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Expression of the *BAR1* Gene in *Saccharomyces cerevisiae*: Induction by the α Mating Pheromone of an Activity Associated with a Secreted Protein

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We have demonstrated and partially characterized the genetic control and pheromonal regulation of a soluble activity, produced only by mating-type **a** cells, that inhibits the action of the α mating pheromone, α-factor, on mating-type **a** cells. This activity was found to be associated with a heat-stable protein and to be secreted by *MAT***a** *BAR1*, *matα2 BAR1*, and *matα1 matα2 BAR1* strains, but not by *MATα BAR1*, *MAT***a**/*MATα BAR1*, *matα1 BAR1*, or *MAT***a** *bar1* strains, demonstrating that it is under the control of both the *MATα2* and the *BAR1* genes. Secretion of this activity was also found to be stimulated to as much as five times the basal level by exposure of the cells to α-factor. This stimulation was maximal after 6 h at a pheromone concentration of approximately 2 U/ml. An assay for this activity was developed by using a refined, quantitative assay for α-factor. The pheromone activity of samples added to wells in an agar plate was related to the size of the halo of growth inhibition produced in a lawn of mutant cells that are abnormally sensitive. The α-factor-inhibiting activity was related to a reduction of the halo size when active samples were added to the lawn. Although the assay for α-factor was found to be relatively insensitive to pH over a range of several units, the α-factor-inhibiting activity displayed a sharp pH optimum at approximately 6.5. The properties of this activity have important implications concerning the role of the *BAR1* gene product in recovery of mating-type **a** cells from cell division arrest by α-factor.

Mating in the yeast *Saccharomyces cerevisiae* involves the concerted action of a multiplicity of unlinked genes (for reviews see references 12, 17, and 22). Genetically, the expression of genes that show the mating-type specificity is controlled by the mating-type locus, *MAT*. In view of the specificity of the response of mating cells to the pheromones secreted by the opposite mating type, it seems plausible that the expression of some of these mating-type specific genes is also controlled by these pheromones, **a**-factor and α-factor. Many of the specific elements of this system and the general features of the control mechanisms have been identified. The alternative mating-types correspond to Mendelian alleles of the *MAT* locus, *MAT***a** and *MATα*; however, the expression of mating-type specific genes is determined primarily by two gene products of the complex *MATα* locus, designated the *MATα1* product and the *MATα2* product (21). Genes that are normally expressed only in *MATα* cells are under positive regulation by the former, whereas at least some of those normally expressed in *MAT***a** cells are under negative regulation by the latter.

Only two of the gene products under this regulation have been isolated and characterized. These are the two mating pheromones, α-factor, which is secreted by mating-type α cells, and **a**-factor, which is secreted by mating-type **a** cells. Both are small peptides. Other mating-specific functions have been defined by mutations that affect mating or mating-related functions, most notably functions associated with the ability of cells of each mating type to respond to the pheromone secreted by the opposite. This response involves a reversible arrest of progression through the cell cycle at a specific point in G1, followed by a sequence of morphological and physiological changes that apparently facilitate mating. In most cases, the gene products associated with these functions remain obscure. An exception is the "Barrier" function, first described by Hicks and Herskowitz (13).

The Barrier function was first defined by the ability of *MAT***a** cells to impede the diffusion of α-factor. This function has also been implicated in the recovery of *MAT***a** cells from α-factor arrest. Chan (4) demonstrated that the recovery of α-factor-arrested **a** cells parallels the disappearance of α-factor activity from the medium. In turn, investigators in three laboratories (7, 11,

16) have demonstrated proteolytic activity associated specifically with *MAT***a** cells that results in the degradation of α-factor and is correlated with their recovery from cell cycle arrest.

Mutations that affect the ability of *MAT***a** cells to inactivate α-factor have been detected by two methods. Strains that carry a *matα2* mutation are defective in the negative control of **a**-specific functions (21). Consequently, they express the Barrier function, which in turn inactivates their α-factor. Sprague and Herskowitz (20) isolated *bar1* mutants as suppressors of the α-factor defect in a *matα2* strain. *MAT***a** *bar1* strains are Barrier negative. Chan and Otte described mutants which are termed "supersensitive" (sst) because they have impaired abilities to recover from α-factor arrest (5, 6). Those belonging to one complementation group, *sst1*, are Barrier negative and are allelic with the *bar1* mutants; they are noncomplementing and map at the same locus, 6.9 centimorgans proximal to the centromere-linked *HIS6* locus on chromosome IX. Chan and Otte also demonstrated that *SST1* strains can help *sst1* mutants recover from α-factor arrest when the two strains are treated together (5).

Clearly, these results indicate that the *BAR1* gene controls a function that is involved in the inactivation of α-factor by *MAT***a** cells, that the *BAR1* gene is itself regulated by the *MATα2* function, and that the *BAR1* function is involved in recovery from α-factor arrest. However, it has not been established that the *BAR1* product is a protease (see below).

In this paper, I will present evidence that mating-type **a** cells secrete a Barrier activity as a soluble, heat-stable protein under the control of the *BAR1* and *MATα2* genes, and that its secretion is also stimulated by exposure of the cells to α-factor.

(These results were reported in preliminary form at the Meeting on Molecular Biology of Yeast at Cold Spring Harbor in 1981.)

MATERIALS AND METHODS

Yeast strains. The yeast strains used in these studies are listed in Table 1, together with their genotypes and sources.

