sequences were amplified with Pfu polymerase and their 3′ ends were modified to include COOH-terminal FLAG tags. They were then cloned into the Bam HI and Eco RI sites of pMAL-c2 (New England Biolabs), respectively. Expression and purification were performed as described [C. M. Chiang and R. G. Roeder, *Pept. Res.* **6**, 62 (1993)], with minor modifications. A portion of this material was purified further by chromatography on beaded amylose (New England Biolabs).
20. ^{32}P-labeled *Ty1* FRE was prepared by annealing two complementary oligonucleotides corresponding to the 27-bp *Ty1* FRE with 8 bp of its natural 5′ and 3′ flanking sequences. The top-strand oligonucleotide contained two additional unpaired G residues at its 5′ end. The fragment was labeled by incubation with ^{32}P-dCTP and Klenow fragment. The *Ty1* competitor DNAs are identical except that the unpaired G residues are not present, and the PRE and TCS mutant competitors contain the appropriate base substitutions. The *TEC1* competitor DNAs correspond precisely to the 17-bp *TEC1* FRE with 8 bp of its native 5′ and 3′ flanking sequences. Protein-DNA complexes were allowed to form for 90 min at 24°C in binding buffer [20 mM tris-HCl (pH 8.0), 40 mM NaCl, 1 mM dithiothreitol, 5% glycerol, bovine serum albumin (1 mg/ml), and Hae III–digested salmon sperm DNA (100 μm/ml)] in a 20-μl volume. Competitor DNAs were added in a 100-fold molar excess before the addition of proteins. Samples were electrophoresed through a 5% (19:1) acrylamide gel for 3 hours at 13 V/cm, dried, and autoradiographed.
21. Probes were synthesized by PCR with a ^{32}P-labeled primer whose 5′ end lies 55 bp upstream of the 5′ end of the FREs present in the *FRE::lacZ* constructs, and a second primer that lies 181 bp downstream of the FRE inserts. Protein samples were incubated with the probe for 10 min in binding buffer in a 50-μl volume. Approximate concentrations were as follows: probe, 2.3×10^{-8} M; MBP-Tec1, 2.9×10^{-7} M; and MBP-Ste12, 2.2×10^{-7} M. Deoxyribonuclease I (USB) was diluted 1:1000 in ice-cold 20 mM tris-HCl (pH 8) and 50 mM $CaCl_2$, and 1 μl was added to each sample. Reactions were incubated at 24°C for 5 min and were quenched by the addition of an equal volume of stop solution [5 M ammonium acetate, yeast tRNA (300 μg/ml), and 50 mM EDTA].
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