Regulation of Mating and Filamentation Genes by Two Distinct Ste12 Complexes in *Saccharomyces cerevisiae*†

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The *Saccharomyces cerevisiae* transcription factor Ste12 controls two distinct developmental programs of mating and filamentation. Ste12 activity is regulated by Fus3 and Kss1 mitogen-activated protein kinases through two Ste12 inhibitors, Dig1 and Dig2. Mating genes are regulated by Ste12 through Ste12 binding sites (pheromone response elements [PREs]), whereas filamentation genes are supposedly regulated by the cooperative binding of Ste12 and Tec1 on a PRE adjacent to a Tep1-binding site (TCS), termed filamentous responsive element (FRE). However, most filamentation genes do not contain an FRE; instead, they all have a TCS. By immunoprecipitation, we show that Ste12 forms two distinct complexes, Ste12/Dig1/Dig2 and Tec1/Ste12/Dig1, both in vivo and in vitro. The two complexes are formed by the competitive binding of Tec1 and Dig2 with Ste12, as Tec1 can complex with Dig2 from Ste12 in vitro and in vivo. In the Tec1/Ste12/Dig1 complex, Tec1 binds to the N terminus of Ste12 and to Dig1 indirectly through Ste12/Dig1/Dig2 complex for mating and also a novel Tec1/Ste12/Dig1 complex, whereas mating genes are occupied by mostly Ste12/Dig1/Dig2 with some Tec1/Ste12/Dig1. We suggest that Tec1 tethers Ste12 to TCS elements upstream of filamentation genes and defines the filamentation genes as a subset of Ste12-regulated genes.

Key regulators of cell fate determination often control multiple developmental pathways in response to different stimuli. In *Saccharomyces cerevisiae*, the transcription factor Ste12 is required for both mating and filamentation (11, 13, 14, 19). During the mating of haploid cells, Ste12 induces the expression of pheromone-responsive genes through Ste12 binding sites, or pheromone response elements [PREs; TGAAAC(A/G)], at the promoters of mating genes (41). Ste12 homodimers can bind cooperatively to tandem PREs in vitro, and the PRE is sufficient to induce pheromone-responsive expression of haploid-specific genes in both mating types (18). During filamentous and invasive growth, Ste12 cooperates with Tec1, a TEA/ATTS-family transcription factor (30). Tec1 was first identified as a regulator of the expression of *Ty1* transposon insertions. Like other TEA/ATTS family members, it binds to CATTCC or CATTCT (termed TCS, for TEA/ATTS consensus sequence) (25, 26, 29). Enhancer elements containing a PRE adjacent to a TCS are termed filamentation/invasion response element (FREs) (29) or sterile response elements (5). FREs have been found in the promoters of *Ty1* and *TEC1* and are necessary and sufficient to confer filamentation-associated expression in *S. cerevisiae* (5, 29). Importantly, recombinant Ste12 and Tec1 bind cooperatively to the FREs of *Ty1* and *TEC1* in vitro (29). Consistent with the cooperative control of filamentation genes by Ste12 and Tec1, a genome-wide study of Ste12 distribution has localized Ste12 to the promoters of pheromone-induced genes and filamentation genes in vivo, and the binding of Ste12 at the promoters of filamentation genes is Tec1 dependent (47).

Ste12 is regulated by the Fus3 and Kss1 mitogen-activated protein (MAP) kinases (2, 41). Fus3 and Kss1 have overlapping functions in mating (12, 39). Both Fus3 and Kss1 can phosphorylate Dig1 and Dig2, two functionally redundant inhibitors of Ste12 (9, 43). Dig1 binds to the N terminus of Ste12 and to Dig1 indirectly through Dig2 complex for mating and also a novel Tec1/Ste12/Dig1 complex, whereas mating genes are occupied by mostly Ste12/Dig1/Dig2 with some Tec1/Ste12/Dig1. We suggest that Tec1 tethers Ste12 to TCS elements upstream of filamentation genes and defines the filamentation genes as a subset of Ste12-regulated genes.

† Supplemental material for this article may be found at http://mcb.asm.org/.

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transcriptional programs of distinct pathways by its association with different cofactors.

**MATERIALS AND METHODS**

**Yeast strains.** Standard yeast manipulation methods were used. Strains used in this study are listed in Table 1. All the strains constructed in this study are derivatives from 10560-4A in a S. cerevisiae background unless otherwise specified. Strain HLY2187 was obtained from a cross between strains 10560-6B and L6149. Strain HLY3324 was transformed with PCR-amplified fragments of TEC1, digested with EcoRI and XhoI sites. To construct plasmid pHL732 (ADH1p-TEC1-(13MYC)) in yeast expression vector pRS314 (40) between the NotI and EcoRI sites to generate pRS314/ADH1p. Then a TCI-3HA cassette with an ADH1 terminator sequence was PCR amplified from the epithio-tagged TCI1-HA strain HLY3324 and cloned into the plasmid pPH314/ADH1p between the EcoRI and XhoI sites. To construct plasmid pHL732 (ADH1p-TEC1-(13MYC)), a TCI-3MYC cassette with the ADH1 terminator sequence was PCR amplified from the strain HLY3324 and cloned into the plasmid pRS314/ADH1p between the EcoRI and XhoI sites.

**Plasmid construction.** The TCS-leu2::HIS3 plasmid (pPL70) was constructed by inserting the sequence AGAATGTGCATTATCGATTCATTCT into the XhoI site in the pEG202 plasmid (J17).