Media and culture conditions. The halo assays for α-factor and Barrier activity employed yeast extract-peptone-dextrose medium (YEPD) supplemented with adenine (80 mg/ml). As indicated, the pH was adjusted with citrate-phosphate buffer (10), dibasic ammonium phosphate, ammonium hydroxide, or acetic acid, added after autoclaving. The final pH of each agar medium was determined by measurements with a glass electrode on samples homogenized in distilled water with a blender. Secreted Barrier activity was measured in defined synthetic (SC) medium that had been clarified by centrifugation. SC medium was prepared by supplementing yeast nitrogen base (Difco Laboratories) as described previously (15, 18).

Liquid cultures were grown in DeLong culture flasks, containing 20% of their nominal volume, with rotary shaking at 200 rpm. All cultures and assays were incubated at 30°C.

Halo assays for α-factor and Barrier. α-Factor and Barrier activities were estimated from the diameter of clear halos of inhibited growth formed in confluent lawns of supersensitive *MAT***a** *bar1* cells. Lawns were

TABLE 1. List of strains

Strain no.	Genotype[a]	Source
X2180	*MAT***a**/*MATα gal2/gal2*	R. K. Mortimer
X2180-1A	*MAT***a** *gal2*	R. K. Mortimer
X2180-1B	*MATα gal2*	R. K. Mortimer
XT1172-S245c	*MATα his6 ade6 leu1 trp5-1 met1 can1 gal2*	This laboratory
VC73	*matα2-1 his6 ade6 leu1 trp5-1 met1 can1 gal2*	V. MacKay
VP1	*matα2-4 his6 ade6 leu1 trp5-1 met1 can1 gal2*	V. MacKay
DC65	*matα1-5 matα2-1 leu2 ade6 leu1 lys2*	J. Strathern
RC629	*MAT***a** *sst1-2 ade2 ura1 his6 met1 can1 cyh2 gal2*	R. Chan
G130D2-18B	*MAT***a** *bar1-1 ade2 met1 cyh2 leu1 rme can1 ura3 gal2*	G. Sprague
G190-4C	*MAT***a** *bar1-1 cyh2 leu1 met1 can1 rme*	G. Sprague
XMB4-12b	*MAT***a** *sst1-1 arg9 ilv3 ura1 gal2(?)*[b]	L. Blair

[a] Gene symbols indicate mutations leading to requirements for the following: *ade* (adenine), *arg* (arginine), *his* (histidine), *ilv* (isoleucine-valine), *leu* (leucine), *lys* (lysine), *met* (methionine), *trp* (tryptophan), *ura* (uracil). Additional symbols include the following: *MAT***a** or *MATα* (mating type), *bar* (Barrier deficient), *can* (resistance to canavanine), *cyh* (resistance to cycloheximide), *gal* (inability to ferment galactose), *rme* (regulator of meiosis), *sst* (supersensitive to α-factor).

[b] We employed a petite derivative of this strain of spontaneous origin.

formed by suspending 2×10^5 cells from stationary YEPD-grown cultures in 2 ml of 0.75% agar at 50°C and immediately pouring the suspension onto the surface of a prewarmed (37°C) agar plate. Plates were allowed to stand at room temperature for 1 to 2 h. Five wells (5.5 mm diameter) were cut in a circular pattern in each plate. Test samples of α-factor (0.075 ml) were placed in the wells with an automatic pipette. After incubation for 2 days at 30°C, the halo diameters were measured with dial calipers. To estimate Barrier activity, test samples were added to the soft agar overlay at the time the lawns were prepared; 1 ml of sample was added to 1 ml of 1.5% agar containing 2×10^5 test cells. A standard amount of α-factor was placed in each well. The halo size is sensitive to the amount of medium in the plate. To assure the necessary uniformity, 27 ml of agar medium was metered into each plate with a peristaltic pump. α-Factor was isolated from strain X2180-1B by the general method described by Duntze et al. (9), modified as described previously (19).

Calibration of α-factor halos. The biological activity of α-factor has been defined by using criteria based on a variety of its physiological effects on *MAT***a** cells, most notably morphological response, agglutination, and recovery time from G1 arrest (17). An additional method that offers some significant advantages makes use of the diffusion of the pheromone through agar and the effectively irreversible inhibition of sensitive *MAT***a** *bar1* (or *sst1*) strains (6). Figure 1a illustrates the clear zones of inhibition ("halos") formed in a lawn of supersensitive cells surrounding a source of α-factor, in this case a well in the agar containing a solution of the pheromone. As the photograph illustrates, the diameter of a halo depends on the concentration of activity in the source, and the edge of the halo is quite sharp, permitting precise measurement of its diameter. In Fig. 1b, the squares of the halo radii (r^2) are plotted against the relative pheromone concentrations placed in the wells, on a logarithmic scale. The resulting linear plot provides a convenient calibration. In practice, the concentration of a test sample can be compared with this reference standard by measuring the diameter of the halo produced at a single concentration. This method permits measurement of relative activities with much greater ease and precision than the earlier methods we have employed. We have defined the unit of activity by this method as the theoretical reciprocal dilution that would yield a halo radius of zero. For any sample (halo radius), this is calculated by using the slope of the calibration curve. The method used for making this calculation and analysis of the errors inherent in this measurement are described in the Appendix.

