Genetic Analysis Reveals That *FLO11* Upregulation and Cell Polarization Independently Regulate Invasive Growth in *Saccharomyces cerevisiae*

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ABSTRACT

Under inducing conditions, haploid *Saccharomyces cerevisiae* perform a dimorphic transition from yeast-form growth on the agar surface to invasive growth, where chains of cells dig into the solid growth medium. Previous work on signaling cascades that promote agar invasion has demonstrated upregulation of *FLO11*, a cell-surface flocculin involved in cell-cell adhesion. We find that increasing *FLO11* transcription is sufficient to induce both invasive and filamentous growth. A genetic screen for repressors of *FLO11* isolated mutant strains that *d*ig *i*nto *a*gar (*dia*) and identified mutations in 35 different genes: *ELM1*, *HSL1*, *HSL7*, *BUD3*, *BUD4*, *BUD10*, *AXL1*, *SIR2*, *SIR4*, *BEM2*, *PGI1*, *GND1*, *YDJ1*, *ARO7*, *GRR1*, *CDC53*, *HSC82*, *ZUO1*, *ADH1*, *CSE2*, *GCR1*, *IRA1*, *MSN5*, *SRB8*, *SSN3*, *SSN8*, *BPL1*, *GTR1*, *MED1*, *SKN7*, *TAF25*, *DIA1*, *DIA2*, *DIA3*, and *DIA4*. Indeed, agar invasion in 20 *dia* mutants requires upregulation of the endogenous *FLO11* promoter. However, 13 mutants promote agar invasion even with *FLO11* clamped at a constitutive low-expression level. These *FLO11* promoter-independent *dia* mutants establish distinct invasive growth pathways due to polarized bud site selection and/or cell elongation. Epistasis with the STE MAP kinase cascade and cytokinesis/budding checkpoint shows these pathways are targets of *DIA* genes that repress agar invasion by *FLO11* promoter-dependent and -independent mechanisms, respectively.

MANY simple fungal organisms are capable of switching between yeast-form growth, where ovoid cells separate subsequent to mitosis, to filamentous growth, in which cells form chains that remain physically attached via a persistent cytoplasmic or cell wall connection. Significantly, hyphal filament formation may be a key virulence factor in pathogenic fungi such as *Candida albicans* and *Ustilago maydis*, contributing to local spread and tissue invasion. Filamentous growth in the budding yeast *Saccharomyces cerevisiae* is pseudohyphal, characterized by chains of polarized, elongated cells that display a prolonged budded period, mitotic delay, apical polar-budding pattern, and increased agar invasion (Kron and Gow 1995). The switch between yeast-form and filamentous growth is a highly regulated process responsive to nutrient availability and other environmental stimuli (Gimeno *et al.* 1992), suggesting that budding yeast filamentation may be a foraging response to nutrient deprivation (Banuett 1998; Gale *et al.* 1998; Mitchell 1998).

In *S. cerevisiae*, physiologic regulation of pseudohyphal differentiation and resulting agar invasion depends upon activation of the Ras2 small GTP-binding protein (Gimeno *et al.* 1992). Ras2 activates both the STE mitogen-activated protein (MAP) kinase and cAMP-dependent protein kinase pathways to promote filamentous differentiation (Gimeno *et al.* 1992; Mosch and Fink 1997; Madhani and Fink 1998; Ahn *et al.* 1999; Mosch *et al.* 1999). Even though the signaling pathways are fairly well characterized (reviewed in Banuett 1998; Madhani and Fink 1998), their receptors remain to be fully described. One candidate, the ammonium permease Mep2, can stimulate Gpa2 to increase cAMP concentrations in the absence of NH_4^+ (Lorenz and Heitman 1998). Gpr1 may also function as a carbon and/or nitrogen starvation sensor during pseudohyphal growth (Lorenz *et al.* 2000b; Tamaki *et al.* 2000). Key outputs of filamentous signaling are likely to be regulated via transcriptional control and may include targets in multiple signal transduction and morphogenetic pathways.

In yeast, cell cycle progression is tightly coupled to bud morphogenesis and cell polarity (reviewed by Kron and Gow 1995; Lew and Reed 1995). Mutations in cell cycle regulators such as the Elm1, Hsl1, or Hsl7 kinases, the Grr1 F-box protein, the mitotic cyclin Clb2, the Cdc28 cyclin-dependent kinase and the Fkh1,2 mitotic transcription factors lead to prolonged budded period, increased cell polarization, constitutive pseudohyphal growth, and increased agar invasion (Blacketer *et al.* 1993; Ahn *et al.* 1999; Edgington *et al.* 1999; Loeb *et al.* 1999; Hollenhorst *et al.* 2000; Zhu *et al.* 2000). Mutating Swe1, a kinase that antagonizes Cdc28's mitotic activity, attenuates filamentous growth and blocks the effects of *elm1*, *hsl1*, and *hsl7* but does not suppress response to filamentous signaling (Kron *et al.* 1994;

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Ahn *et al.* 1999; Edgington *et al.* 1999). Presumably, other cell cycle regulators are *bona fide* targets of the cAMP-dependent kinase and/or STE MAP kinase signaling pathways, but clear links have yet to be established. Apical polar bud site selection in filamentous cells may also be directly regulated by filamentous signaling or may be downstream of the mitotic delay.

Unlike our detailed knowledge of signaling and cell cycle pathways, we understand very little regarding the downstream biophysical mechanisms that permit these processes. During invasion, the individual cells in the colony must displace or degrade the matrix, suggesting the importance of changes in cell-cell and cell-substrate interactions. Indeed, stimulation of the STE MAP kinase (MAPK) pathway increases expression of *FLO11*, a cell-surface flocculin, and *PGU1*, an enzyme that degrades extracellular pectin (Madhani *et al.* 1999). *FLO11* expression is also coupled to *STA1*, *2*, and *3* glycoamylase gene expression, suggesting a link between starch degradation and invasive growth (Lambrechts *et al.* 1996). *FLO11* expression is required for haploid invasive growth as well as diploid pseudohyphal differentiation (Lambrechts *et al.* 1996; Lo and Dranginis 1998)—*flo11*Δ mutant cells do not form chains or invade agar but instead form nonadherent piles on the agar surface. Mep2, cAMP-dependent protein kinase, the MAPK cascade, and other pathways coordinately control *FLO11* expression via a highly complex promoter (Gagiano *et al.* 1999a,b; Galitski *et al.* 1999; Pan and Heitman 1999; Rupp *et al.* 1999).

FLO11 possesses the largest promoter region of any yeast open reading frame (ORF), containing at least four upstream activating sites and nine upstream repression sites (Rupp *et al.* 1999). Several mechanisms of invasive growth repression have already been described. Mutation of the *SFL1* transcriptional repressor derepresses *FLO11* expression and invasive growth (Robertson and Fink 1998). Dig1 and Dig2 cooperate with Kss1 to repress Ste12 and inhibit haploid invasive growth (Cook *et al.* 1996; Tedford *et al.* 1997; Bardwell *et al.* 1998). The *STA10* repressor functions via *FLO8*, a transcriptional activator (Gagiano *et al.* 1999b). In addition, mutations in *IRA1*, a GTPase activating protein gene, lead to hyperactivation of Ras2 and increased *FLO11* transcription (Rupp *et al.* 1999).

To identify additional *FLO11* transcriptional repression mechanisms, we screened for gene disruptions that induce haploid invasive growth and identified mutations in 35 genes. Validating the screen, the majority of these mutations upregulate *FLO11* expression from its endogenous promoter and depend upon this increased expression for their effects. Significantly, we also identified mutations that enhance invasion even when *FLO11* transcription is clamped by a low-level constitutive promoter. This class of mutations has its primary effects on cell polarity rather than cell adhesion. We find that enhancing one or more of the physical processes of cell-cell adhesion, budding polarity, elongation, or other unidentified mechanisms can increase invasion in haploids and diploids.

MATERIALS AND METHODS

Plasmids: To generate plasmid cassettes for promoter replacement with the *adh*$^+$ promoter from *Schizosaccharomyces pombe* (pSP2) or the *CDC28* promoter from *S. cerevisiae* (pSP10), the *Sp adh*$^+$ promoter was amplified from plasmid *spADH-CLB2* (Amon 1997) using primers SalSpADHp and Bgl SpADHp and the *CDC28* promoter was amplified from base pairs −350 to −1 relative to the start site of the *CDC28* ORF from genomic DNA with primers SalCDC28p and BglCDC28p (see Table 2). The PCR products were digested with *Sal*I and *Bgl*II and ligated into pFA6 kanMX6 *GALp* (Longtine *et al.* 1998) digested with *Sal*I and *Bgl*II to excise the *GAL* promoter.

Yeast strains, media, and genetic methods: The yeast strains used in this study are listed in Table 1. Strains were derived in the Σ1278b genetic background (Grenson *et al.* 1966; Liu *et al.* 1993) using standard genetic methods. Standard yeast culture media and filamentous growth media were prepared as previously described (Kron *et al.* 1994; Ahn *et al.* 1999). Yeast media were obtained from United States Biochemical (Cleveland) and other reagents from Fisher Scientific (Pittsburgh) and Sigma (St. Louis). G418 (Life Technologies) was added to YPD agar at 0.2 mg/ml. Synthetic low ammonium medium (SLAD) was prepared with 50 μM ammonium sulfate. Uracil was added to SLAD medium to a concentration of 0.2 mM or histidine to a concentration of 0.3 mM to make SLAD +Ura or SLAD +Ura +His. Haploid matings, diploid sporulations, and tetrad dissections were performed as described (Sherman *et al.* 1986). Yeast were transformed using lithium acetate transformation (Gietz *et al.* 1992) or electroporation (Simon 1993).

PCR disruption (Longtine *et al.* 1998) was used to replace *SWE1*, *BUD8*, *RAS2*, and *KSS1* in both *MAT***a** and *MAT*α Σ1278b cells with the kanMX G418 resistance marker. *SWE1* was deleted from positions +3 to +2372, relative to the start site, *BUD8* from positions +1 to +1851, *RAS2* from positions −76 to +983, and *KSS1* from positions +1 to +1107. Similarly, the defined promoter from −2800 to −1, 5′ to the *FLO11* open reading frame (Rupp *et al.* 1999), was replaced with the promoter of *Sp adh*$^+$ or *CDC28* by amplification of pSP2 or pSP10 with primers FLO11p-1 and FLO11p-2 (Table 2), transformation, and G418 selection. *SIR4* and *HML* deletions were constructed using plasmids pRS42sir4::HIS3+ (Kimmerly and Rine 1987) and pJR826 (J. Rine, personal communication). To construct pseudodiploids, haploid *MAT***a** strain SKY760 was transformed with plasmid B2185 [*CEN MAT*α *URA3*] (Mosch and Fink 1997).

