7. E. U. Selker and J. N. Stevens, *Mol. Cell. Biol.* **7**, 1032 (1987); W. S. Grayburn and E. U. Selker, *ibid.* **9**, 4416 (1989).
8. R. L. Metzenberg, J. N. Stevens, E. U. Selker, E. Morzycka-Wroblewska, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 2067 (1985).
9. E. U. Selker, J. Stevens, P. Garrett-Engele, C. Garrett-Engele, D. Fritz, B. Margolin, R. Metzenberg, unpublished material.
10. E. U. Selker, *Annu. Rev. Genet.* **24**, 579 (1990).
11. Methods for culturing *Neurospora* and performing genetic analyses were standard [R. H. Davis and F. J. De Serres, *Methods Enzymol.* **17A**, 47 (1970)]. For Southern analyses, DNA samples were isolated as described [H. Foss and E. Selker, *Fungal Genet. Newsl.* **36**, 82 (1989)]. Restriction enzymes were used in buffers as suggested by the manufacturers. Genomic DNA was digested with 5 U of enzyme per 0.5 μg of sample. Restriction digests were fractionated by electrophoresis in agarose gels, transferred to Zetabind (Cuno Inc., Meriden, CT) nylon membranes by the method of Southern [E. Southern, *J. Mol. Biol.* **98**, 503 (1975)], and hybridized with radioactive probes as described (*6*). The plasmid pJS63 (*8*) was used to probe the ψ_{63} region in genomic DNA digested with Eco RI and Bam HI. Other probes used in this study include the 0.8-kb Bam HI fragment of pES174, to probe the ζ-η region (*6*); the 3.4-kb Eco RI fragment of the plasmid pKH4 (K. Haack and E. U. Selker, unpublished results), to probe the rDNA spacer region; the plasmids pJS12 and pJS1, to probe the δ_{12} and α_1 regions, respectively (*8*); and the 0.8-kb and ~11-kb Bam HI fragments of plasmid pCSN15, to probe the mating type *a*-specific sequence and its flanking sequence (*mt-flank*), respectively [C. Staben and C. Yanofsky, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 4917 (1990)]. Of the RFLP markers, only ζ-η is normally methylated, and it is distinguishable from its unmethylated M allele whether or not it displays methylation. Nylon membranes were routinely reprobed after stripping with 0.4 M NaOH (42°C for 30 min) and neutralization in 0.1× saline sodium citrate [15 mM sodium chloride, 1.5 mM sodium citrate·$2H_2O$ (pH 7)], 0.5% SDS, and 0.2 M tris (pH 7.5). All conclusions are based on digests that were controlled for completeness of digestion by hybridization with labeled fragments of genes that are not methylated.
12. Vegetative spores from strain N242 [equivalent to the Oak Ridge wild-type strain (N150, FGSC 2489) except for a mutation in *al-2*] were shaken in a buffered solution of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine [MNNG: 80 mM MNNG in 0.12 M KH_2PO_4 and 0.06 M Na_2HPO_4 (pH 7)] for 3.5 or 4 hours at room temperature in the dark. The spores were washed in 0.1% $Na_2S_2O_3$ and assayed for survival (20 to 35% at this dose) and for resistance to *p*-fluorophenylalanine, a measure of mutation in the *mtr* gene, to test the effectiveness of the mutagenesis.
13. H. M. Foss and E. U. Selker, unpublished material.
14. It is possible that the intermediate levels of methylation are due to uneven distribution of nuclei in the heterokaryon. If the *dim*$^+$ product is barely sufficient for normal methylation in wild-type *Neurospora* strains, a regional preponderance of *dim*$^-$ nuclei may cause the heterokaryon to be hypomethylated.
15. F. Gounari, G. R. Banks, K. Khazaie, P. A. Jeggo, R. Holliday, *Genes Dev.* 1, 899 (1987).
