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Transdominant genetic analysis of a growth control pathway

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ABSTRACT **Genetic selections that use proteinaceous transdominant inhibitors encoded by DNA libraries to cause mutant phenocopies may facilitate genetic analysis in traditionally nongenetic organisms. We performed a selection for random short peptides and larger protein fragments (collectively termed "perturbagens") that inhibit the yeast pheromone response pathway. Peptide and protein fragment perturbagens that permit cell division in the presence of pheromone were recovered. Two perturbagens were derived from proteins required for pheromone response, and an additional two were derived from proteins that may negatively influence the pheromone response pathway. Furthermore, three known components of the pathway were identified as probable perturbagen targets based on physical interaction assays. Thus, by selection for transdominant inhibitors of pheromone response, multiple pathway components were identified either directly as gene fragments or indirectly as the likely targets of specific perturbagens. These results, combined with the results of previous work [Holzmayer, T. A., Pestov, D. G. & Roninson, I. B. (1992) *Nucl. Acids. Res.* 20, 711–717; Whiteway, M., Dignard, D. & Thomas, D. Y. (1992) *Proc. Natl. Acad. Sci. USA* 89, 9410–9414; and Gudkov, A. V., Kazarov, A. R., Thimmapaya, R., Axenovich, S. A., Mazo, I. A. & Roninson, I. B. (1994) *Proc. Natl. Acad. Sci. USA* 91, 3744–3748], suggest that transdominant genetic analysis of the type described here will be broadly applicable.**

Transdominant genetic experiments using expression libraries designed to encode inhibitory proteinaceous molecules (peptides and protein fragments, which we refer to as "perturbagens") offer an alternative strategy to classical genetics. This strategy involves (*i*) introduction of perturbagen-encoding libraries into cells; (*ii*) enforcement of specific selection or screening criteria; (*iii*) isolation of perturbagen-induced variants; and (*iv*) characterization of the perturbagens and their targets. Perturbagens are intended to behave in a manner analogous to mutagens; however, instead of producing mutations in genes, perturbagens act at the level of the protein, disrupting specific biochemical interactions in cells to generate a mutant phenocopy.

Perturbagens recovered from a selection are potentially useful as tools for gene identification in two different ways. First, perturbagens may themselves be derived from proteins involved in the process under study and therefore directly identify important genes. Indeed, such dominant negative molecules have been specifically sought and identified in selections in bacteria, yeast, and human cells (1–6). Second, although many perturbagens may not be derived from proteins involved in the process under study (e.g., randomly generated peptides), they may serve as transdominant inhibitors of a process because of serendipitous interactions with proteins that are involved. This type of perturbagen is most useful as a biochemical probe for subsequent identification of important gene products. To date, neither a systematic effort to harness this latter type of perturbagen nor a direct examination of the relative frequencies with which these two types of perturbagen occur has been performed.

The pheromone response pathway of the budding yeast *Saccharomyces cerevisiae* provides an excellent system in which to test the general characteristics of a screen for perturbagen molecules. Haploid yeast respond to pheromones secreted by cells of the opposite mating type in a variety of ways to prepare for mating and diploid formation (for review see ref. 7). These responses include G_1-phase cell cycle arrest and changes in cell morphology. The G_1-phase arrest can be exploited to find yeast harboring mutations that block the pheromone response because escape from cell cycle arrest results in cell division. Because of the extensive study of this pathway, many of the genes involved in pheromone response have been characterized. This wealth of information, combined with the molecular genetic infrastructure available in yeast, such as the complete genome sequence (8), facilitates a test of transdominant genetic analysis.

Here, we report the results of a large-scale selection for random peptide and protein fragment perturbagens that permit escape from α factor-induced cell cycle arrest. Perturbagens that promote escape from cell cycle arrest were recovered from both peptide and protein fragment libraries. Two perturbagens were derived from known pheromone response genes and two from genes that encode proteins that may antagonize the pathway. Furthermore, an additional five perturbagens interacted with proteins involved in pheromone response. Taken together, these data suggest that perturbagen screens may help identify important genes in genetically intractable systems.

MATERIALS AND METHODS

Strains and Media. The *S. cerevisiae* strain used in the selection for α factor-resistant colonies was yVT12 [MATa leu2–3, 112 his3 lys2 sst2Δ ade2–1 HMLa HMRa mfa1::hisG mfa2::hisG ste3::GAL1(uas)-STE3 (strain JRY5312 in ref. 9), a gift from J. Rine, University of California, Berkeley]. Yeast strains were transformed by the method of Gietz and Schiestl (10), and plasmids were maintained by growth in standard selective media. Isolation of plasmids from yeast was accomplished as described (11).