Isolation of mutants that dig into agar: Insertional mutagenesis of a wild-type Σ1278b haploid was performed by homologous recombination with a *Tn3::lacZ::LEU2* transposon-mutagenized yeast genomic DNA library (Burns *et al.* 1994). DNA aliquots from 14 pools of the *Tn3::lacZ::LEU2* library were combined, digested with *Not*I, and used to transform yeast strain SKY760 (*MAT***a** *ura3-52 his3::hisG leu2::hisG*). Twenty independent transformations yielded 5000 and 15,000 colonies per SC −Leu plate, for a total of ~180,000 LEU$^+$ transformants. After 3 days of growth at 22°, the transformation plates were directly screened for *d*igs *i*nto *a*gar (*dia*) mutants. Noninvasive cells were washed away under gently running distilled water and the plates were rubbed with a gloved finger for ~1 min. Using transmitted light and a Zeiss Stemi 2000-C (Thornwood, NY) stereomicroscope, plates were then visually scanned for sites of agar invasion. A total of 388 colonies were

TABLE 1

Yeast strains

Strain	Genotype	Source
SKY760	*MAT***a** *ura3-52 his3::hisG leu2::hisG*	Collection
SKY756	*MAT***a**/α *ura3-52/ura3-52*	Collection
SKY2606	*MAT*α *ura3-52 leu2::hisG*	This study
SKY2607	*MAT***a** *SpADHp-FLO11::kan*^{r} *ura3-52 his3::hisG leu2::hisG*	This study
SKY2608	*MAT*α *SpADHp-FLO11::kan*^{r} *ura3-52 his3::hisG leu2::hisG*	This study
SKY2609	*MAT***a** *CDC28p-FLO11::kan*^{r} *ura3-52 his3::hisG leu2::hisG*	This study
SKY2610	*MAT*α *CDC28p-FLO11::kan*^{r} *ura3-52 his3::hisG leu2::hisG*	This study
SKY2611	*MAT***a** *ura3-52 his3::hisG leu2::hisG sir4::HIS3*	This study
SKY2612	*MAT***a** *ura3-52 his3::hisG leu2::hisG hml::LEU2*	This study
SKY2613	*MAT***a** *ura3-52 his3::hisG leu2::hisG sir4::HIS3 hml::LEU2*	This study
SKY2614	*MAT***a** *ura3-52 his3::hisG leu2::hisG bud8::kan*^{r}	This study
SKY2615	*MAT*α *ura3-52 his3::hisG leu2::hisG bud8::kan*^{r}	This study
SKY2616	*MAT***a** *ura3-52 his3::hisG leu2::hisG kss1::kan*^{r}	This study
SKY2617	*MAT*α *ura3-52 his3::hisG leu2::hisG kss1::kan*^{r}	This study
SKY2618	*MAT***a** *ura3-52 his3::hisG leu2::hisG ras2::kan*^{r}	This study
SKY2619	*MAT*α *ura3-52 his3::hisG leu2::hisG ras2::kan*^{r}	This study
SKY2620	*MAT***a** *ura3-52 his3::hisG leu2::hisG swe1::kan*^{r}	This study
SKY2621	*MAT*α *ura3-52 his3::hisG leu2::hisG swe1::kan*^{r}	This study
SKY2570	*MAT***a** *ura3-52 his3::hisG leu2::hisG bud3-100::LEU2*	This study
SKY2571	*MAT***a** *ura3-52 his3::hisG leu2::hisG bud4-100::LEU2*	This study
SKY2572	*MAT***a** *ura3-52 his3::hisG leu2::hisG bud10-100::LEU2*	This study
SKY2573	*MAT***a** *ura3-52 his3::hisG leu2::hisG axl1-100::LEU2*	This study
SKY2574	*MAT***a** *ura3-52 his3::hisG leu2::hisG bem2-100::LEU2*	This study
SKY2575	*MAT***a** *ura3-52 his3::hisG leu2::hisG elm1-100::LEU2*	This study
SKY2576	*MAT***a** *ura3-52 his3::hisG leu2::hisG hsl1-100::LEU2*	This study
SKY2577	*MAT***a** *ura3-52 his3::hisG leu2::hisG hsl7-100::LEU2*	This study
SKY2578	*MAT***a** *ura3-52 his3::hisG leu2::hisG ydj1-100::LEU2*	This study
SKY2579	*MAT***a** *ura3-52 his3::hisG leu2::hisG zuo1-100::LEU2*	This study
SKY2580	*MAT***a** *ura3-52 his3::hisG leu2::hisG hsc82-100::LEU2*	This study
SKY2581	*MAT***a** *ura3-52 his3::hisG leu2::hisG sir2-100::LEU2*	This study
SKY2582	*MAT***a** *ura3-52 his3::hisG leu2::hisG sir4-100::LEU2*	This study
SKY2583	*MAT***a** *ura3-52 his3::hisG leu2::hisG gcr1-100::LEU2*	This study
SKY2584	*MAT***a** *ura3-52 his3::hisG leu2::hisG pgi1-100::LEU2*	This study
SKY2585	*MAT***a** *ura3-52 his3::hisG leu2::hisG adh1-100::LEU2*	This study
SKY2586	*MAT***a** *ura3-52 his3::hisG leu2::hisG gnd1-100::LEU2*	This study
SKY2587	*MAT***a** *ura3-52 his3::hisG leu2::hisG med1-100::LEU2*	This study
SKY2588	*MAT***a** *ura3-52 his3::hisG leu2::hisG cse2-100::LEU2*	This study
SKY2589	*MAT***a** *ura3-52 his3::hisG leu2::hisG ssn3-100::LEU2*	This study
SKY2590	*MAT***a** *ura3-52 his3::hisG leu2::hisG ssn8-100::LEU2*	This study
SKY2591	*MAT***a** *ura3-52 his3::hisG leu2::hisG srb8-100::LEU2*	This study
SKY2592	*MAT***a** *ura3-52 his3::hisG leu2::hisG taf25-100::LEU2*	This study
SKY2593	*MAT***a** *ura3-52 his3::hisG leu2::hisG gtr1-100::LEU2*	This study
SKY2594	*MAT***a** *ura3-52 his3::hisG leu2::hisG aro7-100::LEU2*	This study
SKY2595	*MAT***a** *ura3-52 his3::hisG leu2::hisG cdc53-100::LEU2*	This study
SKY2596	*MAT***a** *ura3-52 his3::hisG leu2::hisG grr1-100::LEU2*	This study
SKY2597	*MAT***a** *ura3-52 his3::hisG leu2::hisG skn7-100::LEU2*	This study
SKY2598	*MAT***a** *ura3-52 his3::hisG leu2::hisG ira1-100::LEU2*	This study
SKY2599	*MAT***a** *ura3-52 his3::hisG leu2::hisG bpl1-100::LEU2*	This study
SKY2600	*MAT***a** *ura3-52 his3::hisG leu2::hisG msn5-100::LEU2*	This study
SKY2601	*MAT***a** *ura3-52 his3::hisG leu2::hisG dia1-100::LEU2*	This study
SKY2602	*MAT***a** *ura3-52 his3::hisG leu2::hisG dia2-100::LEU2*	This study
SKY2603	*MAT***a** *ura3-52 his3::hisG leu2::hisG dia3-100::LEU2*	This study
SKY2604	*MAT***a** *ura3-52 his3::hisG leu2::hisG dia4-100::LEU2*	This study

recovered from the agar plates with a sharp-tipped toothpick and inoculated onto SC −Leu plates. Putative *dia* mutants and controls were streaked onto YPD and SC −Leu plates, grown for 3 days at 22°, and washed under running water as before. Isolates more invasive than SKY760 wild-type haploids on both YPD and SC −Leu medium were retained. The 194 remaining *dia* mutants were mated back to the wild-type SKY2606 (*MAT*α *ura3-52 leu2::hisG*) and diploids were selected

TABLE 2

Oligonucleotides

Name	Sequence (5′ to 3′)
SalSpADHp	ACTACTAGTCGACCTCTTGCTTAAAGAAAAGCG
BglSpADHp	ACTACTAAGATCTCATGCCCTACAACAACTAAT
SalCDC28p	ACTACTAGTCGACGTTAATTCAGTAAATTTTCG
BglCDC28p	ACTACTAAGATCTCAAAACCCTTAAAAACATATG
FLO11p-1	TTATGTGGTATGATCAGATTGTGTCGCAACGCTCAGCGGGGTTTTGGCTCAATGGGAC CGGAATTCGAGCTCGTTTAAAC
FLO11p-2	CAAAGCCGAGTTAAATAGAAGCGAAAGGACCAAATAAGCGAGTAGAAATGGTCTTTGC ATTTCGTACGCTGCAGGTCGAC
Insertamp1	CGAATCGTAACCGTTCGTACGAGAATCGCT
Insertamp2	CGAATCGTAACCGTTCGTACGAGAATCGCT

on SC −His −Leu medium. Five isolates were sterile and the genomic locus of the *lacZ* insertion was directly determined in these strains. A total of 189 presumed *dia/DIA* heterozygotes were analyzed by sporulation and tetrad dissection. Segregants from at least 4 four-spore tetrads from each cross were analyzed for growth on SC −Leu and for YPD agar invasion. In 95 of the 189 crosses, the *LEU2* marker and hyperinvasive phenotype cosegregated in a 2:2 pattern, confirming a *MAT***a** *ura3-52 his3::hisG leu2::hisG dia::LEU2* genotype. Homozygous diploids of each *dia* mutant were created by crossing *MAT***a** and *MAT*α segregants obtained from the backcross to SKY2606.

Identification of *dia* insertions: The insertion site of the *Tn3::lacZ::LEU2* insertion was determined by vectorette PCR (Botstein laboratory, http://genome-www.stanford.edu/group/botlab/protocols/vectorette.html). Genomic DNA was isolated from *MAT***a** segregants of each *dia* mutant digested with *Rsa*I or *Alu*I and ligated to annealed anchor bubble primers. Sequences adjoining the insertion site were amplified using primers Insertamp1 and Insertamp2 (Table 2). PCR products were gel-purified and sequenced using Insertamp1 and an ABI cycle sequencing kit (Perkin-Elmer, Norwalk, CT). DNA homology searches were performed using the Saccharomyces Genome Database BLAST (http://genome-www2.stanford.edu/cgi-bin/SGD/nph-blast2sgd) and National Center for Biotechnology Information BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) services. In 29 cases at least one of the insertions occurred within the ORF and the disruption is positively identified. In 2 cases (*TAF25* and *GND1*) insertions were within 100 bp of the translational start site. In the remaining 4 cases (*DIA1*, *PGI1*, *CDC53*, and *SKN7*), the insertions were at least 150 bp from any annotated ORF and were named based on the most proximal annotated ORF 3′ to the insertion.

Photomicrography: Microcolonies were imaged through the agar and plastic petri dish using a Zeiss Axiovert 25 with bright-field illumination and a 32× LD Achroplan or 10× CP-Achromat objective. A Photometrics Sensys 1600 charge-couple device camera and IPLab Spectrum image-acquisition software (Signal Analytics, Vienna, VA) were used to capture images. Images were converted to gray scale and filtered to remove noise and enhance contrast in Photoshop (Adobe, Mountain View, CA).