16. A. Vongs, T. Kakutani, R. A. Martienssen, E. J. Richards, *Science* **260**, 1926 (1993).
17. E. U. Selker, unpublished material.
18. Methylation in Dim^{int} progeny from a *dim-2* strain was assayed before and after vegetative propagation by means of vegetative spores (conidia) that were transferred in bulk and allowed to grow on fresh medium. A conservative estimate of the number of mass doublings per transfer is five.
19. T. H. Pittenger, *Genetics* **39**, 326 (1954); D. A. Smith, *ibid.* **76**, 1 (1974); S. F. H. Threlkeld and J. M. Stoltz, *Genet. Res.* **16**, 29 (1970).
20. D. D. Perkins, A. Radford, D. Newmeyer, M. Björkman, *Microbiol. Rev.* **46**, 426 (1982).
21. D. A. Smith, *Genetics* **80**, 125 (1975).
22. A. L. Schroeder and N. B. Raju, *Mol. Gen. Genet.* **231**, 41 (1991).
23. Subsequent experiments confirmed that the Dim$^+$ and Dim$^-$ phenotypes can segregate among vegetative clones from Dim^{int} strains (M. Rountree and H. Foss, unpublished results). All 13 clones from strain N511 that were assayed for methylation, including three Al$^+$, eight Al$^-$, and two mosaic cultures, were Dim$^+$ or nearly so, which suggests that *dim-2* and *al-2* are not closely linked.
24. We use the word aneuploid (and the associated modifiers, heterozygous or homozygous) to describe cultures that were derived from individual ascospores (sexual spores) containing extra chromosomes or chromosome parts, even though these cultures may no longer be aneuploid at the time of analysis.
25. Similarly, strain N510 appears to have transmitted the M alleles of ζ-η and α_1 from its Dim$^+$Al$^+$ *a* parent (N268) to strain N511 (Fig. 4) but lacked evidence of the M allele of ζ-η in its own DNA and revealed the transmitted M allele of α_1 only in a DNA sample from a secondary culture (Fig. 5). This indicates that aneuploidy can be found in both Dim$^-$ and Dim^{int} strains. Studies in progress to determine the incidence and extent of apparent aneuploidy have confirmed heterozygosity among progeny from *dim-2* strains in a variety of crosses. Heterozygosity in Dim$^+$, Dim$^-$, or Dim^{int} strains was observed at more than 10 loci representing five of the seven *Neurospora* chromosomes (H. Foss, M. Rountree, E. U. Selker, unpublished results). Among 38 progeny strains examined from one cross, one Dim^{int} strain was heterozygous for markers on three of five linkage groups scored (linkage groups I, II, and V) (*13*). These results suggest that the behavior of all chromosomes can be affected by *dim-2*.
26. D. Newmeyer and C. W. Taylor, *Genetics* **56**, 771 (1967); D. D. Perkins, *ibid.* **80**, 87 (1975).
27. J. H. Bull and J. C. Wootton, *Nature* **310**, 701 (1984).
28. E. S. Jacobson, G. S. Chen, R. L. Metzenberg, *J. Bacteriol.* **132**, 747 (1977).
29. C. J. Roberts and E. U. Selker, unpublished results.
30. L. A. Culp and P. H. Black, *Biochim. Biophys. Acta* **247**, 220 (1971).
31. D. J. Bond, *Mutat. Res.* **181**, 257 (1987).
32. A. Hagemann and E. U. Selker, unpublished material.
33. E. Viegas-Pequignot and B. Dutrillaux, *Hum. Genet.* **34**, 247 (1976).
34. T. A. Hori, *Mutat. Res.* **121**, 47 (1983).
35. D. A. Shafer and J. H. Priest, *Am. J. Hum. Genet.* **30**, 534 (1984); M. Poot, J. Koehler, P. S. Rabinovitch, H. Hoehn, J. H. Priest, *Hum. Genet.* **84**, 258 (1990).
36. N. Neves *et al.*, *Genome* **35**, 650 (1992).
37. J. M. Cock *et al.*, *J. Phycol.* **26**, 361 (1990).