RESULTS

Demonstration of a soluble Barrier activity. The α-factor assay shown in Fig. 1 is sufficiently simple and precise that a study of the Barrier activity based on the disappearance of α-factor activity seemed feasible. α-Factor was incubated with the cell-free medium in which a *MAT***a** strain had been grown to stationary phase, adjusted to pH 6.5. At intervals, samples were withdrawn and tested for surviving α-factor activity without further treatment. In Fig. 2, the α-factor activity measured in these samples is plotted against the sampling time for two con-

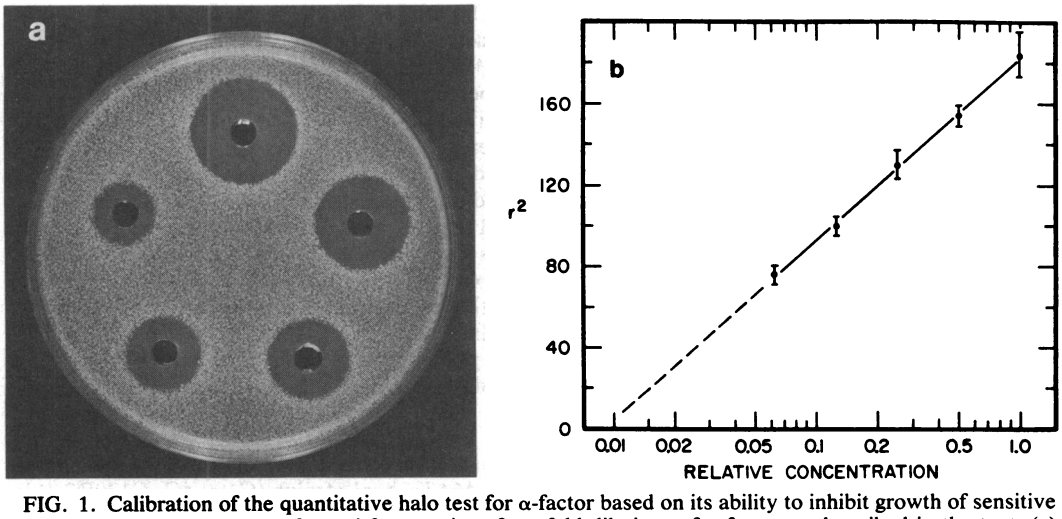

FIG. 1. Calibration of the quantitative halo test for α-factor based on its ability to inhibit growth of sensitive *MAT***a** *bar1* cells. Halos were formed for a series of twofold dilutions of α-factor as described in the text. (a) Photograph of agar plate (10 cm diameter) with clear halos in a lawn of the sensitive strain, XMB4-12b (petite), surrounding wells containing the α-factor samples. The photograph was taken after 2 days of incubation at 30°C. (b) The squares of the halo radii are plotted against the initial relative concentrations of the α-factor put in the wells, on a logarithmic scale. The definition and method for calculating the unit of activity and standard deviations are discussed in the text.

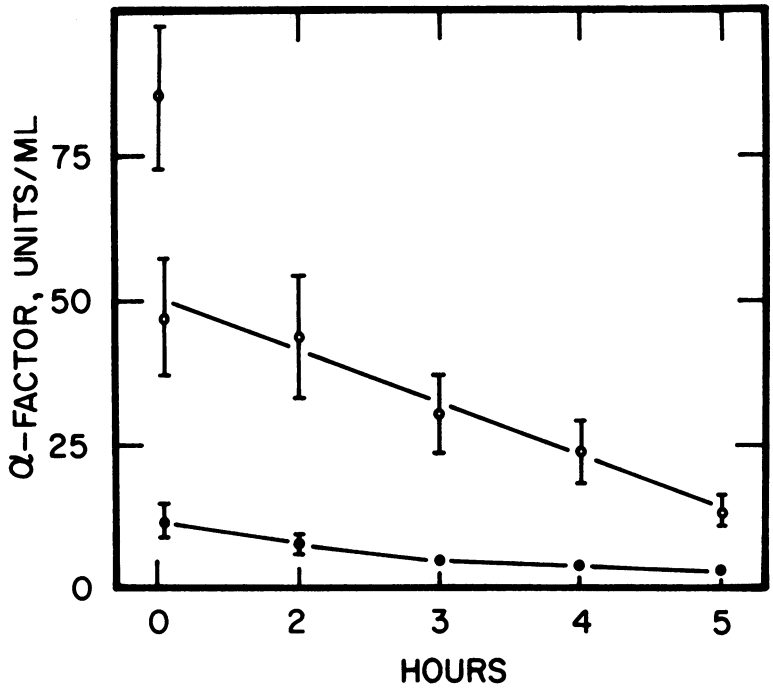

FIG. 2. Inhibition of α-factor by culture medium from a *MATa BAR1* strain. X2180-1A cells were grown overnight by SC medium and harvested by centrifugation, and the pH of the spent medium was adjusted to 6.5 with ammonium hydroxide. α-Factor (final concentration of 86 U/ml [◑]) was added to each of two samples. Immediately after mixing and at 1-h intervals, aliquots were removed and added directly to α-factor halo assay plates. Other symbols: ●, undiluted spent medium; ○, spent medium diluted with an equal volume of fresh SC medium (pH 6.5). Activity and standard deviations (error bars) were calculated as described in the text. Where error bars are omitted, the standard deviation range is less than the height of the symbol.

centrations of medium. The initial pheromone concentration of 86 U/ml is plotted at zero hours. The activity measured in the earliest samples that could be taken after mixing showed substantially decreased α-factor activity, roughly proportional to the concentration of the medium added. This abrupt initial drop was followed by a gradual decline over the next several hours. The rate of this decline did not reflect the relative concentration of medium. When the same experiment was done with medium from *MATα*, *MATa/MATα*, or *MATa bar1* strains (data not shown), there was no significant loss of α-factor activity. These results demonstrate that there is an activity secreted only by *MATa BAR1* strains that inhibits the action of α-factor. One must assume that the reaction continues for an undetermined period of time after the aliquot is placed in the well of the α-factor assay plate. Because we have not yet discovered conditions that will inactivate this activity without also destroying the α-factor, this experiment gives no information about the mechanism of this inhibition, nor does it provide an assay for Barrier activity.