Quantitative invasive growth assay: Cells to be tested for quantitative invasive growth were streaked onto thin (~0.5 cm) YPD plates to obtain colonies derived from single cells. Colonies were grown at 30° for 3 days. A 1-cm-diameter circle was punched out of the agar at the outer edge of the plate where colonies had grown from single cells. The agar plug was rinsed with water and gently scraped with a plastic spatula to remove the cells not penetrating the surface of the agar. Nonadherent cells were collected, sonicated, and counted on a hemacytometer to obtain the number of cells that did not invade the agar. The rinsed piece of agar was placed into a 15-ml polypropylene tube with 5 ml of water and microwaved for 5 sec until the agar was completely melted. The tube was centrifuged at 2000 × *g* for 5 min and the supernatant was carefully removed by Pasteur pipette. The cell pellet was washed once with 5 ml water and recentrifuged, and 50 μl of 1 mg/ml Zymolyase (Seikagaku Corp., Tokyo) was added to resuspend the pellet. The cells were incubated for 15 min at 30°, brought to 1 ml final volume in water, and counted by hemacytometer to determine the number of cells that invaded the agar. The fraction of cells that invaded the agar was calculated as the number of cells that invaded the agar divided by the sum of the number of cells that did not invade the agar and the number that did invade the agar. Invasion was measured in three independent experiments for each *dia* strain and normalized to the invasion of SKY760 (*MAT***a** *ura3-52 his3::hisG leu2::hisG DIA*).

Cell flocculation assay: A flocculation assay was adapted from Bony *et al.* (1998). Yeast strains grown to saturation in 10 ml liquid YPD medium overnight at 30° were deflocculated by two washes in 50 mM sodium citrate, 5 mM EDTA, pH 3.0 buffer followed by sonication for 10 min. Cells were resuspended to a concentration of 10^8 cells/ml in 5 ml of sodium citrate buffer and calcium chloride was added to a final concentration of 20 mM to induce flocculation. Culture tubes were inverted 50 times/min for 10 min and then left standing vertically. After 10 min, 0.2 ml of the cell suspension was removed from just below the meniscus and added to 1 ml of 0.25 M EDTA, pH 8.0. The flocculation level is expressed as the difference in optical density at 600 nm between the deflocculated cell suspension in 0.25 M EDTA and the sample after 10 min of settling. Flocculation for each *dia* strain was normalized to that of strain SKY760 (*MAT***a** *ura3-52 his3::hisG leu2::hisG DIA*). Flocculation was measured in three independent experiments for each strain.

Cell elongation measurements: Yeast strains were incubated overnight in liquid YPD medium at 30°, transferred to fresh YPD, and grown to an OD_{600} of 0.6. Cells were photographed in Nomarski contrast using a Zeiss Axioskop with a 100× oil-immersion objective, a Photometrics Sensys 1600 CCD camera, and IPLab image-acquisition software. NIH Image software was used to manually trace 100 cells and obtain major and minor axis lengths. The reported elongation is the ratio of major to minor axis lengths.

Northern blot analysis: Yeast strains to be analyzed were

incubated in liquid YPD medium overnight at 30°, diluted 100-fold into fresh YPD, and incubated to an OD_{600} of 0.8. Cells were washed in ice water and total RNA was harvested by phenol:chloroform extraction followed by ethanol precipitation. For each sample, 20 μg of total RNA was separated by electrophoresis on a formaldehyde gel and transferred by capillary action to a 0.2-μm-pore-size nylon membrane. DNA probes (1000-bp regions at the 5′ end of the *FLO11* and *ACT1* open reading frames) were amplified and radiolabeled by PCR and then gel-purified. Hybridizations and washes were performed according to Sambrook *et al.* (1989). *FLO11* expression was normalized to *ACT1* expression. For each *dia* strain, this ratio was then normalized to the *FLO11/ACT1* expression ratio of strain SKY760 (*MAT***a** *ura3-52 his3::hisG leu2::hisG DIA*). At least three independent measurements of *FLO11/ACT1* expression levels were measured for each strain.

Bud scar staining: Haploid budding pattern was determined by calcofluor staining as described by Pringle *et al.* (1989). Cells were grown to an OD_{600} of ~0.6 in YPD at 30°. Aliquots of $\sim 10^7$ cells were fixed at room temperature for 1 hr in 3.7% formaldehyde, rinsed twice in water, resuspended in 200 μl of 1 μg/ml calcofluor white (Fluorescent Brightener 28, Sigma) in water, incubated in the dark at 22° for 30 min, washed five times with 1 ml water, and resuspended to a final volume of 25 μl. Stained cells were observed by epifluorescence microscopy using 365-nm excitation and blue emission filters and photographed using a Zeiss Axioskop, a 100× oil-immersion objective, Photometrics Sensys 1600 CCD camera, and IPLab image-acquisition software. Cells with between two and five obvious bud scars were divided into two classes, axial or polar, based on the predominant bud scar distribution. Over 100 cells were analyzed in each of three separate experiments for each strain.

RESULTS

Expression of *FLO11* correlates with agar invasion: To test whether *FLO11* expression directly regulates haploid invasive growth, we constructed strains that express different levels of *FLO11* by replacing the endogenous *FLO11* promoter with the promoters from the *S. pombe adh*$^+$ gene or the *S. cerevisiae CDC28* gene. In addition to altering basal *FLO11* expression, these replacement promoters prevent *FLO11* from being induced or repressed such as by the cAMP-dependent kinase or STE MAPK pathways. As shown in Figure 1, *SpADHp-FLO11* expression in YPD liquid is 0.4 times wild-type expression while *CDC28p-FLO11* expression is 4.2 times greater than that of wild-type *FLO11.* Wild-type haploids invade YPD agar to a minimal extent after 3 days with occasional single cells penetrating the matrix surface, but *SpADHp-FLO11* cells do not invade at all. *CDC28p-FLO11* cells invade the agar significantly more than wild-type cells with microcolonies of cells growing multiple cell layers below the agar surface. We conclude that increasing *FLO11* expression alone is sufficient to induce invasive growth in haploid cells.

Replacement of the endogenous *FLO11* promoter also affects cell filamentation on low ammonia media (Figure 1). *SpADHp-FLO11/SpADHp-FLO11* diploids do not elongate appreciably or form filaments. *CDC28p-FLO11/CDC28p-FLO11* diploids elongate slightly and form pseudohyphae but do not form filaments as well as *FLO11p-FLO11/FLO11p-FLO11* diploids. These results indicate that quantitative levels of *FLO11* expression can affect both cell and colony morphology in response to a low-nitrogen signal and that *FLO11* may possess functions in addition to mediating cell-cell adhesion.

Isolation of mutations that enhance invasive growth: On the basis of our results from modulating *FLO11* expression and its effects on both haploid invasive growth and filamentous differentiation, we hypothesized that regulation of *FLO11* expression from its endogenous promoter may be mediated in part via repression. Thus, we performed a genetic screen to identify potential negative regulators of *FLO11* expression by enhanced agar invasion in the mutants. To isolate *dia* mutants, we mutagenized a Σ1278b-derived *MAT***a** haploid strain (SKY760) by integrating a transposon-mutagenized genomic library containing random *Tn3::lacZ::LEU2* insertions (Burns *et al.* 1994). After 3 days of growth at 30°, 180,000 transformants were directly screened for hyperinvasive growth on the SC-Leu transformation plates. A total of 388 colonies that physically penetrated the surface of the agar were recovered onto SC-Leu medium and then streaked onto SC-Leu and YPD medium. Of the 388 picks, 189 retested as significantly more invasive than wild-type haploids on both synthetic and YPD media. Each of the 189 putative *dia* mutants was analyzed by a cross to a *DIA* strain (SKY2606, *MAT*α *ura3-52 leu2::hisG*) to determine if a single mutation linked to the *LEU2* marker was responsible for the hyperinvasive growth. Five *dia* strains were sterile and these were directly analyzed for insertion locus. In three, the insertions disrupt the *SIR4* gene and two fall in *SIR2. SIR2* and *SIR4* are required for transcriptional silencing of the silent mating type α-locus (Ivy *et al.* 1986).

Each of the remaining 184 strains was analyzed by tetrad dissection. The *LEU2* marker segregated 2:2 and was genetically linked to the hyperinvasive phenotype in 94 of these strains. The insertion sites were determined in these as well. Altogether, the 99 *dia* mutants comprised 60 different insertions in or adjacent to 35 different genes (Table 3). Of these, 31 were previously characterized in other genetic studies while 4 are novel. Of these 4, *DIA1* (YMR316w) does not possess significant homology to any characterized ORFs; *DIA2* (YOR080w) encodes an F-box protein; *DIA3* (YDL024c) is homologous to acid phosphatases such as *PHO5* and *PHO11*; *DIA4* (YHR011w) is homologous to *SES1*, a seryl tRNA synthase.

We measured the magnitude of the increase in invasion of each *dia* strain relative to a wild-type haploid strain (Table 3). For wild-type haploids, 0.02 ± 0.01% of cells invade after 4 days of incubation at 30°. In this screen we were able to detect insertional mutants that were as little as 25 times more invasive than wild type (*e.g.*, *MED1*, *HSL1*, *HSL7*, *MSN5*, *DIA2*, and *DIA4*). At the upper end, we detected an insertion in *SKN7*, which

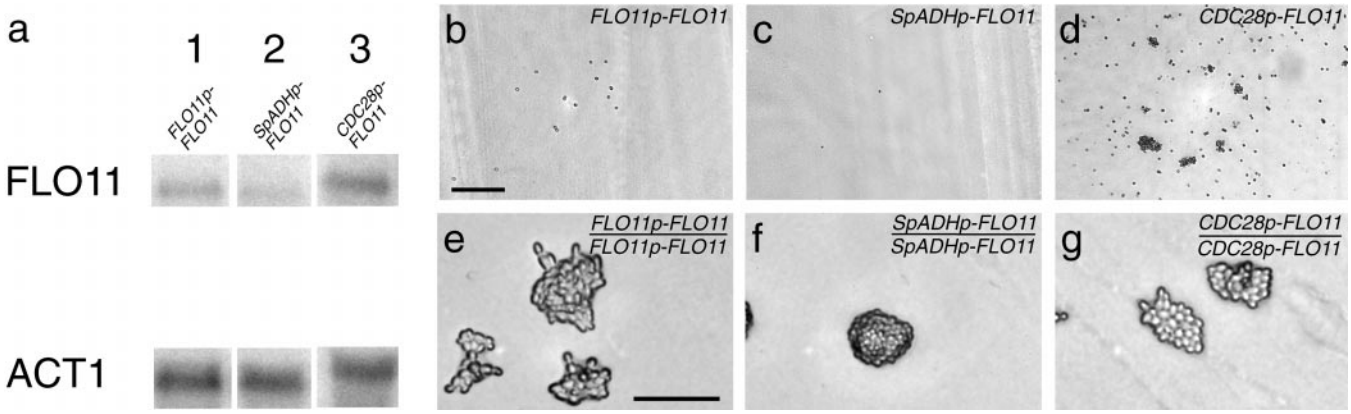

FIGURE 1.—Extent of cell invasion of rich media correlates with *FLO11* expression and the endogenous *FLO11* promoter is required for filamentation in response to low nitrogen. (a) Northern blot analysis of *FLO11* and *ACT1* expression in haploid strains reveals that the *SpADHp-FLO11* is expressed at 0.4 times wild-type *FLO11* expression while *CDC28p-FLO11* is expressed 4.2 times wild-type *FLO11* expression. Haploid strains with *FLO11* under its endogenous promoter (b), the *SpADH* promoter (c), or the *CDC28* promoter (d) were streaked on YPD medium to obtain single cells. YPD plates were incubated at 21° for 96 hr. Cells that did not penetrate the agar were removed by rubbing the plate with a finger under running water. Homozygous diploid strains with *FLO11* under its own promoter (e), the *SpADH* promoter (f), or the *CDC28* promoter (g) were streaked on SLAD medium to obtain single cells. SLAD plates were incubated at 21° for 24 hr. Images of representative colonies are shown. Bar (b), 100 μm for b–d. Bar (e), 50 μm for e–g.

is about 200 times as invasive as wild-type haploids. Images of wild-type and several *dia* mutant strain haploid colonies that have penetrated the surface of the agar are shown in Figure 2.