Library Construction and Analysis. The peptide display library was composed of 15-aa peptides inserted into the green fluorescent protein (GFP) and is detailed in ref. 12. The yeast genomic fragment library was constructed by digesting yeast genomic DNA (strain yVT5, MATa leu2–3, 112 trp1–1 ura3–1 his3–11, 15 ade2–1 can1–100, a gift from J. Rine) with DpnII (New England Biolabs) and ligating size-selected DNA, 100–2,500 bp in length, into a *Bgl*II site located between the GFP coding region and the *PGK1* 3′ untranslated region in plasmid pVT21 (12). The peptide and genomic library were estimated to contain 6.5×10^6 and 7×10^5 individual clones, respectively. The average genomic library insert was estimated to be 400 nucleotides in length.

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Abbreviations: GFP, green fluorescent protein; AD, activation domain; BD, binding domain; STE, sterile.

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Identification of Library Plasmids that Allow Division in the Presence of α Factor. Strain yVT12 was transformed with either the peptide or genomic fragment libraries. Yeast harboring the two libraries were cultured briefly in selective media supplemented with galactose and raffinose and transferred to yeast extract/peptone/galactose/raffinose plates containing 10nM α factor (Sigma). Colonies forming 2–4 days after plating were patched to plates lacking uracil, replica-plated after 2 days to selective plates containing either dextrose or galactose/raffinose, grown for an additional day, and replica-plated to either yeast extract/peptone/dextrose or yeast extract/peptone/galactose/raffinose plates containing 1 μM α factor. Plasmid DNA was isolated from cells that displayed galactose/raffinose-specific growth in the presence of α factor. These plasmids were reintroduced into strain yVT12 to test for linkage between the plasmid and escape from α factor-induced cell cycle arrest.

Colony Formation Assays. "Penetrance" of individual perturbagen clones was determined by growing yeast strains that contained each of the 16 perturbagen plasmids and the parental vector pVT21 in selective media that contained galactose/raffinose as a carbon source to mid-log phase. Identical aliquots containing ≈250 cells of each strain were spread on yeast extract/peptone/galactose/raffinose plates that either contained or lacked 0.5 nM α factor. Penetrance here is described as the fraction of the total cells plated (determined from the plate lacking α factor) that grew in the presence of α factor after 5 days of incubation at 30°C.

Epistasis Tests. pVT68 and pVT36, which contain the entire *STE4* coding region and the *STE11*Δ *N* allele of the *STE11* gene expressed from the *GAL1* UAS, respectively, were constructed by amplifying the entire *STE4* gene from pL19 (13) or nucleotides 1281–2418 of the *STE11* gene from genomic DNA from strain yVT5 by PCR and ligating the resulting fragments into plasmid pVT11 (a gift of A. Adams, University of Arizona). pVT36 and pVT68 harbor the *STE11*Δ*N* and *STE4* genes, respectively, flanked 5′ by the *GAL1* UAS and 5′ untranslated region and 3′ by the *ACT1* 3′ untranslated region, and contain the pRS415 backbone (14). Each perturbagen as well as pVT21 was cotransformed into strain yVT12 with pVT36, pVT68, or pRS415, and growth of transformants on selective media containing galactose and raffinose was monitored.

Halo and Quantitative Mating Assays. Halo assays were performed essentially as described in ref. 15 and halos were monitored over a 2–4 day period. Quantitative mating assays were performed as described in ref. 16.

Two-Hybrid Plasmid Constructions. *Perturbagens.* All perturbagen inserts except pep1(I) and pep2(I) were amplified with *Bam*HI restriction sites by PCR and were ligated into the pACT2 and pAS2–1 vectors (17, 18). These two-hybrid perturbagen inserts contain the carboxy-terminal six amino acids of GFP fused to the N terminus of the perturbagen sequences. The pep1(I) and pep2(I) perturbagens were ligated along with the entire flanking GFP scaffold into the *Eco*RI sites in pACT2 and pAS2–1. Correct reading frames between each perturbagen insert and the *GAL4* DNA binding and activation domains were confirmed by DNA sequencing.