38. We are grateful to M. Culbertson for encouraging us to embark on this project 5 years ago and to A. Bird, R. Metzenberg, R. Myers, N. Raju, F. Stahl, and all the members of our laboratory for useful discussions. The *al-2* mutation in the Oak Ridge wild-type strain was obtained from R. L. Metzenberg. We thank K. Fowler for valuable technical assistance; K. Haack, A. Hagemann, and M. Rountree for sharing unpublished observations; and A. Hagemann, J. Irelan, D. Macleod, R. Metzenberg, V. Miao, M. Rountree, M. Singer, and F. Stahl for comments on the manuscript. This work was supported by U.S. Public Health Service grant GM-35690 from NIH and done during the tenure of an Established Investigatorship of the American Heart Association. C.J.R. was supported in part by a fellowship from the American Heart Association Oregon Affiliate.

21 July 1993; accepted 9 November 1993

Elements of the Yeast Pheromone Response Pathway Required for Filamentous Growth of Diploids

Haoping Liu, Cora Ann Styles, Gerald R. Fink*

Transmission of an external signal from receptors to downstream targets is often mediated by a conserved set of protein kinases that act in sequence (a kinase cascade). In haploid strains of *Saccharomyces cerevisiae*, a signal initiated by peptide pheromones is transmitted through this kinase cascade to a transcription factor STE12, which is required for the expression of many mating-specific genes. Here it was shown that in diploids some of the same kinases and STE12 are required for filamentous growth, but the pheromone receptors and guanosine triphosphate–binding protein are not required for filament formation. Thus, a similar kinase cascade is activated by different signals in haploids and diploids and mediates different developmental outcomes in the two cell types.

In haploid cells of the fungus *S. cerevisiae*, extracellular peptide pheromones control the switch from vegetative growth to the sexual cycle. Each cell type secretes a unique pheromone (**a** cells secrete **a**-factor and α cells α-factor) that binds a cell type–specific receptor on cells of opposite mating type (α-factor to STE2 in *MAT***a** cells and **a**-factor to STE3 in *MAT*α cells) and thereby induces a sequence of events—arrest of cell division in G1, formation of projections, agglutination of two cells of opposite mating type, and cell fusion—that culminate in nuclear fusion (*1*). The signal initiated by the binding of pheromone to its receptor (Fig. 1) is transmitted by a heterotrimeric guanosine triphosphate–binding protein (G protein) encoded by *GPA1*, *STE4*, and *STE18* (*2*) to an ensemble of

Whitehead Institute for Biomedical Research, Massachusetts Institute of Technology, Cambridge, MA 02142.

*To whom correspondence should be addressed.

protein kinases encoded by *STE20*, *STE11*, *STE7*, and *FUS3/KSS1* (*3–8*) that appear to function in series to activate STE12, a transcription factor (*9*) required for the expression of mating-specific genes.

This kinase cascade is a conserved signal transduction module in eukaryotic organisms (*10*). The kinases from *Saccharomyces*, STE11, STE7, and FUS3, are structurally and functionally related to those of an evolutionarily distant fungus, *Schizosaccharomyces pombe*, byr2, byr1, and spk1, respectively (*11*). In *Saccharomyces* the genes in this signal transduction pathway fall into two groups with respect to their expression in haploids and diploids. The first group is transcribed in haploid cells but not in diploid cells. This group includes the genes encoding the pheromones (*MFa1*, *MFa2*, *MFα1*, and *MFα2*), the receptors (*STE2* and *STE3*), all three G protein subunits (*GPA1*, *STE4*, and *STE18*), and mitogen-activated protein (MAP) kinase (*FUS3*) (*2, 7, 12*). The failure of these proteins with mating-specific functions to be expressed in diploids is not surprising because MATa/MATα diploids do not mate. The second group, the genes encoding other protein kinases of the cascade (*STE20*, *STE11*, and *STE7*) as well as the gene encoding the transcriptional activator STE12 are transcribed in both haploids and diploids (*3, 4, 9, 13*). These protein kinases might be required for signal transduction in another developmental event carried out by diploids. However, no phenotype has been observed in diploid strains homozygous for deletions of these genes.

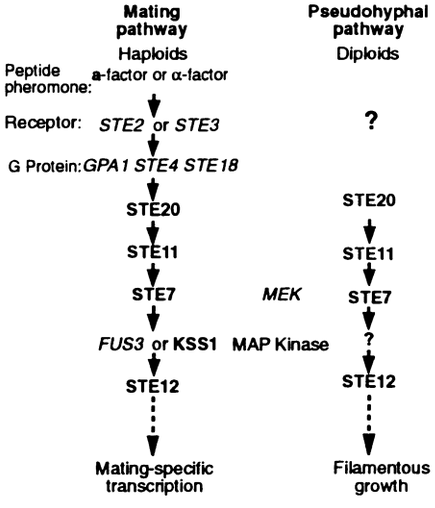

Fig. 1. Elements of the signal transduction pathway required in mating and filament formation. The genes in bold type are transcribed in both haploids and diploids. The genes in italics are expressed only in haploids. It is not known whether the downstream targets of STE12 have a direct or indirect effect on pseudohyphal growth.

Saccharomyces cerevisiae is dimorphic, capable of growing as ellipsoidal yeast cells or as filaments (*14, 15*). On medium containing a low concentration of nitrogen, an elliptical yeast cell can undergo an asymmetric cell division to produce a long thin daughter cell. These long daughters produce a new long daughter on the end opposite the previous mother-daughter junction. The mother and daughter cells remain attached, so reiteration of this unipolar division pattern produces a filament composed of linear chains of elongated cells (a pseudohypha). The yeast form produces colonies on the surface of an agar plate, whereas the pseudohyphae can penetrate beneath the surface.