To construct plasmids pH711 (ADH1p-leu-A-TEC1 HIS3) and pH712 (ADH1p-leu-A-STEL1 HIS3), STE12 and TEC1 ORFs were amplified by PCR and inserted into the yeast vector pEG202 (16) between the EcoRI and XhoI sites. To construct plasmid pH731 (ADH1p-TEC1-HA TRP1), a 1.5-kb ADH1 promoter region was amplified by PCR and inserted into the yeast expression vector pRS314 (40) between the NotI and EcoRI sites to generate pRS314/ADH1p. Then a TCI-3HA cassette with an ADH1 terminator sequence was PCR amplified from the epithio-tagged TCI1-HA strain HLY3324 and cloned into the plasmid pPH314/ADH1p between the EcoRI and XhoI sites. To construct plasmid pHL732 (ADH1p-TEC1-(13MYC)), a TCI-3MYC cassette with the ADH1 terminator sequence was PCR amplified from the strain HLY3324 and cloned into the plasmid pRS314/ADH1p between the EcoRI and XhoI sites.

**Immunoprecipitation.** Cell cultures (50 ml) were grown to an optical density at 600 nm (OD600) of about 1.0, and cells were washed twice with ice-cold water and then broken with FastPrep (Eppendorf) for 40 s twice in 0.7 ml of high-salt breaking buffer (20 mM Tris-HCl, 300 mM NaCl, 0.1% NP-40, 1 mM EDTA plus protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride [PMSF], 2 mM benzamidine, 1 μM leupeptin, 2 μM pepstatin, 2 μM chymostatin, 2.6 μM aprotinin). After a 10-min spin in a microcentrifuge, the lysates were incubated with 1.5 μg of anti-HA antibody (clone 12C5, Roche) at 4°C for 1.5 h, and subsequently 50 μl of a 1:1 slurry of protein A-Sepharose beads was added and incubated for another 1.5 h at 4°C. Bound proteins were washed five times with breaking buffer and eluted with 1% sodium dodecyl sulfate (SDS) in Tris-EDTA buffer, pH 8.0.
In vitro transcription/translation and in vitro binding. Plasmids used for in vitro transcription/translation were constructed by PCR amplification of TEC1, STE12, DIG1, and DIG2 with EcoRI and Xhol on each end and cloned in frame into the EcoRI and Xhol sites of the plasmid pCTE6b(+) (Novagen). TEC1-FLAG was constructed by adding a FLAG sequence in frame in the C terminus of TEC1 in a PCR primer for cloning into the pCTE6b(+) vector. mRNA was transcribed from plasmid DNA for 20 min and subsequently translated for 90 min with the STPS SP6 Single Tube Transcription/Translation System (Novagen). For myc-Ste12, mRNA was transcribed from plasmid pGEM4Z-mcSTE12 (3) using the STPS SP6 Transcription Mix (Novagen). In vitro translated proteins were incubated in IPP150 binding buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, and 1 mM PMSF with rotation at 4°C for 1 h. The proteins were immunoprecipitated with either anti-FLAG M2-agarose (Sigma) or a 1:100 dilution of anti-c-myc (A-14) rabbit polyclonal immunoglobulin G (Santa Cruz Biotechnology) with protein A-Sepharose CL-4B (Amersham Biosciences) overnight at 4°C with rotation. The beads were then washed five times with IPP150 binding buffer before being boiled for 5 min in 5× SDS sample buffer. The proteins were resolved on 9% SDS-polyacrylamide gels, fixed, dried, and detected with a phosphorimage cassette.

β-Galactosidase assay. β-Galactosidase assays were performed as previously described (37) but with the addition of protease inhibitors in the cell-breaking buffer (0.1 M Tris, pH 8.0, 20% glycerol, 0.5 mM PMSF, 2 mM benzamidine, 2 μM leupeptin, 2 μM pepstatin, 4 μM chymostatin, 2.6 μM aprotinin). The following calculation was used: β-galactosidase activity = OD420 × (1.7/0.0045) × 1000/time × volume × concentration, where time is measured in minutes, volume is in microliters, and concentration is in micrograms/microliter.

Comparative promoter analysis of Tc1 and Ste12 sites. Ste12- and Tc1-regulated genes were obtained from two microarray experiments of dig1 dig2 versus wild type (21), available at http://www.rri.com/publications/2000/cell Hughes.html. Only genes whose expression levels in dig1 dig2 were at least twofold higher than wild type in both experiments were used in our analysis. Ty genes or genes whose promoters overlap with a Ty element were also excluded. Sequences of promoters 1,000 bp upstream of the selected Ste12-regulated genes in S. cerevisiae were extracted from the yeast genome database (http://www.yeastgenome.org/). Their coding sequences were blasted with the genome sequences of three other Saccharomyces species (http://www.broad.mit.edu/annotation/fungi/saccharomyces/) to identify their orthologs and obtain 1,000-bp upstream sequences. Comparative promoter analysis of Tc1 and Ste12 sites. Ste12- and Tc1-regulated genes were obtained from two microarray experiments of dig1 dig2 versus wild type (21), available at http://www.rri.com/publications/2000/cell Hughes.html. Only genes whose expression levels in dig1 dig2 were at least twofold higher than wild type in both experiments were used in our analysis. Ty genes or genes whose promoters overlap with a Ty element were also excluded. Sequences of promoters 1,000 bp upstream of the selected Ste12-regulated genes in S. cerevisiae were extracted from the yeast genome database (http://www.yeastgenome.org/). Their coding sequences were blasted with the genome sequences of three other Saccharomyces species (http://www.broad.mit.edu/annotation/fungi/saccharomyces/) to identify their orthologs and obtain 1,000-bp upstream sequences of these orthologs. Promoter sequences from different orthologs were extracted from the yeast genome database (http://www.yeastgenome.org/) to identify their orthologs and obtain 1,000-bp upstream sequences of these orthologs. Promoter sequences from different Saccharomyces species were used to search for Tc1 and Tc1 (CAT TCY) consensus sequences, and maps of potential sites were created using the regulatory sequence analysis tools website at http://rsat.ulb.ac.be/rsat/ (results are compiled in the figures in the supplemental material).