Pheromone response genes. The *STE18* and *STE50* genes were amplified from genomic DNA (strain yVT5) and the *STE4* gene from pL19 (13) by PCR and were ligated into pACT2 and pAS2–1. That each gene was full-length and in-frame with the *GAL4* DNA binding and activation domains was determined by sequencing the 5′ and 3′ ends of each construct. To control for possible errors introduced during PCR amplification, two independently isolated clones of each gene were used in two-hybrid analysis, and reported interactions (19–21) between these and other proteins were confirmed.

pRL222, pSL2091, pSL2168, pSL2289, pSL2175, pSL2122, and pSL2205, which contain the *STE20*, *STE11*, *STE7*, *STE5*, *FUS3*, *KSS1*, and *STE12* genes fused to the *GAL4* activation domain, and pKB84.7, pSL1962, pSL2121, pSL2019, pSL2174, and pSL2120, which contain the *STE20*, *STE7*, *STE11*, *STE5*, *FUS3*, and *KSS1* genes fused to the *GAL4* DNA binding domain, are detailed in ref. 20 and were a gift of G. Sprague (University of Oregon).

Two-Hybrid Methodology. Two-hybrid plasmids carrying activation domain (AD) fusions were transformed into strain Y187 [MATα leu2–3, 112 trp1–901 ura3–52 his3–200 ade2–101 met-gal4Δ gal80Δ URA3::GAL1(uas)-GAL1(TATA)-lacZ] and plasmids carrying binding domain (BD) fusions transformed into strain Y190 [MATa leu2–3, 112 trp1–901 ura3–52 his3–200 ade2–101 lys2–801 cyh2 gal4Δ gal80Δ URA3::GAL1(uas)-GAL1(TATA)-lacZ LYS2::GAL1(uas)-HIS3(TATA)-HIS3] (17). Introduction of BD and AD fusion plasmids into the same cell was accomplished by mating. β-galactosidase expression was initially screened for on plates containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (22) and/or colony-lift filter assays. For the colony-lift filter assays, yeast were transferred to VWR grade 410 sterile filters, were lysed via immersion in liquid nitrogen, and were placed on filters that had been presoaked in Z buffer [phosphate buffered to pH 7 (10 mM KCL/1 mM $MgSO_4$/1.6% X-gal/0.27% β-mercaptoethanol)], and color changes were monitored over an 8-hr period. All interactions initially scored as positive were subjected to quantitative liquid β-galactosidase assays essentially as described in ref. 22.

Gene Disruptions. The YBR059C, YMR086W, YER124C, *PMD1*, YGR179C and YBR186W genes were disrupted in strain yVT8 [MATa leu2–3, 112 trp1 ura3–52 his3 (strain D215U-1BC in ref. 23), a gift of K. Blumer, Washington University) via replacement of their entire coding regions with the *URA3* gene essentially as described in ref. 24, creating strains yVT76, 77, 78, 79, 80, and 81, respectively. DNA fragments used to disrupt each of these genes were composed of the URA3 gene [amplified by PCR from pRS416 (14)] and flanked by a sequence corresponding to the 50 nucleotides immediately upstream and downstream of the start and stop codons, respectively, of the specific gene to be disrupted. The *GIP1* gene was disrupted by using plasmid pJT26-HIS (a gift from A. Neiman, State University of New York, Stony Brook), creating strain yVT82, as described in ref. 25. Yeast that harbored disruptions of each gene were identified via whole colony PCR by using primers homologous to regions within the *URA3* or *HIS3* genes in combination with primers homologous to regions lying outside the regions of homology present in the disruption vectors. Strains yVT76-82 grew as well as parental strain yVT8 at 16, 30, and 37°C, indicating that none of these genes is essential for viability (data not shown). A haploid strain harboring a disruption f the *ECM8* (orf11) gene was not obtained.

DNA Sequencing. All sequence data were obtained by using an ABI373A DNA sequencer (Applied Biosystems, Perkin–Elmer). Nucleotide sequences of regions of each perturbagen insert that were not directly sequenced were obtained from the *S. cerevisiae* genome sequencing database (8).

RESULTS

Isolation of Perturbagens that Promote Escape from Cell Cycle Arrest. To screen for perturbagens that prevent α factor-induced cell cycle arrest in yeast, two expression libraries were constructed. In both cases, library clones were expressed as hybrid proteins with GFP (26). The first library-encoded peptides, 15 aa in length, were displayed on a solvent-exposed loop of GFP (12, 27, 28). This library was intended to serve as a source of nonnative peptide perturbagens that could be used subsequently to identify relevant *in vivo* targets. The second library consisted of short fragments of yeast genomic DNA expressed as carboxy-terminal fusions with GFP (see *Materials and Methods*). This second library was intended to provide a set of protein fragments biased toward native yeast protein domains as well as peptides derived from noncoding sequences. DNA fragments were expressed as GFP fusions to increase expression levels of small protein domains

through stabilization at the protein and mRNA levels. Both the peptide and yeast protein fragment libraries were under the transcriptional control of the *GAL1* upstream activating sequence, which allowed high expression in the presence of galactose and transcriptional repression in the presence of glucose (29).