The existence of this developmental pathway specific to diploid cells raises the possibility that some elements of the mating signal transduction pathway may be required to signal the switch from ellipsoidal cells to long, thin pseudohyphal cells. Therefore, we created Ste$^-$ null mutations in diploid strains from the Σ1278 background (*16*). The signal transduction genes fell into two groups on the basis of the effects of the mutants on pseudohyphal formation. Mutations in the *STE2*, *STE3*, *STE4*, *STE18*, *KSS1*, and *FUS3* genes had no discernible effect on pseudohyphal formation; the abundance and length of pseudohyphae were indistinguishable from those in wild-type controls (Fig. 2, A through E, J, and K). By contrast, mutations in *STE20*, *STE11*, *STE7*, and *STE12* suppressed formation of pseudohyphae (Fig. 2, F through I) (*17*) and invasion into the agar. Northern (RNA) blot analysis showed that the general features of transcription previously established for genes of the signal transduction pathway in ellipsoidal diploid cells were retained in pseudohyphal cells (Fig. 3). Those genes that were not transcribed in cells grown in standard medium (such as *STE2* and *FUS3*) were also not expressed in cells grown in medium containing low concentrations of ammonia. But *STE20*, *STE11*, *STE7*, and *STE12* were transcribed in both standard and low ammonium medium. The pseudohyphal phenotypes caused by mutations in genes from each of the two groups are congruent with the expression patterns of the genes: Mutations in genes that are expressed in diploids (except for *KSS1*, discussed below) blocked pseudohyphal formation, whereas mutations in genes that are not expressed had no effect. This correspondence suggests that elements of the signal transduction pathway are required for pseudohyphal growth. This conclusion is strengthened by the fact that null alleles of *STE20*, *STE11*, *STE7*, and *STE12* have no phenotype in diploids growing on medium with high concentrations of ammonia.

The pseudohyphal phenotype of these *ste* mutants contrasts with that of mutations in *BUD1* (*18, 19*), which result in a random budding pattern (*15*). The *bud1* diploid forms long cells, but fails to form pseudohyphae because the random budding pattern interrupts the sequence of unidirectional extensions required for filament formation. However, microscopic analysis of dividing cells from *ste/ste* diploid strains homozygous for deletions of *ste20*, *ste11*, *ste7*, or *ste12* showed that they have the same polar budding pattern as the *STE* controls. Therefore, these *ste* mutations do not affect the polar budding pattern of diploids but fail to form the cell type necessary for filament formation.

Neither of the putative MAP kinase

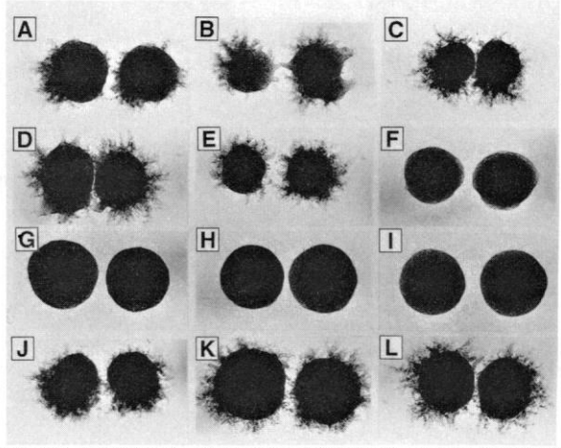

Fig. 2. Suppression of filamentous growth by mutations in genes encoding proteins of the mating kinase cascade. The strains were constructed from 10480-5C and 10480-5D in the Σ1278 background (*30*). Wild-type *STE/STE* and mutant *ste/ste* diploids were streaked on synthetic low-ammonia dextrose (SLAD) medium (*15*), and the resulting colonies were photographed after 3 days of growth at 30°C. (**A**) *STE/STE* (L5366), (**B**) *ste2/ste2* (HLY353), (**C**) *ste3/ste3* (HLY509), (**D**) *ste4/ste4* (HLY399), (**E**) *ste18/ste18* (HLY463), (**F**) *ste20/ste20* (HLY492), (**G**) *ste11/ste11* (HLY506), (**H**) *ste7/ste7* (HLY351), (**I**) *ste12/ste12* (HLY352), (**J**) *fus3/fus3* (HLY398), (**K**) *kss1/kss1* (HLY349), and (**L**) *fus3/fus3 kss1/kss1* (HLY477). Every diploid contained a vector carrying a *URA3* gene to complement the *ura3* auxotrophy of the strain. This plasmid was required for growth without supplementation because uracil and some other amino acids affect the extent of filament formation. The extent of suppression varies with time and with the individual mutant (*17*). Strains carrying a null allele of *KSS1* (either *kss1* or *fus3 kss1*) have a slightly different pattern of pseudohyphal formation from wild-type strains.