ChIP assay. Chromatin immunoprecipitation (ChIP) analysis was performed as previously described (22, 42). The same set of protease inhibitors as described previously for immunoprecipitation was added to the ChIP lysis buffer. Two micrograms of anti-myc antibody (Santa Cruz) was incubated with the cell extract from 50 ml of cells grown to an OD600 of 1.0. PCR primers used in the ChIP analysis are listed in Table 2.

RESULTS

Filamentation genes are regulated through TCS elements. Based on the current model that the cooperative binding of Ste12 and Tc1 to FRES regulates the transcription of filamentation genes (29), promoters of filamentation genes should contain FRES (a PRE adjacent to a TCS). However, we noticed that many filamentation genes do not have a sequence that resembles an FRES in their promoter region. To further address this, we extracted 1,000-bp upstream sequences of Ste12-regulated mating and filamentation genes that have been identified by genome-wide transcription analyses (dig1 dig2 versus wild type) (21) and determined the existence of PRE (TGAACAR) and TCS (CATTCY) elements in these promoters. Because both motifs are rather short and can appear randomly at high frequency (PRE, 0.34 sites per 1,000 bp; TCS, 1.04 sites per 1,000 bp), we compared the promoter regions of these genes with the promoters of their orthologs from three other closely related Saccharomyces species (23). The information on PRE and TCS positions in these promoters is compiled in three figures that are available in the supplemental material. Genuine transcription factor binding motifs are likely to be conserved among all four species, while a random occurrence is generally not conserved (23). PREs and TCS that are conserved among Ste12-regulated genes in all four species are compiled in Table 3. All Ste12-regulated genes have either PREs, TCS, or both elements in their promoters. Not surprisingly, all the genes that have only PREs are involved in mating (Table 3; see Fig. S1 in the supplemental material). Only a few mating genes have both PREs and TCS in their promoters, including FUS1, FUS3, and PRM1 (Table 3; see Fig. S3 in the supplemental material). In contrast, most genes that contain only TCS are involved in filamentation (Table 3; see Fig. S2 in the supplemental material). A few filamentation genes also have PREs in their promoters (Table 3; see Fig. S3 in the supplemental material). Therefore, it appears that the major difference between mating and filamentation genes lies in the presence of PREs versus TCS in their promoters.

We searched further for potential FREs among the genes that have both a PRE and a TCS in their promoter. In addition to two previously reported genes, TEC1 and Ty1 (5, 29), we found only two additional genes, YDR294C and SVS1, that contain a potential FRES (Table 3; see Fig. S3 in the supplemental material). S. cerevisiae FLO11 contains a single nucleotide mismatch in a potential FRE right upstream of a TCS, and the combination was previously suggested to be a potential FRE. However, the potential FRE is not in the promoters of FLO11 orthologs in the other three species (see Fig. S2 in the supplemental material) and, thus, might not be functional. Because the FLO11 promoter has been shown to be much larger than 1,000 bp (38), we extended our sequence comparison of FLO11 promoters to 3,000 bp. No FREs but only two conserved TCS and a separate PRE were found. Therefore, FLO11 is more likely regulated through TCS than through a FRE, although the upstream FRE may play a role in FLO11.

<table>
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<th>TABLE 2. Primers used in ChIP assays</th>
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</tr>
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4796 CHOU ET AL. MOL. CELL. BIOL.
regulation, too. In total, all filamentation genes have TCS in their promoters, but only four of them (Ty1, TEC1, YDR249C, and SVS1) contain an FRE. Our promoter analysis suggests that TCS and not FREs are the prevailing cis elements upstream of filamentation genes.

Our promoter analysis is in agreement with transcription patterns of these genes. Fus3 plays a positive role in the transcriptional activation of Ste12, whereas it specifically phosphorylates Tec1 and triggers ubiquitin-mediated Tec1 degradation during the pheromone response (1, 7, 8). Therefore, transcription of TCS-driven genes in a fus3 mutant is expected to be higher than that in wild type, while PRE-driven transcription in fus3 mutants should not be higher than that in wild type during the pheromone response. Ratios of gene expression in fus3 mutants treated with 50 nM \( \text{H}9251 \) versus wild type should not be higher than that in wild type during the pheromone response (1, 7, 8). Therefore, transcriptional activation of Ste12, whereas it specifically phosphor-

### Table 3. Comparative promoter analysis for conserved Ste12 and Tec1 binding sites in four closely related Saccharomyces species

<table>
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<tr>
<th>Gene</th>
<th>No. of PREs</th>
<th>No. of TCS</th>
<th>Pathway</th>
<th>Expression ratio</th>
<th>Gene</th>
<th>No. of PREs</th>
<th>No. of TCS</th>
<th>Pathway</th>
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<th>No. of TCS</th>
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<td>?</td>
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* Microarray data of *dig1 dig2* versus wild-type were used to identify Ste12-regulated genes (21). Genes that were upregulated at least twofold in *dig1 dig2* versus wild-type were included in this study. Ty1 genes were excluded from the list. In addition, FLO11, CLN1, CWPI, and TEC1 were added as they are shown to be regulated by the filamentation pathway and Ste12/Tec1 in other studies (31, 47). The 1,000-bp upstream sequences of each gene from all four *Saccharomyces* species were extracted (23) and compared to determine potential conserved PREs (TGAAACR) and TCS sites (CATTCY). Functional assignments to either the mating (M) or filamentation (F) pathway are based on functional studies and/or transcriptional patterns (27, 31, 36, 45, 47). Ratios of their expression in *fus3* mutants treated with 50 nM \( \text{H}9251 \) versus wild type treated with 50 nM \( \text{H}9251 \) are from Hughes et al. (21). NA, not available; ?, not determined.