The soluble Barrier protects *MATα bar1* from α-factor. It is commonly presumed that the defect in *MATa bar1* mutant strains that makes them supersensitive to α-factor is their lack of an active product that inactivates or inhibits the pheromone. If this product is secreted into the medium, as the above experiment suggests, then medium from *MATa BAR1* cells should protect *MATa bar1* cells. The results (Fig. 3) show that this is the case. In this experiment, we have used a fixed amount of α-factor to compare the sizes of the halos produced in lawns of *MATa BAR1* cells, *MATa bar1* cells, and *MATa bar1* cells in the presence of medium from *MATa BAR1* cells, at different pH values. Unfortunately, we did not have isogenic strains that differed only by the *BAR1* function. We did, however, observe substantially the same behavior with four different *MATa bar1* strains (G190-4C, G130D2-18B, RC629, and XMB4-12b). The difference between the upper and lower curves represents the effect of the normal *BAR1* function. The intermediate curve shows that when the secreted Barrier activity is added to the supersensitive mutant cells at the higher pH values, it reduces their

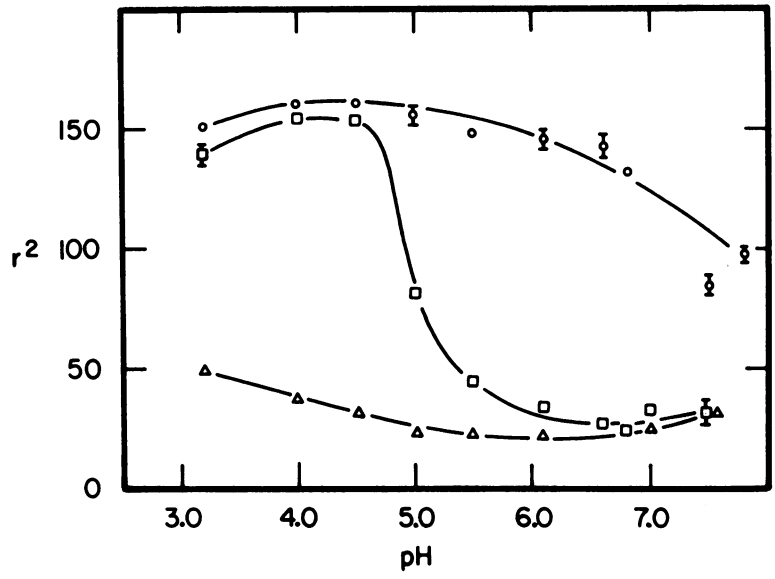

FIG. 3. Effect of secreted Barrier on the response of *MAT***a** *BAR1* and *MAT***a** *bar1* cells to α-factor. Halos were formed on agar plates that had been adjusted to various pH values. The squares of the halo radii, r^2, are plotted against the pH of the medium. Symbols indicate compositions of the soft agar overlays: ○, *MAT***a** *bar1* cells (G190-4C); □, the same cells with medium from *MAT***a** *BAR1* cells added to the overlay as a source of soluble Barrier activity; △, *MAT***a** *BAR1* cells. The error bars represent the standard deviations of the r^2 values. Where error bars are omitted, the standard deviation range is less than the height of the symbol.

sensitivity, as judged by the halo diameters, to that of the normal *a* strain.

Development of an assay for Barrier activity. The observations shown in Fig. 3 provide the basis for an indirect yet simple assay. When Barrier was added to the soft agar overlay of *bar1* cells on the assay plate, between pH5 and 7, the sizes of the resulting α-factor halos were reduced. The use of this effect as an assay for Barrier activity is demonstrated in Fig. 4. The squares of the halo radii (r^2) are plotted against the relative concentrations of Barrier activity added to the test cells, on a logarithmic scale. Each well in this case received the same amount of α-factor. Once again, a linear plot was obtained, providing a simple, relatively precise empirical assay. We have defined the unit of Barrier activity measured by this method as the relative concentration of the sample that, by extrapolation, would yield a halo radius of zero for an arbitrary amount of α-factor. We have not attempted to establish an absolute standard of activity for the experiments reported in this paper. Instead, the results of each experiment are presented in arbitrary relative units. However, we have used the same concentration of α-factor throughout, so the results in different experiments can be compared. As in the case of the α-factor assay, the Barrier activity can be calculated from the halo radius for a single concentration and the slope of the appropriate calibration curve (Fig. 4). The method used for making this calculation and analysis of the errors inherent in this measurement are described in the Appendix.

pH dependence of Barrier activity and α-factor. Any assay method for α-factor that is based on the response of normal *MAT***a** cells will depend, at least in part, on the pH dependence of the recovery mechanism, including Barrier, as well as the pH dependence of the initial response to the pheromone. This is because the manifestations of the response are reversible and, therefore, their quantitation is always a measure of the net effect at the time of the measurement. In Fig. 3, the halo sizes in the lawn of normal **a** cells (bottom curve) appear to have little pH dependence, but this may reflect saturation of the contribution of their Barrier under these conditions. However, the Barrier-negative *bar1* cells (top curve) also exhibit relatively little pH dependence compared with the striking effect when soluble Barrier activity is added. Figure 5 shows a more precise determination of the pH dependence of this activity, determined by the method of Fig. 4, using four dilutions of Barrier activity at each pH. These results indicate an optimum at approximately pH 6.5. The concentration of Barrier was chosen to give the optimal sensitivity for the assay in the