homologs from *Saccharomyces*, FUS3 or KSS1, appear to be required for pseudohyphal formation. In haploid cells *FUS3* and *KSS1* are redundant; *fus3* or *kss1* single mutants are fertile, but the *fus3 kss1* double mutant is sterile. However, in diploid cells the *fus3*, *kss1*, and *fus3 kss1* strains were all capable of forming pseudohyphae (Fig. 3, J through L). The failure of *kss1* mutants to affect pseudohyphal formation could be explained if there were another MAP kinase–like gene, as yet unidentified, whose product transduced the signal from STE7 to STE12 to stimulate pseudohyphal growth. Such a gene might be redundant with KSS1 in diploids just as FUS3 is redundant with KSS1 in the haploid mating pathway (*20*). This hypothetical gene is unlikely to be functional in the haploid mating pathway because a *fus3 kss1* double mutant is sterile.

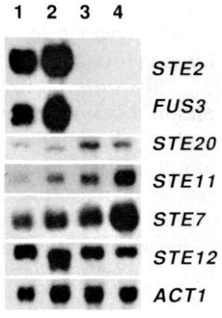

Fig. 3. Northern (RNA) analysis of *STE* gene transcripts in nitrogen-starved diploid cells. *MAT***a** haploid cells (HLY333) were grown to early logarithmic phase in yeast extract (1%), peptone (2%), and dextrose (2%) medium (YPD), and half of the cells were collected, resuspended in fresh YPD medium at pH 4 containing α-factor (5 μM), and incubated at 30°C for 2 hours. Diploid cells (HLY444) were grown either in synthetic complete (SC) medium (*15*) or SLAD medium. Total RNAs were extracted from these cells and 10 μg of each was loaded in the gel. 1, *MAT***a** cells; 2, α-factor–treated *MAT***a** cells; 3, diploid cells grown in SC; and 4, diploid cells grown in SLAD. Each filter was probed with labeled DNA from *STE2*, *FUS3*, *STE20*, *STE11*, *STE7*, *STE12*, or *ACT1* (actin).

Dominant gain of function mutants in *STE11* and overexpression of *STE12* have been used to order steps in the signal transduction pathway (*21–23*). *STE11-4* and overexpression of *STE12* led to enhanced pseudohyphal growth in wild type (Fig. 4, A through C). Experiments with double mutants indicate that the kinases we have identified as necessary for pseudohyphal growth can be roughly ordered in a pathway. *STE11-4* bypassed *ste20* but not the *ste7* or *ste12* mutations for pseudohyphal growth (Fig. 4, E, H, and K). These data suggest that STE11 acts downstream of STE20 and upstream of STE7. Overexpression of *STE12* appeared to bypass *ste20*, *ste7*, and *ste12* mutations, indicating that STE12 acts downstream of STE7 (Fig. 4, F, I, and L). Thus, the order of steps in the pseudohyphal pathway appears to be similar to that deduced for the mating pathway (Fig. 1).

Although this kinase cascade is conserved in several fungi, the mode of activation of the cascade differs among them. In *Saccharomyces* the signal for activation of mating functions originates with the binding of the peptide pheromones to the receptor; however, the pheromones and receptors are not required for the induction of filaments. In *Ustilago maydis*, the mating pheromone and receptor (encoded by the **a** locus) seem to be required for filamentous growth in diploids and dikaryons (*24–27*). The pheromone may act through its receptor in an autocrine manner to stimulate cells to form filaments. Because the pheromones and receptors are not elements of the signal transduction cascade for filament formation in *Saccharomyces*, this organism must utilize other molecules to generate the signals carried by the kinase cascade.