Yeast strain yVT12 was transformed with the two libraries, and selections for resistance to α factor were carried out on plates containing α factor plus galactose. Plasmid DNA was isolated from yeast that displayed galactose-specific escape from cell cycle arrest, and these plasmids were reintroduced into strain yVT12 to test for the ability to confer resistance to α factor. DNA sequences of perturbagens that passed this final test were determined (see *Materials and Methods*). In total, 14 different perturbagen sequences were identified from the protein fragment library, and two different sequences were identified from the peptide library.

Sequence Analysis of Perturbagens. Based on sequence, the 16 perturbagens could be divided into two groups: peptides that shared no recognizable homology with yeast proteins (group I), and protein fragments derived from either known yeast proteins or predicted ORFs from yeast genomic DNA (group II). Group I was composed of the two perturbagens obtained from the peptide display library [pep1–2(I); Table 1] and three perturbagens obtained from the genomic fragment library [pep3–5(C); Table 1]. In the case of the three peptide perturbagens from the genomic library, none of the sequences created in-frame translational fusions between GFP and any yeast ORFs. Rather, the predicted translation products were short peptides (21–59 aa in length) appended to the carboxy-terminus of GFP.

A similarity search of GenBank by using the five peptides encoded by the group I sequences revealed no obvious homologies. In addition, sequence motif searches did not uncover any significant features. The absence of such features may indicate that these peptides do not affect pheromone response by mimicking the structure of known proteins in the pheromone response pathway or other yeast proteins.

Two of the group II sequences (orf1 and orf2; Table 1) encoded portions of the *STE11* and *STE50* genes, respectively, both of which participate in the pheromone response pathway (30–33). Indeed, overexpression of either the amino-terminal half of STE11p or a carboxy-terminal truncation allele of *STE50* (*ste50–2*), both of which are similar to the regions overexpressed in orf1 and orf2 (Table 1), have been reported to decrease sensitivity to pheromone (ref. 31 and cited in ref. 34). Thus, the perturbagen screen resulted in recovery of portions of at least two genes involved in activation of the pheromone response pathway.

None of the other ORF segments in group II were derived from known pathway members. However, portions of three previously identified yeast genes, *GIP1*, *PMD1*, and *ECM8*, were among this set (orfs 3, 4, and 11;Table 1). *GIP1* and *PMD1* encode proteins required for proper timing and execution of sporulation but have no reported functions in pheromone response (25, 35). The *ECM8* gene product is required for proper cell wall formation but, like Gip1p and Pmd1p, has no known function in pheromone response (36).

Perturbagen Penetrance. Perturbagen behavior was characterized further through colony formation assays to determine the genetic penetrance of each perturbagen. Yeast harboring 1 of each of the 16 perturbagen-encoding plasmids or the parental vector pVT21 were plated onto media that either contained or lacked α factor (see *Materials and Methods*). Colonies on the various plates were counted after 5 days, and the fractions of the total number of cells plated that were able to form colonies in the presence of α factor were determined. The penetrance of the perturbagens ranged from 90 to 6% (see Table 2). Thus, perturbagens displayed a wide phenotypic range, with some closely approximating the behavior of a null mutation in a gene essential to pheromone response and others behaving as weaker alleles.

Epistasis Analysis of Perturbagens. To determine the approximate point at which the perturbagens inhibit the pheromone pathway, epistasis analysis was performed. Perturbagens were expressed in the presence of dominant pathway-activating alleles of the *STE4* and *STE11* genes [*GAL-STE4* (13) and *STE11ΔN* (5, 37); see *Materials and Methods*]. Perturbagens that block pheromone response downstream of STE11p should suppress slow growth caused by both the *GAL-STE4* and *STE11ΔN* alleles whereas perturbagens that inhibit pheromone response at a point upstream of STE11p but downstream of STE4p should suppress growth inhibition caused by *GAL–STE4* only. The pep1(I), pep4(C), and orf5 perturbagens suppressed the slow-growth phenotype caused by both the *GAL–STE4* and *STE11ΔN* alleles. In contrast, the pep3(C), orf4, and orf8 perturbagens suppressed only the *GAL–STE4* allele (Table 2). These results indicated that the perturbagens