Most *dia* mutations increase *FLO11* expression: Ectopic induction of *FLO11* can enhance haploid invasive growth (Figure 1). To determine which of the *dia* mutations may enhance invasion by upregulating *FLO11*, we measured *FLO11* transcription by Northern blot analysis (Figure 3 and Table 4). In each *dia* strain, the expression of *FLO11* was normalized to the expression of *ACT1* and this ratio was normalized to the *FLO11*:*ACT1* expression ratio of wild-type cells. Of the 35 *dia* mutants, 25 display elevated *FLO11* expression (>1.5-fold wild type) while 10 do not significantly upregulate *FLO11*. These 10 *dia* mutations likely enhance invasive growth through a mechanism other than induction of *FLO11* expression.

An increase in *FLO11* expression in *dia* mutants may be a determinant of their hyperinvasive phenotype or a secondary effect and not required for agar invasion. To address whether upregulation of *FLO11* is necessary for haploid invasion in each *dia* strain, we crossed *SpADHp-FLO11* into each *dia* mutant background. While the *S. pombe adh*$^{+}$ promoter yields a reduced level of *FLO11* expression (Figure 3a), importantly, this mutation likely prevents the cell from stimulating or repressing *FLO11* transcription through any of its normal physiologic regulatory mechanisms. Indeed, by Northern analysis, the *SpADHp-FLO11* construct results in the same, low level of *FLO11* expression in each *dia* mutant as in the wild-type *DIA* haploid strain (data not shown).

As in a wild-type *DIA* strain, *SpADHp-FLO11* completely abolishes haploid invasion in 22 of the *dia* mutants (Table 4 and Figure 3). In many of these mutants, it is likely that derepression of *FLO11* expression is both necessary and sufficient for their hyperinvasive phenotype. However, within this group, *gtr1-100*, *med1-100*, and *taf25-100* do not upregulate *FLO11* from the endogenous promoter. Such strains appear to require more *FLO11* than that supplied by the *SpADH* promoter and/or may induce invasion through a *FLO11*-independent mechanism.

In 12 *dia* strains, although invasion is attenuated when expression of *FLO11* is clamped by *SpADH-FLO11*, it is not abrogated. Further, *elm1-100* can invade agar as well with *SpADH-FLO11* as the native *FLO11* promoter. These 13 *dia* mutants presumably invade by a pathway that does not require enhanced *FLO11* expression. Not surprisingly, 7 of these *dia* strains, *ax1-100*, *bud10-100*, *bud3-100*, *bud4-100*, *sir2-100*, *sir4-100*, and *bem2-100*, are among those that do not increase *FLO11* transcription from the native promoter. However, 6 of these *dia* strains, *elm1-100*, *dia2-100*, *bpl1-100*, *aro7-100*, *pgi1-100*, and *gnd1-100*, are among those that were found to upregulate *FLO11*. Save for *elm1-100*, this *FLO11* induction likely promotes invasion, but a *FLO11*-independent mechanism must contribute. Thus, while regulation of *FLO11* transcription provides an important mechanism of invasive growth control, *FLO11* upregulation is not essential for invasive growth. One or more *FLO11*-independent mechanisms are likely to be sufficient for invasive growth.

Secondary phenotypic characterization of *dia* mutants: We examined the *dia* mutants further to determine if cellular processes other than agar invasion were affected and to investigate potential mechanisms for *FLO11*-independent agar invasion. We hypothesized that elongated cell shape and polarized bud site selec-

TABLE 3

Identification of *dia* mutants

Gene	ORF	Mutant allele	Insertion site[a]	Haploid invasion ± SEM[b]
BUD3	YCL014W	*bud3-100*	+2242	44 ± 13
		bud3-101	+2343	
BUD4	YJR092W	*bud4-100*	−195	43 ± 7
		bud4-101	−175	
		bud4-102	−7	
		bud4-103	+55	
		bud4-104	+185	
		bud4-105	+545	
		bud4-106	+702	
		bud4-107	+989	
		bud4-108	+2070	
BUD10	YIL140W	*bud10-100*	+786	72 ± 18
		bud10-101	+870	
		bud10-102	+1064	
		bud10-103	+1393	
AXL1	YPR122W	*axl1-100*	+128	47 ± 12
BEM2	YER155C	*bem2-100*	+3806	41 ± 22
ELM1	YKL048C	*elm1-100*	+58	85 ± 9
HSL1	YKL101W	*hsl1-100*	+1163	25 ± 7
HSL7	YBR133C	*hsl7-100*	+590	26 ± 5
YDJ1	YNL064C	*ydj1-100*	+195	86 ± 8
		ydj1-101	+1070	
ZUO1	YGR285C	*zuo1-100*	+159	92 ± 46
HSC82	YMR186W	*hsc82-100*	+64	39 ± 15
SIR2	YDL024C	*sir2-100*	+1076	82 ± 26
SIR4	YDR227W	*sir4-100*	+3120	71 ± 18
GCR1	YPL075W	*gcr1-100*	−61	79 ± 29
		gcr1-101	+28	
		gcr1-102	+515	
PGI1[c]	YBR196C	*pgi1-100*	−271	100 ± 45
ADH1	YOL086C	*adh1-100*	+489	154 ± 6
GND1[c]	YHR183W	*gnd1-100*	−85	79 ± 17
		gnd1-101	−58	
MED1	YPR070W	*med1-100*	+447	44 ± 11
CSE2	YNR010W	*cse1-100*	+306	45 ± 20
SRB8	YCR081W	*srb8-100*	+1323	116 ± 47
SSN3	YPL042C	*ssn3-100*	+1111	115 ± 28
SSN8	YNL025C	*ssn8-100*	−126	85 ± 9
		ssn8-101	−107	
		ssn8-102	+49	
		ssn8-103	+76	
		ssn8-104	+405	
TAF25[c]	YDR167W	*taf25-100*	−22	54 ± 17
GTR1	YML121W	*gtr1-100*	+56	68 ± 9
ARO7	YPR060C	*aro7-100*	+617	38 ± 5
CDC53[c]	YDL132W	*cdc53-100*	−357	49 ± 3
GRR1	YJR090C	*grr1-100*	+1145	173 ± 53
		grr1-101	+1203	
		grr1-102	+2199	

(*continued*)

tion, in addition to increased cell-cell adhesion, may be independent determinants of agar invasion in the *dia* mutants. Thus, we measured the ratio of major to minor axes in individual cells of each *dia* strain and the fraction of cells that exhibit polar bud site selection as opposed to the expected axial budding. On the basis of these results (Table 5), we separated the *dia* mutations into four classes. Class 1 mutations (3 genes) exhibit both significant polar bud site selection and cell elongation as compared to wild-type *DIA* haploids. Class 2 mutations (12 genes) perform polarized budding but remain round while class 3 mutants (2 genes) are elongated

TABLE 3

(Continued)

Gene	ORF	Mutant allele	Insertion site[a]	Haploid invasion ± SEM[b]
SKN7[c]	YHR205W	*skn7-100*	−579	196 ± 70
IRA1	YBR140C	*ira1-100*	+8936	116 ± 15
BPL1	YDL141W	*bpl1-100*	+2007	61 ± 17
MSN5	YDR335W	*msn5-100*	+400	29 ± 17
		msn5-101	+1268	
		msn5-102	+1338	
		msn5-103	+2078	
DIA1[c]	YMR316W	*dia1-100*	−150	44 ± 17
DIA2	YOR080W	*dia2-100*	+803	30 ± 15
DIA3	YDL024C	*dia3-100*	+256	54 ± 24
DIA4	YHR011W	*dia4-100*	+138	27 ± 7

[a] Approximate position of *Tn3::LEU2* insertions relative to the ATG translational start site.
[b] Invasion was quantified as described in MATERIALS AND METHODS. Values listed are the ratio of cells that invaded the plates, normalized to wild-type levels of invasion.
[c] See MATERIALS AND METHODS.

but display predominantly axial budding. Class 4 mutants (18 genes), like wild-type *DIA* cells, bud in an axial pattern and remain round. Examples of bud scar staining that illustrate budding pattern and individual cell morphology for representative members of each class are shown in Figure 4.

Not surprisingly, among the class 4 genes, where mutants display budding and cell shape similar to that of a *DIA* wild-type strain, most increase *FLO11* expression and require the native promoter for their phenotype. We found that bud site selection had a significant effect on invasion independent from derepression of the *FLO11* promoter. The majority of class 1 and 2 *dia* strains, all of which exhibit an increase in polar budding, are able to invade agar without upregulating *FLO11*. In turn, nearly all class 3 and 4 *dia* mutant strains, those that do not display an increase in polar budding, no longer invade agar at the low levels of *FLO11* expression permitted by the *SpADH* promoter. In detail, most polar-budding but round, class 2 haploids—*bud3-100*, *bud4-100*, *bud10-100*, *axl1-100*, *bem2-100*, *sir2-100*, and *sir4-100*—tend neither to upregulate *FLO11* expression nor to require the native promoter for their invasive phenotype (Tables 4 and 5) while *pgi1-100* and *gnd1-100* mutations enhance both polar budding as well as *FLO11* expression. By contrast to polar budding, cell elongation alone, as in class 3 mutants, is insufficient to cause agar invasion as evidenced by the lack of invasion in

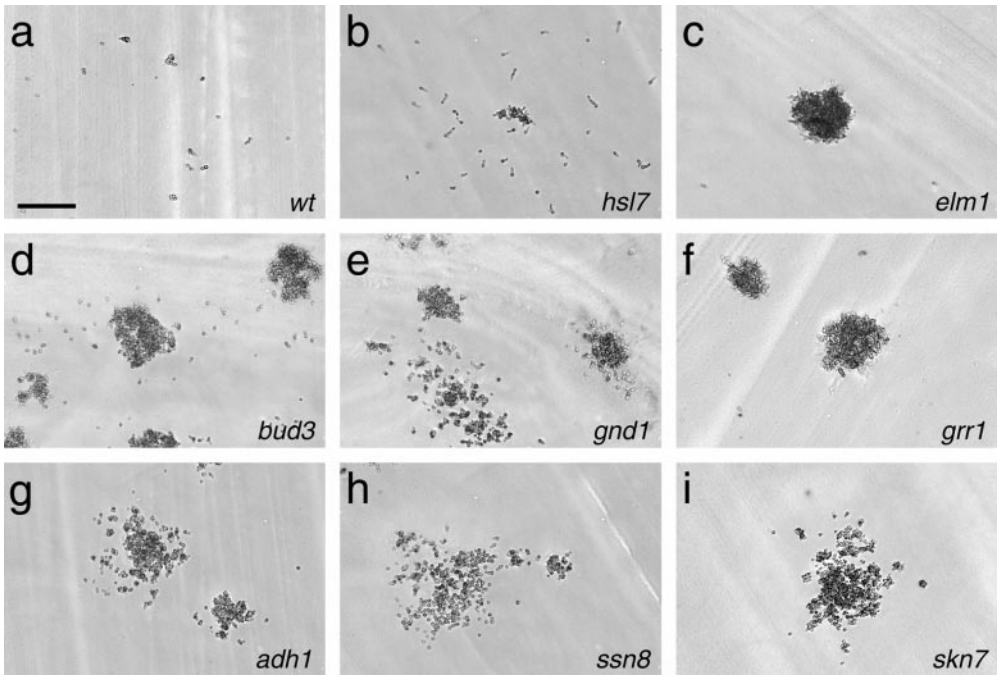

FIGURE 2.—Haploid *dia* mutant colonies invade agar. *dia* strains were streaked on YPD medium to obtain single cells and grown for 96 hr at 21°. Cells that did not penetrate the agar were removed by rubbing the plate with a finger under running water. Representative colonies were imaged. Bar, 100 μm.