The finding that the kinase cascade from the mating signal transduction pathway is required for pseudohyphal growth provides a clear rationale for the expression of these genes in *Saccharomyces* diploids (*28*) and shows that this signaling pathway can mediate two different input signals in the same organism. These results parallel those for animal cells where the MAP kinase pathway has been shown to connect a great variety of external signals with proliferative or differentiative responses. Of course, in yeast it is possible that the *STE* gene products affect pseudohyphal growth indirectly. Nevertheless, the target of this kinase cascade in both morphogenetic pathways is the same transcription factor, STE12. Although STE12 was thought only to function during mating (*29*, *30*), our data show that STE12 also functions in vegetative cells. Because some of the genes activated by STE12 for mating are not expressed in diploid cells (such as *FUS1* and *FUS2*) (*31*), there may be genes uniquely required for the formation of filaments that are activated by STE12 during vegetative growth.

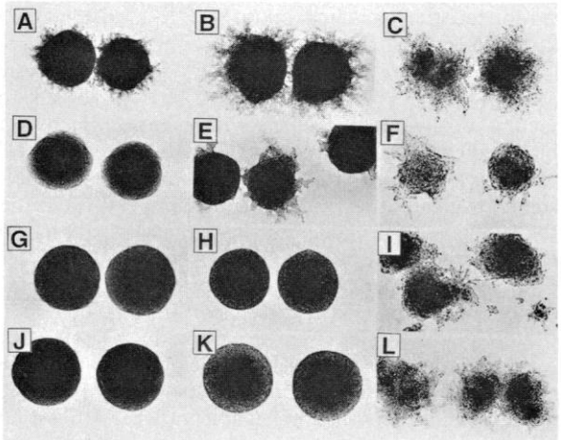

Fig. 4. Enhanced pseudohyphal growth by a *STE11* dominant mutant and *STE12* overexpression. (**A** through **C**) *STE/STE* diploids (L5366) containing, respectively, a vector, *STE11-4*, or *GAL-STE12*. (**D** through **F**) *ste20/ste20* diploids (HLY492) carrying, respectively, a vector, *STE11-4*, or *GAL-STE12*. (**G** through **I**) *ste7/ste7* diploids (HLY351) carrying, respectively, a vector, *STE11-4*, or *GAL-STE12*. (**J** through **L**) *ste12/ste12* diploids (HLY352) carrying, respectively, a vector, *STE11-4*, or *GAL-STE12*. Strains carrying the vector or *STE11-4* were streaked for single cells on SLAD medium; cells carrying *GAL-STE12* were streaked on synthetic low-ammonia medium containing 2% galactose (G) and 0.13% glucose (D) (SLAGD). Overexpression of the *GAL-STE12* construct on SLAG (SLAGD without glucose) is lethal. The resulting colonies were photographed after 3-days growth at 30°C. The photographs of the *GAL-STE12* colonies were enlarged about ×1.5 as compared with the others.

REFERENCES AND NOTES

1. G. F. Sprague and J. Thorner, in *The Molecular Biology of the Yeast* Saccharomyces, J. R. Broach, J. R. Pringle, E. Jones, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1992), pp. 657–744.
2. M. Whiteway *et al.*, *Cell* **56**, 467 (1989).
3. E. Leberer, D. Dignard, D. Harcus, D. Y. Thomas, M. Whiteway, *EMBO J.* **11**, 4815 (1992).
4. S. W. Ramer and R. W. Davis, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 452 (1993).
5. N. Rhodes, L. Connell, B. Errede, *Genes Dev.* **4**, 1862 (1990).
6. M. A. Teague, D. T. Chaleff, B. Errede, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 7371 (1986).
7. E. A. Elion, P. L. Grisafi, G. R. Fink, *Cell* **60**, 649 (1990).
8. W. E. Courchesne, R. Kunisawa, J. Thorner, *ibid.* **58**, 1107 (1989).
9. S. Fields and I. Herskowitz, *ibid.* **42**, 923 (1985).
10. A. M. Neiman *et al.*, *Mol. Biol. Cell* **4**, 107 (1993).
11. S. A. Nadin-Davis and A. Nasim, *EMBO J.* **7**, 985 (1988); Y. Wang, H. P. Xu, M. Riggs, L. Rodgers, M. Wigler, *Mol. Cell. Biol.* **11**, 3554 (1991); T. Toda, M. Shimanuki, M. Yanagida, *Genes Dev.* **5**, 60 (1991).
12. N. Nakayama, A. Miyajima, K. Arai, *EMBO J.* **4**, 2643 (1985).
13. D. T. Chaleff and K. Tatchell, *Mol. Cell. Biol.* **5**, 1878 (1985).
14. C. J. Gimeno and G. R. Fink, *Science* **257**, 626 (1992).
15. C. J. Gimeno, P. O. Ljungdahl, C. A. Styles, G. R. Fink, *Cell* **68**, 1077 (1992).
16. Isogenic strains in the Σ1278 background, *leu2::hisG ura3-52* (HLY335, **a**; HLY336, α) and *trp1::hisG ura3-52* (HLY338, **a**; HLY337, α) were used for strain constructions. Disruptions were made by transformation with the cognate *ste::LEU2*, *ste::URA3*, or *ste::TRP1* plasmid to disrupt the resident *STE* gene. These disruptions were confirmed by Southern (DNA) hybridization. The *ste20* disruption in the Σ1278 background was not completely sterile; moreover, some of the restriction sites in the region hybridizing with *STE20* in Σ1278 differ from those in S288C. To make diploids, we transformed each of the sterile