a See Fig. S1 in the supplemental material.

b See Fig. S2 in the supplemental material.

c See Fig. S3 in the supplemental material. Among the genes with both PREs and TCS, *TEC1* contains an FRE, which is indicated with an asterisk. In addition, *YDR249C* and *SVS1* also have a potential FRE with 11 bp and 4 bp between the PRE and TCS, respectively. *GFA1* promoter contains a PRE and a TCS with 28-bp spacing.
FIG. 1. Tec1 forms a complex with Ste12 and Dig1 but not with Dig2. (A) Immunoprecipitation of Tec1-HA. Protein lysates were subjected to immunoprecipitation with an anti-HA antibody, and the precipitation products were resolved by SDS-polyacrylamide gel electrophoresis and probed with an anti-myc antibody. As controls, cell lysates were subjected to Western blotting with anti-myc and anti-HA antibodies. The following yeast strains were used: lane 1, HLY3334 (TEC1-HA STE12-215); lane 2, HLY3335 (TEC1-HA DIG1-myc); lane 3, HLY3336 (TEC1-HA DIG2-myc); lane 4, HLY3320 (TEC1-myc); lane 5, HLY3321 (STE12-myc); lane 6, HLY3322 (DIG1-myc); and lane 7, HLY3323 (DIG2-myc). The lower band in lane 2 is a breakdown product of Dig1-myc. (B) Immunoprecipitation of Dig2-HA, as in panel A. The yeast strains in each lane are as follows: lane 1, HLY3337 (DIG2-HA STE12-myc); lane 2, HLY3338 (DIG2-HA DIG1-myc); lane 3, HLY3339 (DIG2-HA TEC1-myc); lane 4, HLY3327 (DIG2-HA); lane 5, HLY3321 (STE12-myc); lane 6, HLY3322 (DIG1-myc); and lane 7, HLY3324 (TEC1-HA). The lower bands in lanes 2 and 6 are a breakdown product of Dig1-myc. (C) Tec1 binds to the N-terminal region of Ste12. The yeast strain HLY3350 (ste12 tec1) carrying plasmid pHL754 (ADH1p-Ste121-215myc) was transformed with either pHL731 (ADH1p-TEC1-HA) or a vector (pRS313), and the transformed strains were used in immunoprecipitation with an anti-HA antibody. The precipitated proteins were analyzed by Western blotting with an anti-myc antibody. IB, immunoblot.

FIG. 2. Differential regulation of Ste12 and Tec1 transcriptional activities by Dig1 and Dig2. Relative β-galactosidase activities of PRE (FUS1)-lacZ, FRE(Ty1)-lacZ, and TCS-lacZ (pHL710) in wild-type (10560-4A), dig1 (HLY3315), dig2 (HLY3316), and dig1 dig2 (HLY3317) strains and relative β-galactosidase activities of LexA-Tec1 (pHL711) and LexA-Ste12 (pHL712) in lexAops-lacZ-integrated wild-type (HLY3328), dig1 (HLY3329), dig2 (HLY3330), and dig1 dig2 (HLY3331) strains are shown. The activity for each strain was an average from three independent transformants, and the relative activity was calculated by dividing activity by that of the dig1 dig2 strain for each reporter.

reported (43), as well as Dig1-myc (Fig. 1B), but the amount of Tec1 in the Dig2 IP was barely detectable, despite the fact that similar levels of Ste12 and Tec1 were in the cell lysate (Fig. 1). Therefore, Tec1 and Dig2 are likely in two different complexes with Ste12 and Dig1. The Ste12/Dig1/Dig2 complex is the known Ste12 complex for the mating program (34), while the newly identified Tec1/Ste12/Dig1 complex is likely for the filamentation program.

TCS-lacZ expression and LexA-Tec1 transcriptional activity are inhibited by Dig1 but not Dig2. Dig1 and Dig2 are two functionally redundant inhibitors of Ste12, and PRE-lacZ expression or Ste12 activity is high in dig1 dig2 double mutants but not in either dig1 or dig2 single mutants (9, 43). FRE-lacZ is also highly expressed in a dig1 dig2 double mutant (4), but its expression in dig1 or dig2 single mutants has not been reported. If only Dig1, but not Dig2, is present in the complex with Tec1 and Ste12, TCS-driven expression is expected to be high in a dig1 strain. To investigate whether Dig1 and Dig2 play different roles in the regulation of TCS-driven transcription, we assayed TCS-lacZ (8) expression in dig1, dig2, and dig1 dig2 mutants. For comparison, we also assayed the expression of PRE(FUS1)-lacZ (44) and FRE(Ty1)-lacZ (5, 29, 32) reporters in the dig1, dig2, and dig1 dig2 mutants. Expression of TCS-lacZ and FRE(Ty1)-lacZ, but not PRE(FUS1)-lacZ, was significantly elevated in dig1 mutants (Fig. 2). In contrast, TCS-lacZ and FRE(Ty1)-lacZ expression was not increased in dig2 mutants. Deletion of both DIG1 and DIG2 increased the expression of all three reporters, as expected for TCS-lacZ and as previously reported for FRE(Ty1)-lacZ and PRE(FUS1)-lacZ (4, 34). Therefore, a dig1 single mutant is able to release the inhibition on FRE- or TCS-driven expression. This is consistent with a genome-wide transcription analysis which shows that Dig1 is the primary negative regulator for the expression of filamentation genes (6).