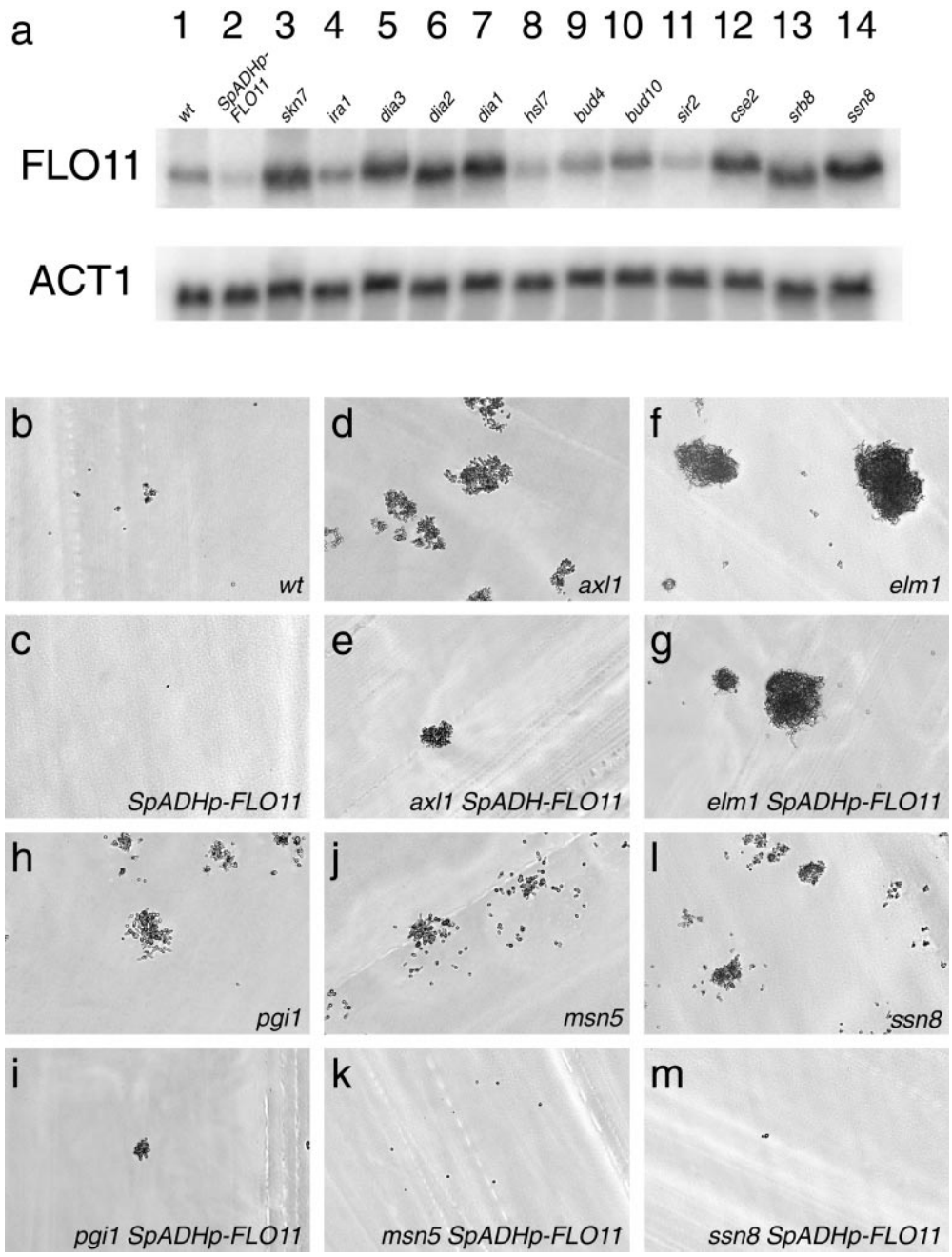

FIGURE 3.—Upregulation of *FLO11* transcription is required for hyperinvasive phenotype of some *dia* strains. (a) Northern analysis of *FLO11* and *ACT1* expression in haploid strains reveals that many *dia* strains upregulate *FLO11* (lanes 3, 5, 6, 7, 12, 13, and 14) while others do not (lanes 4, 8, 9, 10, and 11). (b–m) Haploid *dia* strains were streaked on YPD medium to obtain single cells. YPD plates were incubated at 21° for 96 hr. Cells that did not penetrate the agar were removed by rubbing the plate with a finger under running water. Images of representative colonies are shown.

the *SpADHp-FLO11 grr1-100* and *SpADHp-FLO11 cdc53-100* strains. Nonetheless, cell elongation may augment agar invasion given a critical level of *FLO11* expression. *taf25-100*, *gtr1-100*, and *med1-100* are the only three *dia* mutants that increase haploid invasion without increasing *FLO11* expression or cell polarity.

In addition to measuring changes in cell polarity, we also assayed each *dia* mutant for flocculation (Table 5) to determine if the mutations affect cell-cell adhesion. *FLO11* has been implicated in both invasive growth and calcium-dependent flocculation (LO and DRANGINIS 1996). Enhanced flocculation tends to correlate with polarized budding, but there are several notable exceptions. In fact, several of the class 2 mutations that do not upregulate *FLO11* expression (*bud3-100*, *bud4-100*, *bud10-100*, and *axl1-100*) still display increased flocculence. Among the 12 class 2 mutants, only 4 (*sir2-100*, *sir4-100*, *gnd1-100*, and *dia2-100*) do not show at least a fourfold increase in flocculation. Of the 3 class 1 mutants, *elm1-100* is 20 times as flocculent as wild-type cells while *hsl1-100* and *hsl7-100* show no increase in flocculence. *grr1-100* is hyperflocculent while *cdc53-100* is not. On the other hand, 11 of the 18 class 4 mutants that are neither polar budding nor elongated are no more flocculent than wild-type haploids. Thus, some of the pathways that repress agar invasion may also repress flocculation, but flocculation is not necessary for agar invasion.

TABLE 4

***FLO11* expression in *dia* mutants**

Strain	Relevant genotype	*FLO11* expression ± SEM	*dia SpADHp-FLO11* invasion[a]
SKY760	Wild type	1 ± 0.17	−
SKY2575	*elm1-100*	1.7 ± 0.2	+++
SKY2602	*dia2-100*	3.6 ± 1.0	++
SKY2599	*bpl1-100*	2.1 ± 0.6	++
SKY2594	*aro7-100*	2.0 ± 0.4	++
SKY2584	*pgi1-100*	1.9 ± 0.3	++
SKY2573	*axl1-100*	1.29 ± 0.22	++
SKY2572	*bud10-100*	1.23 ± 0.12	++
SKY2570	*bud3-100*	0.92 ± 0.10	++
SKY2571	*bud4-100*	0.84 ± 0.15	++
SKY2581	*sir2-100*	0.75 ± 0.10	++
SKY2582	*sir4-100*	0.64 ± 0.24	++
SKY2586	*gnd1-100*	2.2 ± 0.6	+
SKY2574	*bem2-100*	0.83 ± 0.11	+
SKY2580	*hsc82-100*	5.6 ± 1.1	−
SKY2590	*ssn8-100*	3.4 ± 0.4	−
SKY2589	*ssn3-100*	3.2 ± 0.3	−
SKY2601	*dia1-100*	3.1 ± 0.8	−
SKY2588	*cse2-100*	2.7 ± 0.7	−
SKY2591	*srb8-100*	2.6 ± 0.9	−
SKY2578	*ydj1-100*	2.6 ± 0.5	−
SKY2596	*grr1-100*	2.4 ± 0.4	−
SKY2600	*msn5-100*	2.3 ± 0.3	−
SKY2597	*skn7-100*	2.3 ± 0.5	−
SKY2603	*dia3-100*	2.2 ± 0.3	−
SKY2595	*cdc53-100*	2.1 ± 0.6	−
SKY2585	*adh1-100*	2.1 ± 0.4	−
SKY2583	*gcr1-100*	1.9 ± 0.3	−
SKY2604	*dia4-100*	1.7 ± 0.4	−
SKY2577	*hsl7-100*	1.7 ± 0.2	−
SKY2576	*hsl1-100*	1.5 ± 0.3	−
SKY2598	*ira1-100*	1.5 ± 0.4	−
SKY2579	*zuo1-100*	1.3 ± 0.2	−
SKY2593	*gtr1-100*	0.93 ± 0.16	−
SKY2587	*med1-100*	0.90 ± 0.08	−
SKY2592	*taf25-100*	0.83 ± 0.15	−

[a] For *dia SpADHp-FLO11* strain invasion scores, +++ signifies no reduction in invasion as compared to *dia* strains, ++ signifies a slight reduction in invasion compared to *dia* strains, + signifies a great reduction in invasion compared to *dia* strains, and − signifies no detectable invasion.

Surprisingly, *SpADHp-FLO11* does not affect flocculation of any of the *dia* mutant strains (data not shown), suggesting that upregulation of another adhesin or other factors may be responsible for increased flocculation in these strains. In turn, the decreased agar invasion in *SpADHp-FLO11 dia* strains suggests that increased cell-cell adhesion may not be sufficient to explain *FLO11*-induced agar invasion.

Haploid invasive growth is distinct from diploid invasive growth and pseudohyphal differentiation: Haploid invasive growth pathways have been linked to diploid pseudohyphal growth through their common regulation via the cAMP-dependent kinase and STE MAPK pathways. We constructed homozygous diploids of each *dia* mutation and measured agar invasion of each *dia/dia* strain on rich YPD medium as well as cell elongation in response to low nitrogen on SLAD medium to determine whether enhanced haploid invasive growth correlates with altered diploid invasion and/or pseudohyphal differentiation. Table 5 shows complete results of these assays while Figure 5 provides representative images of colonies that penetrated the surface of YPD medium or colonies growing on SLAD medium.

Wild-type diploid cells are significantly more invasive than wild-type haploid cells, possibly resulting from a switch to polarized bud site selection in diploids. Invasive growth of *dia/dia* mutant strains is greater than that of wild-type diploids for 17 of the mutants. Invasion is decreased relative to the wild-type diploid in 4 of the *dia/dia* mutant strains (*ydj1-100/ydj1-100*, *taf25-100/taf25-100*, *gtr1-100/gtr1-100*, and *bpl1-100/bpl1-100*). In the remaining 14 *dia/dia* strains, invasion is not significantly different between *dia/dia* mutants and wild-type diploids. Every elongated haploid strain (classes 1 and 3) is also hyperinvasive as a diploid yet only 3 class 2 mutants (polar budding, round morphology) are hyperinvasive as diploids.