Table 1. Perturbagens

Perturbagens	Chromosome	Position	GFP fusion partner
pep1(I)	NA	NA	15-aa peptide (internal)
pep2(I)	NA	NA	13-aa peptide (internal)
pep4(C)	12	345,284–345,535	59-aa peptide
pep4(C)	13	17,605–17,785	21-aa peptide
pep5(C)	7	954,846–955,084	31-aa peptide
orf1	12	849,840–850,463	amino acids 14–221 of *STE11*
orf2	3	63,438–64,244	amino acids 32–279 of *STE50*
orf3	2	329,957–329,565	amino acids 32–160 of *GIP1*
orf4	5	425,915–424,661	amino acids 1512–1753 of *PMD1*
orf5	5	408,993–408,253	amino acids 23–269 of YER124C
orf6	2	600,538–600,774	amino acids 11–89 of YBR186W
orf7	13	441,164–443,186	amino acids 653–960 of YMR086W
orf8	2	357,343–355,292	amino acids 934–1108 of YBR059C
orf9	7	854,410–854,195	amino acids 161–231 of YGR179C
orf10	14/5	409,769–409,846/ 81,490–81,418	amino acids 477–502 of YNL115+ amino acids 37–395 of *RAD23*
orf11	2	390,347–390,624	18-aa peptide + amino acids 26–98 of *ECM8*

Shown are the chromosomes from which each perturbagen-encoding sequence was derived (Chromosome), the numerical positions of the first and last nucleotides of each genomic sequence fused to GFP (Position, see ref. 8), and the predicted GFP-fused peptides comprising each perturbagen (GFP fusion partner). Sequences of the two internal peptides are as follows: pep1(I), WKYIRCIMPWHRFWF and pep2(I), RGYFNDRWYGCPG. The orf10 perturbagen is composed of tandemly ligated genomic fragments from YNL116W and RAD23 (56), both of which are required for the perturbagen to function (data not shown). The orf11 insert contains a deletion of the G residue present in the genomic sequence at position 390,403. This deletion restores the reading frame of the *ECM8* gene (36).

Table 2. Perturbagen penetrance and genetic epistasis

Perturbagen	Percentage of cells plated that formed colonies in the presence of α factor		Suppression of slow-growth phenotypes	
	Dextrose	Galactose	*GAL–STE4*	*STE11ΔN*
pVT21	<0.5	<0.5	−	−
pep1(I)	<0.5	60 ± 2	−	−
pep2(I)	<0.5	69 ± 3	+	+
pep3(C)	<0.5	90 ± 7	+	−
pep4(C)	<0.5	71 ± 9	+	+
pep5(C)	<0.5	6 ± 2	−	−
orf1	<0.5	55 ± 9	−	−
orf2	<0.5	19 ± 10	−	−
orf3	<0.5	18 ± 4	−	−
orf4	<0.5	23 ± 4	+	−
orf5	<0.5	65 ± 4	+	+
orf6	<0.5	47 ± 6	−	−
orf7	<0.5	34 ± 9	−	−
orf8	<0.5	65 ± 9	+	−
orf9	<0.5	9 ± 4	−	−
orf10	<0.5	59 ± 5	−	−
orf11	<0.5	40 ± 4	−	−

Expression of the *GAL-STE4* and *STE11ΔN* alleles resulted in slow growth rather than complete growth arrest when coexpressed with the parental plasmid pVT21 (both the *GAL–STE4* and *STE11ΔN* alleles caused complete growth arrest in the absence of pVT21). This slow growth may have resulted from limiting levels of the *GAL4* transcription factor because binding sites for GAL4p were present in pVT21, its derivatives, and the *GAL–STE4* and *STE11ΔN* expression plasmids. Failure of some perturbagens (e.g., orf2) to suppress growth defects caused by the *GAL–STE4* and *STE11ΔN* alleles was likely caused by the generally low penetrance of these perturbagens. However, this failure also could result from inhibition of targets that act at the same point as or upstream of STE4p or from an inability of particular perturbagens to inhibit these specific dominant alleles.

isolated in the screen collectively act at a variety of points in the pheromone response pathway (Fig. 1).

Two-Hybrid Analysis of Perturbagens. Inhibition of the pheromone response pathway by a perturbagen is likely to depend on an interaction between the perturbagen and a protein involved in the response (i.e., a target protein). This interaction in turn results in either a partial or complete impairment of the function of the target protein. To identify potential targets of the various perturbagens, we used the two-hybrid technique of Fields and Song (38), a method that detects protein–protein interactions in yeast.