haploids with an autonomously replicating plasmid containing the cognate *STE* gene and a selectable marker. The resulting fertile transformants were crossed to produce a diploid; strains that had lost the plasmid were obtained as mitotic segregants.
17. Suppression of pseudohyphal growth is defined by failure to observe pseudohyphae after 3 days. A further test was to wash away surface colony cells and observe whether any cells in the colony had penetrated the agar. Wild-type and Ste2$^-$, Ste3$^-$, Ste4$^-$, and Ste18$^-$ mutants left a florid network of long pseudohyphal filaments. The Ste20$^-$ mutants were completely suppressed; cells did not penetrate the agar but grew on the surface as elliptical cells. Null mutations in *STE11*, *STE7*, and *STE12* are somewhat leaky. Washing away surface colony cells exposed a few cells that penetrated the agar. Some formed short pseudohypha-like chains made up of round cells; rarely was a long cell observed. The presence of these short structures in kinase cascade mutants suggests the possibility of an alternate pathway for activation of pseudohyphal growth.
18. A. Bender and J. R. Pringle, *Cell* **65**, 1203 (1991).
19. J. Chant, K. Corrado, J. R. Pringle, I. Herskowitz, *ibid.*, p. 1213.
20. E. A. Elion, J. A. Brill, G. R. Fink, *Cold Spring Harbor Symp. Quant. Biol.* **56**, 41 (1991).
21. D. Blinder, S. Bouvier, D. D. Jenness, *Cell* **56**, 479 (1989).
22. B. J. Stevenson, N. Rhodes, B. Errede, G. Sprague Jr., *Genes Dev.* **6**, 1293 (1992).
23. J. W. Dolan and S. Fields, *ibid.* **4**, 492 (1990).
24. F. Banuett and I. Herskowitz, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5878 (1989).
25. M. Bolker, M. Urban, R. Kahmann, *Cell* **68**, 441 (1992).
26. B. Gillissen *et al.*, *ibid.*, p. 647.
27. F. Banuett, *Trends in Genet.* **8**, 174 (1992).
28. The failure to detect a morphological phenotype for kinase cascade mutations in haploid cells is probably a consequence of the inability of wild-type haploid cells to form filaments. Only diploid cells have a polar budding pattern that allows filament formation; most haploid strains have an axial pattern that is inconsistent with filament formation. However, some haploid strains in standard use (*32*), as well as some *bud* mutants, produce a high proportion of polar budding cells. These polar budding haploid strains are capable of producing short filaments. It is likely that in these haploid strains, the kinase cascade is also required for filament formation.
29. J. W. Dolan, C. Kirkman, S. Fields, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5703 (1989).
30. B. Errede and G. Ammerer, *Genes Dev.* **3**, 1349 (1989).
31. J. Trueheart, J. D. Boeke, G. R. Fink, *Mol. Cell. Biol.* **7**, 2316 (1987).
32. R. M. Wright, T. Repine, J. E. Repine, *Curr. Genet.* **23**, 388 (1993).
33. We thank C. Berkower, E. Elion, B. Errede, S. Fields, E. Leberer, S. Michaelis, G. F. Sprague Jr., and M. Whiteway for the *STE* plasmids. We also thank J. Brill, K. Cunningham, C. Gimeno, S. Kron, and other members in the Fink laboratory for helpful discussions. Supported by a Jane Coffin Childs Fellowship (H.L.) and USPHS grant GM 35010 (G.R.F.). G.R.F. is an American Cancer Society Research Professor.