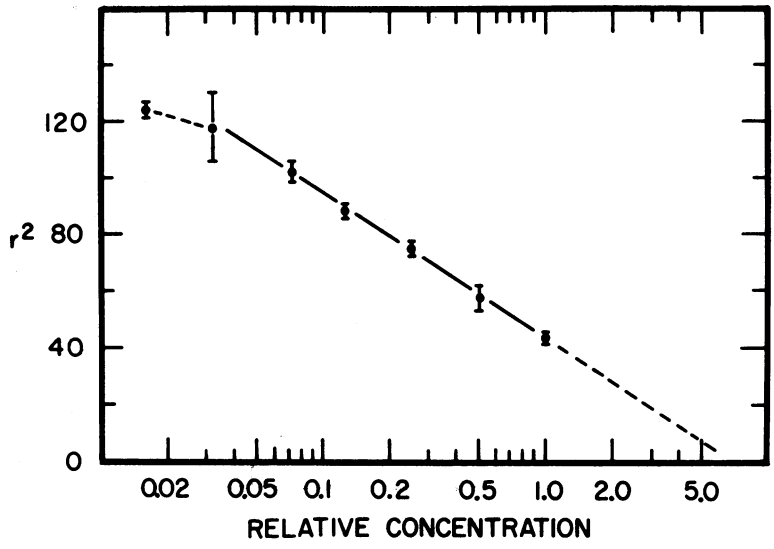

FIG. 4. Calibration of the quantitative halo test for the soluble Barrier activity, based on its ability to inhibit formation of α-factor halos. The test is identical to that shown in Fig. 1, except that the sample being tested for Barrier activity is added to the test cells in the soft agar overlay before the lawn is formed. A fixed amount of α-factor is placed in each well. In this case, the squares of the halo radii, r^2, are plotted against the initial relative concentrations of the Barrier test sample added to the overlay on a logarithmic scale. The methods for calculating relative Barrier activities and their standard deviations are discussed in the text.

range of the pH optimum, but at higher concentrations, there was appreciable Barrier activity at lower pH values (data not shown). Consequently, these data do not resolve the question of whether the secreted activity can account for the entire difference observed between the two strains in Fig. 3 or whether the difference at the lower pH values reflects an activity that is not secreted into the medium.

Barrier activity is associated with a heat-stable

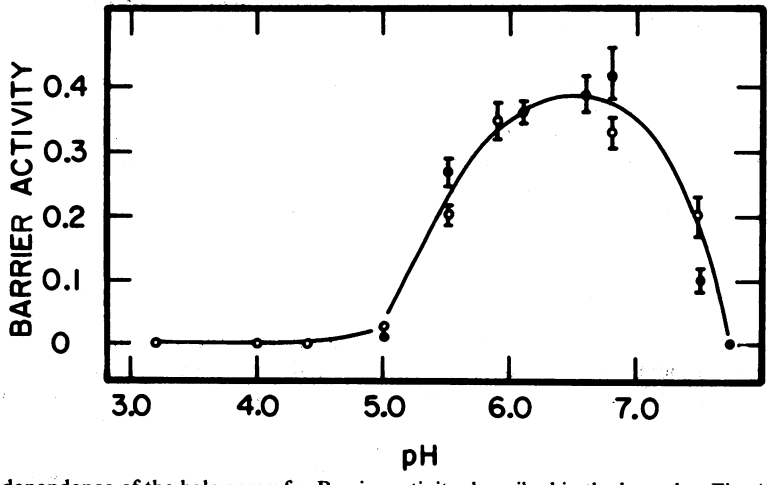

FIG. 5. pH dependence of the halo assay for Barrier activity described in the legend to Fig. 4 and the text. A standard sample of Barrier activity was prepared by growing strain X2180-1A to stationary phase in liquid SC medium, incubating it overnight with 4 U of α-factor per ml, centrifuging it, and heating the clarified medium in boiling water for 5 min. The pH of YEPD medium was adjusted as described in the text. For each point, the relative Barrier activity and standard deviation (error bars) were estimated from four dilutions of the standard sample as described in the text. Open and closed cirles represent separate experiments. Where error bars are omitted, the standard deviation range is less than the height of the symbol.

protein. On the premise that Barrier activity is a protease, we attempted to use heat inactivation to stop its reaction with α-factor, taking advantage of the heat stability of α-factor. To our surprise, we found the activity resistant to boiling. We were able to make use of this property to demonstrate that it is inactivated by pronase (Table 2). Samples 1 and 2 show that the Barrier activity survives up to 15 min of immersion in boiling water. Sample 3 shows that pronase mimics Barrier in this assay, presumably by inactivating the α-factor (8), but sample 4 shows that this effect is totally inactivated by boiling. Consequently, the loss of the Barrier activity in sample 5 is most simply interpreted as inactivation by pronase.

Stimulation of Barrier secretion by α-factor. In view of the profound changes that occur in *MAT***a** cells in response to α-factor, we anticipated that the secretion of Barrier could be part of the response to α-factor. Clearly, the observations described above demonstrate that at least some secretion is constitutive. However, we have found that exposure of *MAT***a** cells to α-factor results in a significant stimulation of secreted Barrier activity. Table 3 shows the dependence of the amount of activity secreted on the concentration of α-factor to which the cells were exposed. When the culture was grown to a stationary cell density and then exposed to the pheromone, there was a concentration-dependent stimulation of Barrier activity which saturated between 2 and 4 α-factor U/ml, yielding a level of Barrier activity approximately five times the constitutive level. The time course of this stimulation is shown in Table 4. When the saturating pheromone concentration of 4 U/ml was added, the maximum stimulation of Barrier activity (approximately threefold) was observed between 4 and 6 h.