Each of the 16 perturbagens was fused in-frame with either the *GAL4* DNA BD or AD transcription (see *Materials and Methods*). In the case of the two perturbagens isolated from the peptide display library, the entire GFP coding sequences containing the random peptide inserts were cloned in-frame with BD- and AD-encoding sequences (see *Materials and Methods*). Each of the BD- and AD-perturbagen fusions then was coexpressed with various AD- and BD-pheromone response gene fusions in a yeast strain that carried the lacZ gene as a reporter such that potential interactions could be examined pairwise (see Table 3 for pairings and see *Materials and Methods*).

As judged by the two-hybrid assay, seven of the perturbagens interacted with individual members of the pheromone response pathway (Table 3). Proteins with which perturbagens interacted included the products of the *STE5*, *STE18*, and *STE11* genes (30, 32, 39–41). The simplest interpretation of these findings is that the sterile (STE) gene product with which a particular perturbagen interacts is the protein inhibited by the perturbagen *in vivo*. This interpretation is bolstered by the observation that the Ste5p and Ste11p proteins with which the pep2(I), orf4, and orf5 perturbagens interact function in the pathway at points consistent with the sites of inhibition determined for these perturbagens through epistasis analysis [the *STE5* protein has been found to interact with pathway components that function both upstream and downstream of *STE11* (refs 20 and 42–44; see Table 2)].

Phenotypic Analysis of Perturbagen Gene Disruption Strains. Two general types of perturbagens that inhibit pheromone response can be envisioned. One type of perturbagen derives from genes involved in pheromone response (e.g., *STE11* and *STE50*) and therefore directly identifies pathway components. These perturbagens most likely act in a dominant negative manner. The second type of perturbagen arises from either fragments of proteins not directly involved in the pathway or random peptides that have a fortuitous affinity for pathway components. The value of these perturbagens lies in their use as probes to identify pathway components (e.g., pep2(I); Table 3).

To help place the remaining ORF-derived perturbagens into one of the two classes, we tested whether the proteins encoded by the wild-type genes function in the pheromone response pathway. The coding regions of the *GIP1*, *PMD1*, YER124C, YBR186W, YMR086W, YBR059C, and YGR179C genes were disrupted in haploid strain yVT8 by homologous recombination (see *Materials and Methods*). The disruption strains, all of which were viable, were compared with the wild-type strain in standard halo assays to determine directly whether sensitivity to α factor was altered (15). The disruption strains and the congenic wild-type strain yVT8 were spread separately on plates, and filter disks spotted with varying amounts of α factor were placed on the surface of each plate (see *Materials and Methods*). Diffusion of α factor from the disks created a gradient of pheromone and a resulting zone of growth inhibition, or halo, the diameter of which was measured and compared among strains. Halo diameters resulting from strains disrupted for the *GIP1*, *PMD1*, YER132C, YBR186W, and YMR086W genes were identical to those of the wild-type strain, indicating that none of these gene products was required for normal response to α factor (data not shown). In contrast, halo diameters measured for strains harboring disruptions of the YBR059C and YGR179C genes were slightly larger than those measured for the wild-type strain (Fig. 2). Thus, disruptions of the YBR059C and YGR179C genes resulted in small but reproducible increases in α factor sensitivity, ≈2- and 4-fold, respectively, as compared with the parental strain (Fig. 2). These results suggest that the wild-type products of the YBR059C and

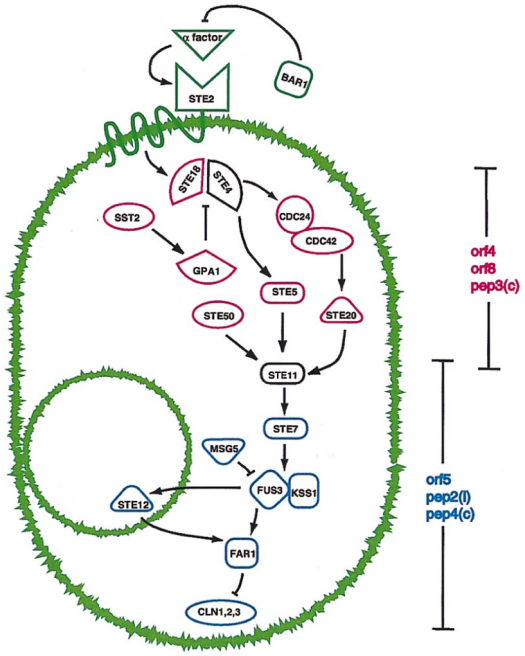

FIG. 1. Pheromone response pathway in yeast and summary of epistatic interactions with *STE4* and *STE11*.