In addition to failing to induce diploid invasive growth, class 2 *dia/dia* mutations typically do not affect pseudohyphal growth. Only *pgi1-100/pgi1-100* and *gnd1-100/gnd1-100* strains are hyperfilamentous. However, each *dia* mutation that induces cell elongation in haploids (class 1 and 3 strains) also enhances diploid *dia/dia* filamentation on SLAD medium. Among the class 4 *dia/dia* mutants, diploid invasive growth does not appear to be linked to low-nitrogen stimulated filamentation. In some strains, such as *ira1-100/ira1-100*, filamentation can be induced without hyperinvasion but in other strains, such as *ssn3-100/ssn3-100*, invasiveness increases while filamentation remains unchanged. In fact, reciprocal regulation of invasion and pseudohyphal differentiation can occur as in a *gcr1-100/gcr1-100* strain (Figure 5).

Polar bud site selection enhances agar invasion: Diploids require Bud8 for bipolar budding (Zahner *et al.* 1996) and filamentous growth (Mosch and Fink 1997). Mutants bud proximal to their birth scars, in a pattern similar to normal haploid axial bud site selection. We assayed the effects of a *bud8* deletion on the invasiveness of each *dia* mutant. Deletion of *BUD8* switches all of the *dia* mutant strains except *elm1-100* and *bem2-100* to predominantly proximal budding. *elm1-100* bud site selection remains polar in a *bud8*Δ while *bem2-100* budding is random in both *BUD8* and *bud8*Δ backgrounds (data not shown). Each *dia* mutant strain that buds in a polar manner, except *elm1-100* and *bem2-100*, requires *BUD8* expression for invasion (Figure 6 and Table 6). Several of these strains (*bud3-100*, *bud4-100*, *bud10-100*, *axl1-100*, *sro4-100*, *hsl1-100*, *hsl7-100*, *sir2-100*, and *sir4-*

TABLE 5

Classification of *dia* mutant strains based on secondary phenotypes

Class	Strain	Relevant genotype	Fraction polar budding ± SEM	Elongation ± SD	Flocculation ± SEM	*dia/dia* diploid invasive growth[a]	*dia/dia* diploid filamentous growth[b]
Wild type	SKY760	*DIA*	0.045 ± 0.012	1.19 ± 0.09	1.0 ± 0.2	+	+
1	SKY2575	*elm1-100*	0.94 ± 0.03	2.9 ± 0.6	20.8 ± 6.7	++	+++
	SKY2576	*hsl1-100*	0.64 ± 0.13	2.8 ± 1.0	1.4 ± 0.3	++	++
	SKY2577	*hsl7-100*	0.55 ± 0.15	3.0 ± 1.4	1.3 ± 0.3	++	++
2	SKY2570	*bud3-100*	0.89 ± 0.02	1.16 ± 0.02	5.7 ± 1.7	+	+
	SKY2571	*bud4-100*	0.88 ± 0.04	1.16 ± 0.04	10.6 ± 3.0	+	+
	SKY2572	*bud10-100*	0.83 ± 0.05	1.4 ± 0.3	5.2 ± 1.2	+	+
	SKY2573	*axl1-100*	0.86 ± 0.05	1.17 ± 0.10	5.9 ± 0.9	+	+
	SKY2581	*sir2-100*	0.89 ± 0.02	1.22 ± 0.11	1.1 ± 0.2	+	+
	SKY2582	*sir4-100*	0.93 ± 0.04	1.15 ± 0.08	1.1 ± 0.1	+	+
	SKY2574	*bem2-100*	0.70 ± 0.10	1.3 ± 0.3	7.3 ± 2.5	++	+
	SKY2584	*pgi1-100*	0.31 ± 0.10	1.26 ± 0.15	4.3 ± 1.1	++	++
	SKY2586	*gnd1-100*	0.45 ± 0.03	1.32 ± 0.13	1.6 ± 0.2	++	++
	SKY2578	*ydj1-100*	0.67 ± 0.11	1.23 ± 0.11	13.7 ± 3.6	−	+
	SKY2594	*aro7-100*	0.84 ± 0.05	1.13 ± 0.10	6.2 ± 2.1	+	+
	SKY2602	*dia2-100*	0.74 ± 0.08	1.5 ± 0.5	1.5 ± 0.1	+	+
3	SKY2596	*grr1-100*	0.095 ± 0.055	3.6 ± 0.3	15.8 ± 4.9	++	+++
	SKY2597	*cdc53-100*	0.15 ± 0.04	1.8 ± 0.4	1.9 ± 0.3	++	++
4	SKY2580	*hsc82-100*	0.008 ± 0.008	1.27 ± 0.14	1.2 ± 0.1	++	+
	SKY2579	*zuo1-100*	0.10 ± 0.04	1.14 ± 0.08	1.2 ± 0.3	+	+
	SKY2585	*adh1-100*	0.049 ± 0.027	1.19 ± 0.18	2.3 ± 0.5	++	++
	SKY2588	*cse2-100*	0.075 ± 0.029	1.11 ± 0.07	4.7 ± 0.7	++	+
	SKY2601	*dia1-100*	0.096 ± 0.018	1.21 ± 0.13	1.0 ± 0.2	+	++
	SKY2603	*dia3-100*	0.12 ± 0.05	1.28 ± 0.18	1.1 ± 0.2	+	++
	SKY2604	*dia4-100*	0.019 ± 0.018	1.18 ± 0.12	0.9 ± 0.2	+	++
	SKY2583	*gcr1-100*	0.13 ± 0.07	1.23 ± 0.13	1.0 ± 0.1	++	+
	SKY2598	*ira1-100*	0.14 ± 0.05	1.16 ± 0.09	1.7 ± 0.4	+	+++
	SKY2600	*msn5-100*	0.054 ± 0.010	1.19 ± 0.09	1.1 ± 0.1	+	+
	SKY2591	*srb8-100*	0.16 ± 0.04	1.12 ± 0.09	3.7 ± 1.1	++	++
	SKY2589	*ssn3-100*	0.091 ± 0.024	1.17 ± 0.09	3.5 ± 1.2	++	+
	SKY2590	*ssn8-100*	0.13 ± 0.13	1.4 ± 0.4	3.1 ± 0.5	++	+
	SKY2599	*bpl1-100*	0.068 ± 0.032	1.21 ± 0.15	2.5 ± 1.1	−	−
	SKY2593	*gtr1-100*	0.17 ± 0.08	1.15 ± 0.07	1.1 ± 0.2	−	+
	SKY2587	*med1-100*	0.054 ± 0.030	1.17 ± 0.11	1.0 ± 0.2	++	+
	SKY2597	*skn7-100*	0.12 ± 0.04	1.14 ± 0.09	7.1 ± 4.2	++	+
	SKY2592	*taf25-100*	0.22 ± 0.08	1.6 ± 0.6	1.3 ± 0.2	−	−

[a] For diploid invasion scores, + signifies extent of wild-type invasion, ++ signifies an increased invasion relative to wild-type strains, and − signifies no detectable invasion.

[b] For diploid filamentation scores, + signifies slight elongation and filament formation of some cells, ++ signifies extensive elongation of filament formation of some of the cells, +++ signifies extensive elongation and filament formation of all of the cells, and − signifies no elongation or filament formation.

100) do not overexpress *FLO11* whereas others (*ydj1-100*, *pgi1-100*, *gnd1-100*, *aro7-100*, and *dia2-100*) do. Therefore, polarity is an important factor in allowing each of these strains to invade, regardless of the level of *FLO11* expression. *BUD8* deletion does not affect invasion in axial-budding strains (Table 6) or cell elongation in any of the strains tested (data not shown).

To further test our hypothesis that polar budding is sufficient to cause agar invasion given a basal level of *FLO11* expression, we measured agar invasion in diploid (*MAT***a**/*MAT*α) and pseudodiploid (*MAT***a** [*MAT*α *CEN URA3*]) strains (Figure 7). Diploid cells bud at the distal pole 95 ± 4% of the time while pseudodiploids form 93 ± 5% of new buds at the distal pole. Diploids invade agar more avidly than haploids, while pseudodiploids are more invasive than either haploids or diploids. This increased invasiveness requires *BUD8* since *MAT***a** *bud8::kan*^{r} [*MAT*α *CEN URA3*] and *MAT***a**/*MAT*α *bud8::kan*^{r}/*bud8::kan*^{r} strains do not invade, consistent with the notion that polar budding causes invasiveness.

Sir2 and Sir4 repress the silent mating locus. Therefore, the *sir2-100* and *sir4-100* strains express both mating type **a** and α-specific genes and should behave as pseudodiploids. These two *dia* mutants perform polar

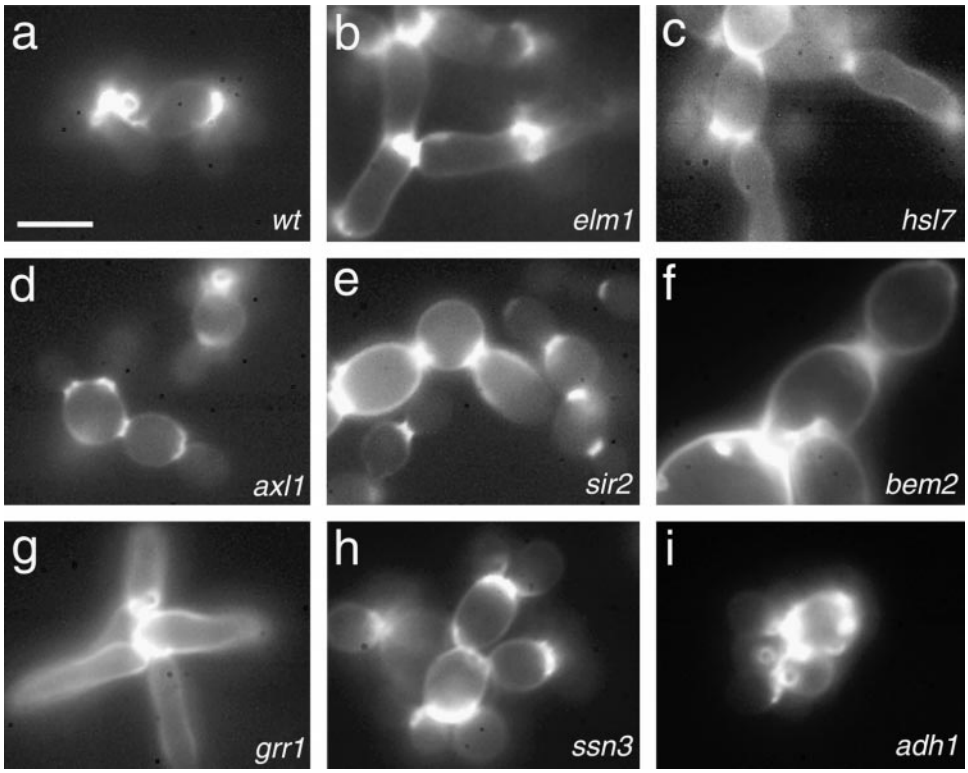

FIGURE 4.—Some *dia* mutations affect cell morphology and bud site selection. Haploid *dia* strains were grown to log growth phase in YPD at 30°, fixed in formaldehyde, and stained with calcofluor white to visualize bud scars. Images of representative cells are shown. Class 1 mutations (b and c) elongate cells and cause predominantly polar bud site selection. Class 2 mutations (d–f) cause polar budding but not cell elongation. Class 3 (g) mutations elongate cells but maintain axial budding. Class 4 mutations (h and i) do not affect bud site selection or cell morphology. Bar, 5 μm.

budding and require *BUD8* for invasion. To test whether the expression of the silent mating locus is responsible for their phenotypes, we deleted the *HML* silent locus in a *MAT***a** *sir4::HIS3* strain. This *sir4*Δ *hml*Δ strain mates as *MAT***a**, no longer invades agar, and resumes axial budding in 98 ± 1% of new buds, suggesting that expression of both mating type loci promotes agar invasion by inducing polar budding.