To determine whether the high TCS-lacZ expression in the dig1 strain reflects the inhibitory effect of Dig1 on Tec1 transcriptional activity, we constructed a fusion of Tec1 to the DNA-binding domain of bacterial LexA, and the lexA-TEC1
expression was under the control of the \textit{ADH1} promoter so that the \textit{lexA-TEC1} expression was not influenced by Ste12 activity. Tec1 transcriptional activity was assayed in strains carrying a \textit{lacZ} reporter under the regulation of \textit{lex4} operators. Similar to the TCS reporter, LexA-Tec1 activity was up by \(~45\)-fold in the \textit{dig1} mutant (Fig. 1). In contrast to LexA-Tec1, LexA-Ste12 activity was high only in the \textit{dig1 dig2} double mutant and not in the \textit{dig1} single mutant, as previously reported (34). Therefore, Dig1 is the major inhibitor of Tec1 activity. Since \textit{PRE-lacZ} expression or LexA-Ste12 activity was similar in \textit{dig1} and \textit{dig2} single mutants, the observed difference in \textit{TCS-lacZ} expression or LexA-Tec1 activity between \textit{dig1} and \textit{dig2} strains is unlikely due to the difference in Dig1 and Dig2 protein abundance but could be explained by the protein composition in the Tec1/Ste12/Dig1 and Ste12/Dig1/Dig2 complexes.

**Tec1 binds to the same region of Ste12 as Dig2.** Since the two Ste12 complexes differ in Tec1 and Dig2, a possible mechanism that could give two distinct Ste12 complexes is one whereby Tec1 and Dig2 bind to the same region on Ste12 in a mutually exclusive way. Dig2 is known to bind to the N-terminal DNA binding region of Ste12 (34). We found that Tec1 also associated with the N-terminal region of Ste12 by immunoprecipitation (Fig. 1C). A myc-tagged N-terminal fragment of Ste12(1–215) in an \textit{ste12} mutant was used for IP to avoid the potential interaction of Ste12 N terminus with full-length Ste12. Because Ste12(1–215) is not sufficient for the expression of Ste12-regulated genes, HA-tagged Tec1 was expressed from the \textit{ADH1} promoter. The N-terminal DNA binding region (residues 1 to 215) of Ste12 is sufficient for interaction with Tec1; other regions of Ste12 were not required for the interaction with Tec1, as immunoprecipitation of Ste12 with deletions between residues 253 to 355, 387 to 512, and 512 to 669 could still pull down Tec1 (data not shown).

**Dig1 interaction with Tec1 requires Ste12.** Because Dig1 can bind Ste12 and inhibit Ste12 activity (9, 43) and because Ste12 is a component of the Tec1 immunocomplex, it is possible that the interaction of Dig1 with Tec1 is not direct but is mediated through Ste12. To test this possibility, we examined whether Dig1 and Tec1 still interact in the absence of Ste12. Because \textit{TEC1} expression is Ste12 dependent, we placed \textit{TEC1-HA} under the control of the \textit{ADHI} promoter. Dig1-myc was detected in the Tec1-HA immunoprecipitation in wild type but not in an \textit{ste12} mutant (Fig. 3A). Therefore, Ste12 is required for Tec1 interaction with Dig1.

**Tec1 transcriptional activity is dependent on its association with Ste12.** Not only was the interaction of Dig1 and Tec1 dependent on Ste12, but deleting \textit{STE12} in a \textit{dig1} mutant also blocked the elevated LexA-Tec1 transcriptional activity that was otherwise observed in a \textit{dig1} strain (Fig. 3B, \textit{dig1} versus \textit{dig1 ste12}). It is possible that Tec1-associated Ste12 is directly responsible for the induction of Tec1 transcriptional activity in the \textit{dig1} strain. In this case, the region of Tec1 that interacts with Ste12 should be required for Tec1 transcriptional activation. To test this possibility, we generated deletions of Tec1 from either the N or the C terminus. Deletions within the first 300 residues did not affect its association with Ste12, whereas a deletion of up to 400 residues was unable to bind Ste12 (Fig. 3C). A deletion from residue 401 to the C terminus was able to bind to Ste12, but deleting to residue 301 abolished Ste12...
binding (Fig. 3C). These data suggest that the region between residues 301 to 400 of Tec1 is required for Tec1 to interact with Ste12. Although required, the region was not sufficient for binding with Ste12 (data not shown). The Tec1 deletions were then fused in frame to the DNA binding domain of LexA, and the LexA-Tec1 fusions were analyzed for Tec1 transcriptional activity in the \( \text{dig}1 \) strain. We found that C-terminal deletions of Tec1 abolished the elevated LexA-Tec1 transcriptional activity in the \( \text{dig}1 \) strain (Fig. 3D). In contrast, the region from the N terminus to residue 300 was not required for the high LexA-Tec1 activity in the \( \text{dig}1 \) mutant (Fig. 3D, Tec1 regions 101 to 486, 201 to 486, and 301 to 486). These data suggest that the Ste12 binding region (residues 301 to 400) is required for LexA-Tec1 transcriptional activity. Therefore, Tec1 transcriptional activity is dependent on its association with Ste12, which is under the negative regulation of Dig1. Although the C terminus of Tec1 (from 401 to 486) was not essential for Ste12 binding, it was still required for LexA-Tec1 transcriptional induction in the \( \text{dig}1 \) strain (Fig. 3D). This suggests that, besides Ste12, there might be additional regulations on the C terminus of Tec1.