Table 3. Two-hybrid analysis

	STE4	STE18	STE50	STE20	STE5	STE11	STE7	FUS3	KSS1	STE12
pep1(I)	–	–	–	–	–	–	–	–	–	–
pep2(I)	–	–	–	–	9	–	–	–	–	–
pep3(C)	–	–	–	–	–	–	–	–	–	–
pep4(C)	–	–	–	–	–	–	–	–	–	–
pep5(C)	–	–	–	–	–	–	–	–	–	–
orf1	–	–	–	–	+	+	–	–	–	–
orf2	–	–	-	–	134	17	–	–	–	–
orf3	–	–	–	–	13	–	–	–	–	–
orf4	–	–	–	–	19	–	–	–	–	–
orf5	–	–	–	–	–	9	–	–	–	–
orf6	–	–	–	–	–	–	–	–	–	–
orf7	–	–	–	–	–	–	–	–	–	–
orf8	–	–	–	–	–	–	–	–	–	–
orf9	–	–	–	–	14	–	–	–	–	–
orf10	–	18	–	–	–	–	–	–	–	–
orf11	–	–	–	–	–	–	–	–	–	–

Shown are the results of pairwise examinations of each perturbagen with various known members of the pheromone response pathway. Numbers given for each positive interaction represent units of β-galactosidase activity (micromoles of o-nitrophenyl-β-D-galactopyranoside hydrolysed min^{-1} $cell^{-1}$) determined in quantitative liquid assays according to the methods described (ref. 22 and *Materials and Methods*). Units of activity were standardized to the level of activity measured for each binding domain partner combined with an activation domain SV40-large-T antigen fusion (standardized to 1 unit). In all cases, units of activity were determined from two independent pairings, each measured in triplicate. The average SD was <20% with a maximum of 41% (the orf9/STE5 pairing). In all cases except orf10, the numbers shown were determined from the orf-AD fusion/STE-BD fusion pairing. The positive interactions marked with a + for orf1 were not observed in this study and are extrapolated from ref. 20.

YGR179C genes may regulate the pathway in a negative manner to ameliorate the arrest caused by α factor.

The observation that disruption of the YBR059C and YGR179C genes increased α factor sensitivity, combined with previous results (30–32) demonstrating that the *STE11* and *STE50* genes are required for pheromone response, indicate that four of the ORF-derived perturbagens originate from genes that play a role in pheromone response. Therefore, perturbagens themselves directly identified both positive (*STE11* and *STE50*) and putative negative (YBR059C and YGR179C) regulators of the pheromone response pathway (see *Discussion*).

DISCUSSION

We have tested a general method for genetic analysis by using the yeast pheromone response pathway, a model growth control system whose components are well understood. The results of a selection for perturbagens that promote cell division in the presence of α factor suggests that the method is an efficient way to dissect biological processes, especially when traditional modes of genetic analysis are not feasible. Multiple perturbagens were recovered in two parallel selections. These perturbagens were well-behaved genetic entities whose activity could be quantified in penetrance assays and mapped by epistasis tests. The perturbagens themselves identified elements of the α factor response pathway, both known genes and to our knowledge previously uncharacterized genes. In addition, two-hybrid analysis revealed specific interactions between individual perturbagens and known components of the pathway.

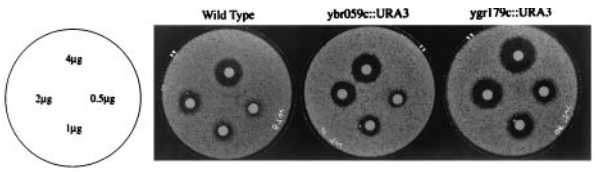

FIG. 2. Halo assays of wild-type and congenic yeast strains. Shown are the results of halo assays performed on a wild-type strain (yVT8) and congenic yeast strains harboring disruptions of either the YBR059C gene or YGR179C gene (strains yVT76 and yVT80, respectively) after 4 days. The sensitivity to α factor of the mutant strains relative to the wild-type strain was determined by measuring halo diameters caused by different α factor concentrations and then comparing diameter widths between the strains. The amounts of α factor spotted to the disks at corresponding positions on each plate are shown on the schematic to the left.