Genetic control of the soluble Barrier activity. We have used this assay to demonstrate that this soluble activity is under the control of both *BAR1* and *MAT*α*2*, as has been reported for the Barrier phenotype by other workers (20, 21). Table 5 shows the secreted Barrier activity from a variety of strains with and without exposure to α-factor. Comparison of the isogenic strains X2180 (*MAT***a**/*MAT*α), X2180-1A (*MAT***a**), and X2180-1B (*MAT*α) shows that the activity is secreted only by the *MAT***a** strain. VP1 and VC73 (which were isolated from XP1172-S245c) are defective in the *MAT*α*2* function and therefore express both **a**-specific and α-specific functions. They also secrete Barrier activity at normal or slightly elevated *MAT***a** levels but show significantly less stimulation by added α-factor. This probably reflects the fact that these strains also produce α-factor and are therefore partially self-stimulated. This interpretation is supported by the behavior of DC65, which is defective in both the *MAT*α*1* and *MAT*α*2* functions and therefore expresses only **a**-specific functions. This double mutant, which does not secrete α-factor, exhibits a significantly higher stimulation by added pheromone. Finally, the results for the two independent *bar1* mutants, RC629 and G190-4C, further demonstrate the control of *BAR1*.

Although these experiments suffer from some uncertainty, owing to the lack of isogenic backgrounds in some cases and the variation in the amount of growth observed, the results provide strong confirmation that the secreted activity measured by this assay is under the control of both *MAT*α*2* and *BAR1*.

DISCUSSION

The ability of *MAT***a** cells to recover from arrest by α-factor is at least partly a conse-

TABLE 2. Effects of pronase and boiling on soluble Barrier activity[a]

Sample				Barrier activity	
No.	Barrier	Pronase	Boiled	U/ml	SD
1	+	−	−	0.19	±0.05
2	+	−	+	0.20	±0.04
3	−	+	−	0.11	±0.04
4	−	+	+	0.001	±0.001
5	+	+	+	0.001	±0.001

[a] Samples of liquid SC medium from α-factor–treated cultures of X2180-1A were adjusted to pH 6.0 with ammonium hydroxide. Pronase was added at a final concentration of 0.1 mg/ml. Liquid SC medium (pH 6.0) was used for Barrier minus controls. All samples were incubated at 37°C for 10.5 h and then were placed in boiling water for 15 min. Barrier activity was measured as described in the text and the legend to Fig. 4.

TABLE 3. Effect of α-factor concentration on the production of soluble Barrier activity in a *MAT***a** *BAR*1 strain[a]

α-Factor concn (U/ml)	Barrier activity			
	U/ml	SD	Increase	
			Ratio	SD
0.0	0.04	±0.01	1.0	
1.0	0.08	±0.02	2.0	±0.7
2.0	0.18	±0.03	4.5	±1.4
4.0	0.22	±0.06	5.5	±2.0
8.0	0.19	±0.03	4.8	±1.4

[a] Strain X2180-1A grown for 24 h in liquid SC medium and then was incubated for 10 h in the concentration of α-factor shown in the table. Barrier activity was measured in the growth medium as described in the text and the legend to Fig. 4.

TABLE 4. Time dependence of α-factor stimulation of production of soluble Barrier activity in a *MAT***a** *BAR*1 strain[a]

Time of exposure to α-factor (h)	Barrier activity			
	U/ml	SD	Increase	
			Ratio	SD
Control	0.07	±0.02	1.0	
0.5	0.07	±0.01	1.0	±0.03
1.0	0.09	±0.02	1.3	±0.5
2.0	0.11	±0.02	1.6	±0.5
4.0	0.16	±0.02	2.3	±0.7
6.0	0.24	±0.04	3.4	±1.1
8.0	0.21	±0.02	3.0	±1.0

[a] Strain X2180-1A was grown for 24 h in liquid SC medium, and then α-factor was added to each culture (except for the control) at a concentration of 4 U/ml. Barrier activity was measured in the growth media as described in the text and the legend to Fig. 4.

quence of their ability to inactivate it. In turn, this ability has been thought to depend largely on the **a**-specific Barrier function, which is under the control of the *BAR1* gene (6, 13, 20, 21). We have demonstrated an activity associated with a heat-stable protein that inhibits the response of *MAT***a** cells to α-factor. By both genetic and physiological criteria, this activity is under the control of the *BAR1* gene, and its absence accounts, at least in part, for the *bar1* phenotype. We have further demonstrated that the secretion of this activity is stimulated by α-factor.

Is Barrier a protease? It is well documented (7, 11, 16) that *MAT***a** cells, but not *MAT*α cells, degrade α-factor by proteolytic action, so it has been commonly assumed that the *BAR1* product is a protease. There is also an undocumented report that *bar1* mutants degrade α-factor at reduced rates, relative to wild type (E. Ciejek and J. Thorner, personal communication cited in reference 5). Although we do not know whether the protein whose activity we have described is a protease, its properties leave room for serious doubt. In particular, its great heat stability is not a property usually associated with proteases. The strongest hint, however, is given by the data in Fig. 2. Owing to the uncertainty in the effective reaction time, it is difficult to interpret the kinetics of these data. The initial drop in α-factor activity, however, is more suggestive of a stoichiometric relationship than of a catalytic one. This observation by itself does not rule out the possibility of a protease activity, but taken with the heat stability of the Barrier protein, it suggests that other possibilities warrant consideration. Two simple alternatives come to mind. (i) The activity could be an α-factor binding protein that mediates proteolysis by either a specific or a nonspecific protease. (ii) It could be a protein that binds to the cell or the cell–α-factor complex, thereby inhibiting or reversing the cell response, possibly by mediating proteolysis.