Most *dia* strains do not require *RAS2* activity for hyperinvasion: To test whether any of the *dia* mutations activate *RAS2* to cause hyperinvasive growth, we crossed each *dia* strain to a *ras2::kan*^{r} strain to obtain *dia ras2* segregants. Then we compared agar invasion by *dia RAS2* colonies and *dia ras2* colonies (Table 6 and Figure 8). Surprisingly, only three mutants, *ira1-100*, *bpl1-100*, and *ssn3-100*, require Ras2 activity for hyperinvasion. Ira1 is a GTPase-activating protein for Ras2 (TANAKA *et al.* 1989) and may inhibit agar invasion by inactivating Ras2. Bpl1 catalyzes biotinylation of proteins and its relation to Ras2 is unclear (CRONAN and WALLACE 1995). Ssn3 is a cyclin-dependent kinase homolog and a subunit of the RNA polymerase II mediator complex involved in Mig1-independent glucose repression along with Ssn8 and Srb8 (BALCIUNAS and RONNE 1995). Nonetheless, *ssn8-100* and *srb8-100* do not require Ras2 for agar invasion. The remainder of the *dia* strains activate invasive growth either downstream of *RAS2* or via a parallel pathway.

***DIA* gene interactions with Kss1 MAPK invasive growth signaling:** Kss1 is a MAP kinase that can both stimulate and inhibit the filamentous and hyperinvasive growth pathway (MADHANI *et al.* 1997; BARDWELL *et al.* 1998). Unphosphorylated Kss1 binds and represses Ste12, a transcriptional activator involved in pheromone signaling and filamentous response. Kss1 phosphorylation by Ste7 results in phosphorylation and activation of Ste12 and decreases the affinity of Kss1 for Ste12 and Dig1, relieving repression. By crossing each *dia* mutant strain to a *kss1*Δ strain, we determined whether the stimulatory and/or inhibitory effects of Kss1 are required for their hyperinvasive growth. Pathway analysis with *kss1*Δ is potentially superior to *ste12*Δ or other mutations that abrogate signal transduction as *kss1*Δ confers a low constitutive level of signaling, reducing the likelihood of false epistasis (BARDWELL *et al.* 1998).

We compared invasive growth of *dia KSS1* strains with *dia kss1*Δ strains (Table 6 and Figure 9). *KSS1* deletion reduces invasion in six *dia* strains: *ydj1-100*, *zuo1-100*, *hsc82-100*, *pgi1-100*, *gnd1-100*, and *gtr1-100*. Ydj1, Zuo1, and Hsc82 are involved in protein folding and cell stress response (LOUVION *et al.* 1998; LU and CYR 1998; YAN *et al.* 1998). Pgi1 catalyzes the isomerization of glucose-6-phosphate to fructose-6-phosphate while Gnd1 catalyzes the reduction of 6-phosphogluconate to ribulose-5-phosphate (LOBO and MAITRA 1982; DICKINSON 1991). Gtr1 is a GTP-binding protein required for phosphate transport (BUN-YA *et al.* 1992). These proteins may provide links between metabolic regulation and invasive growth. Paradoxically, *gtr1-100* does not increase *FLO11* expression, a known target of the MAPK invasive growth signaling pathway, suggesting *KSS1* may affect invasive growth through *FLO11*-independent mechanisms in ad-

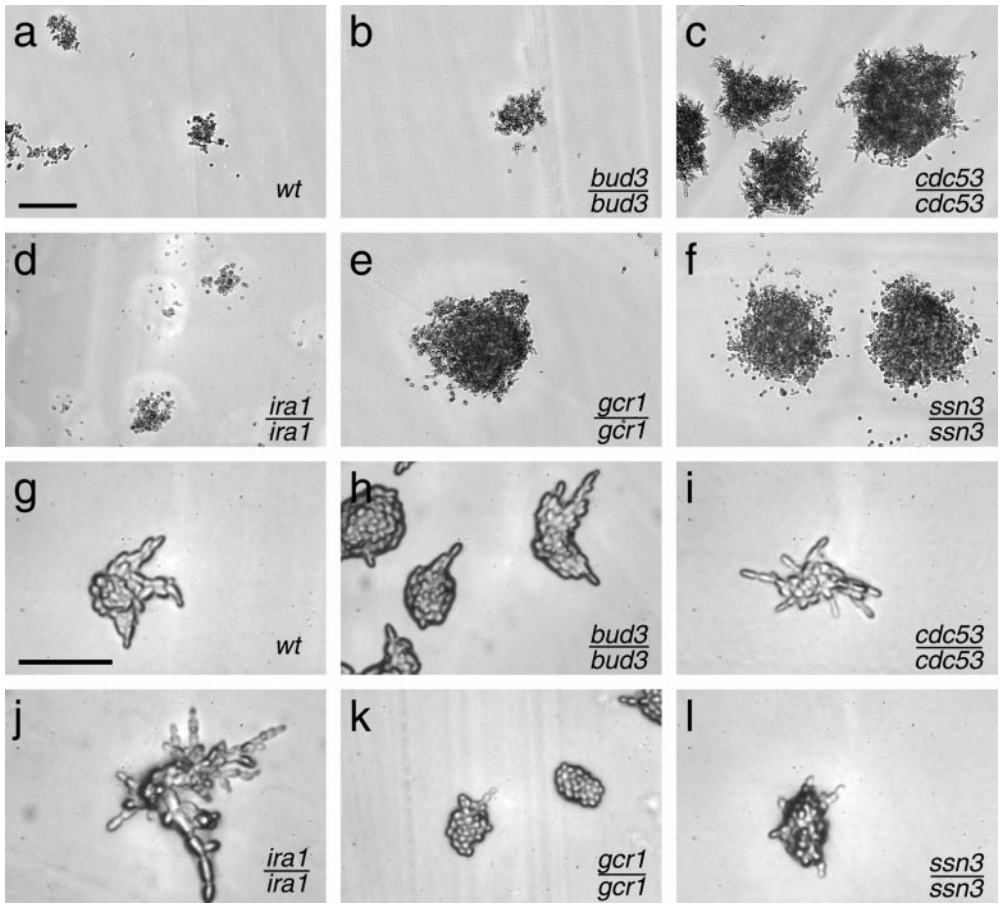

FIGURE 5.—Some *dia/dia* mutations enhance diploid agar invasion and/or pseudohyphal differentiation. Diploid *dia/dia* strains were streaked on YPD medium (a–f) or SLAD +Ura medium (g–l) to obtain single cells. YPD plates were incubated at 21° for 96 hr. Then cells that did not penetrate the agar were removed by rubbing the plate with a finger under running water. SLAD+Ura plates were incubated at 21° for 36 hr. Representative colonies were imaged. Bar (a), 100 μm for a–f. Bar (g), 50 μm for g–l.

dition to inducing *FLO11* transcription. However, it is unclear whether each of these genes requires the stimulatory or inhibitory activity of Kss1.

***SWE1* mediates the enhanced invasive growth in *hsl1*, *hsl7*, and *elm1* mutant strains:** Elm1, Hsl1, and Hsl7 repress cell elongation, filamentous growth, and invasive growth by negatively regulating Swe1, which in turn phosphorylates Cdc28 to inhibit mitosis (EDGINGTON *et al.* 1999). As expected, *SWE1* deletion suppresses the cell elongation and hyperinvasion phenotypes of *elm1-100*, *hsl1-100*, and *hsl7-100* strains (Figure 10).

DISCUSSION

The flocculin gene *FLO11* is upregulated by activation of filamentous signaling to mediate cell-cell adhesion during flocculation, invasive growth, and filament formation (LO and DRANGINIS 1998). Although a precise role for Flo11 in yeast invasion remains unclear, this flocculin probably contributes to cell-cell and/or cell-matrix adhesion so the multicellular colony can generate sufficient traction force to burrow into the substratum. Previous studies have shown that *flo11* deletion mutant cells are completely deficient for agar invasion and filamentous growth (LAMBRECHTS *et al.* 1996; LO and DRANGINIS 1996). However, study of the significance of regulated expression of *FLO11* is confounded by the complexity of the *FLO11* promoter and its responsiveness to multiple stimuli (RUPP *et al.* 1998; GAGIANO *et al.* 1999b). Within the microenvironment of the invasive yeast colony, it is difficult to predict the pattern of expression of *FLO11* on a cell-by-cell basis, yet this expression may be critical to the phenotype. By replacing the endogenous *FLO11* promoter with the *S. pombe adh*$^+$ and *S. cerevisiae CDC28* promoters, we clamped *FLO11* expression at a level somewhat below and somewhat above the basal expression of *FLO11* in rich liquid media. This constitutive expression had significant effects on both haploid cell invasion as well as diploid cell morphology and filamentation, suggesting that the quantitative level of *FLO11* expression and presumably abundance of Flo11 protein on the cell surface are important determinants of both invasive growth and pseudohyphal development. Further, based on the filamentous growth response of diploid cells carrying clamped promoter alleles of *FLO11*, Flo11 appears to have a role in determining cell shape in addition to its functions in cell adhesion.