20 July 1993; accepted 23 September 1993

Mutations That Allow Disulfide Bond Formation in the Cytoplasm of *Escherichia coli*

Alan I. Derman, William A. Prinz, Dominique Belin, Jon Beckwith*

Disulfide bonds are rarely found in cytoplasmic proteins. Mutations were selected for in *Escherichia coli* that allow disulfide bond formation in the cytoplasm. In the presence of these mutations, export-defective versions of alkaline phosphatase and mouse urokinase were able to fold into their enzymatically active conformations in the cytoplasm because their disulfide bonds were formed. The mutations were mapped to the gene for thioredoxin reductase and diminish or eliminate the activity of this enzyme. Thioredoxin itself was found to be unnecessary for this disulfide bond formation. Thioredoxin reductase, but not thioredoxin, is thus implicated in keeping cysteines reduced in cytoplasmic proteins.

Disulfide bonds are important for the folding and structure of many proteins (*1*), most of which are exported from the cytoplasm (*2*). The cytoplasmic proteins that do have disulfide bonds are typically sulfhydryl oxidoreductases, which undergo redox interconversion between free sulfhydryl and disulfide-bonded conformations (*3*). The absence from the cytoplasm of proteins with stable disulfide bonds and their presence among exported proteins is generally attributed to the differing reducing environments of subcellular compartments (*4*, *5*). The reducing potential of the cytoplasm is substantially greater than that of the lumen of the endoplasmic reticulum or the extracellular environment (*5*). Indeed, the formation of disulfide bonds in exported proteins occurs only during or after their export from the cytoplasm (*6*, *7*). However, an oxidizing environment is not itself sufficient for the formation of disulfide bonds in exported proteins. A system consisting of at least two cellular envelope proteins, DsbA and DsbB, is necessary for the formation of disulfide bonds in exported proteins in *E. coli* (*8*).

With this background in mind, one can imagine two possible explanations for the scarcity of disulfide bonds in cytoplasmic proteins. The absence from the cytoplasm of a system such as the Dsb proteins may suffice to explain the fact that disulfide bonds are rarely found in cytoplasmic proteins. Alternatively, or in addition, a mechanism may exist that actively prevents the formation of disulfide bonds in the cytoplasm. We have taken a genetic approach in order to assess these possible explanations. We have obtained mutants of *E. coli* that allow the formation of disulfide bonds in the cytoplasm. That we have been able to obtain these mutants argues for the existence of a mechanism that actively prevents the formation of disulfide bonds in the cytoplasm. Characterization of these mutants provides insight into how disulfide bond formation is ordinarily prevented in the cytoplasm.

We have carried out a genetic selection for mutants in which disulfide bonds are formed in a protein localized to the cytoplasm. Alkaline phosphatase (AP), a nonspecific phosphomonoesterase, is normally found in the *E. coli* periplasm (*9*). AP has two intrachain disulfide bonds that are required for its native structure (*10*) and therefore for its enzymatic activity (*11*). However, when AP is retained in the cytoplasm, as when expressed without its signal sequence, its disulfide bonds are not formed (*6*, *12*). The inability of these disulfide bonds to be formed is the most likely explanation for the finding that AP is enzymatically inactive when retained in the cytoplasm (*6*, *13*). Selection for mutants in which AP that is localized to the cytoplasm acquires enzymatic activity is therefore equivalent to selection for the formation of disulfide bonds in the cytoplasm.

We required that AP substitute for a phosphomonoesterase that is normally present in the cytoplasm and whose activity is required for a particular metabolic function. Fructose-1,6-bisphosphatase is a phosphomonoesterase that is required for growth of *E. coli* on gluconeogenic carbon sources such as glycerol (*14*). If in a mutant lacking fructose-1,6-bisphosphatase (*fbp*$^-$), AP were able to acquire enzymatic activity in the cytoplasm, AP could substitute for fructose-1,6-bisphosphatase and restore growth on glycerol (*15*).

We constructed a strain that carries a deletion in the *fbp* gene (*16*) and expresses AP without its signal sequence under control of the *tac* promoter (*12*). We isolated 10 independent spontaneous mutants of this strain that were able to grow on glycerol (Glyc$^+$) at 37°C only if expression of the signal-sequenceless AP was induced with isopropyl *thio*-β-D-galactopyranoside (IPTG). These mutants satisfied another selection for active cytoplasmic AP as well.

A. I. Derman, W. A. Prinz, J. Beckwith, Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115.
D. Belin, Département de Pathologie, Centre Médical Universitaire, Geneva, Switzerland.
*To whom correspondence should be addressed.