**Stoichiometry of Ste12 interaction with Tec1, Dig1, and Dig2 in vitro.** To further characterize the interaction between Tec1 and Ste12, we generated Tec1-FLAG and Ste12 by in vitro transcription/translation and examined whether Tec1-FLAG could interact with Ste12 in vitro. As shown in Fig. 4A, lane 4, IP with an anti-FLAG antibody brought down similar levels of \(^{35}\text{S}\)-labeled Tec1-FLAG and Ste12. Reciprocally, IP of \(^{35}\text{S}\)-labeled myc-Ste12 also brought down 35S-Tec1 at the molar ratio of about 1 Ste12 to 1.3 Tec1 (Fig. 4D, lane 8).

We also used in vitro translated proteins to confirm that Ste12 is required to mediate the interaction between Tec1 and Dig1. IP of Tec1-FLAG did not bring down Dig1 in the absence of Ste12 (Fig. 4B, lane 8); the weak Dig1 band in lane 8 was nonspecific, as a similar level of Dig1 was also seen in the IP with beads without Tec1-FLAG in lane 5. However, a significant amount of Dig1 was precipitated with Tec1-FLAG in the presence of Ste12 (Fig. 4B, lane 10). Therefore, Tec1 does not interact with Dig1 directly; rather, Ste12 bridges Tec1 and Dig1 in the complex.

In contrast to Dig1, Dig2 was not detected in the Tec1-
FLAG immunoprecipitation even in the presence of Ste12 (Fig. 4B, lane 11). Interestingly, when both Ste12 and Dig1 were present, Tec1-FLAG could bring down a small, but detectable amount of Dig2 (Fig. 4B, lane 12 as indicated by the arrow). Because 35S-Ste12 IP also produced a faint band at the same position as Dig2 (Fig. 4B, lanes 7, 10, and 11), we repeated the Tec1-FLAG IP experiment with unlabeled Ste12 (Fig. 4C). IP of Tec1-FLAG in the presence of Ste12 could bring down Dig1 (lane 7) but not Dig2 (lane 5). In the presence of both Ste12 and Dig1, Tec1-FLAG could bring down a small amount of Dig2 (Fig. 4C, lane 6). Therefore, a small amount of Dig2 was tethered to the Tec1/Ste12/Dig1 complex through its association with Dig1.

We also investigated the ability of Ste12 to interact with Dig1 and Dig2 by using a myc-tagged Ste12 for IP (3). As shown by Bardwell et al., a small amount of Dig1 bound to myc-Ste12 (Fig. 4D, lane 9). The molar ratio of this binding is about 1 Ste12 to 0.3 Dig1. The amount of Dig2 in the myc-Ste12 IP was also very small, at about 1 Ste12 to 0.2 Dig2 (Fig. 4D, lane 10). Surprisingly, in the presence of both Dig1 and Dig2, the amount of Dig1 and Dig2 associated with myc-Ste12 reached a molar ratio of 1.4 Dig1 and 1.5 Dig2 to 1 Ste12 (Fig. 4D, lane 11). Therefore, there is synergy between Dig1 and Dig2 in binding to myc-Ste12 in vitro. Identical results, both in terms of low levels of binding with Dig1 or Dig2 and the amount of Dig2 (Fig. 4C, lane 6). Therefore, a small amount of Dig2 was tethered to the Tec1/Ste12/Dig1 complex through its association with Dig1.

FIG. 5. Tec1 competes with Dig2 for Ste12 binding both in vitro and in vivo. (A) Tec1 can compete off Dig2 from Ste12. Twenty microliters of myc-Ste12 and 10 μl of in vitro translated and 35S-labeled Dig1 and Dig2 were used in each immunoprecipitation as described in the legend of Fig. 4D, and 1 μl of Dig1 or Dig2 was loaded as input. Unlabeled in vitro translated Ste12 was added to each IP in increasing amounts: 0 μl, 5 μl, 10 μl, 20 μl, 50 μl, and 100 μl. (B) Dig2 cannot compete off Tec1 from Ste12. Twenty microliters of myc-Ste12 and 10 μl of 35S-labeled Tec1 were used in each IP as described in the legend of Fig. 4D, and 1 μl of Tec1 was loaded as input. Increasing amounts (0 μl, 5 μl, 10 μl, 20 μl, 50 μl, and 100 μl) of both Dig2 and Dig1 were added to each IP for competition. (C) Tec1 competes with Dig2 for Ste12 binding in vivo. Strain HLY3340 (STE12-HA DIG2-myc tec1) carrying either a GAL-TEC1 (27c-2A) (33) or a vector was grown in YEPD until log phase; cells were washed several times with water and resuspended into YEP +2% raffinose to grow overnight. Galactose (2%) was added, and cells were grown for an additional 4.5 h before harvest for IP with an anti-HA antibody. The lysate and IP eluate were blotted with either anti-HA (anti-Ste12) or anti-myc (anti-Dig2) antibodies. IB, immunoblot.

for Ste12 binding in vivo by an Ste12 IP in cells with and without overexpression of TEC1. When overexpressed from the GAL1 promoter, Tec1 significantly reduced the amount of Dig2 that was associated with Ste12 in yeast cells (Fig. 5C). Thus, our data show that Tec1 competes with Dig2 in binding with Ste12 both in vivo and in vitro.

The Tec1/Ste12/Dig1 complex binds to TCS of filamentation genes and PREs of mating genes. If filamentation genes are regulated by the Tec1/Ste12/Dig1 complex via the TCS and mating genes are regulated by the Ste12/Dig1/Dig2 complex via the PRE, we would expect to find Dig2 at the promoters of
mating genes and Tec1 at those of filamentation genes. Ste12 and Dig1 should be present at the promoters of both groups. A study of the genome-wide location of Ste12, Dig1, and Tec1 has shown that Ste12 and Dig1 are present at both mating and filamentation genes, and Tec1 is present at filamentation genes as well as at some of the mating genes (47). The localization of Dig2 in these genes is not known. Therefore, we compared the distribution of Tec1 and Dig2 at the promoters of mating and filamentation genes using ChIP analysis. Ste12 and Dig1 were included as controls.