Perturbagen Targets and Mechanisms of Inhibition. Several observations suggest that a subset of the perturbagens isolated in this screen act by directly inhibiting an essential component of the pheromone response pathway. First, two pertubagens were derived from pheromone response genes (orf1 and orf2; Table 1). Second, by two-hybrid analysis we found that six additional perturbagens interacted with pathway proteins encoded by the *STE5*, *STE18*, and *STE11* genes (Table 3). Furthermore, of these latter perturbagens, the pep2(I), orf4, and orf5 perturbagens inhibited the pheromone response pathway at points consistent with the sites of action determined for the STE proteins with which they interacted (Tables 2 and 3). The simplest interpretation of these data is that at least half of the perturbagens impair pheromone response by directly blocking the function of specific STE genes.

Three general mechanisms may explain how perturbagens act as proteinaceous inhibitors of pheromone response. First, these agents may act as dominant negatives, inhibiting pheromone response by directly interfering with the function of the wild-type proteins from which they are derived (4). The perturbagens derived from the *STE50* and *STE11* genes likely act in this fashion. Second, perturbagens not derived from proteins involved in pheromone response may inhibit the function of proteins in the pathway in a transdominant manner through direct protein–protein interactions. Interactions between perturbagens and pheromone response gene products could arise fortuitously, as with the peptide perturbagens [e.g., pep2(I), which interacts with STE5p; Tables 1 and 3] and possibly some of the perturbagens derived from genes that apparently are not involved in pheromone response (Table 3 and, for example, orf3). Third, interactions between perturbagens and various STE gene products may reflect physiologically relevant interactions between the full-length protein from which a perturbagen is derived and its STE binding partner, which occur during a phase of the yeast life cycle other than mating, such as pseudohyphal growth (for reviews see refs. 45 and 46).

The potential *in vivo* targets, and thus the mechanisms of action, for eight perturbagens remain obscure. DNA sequence

analysis and two-hybrid studies provided no hints about how they inhibit the pathway. Despite the lack of evidence, these pertubagens may inhibit components of the pheromone response pathway. For example, it is possible that insensitivity of the two-hybrid screen may limit the ability to detect the *in vivo* target. Indeed, we observed that certain perturbagens were unstable as two-hybrid fusion proteins (data not shown). In addition, we examined only a subset of pheromone response components in the two-hybrid analysis (6, 47–50). Another possibility is that these perturbagens may negatively affect the pheromone response pathway in a manner not related directly to pheromone response, for instance, by influencing general cell cycle control through boosting cyclin levels (6). A final possibility is that inhibitory agents isolated in this selection may not function as transdominant proteinaceous inhibitors. For instance, inhibition of pheromone response may have occurred because of overexpression of the native function of a protein rather than through inhibition of other proteins. The orf8 perturbagen may act this way because it is derived from a protein that may down-regulate the pheromone response pathway (Fig. 2). Inhibition also could result from a reduction in the level of expression of an important gene because of antisense effects (3). The only candidate for such a molecule is the pep3(C) perturbagen, which is expressed from the noncoding strand of the *CDC45* gene (51–53). However, it is unlikely that the pep3(C) perturbagen inhibits pheromone response through a negative effect on *CDC45* expression because cdc45p is required for proper cell cycle progression because of its essential function in the initiation of DNA replication (52, 53).

Perturbagens as a Tool to Identify Pathway Components. Based on the phenotypes of strains harboring loss-of-function alleles of the genes from which the orf1–9 perturbagens are derived, two of the perturbagens encoded portions of genes required for pheromone response (refs. 30–32; Fig. 2 and data not shown). In contrast, six perturbagens interacted with three different pheromone response genes as judged by the two-hybrid assay (Table 3). These data suggest that the primary value of transdominant agents may lie in their use as probes for the subsequent identification of important genes rather than as direct identifiers of important genes.

Two genes defined by perturbagens, the YBR059C and YGR179C genes, encode proteins that may down-regulate the response to pheromone based on the phenotype that yeast lacking these genes are ≈2- and 4-fold more sensitive to pheromone than congenic wild type yeast (Fig. 2). Despite extensive study of the pheromone response pathway, roles for the YBR059C and YGR179C gene products, to our knowledge, have not been reported. Prior identification of these genes may have been hindered by the relatively weak phenotypes displayed by strains harboring disruptions of either of these two genes (Fig. 2). In addition, fewer screens specifically designed to identify supersensitive mutants have been performed (47, 54, 55). The identification of two additional, albeit nonessential, pheromone response genes through perturbagens suggests that perturbagen-based strategies complement other genetic methodologies. Furthermore, although the selection demanded inhibition of the pheromone response pathway, perturbagens themselves identified both positive (*STE11* and *STE50*) and putative negative (YBR059C and YGR179C) pathway elements. Perturbagen-based approaches may therefore identify a wider range of genes on a per-screen basis than traditional approaches.

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