Any of these possibilities—inactivation by a protease, binding to a larger protein, or reducing the responsiveness of the cell—could lead to a decrease in the apparent activity. However, none of these possibilities is inconsistent with the observed proteolysis of α-factor by *MAT***a** cells, for either of the last two alternatives could mediate the proteolysis and in turn provide the mating-type specificity of the process. This is in accord with the observations that the mating-type specificity of α-factor proteolysis is not stringent (7) and is lost altogether when cells are converted to spheroplasts (16).

Is there a cell-bound Barrier function? We

TABLE 5. Effect of α-factor on production of soluble Barrier activity in various strains[a]

Strain	Genotype	α-Factor treated	Barrier activity		Ratio	Cells per ml (10^8)
			U/ml	SD		
X2180-1A	*MAT***a** *BAR1*	−	0.10	±0.01		0.96
X2180-1A	*MAT***a** *BAR1*	+	0.30	±0.02	3.0	1.02
X2180-1B	*MAT*α *BAR1*	−	0.002	±0.001		1.31
X2180	*MAT***a** *BAR1* / *MAT*α *BAR1*	−	0.01	±0.003		0.77
XT1172-S245c	*MAT*α *BAR1*	−	0.0001	±0.001		0.90
VP1	*matα2-4 BAR1*	−	0.17	±0.02		0.82
VP1	*matα2-4 BAR1*	+	0.23	±0.02	1.4	0.96
VC73	*matα2-1 BAR1*	−	0.29	±0.02		0.81
VC73	*matα2-1 BAR1*	+	0.43	±0.02	1.5	0.75
DC65	*matα1 matα2 BAR1*	−	0.08	±0.01		0.14
DC65	*matα1 matα2 BAR1*	+	0.30	±0.02	3.8	0.15
RC629	*MAT***a** *sst1-2*	−	0.01	±0.003		0.53
G190-4C	*MAT***a** *bar1-1*	−	0.01	±0.003		0.86

[a] Cultures were grown for 24 h in liquid SC medium to the cell density shown in the last column. α-Factor-treated samples received α-factor at a final concentration of 8 U/ml 16 h before the cells were harvested.

believe our results demonstrate unambiguously that at least part of the Barrier activities described previously (4–6, 13, 20) are accounted for by this secreted activity. The specific mechanism by which this activity is secreted can be determined only by further characterization of the secreted product and any possible precursors. Barrier activity was first identified by Hicks and Hershkowitz (13) as a property specific to *MAT***a** cells that acted as a barrier to the diffusion of α-factor through agar. They also demonstrated that this activity could be accounted for, at least in part, by a diffusible inhibitor of the response to α-factor. The question of whether there is also a Barrier activity that is bound to the cell remains. Three groups have reported independent observations of cell-bound proteolytic activities that inactivate α-factor (7, 11, 16). However, there have been no definitive reports to indicate whether these proteolytic activities are under the genetic control of *BAR1*.

There is a temptation to interpret the data shown in Fig. 3 as evidence for a cell-bound activity, as the pH dependence of the difference between *bar1* and *BAR1* cells does not parallel the pH dependence of the soluble Barrier activity. The difference between *BAR1* cells and *bar1* cells at lower pH values could indicate a cell-bound activity with a lower pH optimum. However, the difference could be merely a quantitative effect. Similar experiments involving higher concentrations of Barrier show that the secreted form retains some activity at lower pH values (data not shown). Therefore, we cannot rule out the possibility that even higher levels of Barrier could completely account for the *bar1* defect. We have attempted to detect a bound activity directly by measuring the disappearance of α-factor activity incubated with intact cells. The protocol was similar to the one used to obtain the data in Fig. 2, except that the incubation mixture contained washed cells in place of the spent medium. The results of these experiments have been positive, and they strongly suggest that there is such a bound activity, and that it is a significant component of the response. However, we have not ruled out the possibility that this can be accounted for by secretion of soluble activity during the assay.

Regulation of the Barrier function. There is, as yet, no evidence that establishes *BAR1* as the structural gene for the Barrier activity. It could be a regulatory gene. There is no obvious pleiotropy associated with the phenotype, so it is quite possibly the structural gene. On the other hand, it could be a regulatory gene controlling different related activities, such as binding protein(s), protease(s), or inhibitor(s). There is good evidence, however, that *BAR1* is itself negatively controlled by *MAT*α*2* (21). The results presented in this paper strongly support this conclusion. In addition, we can now say that the expression of *BAR1* is under the control of α-factor. This stimulation may therefore be viewed as part of the response of *MAT***a** cells to this pheromone. A similar α-factor stimulation of the secretion of **a**-factor has been observed by J. R. Strazdis and V. MacKay (personal communication). The parallel regulation of these two **a**-specific functions is striking. Both functions, which involve secreted polypeptide products (1, 2), are under negative regulation by *MAT*α*2* (19, 21), are expressed constitutively at basal levels and are expressed at higher levels after exposure of the cells to α-factor. In addition, they are both under negative control of the still-obscure *TUP1* function (14, 19).

Control of *BAR1* by *MAT*α*2* is clearly demonstrated by the data in Table 5. The quantitative nature of this control (19) is illustrated by the difference between VP1 and VC73. The former, which secretes less Barrier activity, has a less extreme *mat*α*2* phenotype than the latter, as evidenced by its ability to sporulate when crossed with *MAT***a** (15).