STE12-myc, TEC1-myc, DIG1-myc, and DIG2-myc strains were grown in YEPD (yeast extract, peptone, and dextrose) medium and harvested for ChIP analysis. The immunoprecipitated DNA was analyzed by PCR using primer pairs that are located about 100 bp upstream and downstream of either the TCS elements of filamentation genes or PRE elements of mating genes. Because we wanted to determine the differences between Ste12 complexes at PREs and at TCS, promoters containing both PRE and TCS sites were excluded from the analysis. In addition, only promoters that were efficiently bound by Ste12 and Tec1 in the whole-genome ChIP experiment were used (47). Ste12, Tec1, and Dig1 were present in nearly equal amounts at the TCS of filamentation genes, whereas Dig2 was detected at a lower level (Fig. 6A). Since our in vitro binding experiments have shown that Ste12, Ste12, and Dig1 were present at a near equal ratio in the Tec1/Ste12/Dig1 complex and that limited Dig2 was bound to the complex through the association with Dig1, the relative amounts of the four proteins detected at the filamentation genes are consistent with the localization of the Tec1/Ste12/Dig1 complex to the filamentation genes.

To test whether the presence of Dig2 at the promoters of the filamentation gene promoters was due to its association with Dig1 in the Tec1/Ste12/Dig1 complex, instead of with Ste12, we compared Dig2-myc localization at the promoters of filamentation genes in a wild-type strain and a dig1 mutant by ChIP (Fig. 6B). The deletion of DIG1 greatly decreased the amount of Dig2 bound to the promoter of CHS7 (Fig. 6B) as well as CWP1 (data not shown), indicating that Dig2 associates with Dig1 to TCS. The observed decrease in Dig2 at the CHS7 promoter in the dig1 mutant was not due to a potential Dig1-dependent interaction of Dig2 with Ste12, as the same amount of Dig2 was detected at the PREs of FUS1 in wild-type and dig1 mutant cells (Fig. 6B).

PCR of selected mating genes from the above ChIPs shows that Tec1 was present, but at a much lower level than Ste12, Dig1, and Dig2, at the promoters of mating genes (Fig. 6A). Because Tec1 is only present in the Tec1/Ste12/Dig1 complex, the detection of the small amount of Tec1 at the mating genes suggested that there was some Tec1/Ste12/Dig1 complex at the promoters of mating genes via Ste12 binding to PREs. We suggest that both types of Ste12 complexes are present at the promoters of mating genes. The Ste12/Dig1/Dig2 complex is the major form, while the Tec1/Ste12/Dig1 complex is the minor form.

The biological significance for the presence of the Tec1/Ste12/Dig1 complex at the PREs of mating genes is not clear. But we did find that the PRE(FUS1)-lacZ expression level was slightly elevated in tec1 and was even higher in a tec1 dig1 double mutant (Fig. 6C). The synergistic effect between tec1 and dig1 was specific, as PRE(FUS1)-lacZ expression was not increased in a tec1 dig2 mutant, suggesting that Tec1 might function together with Dig1 in inhibiting Ste12 activity at the promoters of mating genes.
DISCUSSION

Formation of two distinct Ste12 complexes by competitive binding of Tec1 and Dig2 to Ste12. In this study, we show that Tec1 and Ste12, along with Dig1, form an Ste12 complex that is different from the known Ste12/Dig1/Dig2 complex that regulates mating genes (Fig. 7). In vivo IP of Tec1 or Dig2 could pull down Ste12 and Dig1 but not much of each other. Using in vitro translated proteins, we show that Tec1 can bind directly to Ste12 at a nearly 1:1 molar ratio but cannot bind to Dig1 or Dig2. Dig1 is associated with the Tec1-Ste12 complex through Ste12, probably with the middle region of Ste12 as previously defined for the Ste12/Dig1/Dig2 complex (34). Limited Dig2 is tethered to the Tec1 complex through its interaction with Dig1. This explains why residual Dig2 is found in the Tec1 complex in vivo in both IP and ChiP experiments.

Ste12 can form two distinct complexes because Tec1 and Dig2 bind to Ste12 in a competitive manner so that Ste12 interacts with either Tec1 or Dig2 but not both. Like Dig2 (34), Tec1 binds to the N-terminal DNA binding region of Ste12. Excess Tec1 can replace Dig2 from binding to Ste12 both in vitro and in vivo. This provides an underlying mechanism for the existence of two distinct Ste12 complexes. Interestingly, excess Dig2 cannot replace Tec1 from the associated Ste12, indicating that Tec1 may have a higher affinity for Ste12 than Dig2. The different Ste12 binding affinities between Dig2 and Tec1 may imply that the amount of Tec1 in a cell determines the ratio of the two Ste12 complexes. The number of Tec1, Dig2, Dig1, and Ste12 molecules inside a \textit{MATa} cell, not exposed to alpha pheromone, is estimated to be about 530, 1310, 1460, and 1920 molecules/cell, respectively (15). Therefore, Tec1 seems to be the limiting protein among the three Ste12 binding proteins. Considering that Tec1 transcription and protein stability are tightly regulated by the mating and filamentation MAP kinase pathways and that Tec1 has a higher affinity for Ste12 than Dig2, the amount of Tec1 may determine the ratio of the two Ste12 complexes in a cell, thus facilitating either mating or filamentation.