The stimulation of Barrier secretion by α-factor is demonstrated by the data in Tables 3, 4, and 5. The maximum activity, equal to three to five times the basal level, was reached within approximately 6 h after the addition of 4 U of pheromone per ml. This maximum activity was produced by pheromone concentrations of approximately 2 U/ml or more. The results shown in Table 5 further demonstrate the defect in *bar1* mutants. Comparison of the behavior of the *mat*α*2* mutants, VC73 and VP1, with the *mat*α*1-mat*α*2* double mutant, DC65, suggests that the secretion of Barrier is also stimulated by pheromone in these strains, and that in the former it is stimulated by the endogenous α-factor produced by these mutants.

These results give no information about the molecular level at which the regulation by either *MAT*α*2* or α-factor occurs. However, preliminary studies, using a cloned copy of the *BAR1* gene as a probe, indicate that transcription of this gene is under similar regulation (MacKay and Manney, unpublished data).

Physiological implications. Regardless of the mechanism by which Barrier mediates the recovery of *MAT***a** cells from α-factor arrest, the stimulation of its secretion by the pheromone must play a role in the kinetics of the process. We have previously described the puzzling observation that the period of inhibition of *MAT***a** cells by very low concentrations of α-factor is almost independent of the pheromone concentration and occurs within less than one doubling time (17). If under physiological conditions there

is a stoichiometric relationship between Barrier activity and the inhibition of *MAT***a** cells by α-factor, then the recovery kinetics may directly reflect the concentration of Barrier. The prompt, α-factor concentration-independent recovery at low concentrations would reflect the basal level, whereas the concentration-dependent inhibition times at higher concentrations could reflect the pheromone-stimulated secretion. Consequently, the kinetics of recovery from α-factor arrest do not necessarily reflect the kinetics of α-factor degradation, but may instead reflect the pheromone stimulation of Barrier secretion.

APPENDIX

Calculation of activity units and error analysis for quantitative halo assays. Both of the assays illustrated in Fig. 1 and 4 depend on the dilution of α-factor by diffusion through agar to a concentration that will not irreversibly inhibit the growth of the sensitive test cells forming a lawn on the surface of a plate. The size of the clear halo is a measure of the activity. Proportionality between the square of the halo radius and the logarithm of the relative concentration of α-factor placed in the well is predicted by a model based on diffusion of the pheromone through the agar. The assay for Barrier is more complicated, since the Barrier sample is added to the overlay of sensitive cells and becomes part of the environment through which the α-factor diffuses. Although it is clear that diffusion of the pheromone is the basis of both assays, we have defined units of activity empirically, so that they do not depend upon assumptions concerning the specific mechanisms involved.

The linear relationship illustrated in Fig. 1b can be described by the equation

$$r^2 = a + b \log C_{rel} \quad (1)$$

where r^2 is the square of the halo radius, C_{rel} is the relative concentration of α-factor added to the well, and a and b are constants. This can be extrapolated to the abscissa to find the value of C_{rel} corresponding to $r^2 = 0$, which equals the theoretical threshold concentration, C_0, for production of a halo. This yields the equation

$$0 = a + b \log C_0 \quad (2)$$

We define the concentration C_0 to be 1 U/ml. Then the activity, A, of the undiluted sample (i.e., $C_{rel} = 1$) is the reciprocal of C_0, which may be calculated from the equation:

$$A = e^{a/b} \text{ (units per milliliter)} \quad (3)$$

In practice, each value of r^2 and its standard deviation is determined from the average diameter of five independent halos. The parameters a and b and their standard deviations, s_a and s_b, are determined by the method of least squares, with instrumental weighting, as described by Bevington (reference 3, p. 92-113). The activity is then calculated from equation 3, and the standard deviation of the activity, s_A, is calculated by propagation of errors (reference 3, p. 56–60) from the equation

$$s_A = A(a^2 s_b^2/b^4 + s_r^2 2/b^2)^{1/2} \quad (4)$$

When a calibration curve and its slope, b, have been determined, the activities and standard deviations of additional samples can be determined from individual values for r^2, using the equations

$$A = e^{r^2/b} \text{ (units per milliliter)} \quad (5)$$

$$s_A = A(r^4 s_b^2/b^4 + s_r^2 2/b^2)^{1/2} \quad (6)$$

Mathematical analysis of the assay for Barrier is analogous to that described for α-factor. In this case, C_{rel} is the relative concentration of Barrier added to the soft agar overlay. Equal amounts of α-factor are placed in each well, so differences in the sizes of the halos depend on the extent to which the Barrier activity inhibits the response of the test cells to the pheromone during its diffusion. It is apparent from a comparison of Fig. 1b and 4 that the mathematics of these relationships are identical, except that in Fig. 4 (Barrier assay) the slope, b, is negative. In this case, extrapolation to $r^2 = 0$ yields the relative concentration of Barrier activity that would reduce the α-factor concentration to 1 U/ml, as defined above. Accordingly, 1 U of Barrier activity is defined as the amount that reduces the activity of an arbitrary concentration of α-factor to 1 U/ml in this assay. (For the present studies, we have not found it necessary or practical to define an absolute unit.) In practice, then, Barrier activities are calculated as fractions of the theoretical activity that would reduce the amount of α-factor used in that experiment to 1 U/ml. These values and their standard deviations are calculated as described above.

A program, written in BASIC 4.0 for the Commodore model 8032 microcomputer, which evaluates these equations from experimental data, is available from the author upon request.

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