The Tec1/Ste12/Dig1 complex regulates TCS-driven transcription. We show that TCS-driven transcription is regulated by the Tec1/Ste12/Dig1 complex. Tec1 by itself has minimal transcriptional activity. Its activity is determined by the associated Ste12 because Tec1 transcriptional activity is low in \textit{ste12} mutants, and \textit{STE12} deletion abolishes all elevated Tec1 activity in a \textit{dig1} mutant. This result is different from the reported Ste12-independent transcriptional activity of Tec1 in TCS control (24). The Ste12-independent activity of Tec1 appears to be significant only when Tec1 is highly overproduced (24), whereas we showed Ste12-dependent Tec1 transcriptional activity and TCS transcription under normal circumstances and in a \textit{dig1} strain. The Ste12-independent activation of Tec1 could be mediated through the C terminus (residues 401 to 486) of Tec1, as we found the region is not essential for Ste12 interaction but is required for Tec1 transcriptional activity. In the Tec1/Ste12/Dig1 complex, the main function of Tec1 is to associate with Ste12 through its C-terminal region to bring Ste12 and its transcriptional activity to TCS sites. Removal of the Tec1 interaction domain with Ste12 abolishes its transcriptional activity. The same C-terminal domain of Tec1 is termed the TCS-control region by Kohler et al. (24). We suggest that Ste12 acts as the transcriptional activator in the Tec1/Ste12/Dig1 complex and, thus, places the TCS control under the filamentation MAP kinase pathway. Active Kss1 removes Dig1 inhibition on Ste12 and allows transcription from a TCS. In fact, Fus3 and Kss1 have an equal and exchangeable role in activating transcription from TCS, as revealed by a stable Tec1 mutant in \textit{kss1} and \textit{fus3} (8), which is also consistent with the finding that both Fus3 and Kss1 are able to phosphorylate Dig1 (9, 43).

A limited amount of Dig2 is present in the Tec1/Ste12/Dig1 complex via interaction with Dig1, based on our in vitro IP (Fig. 4D) and ChiP analysis of Dig2 in \textit{dig1} cells (Fig. 6B). The lack of direct Dig2 interaction on Ste12 in the Tec1/Ste12/Dig1 complex may explain the difference in basal transcription levels of mating and filamentation genes, as filamentation genes are moderately expressed in vegetative growing cells, whereas mating genes are more stringently regulated with very low levels of basal expression. It also explains why Dig1 is the major inhibitor for the expression of filamentation genes, while Dig2 has minimal effects (6).

Transcriptional regulation of most filamentation genes is through TCS. The transcription of filamentation genes is thought to be regulated by cooperative interaction of Ste12

FIG. 7. Proposed regulation of mating and filamentation genes by Ste12/Dig1/Dig2 and Tec1/Ste12/Dig1 complexes in \textit{S. cerevisiae}. Ste12 forms two distinct transcriptional complexes: Ste12/Dig1/Dig2 at the PREs of mating genes and Tec1/Ste12/Dig1 at the TCS of filamentation genes. A small amount of Tec1/Ste12/Dig1 is also present at the PREs of mating genes. The expression of filamentation genes is regulated by Tec1-associated Ste12 on TCS, which is inhibited by Dig1. A smaller amount of Dig2 is associated with the Tec1 complex through Dig1; thus, Dig2 does not regulate Ste12 activity.
and Tec1 via the Fkc cis elements (29). However, most filamentation genes do not have FREs in their promoters (Table 3). Many filamentation genes do not even have a PRE; rather, they all have TCS. Since a single TCS is sufficient to provide the expression pattern of filamentation genes (24), most filamentation genes are likely regulated through TCS by the Tec1/Ste12/Dig1 complex. In support of this, we show by ChIP assay that Tec1, Ste12, and Dig1 are present at the promoters of filamentation genes, while Dig2 is detected at a lower level, and the binding is Dig1 dependent. Our model of the Tec1/Ste12/Dig1 complex binding to TCS elements gives an alternative and more general explanation for why the binding of Ste12 to filamentation genes is Tec1 dependent (47).

Ste12 controls diverse transcriptional programs by association with different cofactors that are differentially regulated at the transcription and protein stability levels. The regulation of Ste12-mediated transcriptional programs provides a prime example for us to understand how a transcription factor selectively activates distinct developmental programs in response to different stimuli. In the case of Ste12, it controls different of Ste12-mediated transcriptional programs provides a prime target at the transcription and protein stability levels.

The regulation of Ste12-mediated transcriptional programs provides a prime example for us to understand how a transcription factor selectively activates distinct developmental programs in response to different stimuli. In the case of Ste12, it controls different transcriptional programs by selective partnership with different cofactors. We show that Tec1 brings Ste12 to TCS elements to activate filamentation genes. This mechanism is similar to the regulation of α-specific genes, where Ste12 is brought by α1 to the promoters of α-specific genes (46). Ste12 activation by the pheromone-responsive MAP kinase pathway is responsible for the induction of a α-specific, α-specific, and haploid-specific genes. Active Ste12, therefore, is capable of activating all genes under its control through interactions with its cofactors. Thus, a key to the signaling specificity is selective regulation of the associated cofactors. Degradation of Tec1 specifically disassociates Ste12 from the promoters of filamentation genes and turns off the filamentation transcriptional program (1, 7, 8). Degradation of Tec1 may also allow maximal induction of mating genes (7). Therefore, signaling specificity for Ste12 in regulating multiple developmental pathways is achieved by its ability to associate with other transcription factors and by selective regulation of the associated factors.

Conclusion. Ste12 forms two distinctive complexes, Ste12/Dig1/Dig2 and Tec1/Ste12/Dig1, by competitive binding of Dig2 and Tec1 to the N terminus of Ste12. Most filamentation genes are regulated by the Ste1/Ste12/Dig1 complex via Tec1 binding sites.

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