Based upon the clear relationship between levels of *FLO11* expression and invasive growth, we performed a genetic screen for mutants that dig into agar. We visually screened transposon insertion mutants for clones that display enhanced agar invasion and then measured the *FLO11* expression in each of these mutant strains. We

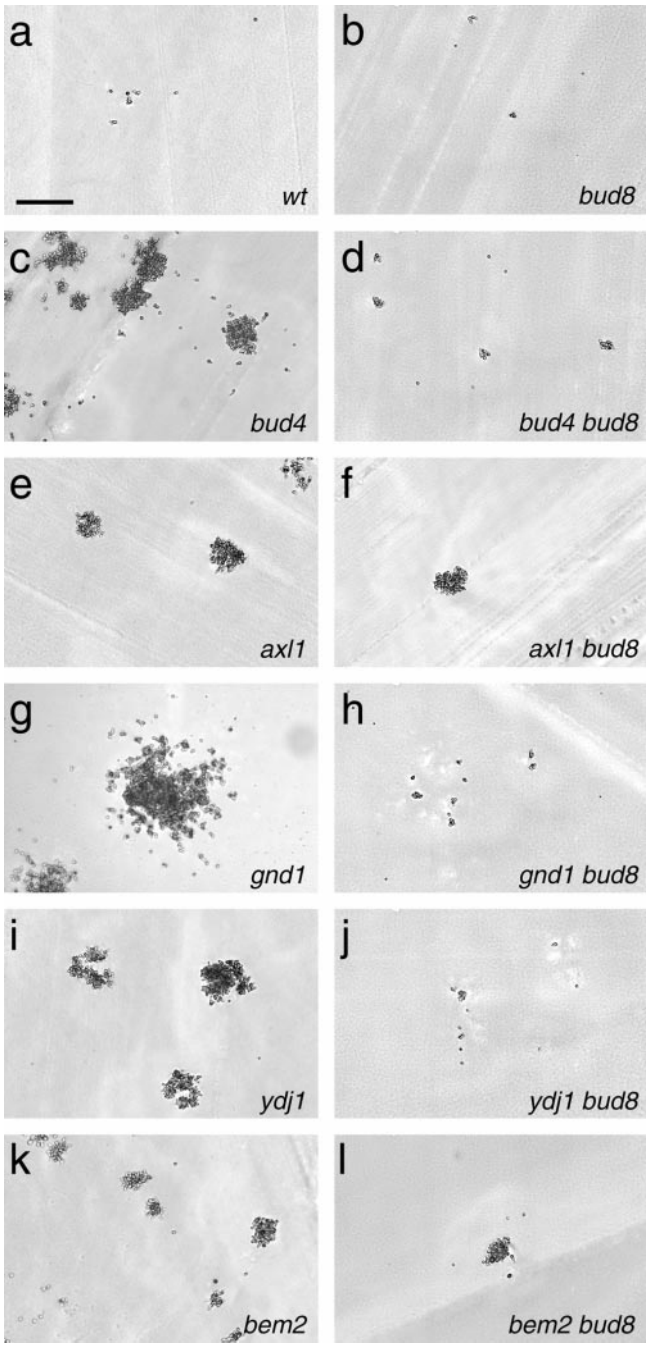

Figure 6.—Class 2 *dia* mutations require polar bud site selection for cell invasion. Haploid *dia* strains were streaked on YPD to obtain single cells. The plates were incubated at 21° for 96 hr. Cells that did not penetrate the agar were removed by rubbing the plate with a finger under running water. Representative colonies were imaged. Bar, 100 μm.

identified 35 *DIA* genes that when mutated confer markedly enhanced invasive growth. The mutations fall in elements of multiple signaling, metabolic, and morphogenesis pathways. Although most *DIA* genes are novel regulators of invasion and filamentation, among them are several genes previously implicated as negative regulators of agar invasion and/or filamentous growth. Nonetheless, we did not identify several genes whose deletion has previously been reported to promote haploid invasive growth such as *CLB2*, *BCY1*, *TPK3*, *SFL1*, *CTS1*, and *ACE2* (King and Butler 1998; Robertson and Fink 1998; Ahn *et al.* 1999; Pan and Heitman 1999). That our screen was not performed to saturation, that we only selected highly invasive mutants, and our use of a transposable element as a mutagen may each have contributed to lack of complete coverage of this phenotype.

Regulation of *FLO11* in haploid invasive growth: A well-described phenotype of *flo11* deletion is a nearly complete deficit in diploid filamentous growth. Similarly, *FLO11* has been shown necessary for haploid invasion. Our results with *S. pombe adh*$^+$ promoter and *CDC28* promoter-driven *FLO11* suggest that *FLO11* upregulation is also sufficient to drive invasion. As expected, we found that *FLO11* induction likely plays a key role in the enhanced invasion displayed by the *dia* mutants; 26 of the 35 mutations we identified confer significantly increased *FLO11* expression. However, to address whether *FLO11* upregulation was essential for the enhanced invasion conferred by the *dia* mutants, we assayed for invasive growth in strains where the endogenous *FLO11* promoter was replaced with the *S. pombe adh*$^+$ promoter and thereby clamped at a low, constitutive expression level.

Bypassing the effects of *SpADH* promoter-driven *FLO11* is a relatively high bar to clear. This construct permits only ~40% of the *FLO11* transcription of haploids grown in YPD with *FLO11* under its own promoter. *FLO11* expression may fall below a "critical threshold" required for cell invasion. Nonetheless, we did find that many *dia* strains, including some that upregulate *FLO11* expression, could still invade despite this low *FLO11* expression level. In particular, strains that bud in a polar manner do not require *FLO11* upregulation to be invasive. The only exceptions are *hsl1*, *hsl7*, and *ydj1*, suggesting that increased cell-cell adhesion from higher *FLO11* expression is crucial in these strains along with polar bud site selection. In the other polar-budding strains the level of cell-cell adhesion provided by *FLO11* may contribute a basal level of cell invasion, which is enhanced by cell elongation or polarized bud site selection.

Agar invasion, flocculation, and filamentation are genetically separable processes: Among our findings is that haploid agar invasion, flocculation, diploid invasion, and diploid filamentation can be genetically separated. Each process can be activated by the MAP kinase signaling pathway, but pathways unique to each process also exist. For example, mutations that increase polar budding in haploids but do not increase *FLO11* expression (*e.g.*, *bud3*, *bud4*, *bud10*, *axl1*, *sir2*, and *sir4*) do not enhance agar invasion or diploid elongation. This class of genes identifies a haploid-specific invasive growth pathway based on polarized bud site selection. In contrast, mutations that enhance cell elongation also in-

TABLE 6

Epistasis analysis of *dia* mutants

Class	Strain	Relevant genotype	*dia bud8* invasion[a]	*dia ras2* invasion[a]	*dia kss1* invasion[a]
Standard	SKY760	*DIA*	+/−	+/−	+/−
1	SKY2575	*elm1-100*	+/−[b]	+/−	+/−
	SKY2576	*hsl1-100*	−	+/−	+/−
	SKY2577	*hsl7-100*	−	+/−	+/−
2	SKY2570	*bud3-100*	−	+/−	+/−
	SKY2571	*bud4-100*	−	+/−	+/−
	SKY2572	*bud10-100*	−	+/−	+/−
	SKY2573	*axl1-100*	−	+/−	+/−
	SKY2581	*sir2-100*	−	+/−	+/−
	SKY2582	*sir4-100*	−	+/−	+/−
	SKY2574	*bem2-100*	+/−[c]	+/−	+/−
	SKY2584	*pgi1-100*	−	+/−	−
	SKY2586	*gnd1-100*	−	+/−	−
	SKY2578	*ydj1-100*	−	+/−	−
	SKY2594	*aro7-100*	−	+/−	+/−
	SKY2602	*dia2-100*	−	+/−	+/−
3	SKY2596	*grr1-100*	+/−	+/−	+/−
	SKY2597	*cdc53-100*	+/−	+/−	+/−
4	SKY2580	*hsc82-100*		+/−	−
	SKY2579	*zuo1-100*		+/−	−
	SKY2585	*adh1-100*		+/−	+/−
	SKY2588	*cse2-100*		+/−	+/−
	SKY2601	*dia1-100*		+/−	+/−
	SKY2603	*dia3-100*	+/−	+/−	+/−
	SKY2604	*dia4-100*		+/−	+/−
	SKY2583	*gcr1-100*		+/−	+/−
	SKY2598	*ira1-100*		+/−	+/−
	SKY2600	*msn5-100*		+/−	+/−
	SKY2591	*srb8-100*		+/−	+/−
	SKY2589	*ssn3-100*	+/−	−	+/−
	SKY2590	*ssn8-100*		+/−	+/−
	SKY2599	*bpl1-100*		−	+/−
	SKY2593	*gtr1-100*		+/−	−
	SKY2587	*med1-100*		+/−	+/−
	SKY2597	*skn7-100*		+/−	+/−
	SKY2592	*taf25-100*		+/−	+/−

[a] For *dia bud8*, *dia ras2*, and *dia kss1* strain invasion scores, +/− signifies no change from *dia* strain invasion while − signifies a lower level of invasion with respect to *dia* strains.

[b] *elm1-100 bud8* strains bud in a polar manner.

[c] *bem2-100 bud8* strains bud randomly.

crease diploid invasion and filamentation but have no effect on flocculation.

Mutations that do not affect cell elongation or bud site selection have more complex effects on diploid phenotypes. Mutations that derepress *FLO11* generally also induce hyperinvasive growth in diploids. Pseudohyphal growth relies on many of the same physical processes as agar invasion including cell adhesion and polarization, so intersection of these pathways is not surprising. For example, *ira1/ira1* mutants are extremely filamentous; *adh1/adh1* mutations also enhance filamentation. However, the regulatory pathways that affect these processes also diverge. These distinctions can be quite subtle; *srb8/srb8* displays increased pseudohyphal growth but *ssn3/ssn3* and *ssn8/ssn8* do not, even though the three genes appear to function together in the RNA polymerase II complex (Balciunas and Ronne 1995). Invasion-specific pathways may diverge from pseudohyphal growth downstream or act independently of Ras2 or Kss1 as *ras2* or *kss1* mutations are readily bypassed by many *dia* mutants.

Bud site selection as a determinant of agar invasion: This screen identified polar bud site selection as a novel mechanism for inducing or enhancing yeast haploid invasive growth that can promote haploid cell invasion without upregulation of *FLO11*. We identified mutations in 15 different genes that both increase polar budding and induce invasive growth in haploids. As expected,

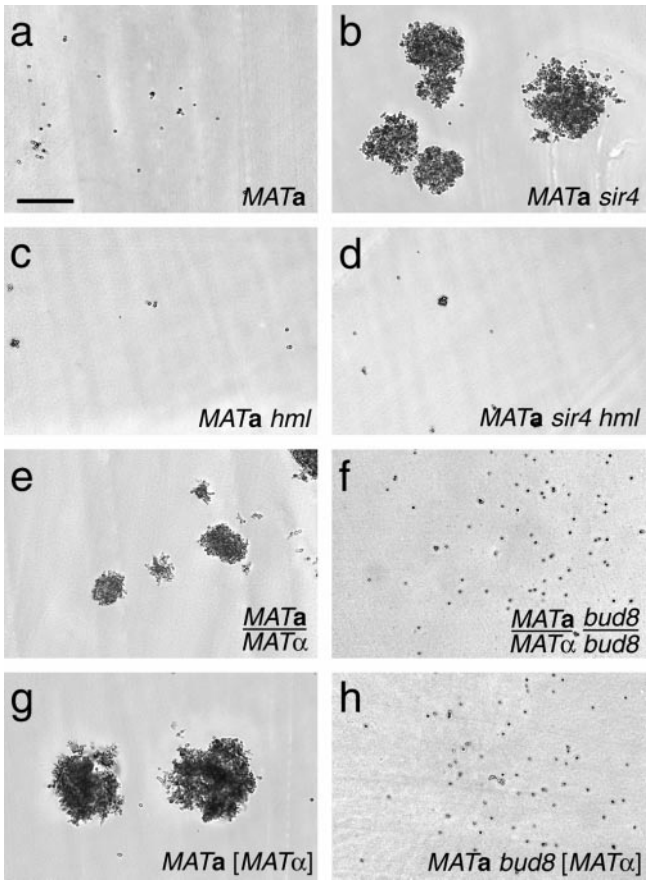

FIGURE 7.—Polar bud site selection is responsible for agar invasion in pseudodiploids and diploids. Haploid (a–c), diploid (d and e), and pseudodiploid carrying plasmid B2185 [*MAT*α *CEN URA3*] (g and h) strains were streaked on YPD to obtain single cells. The plates were incubated at 21° for 96 hr. Cells that did not penetrate the agar were removed by rubbing the plate with a finger under running water. Representative colonies were imaged. Bar, 100 μm.

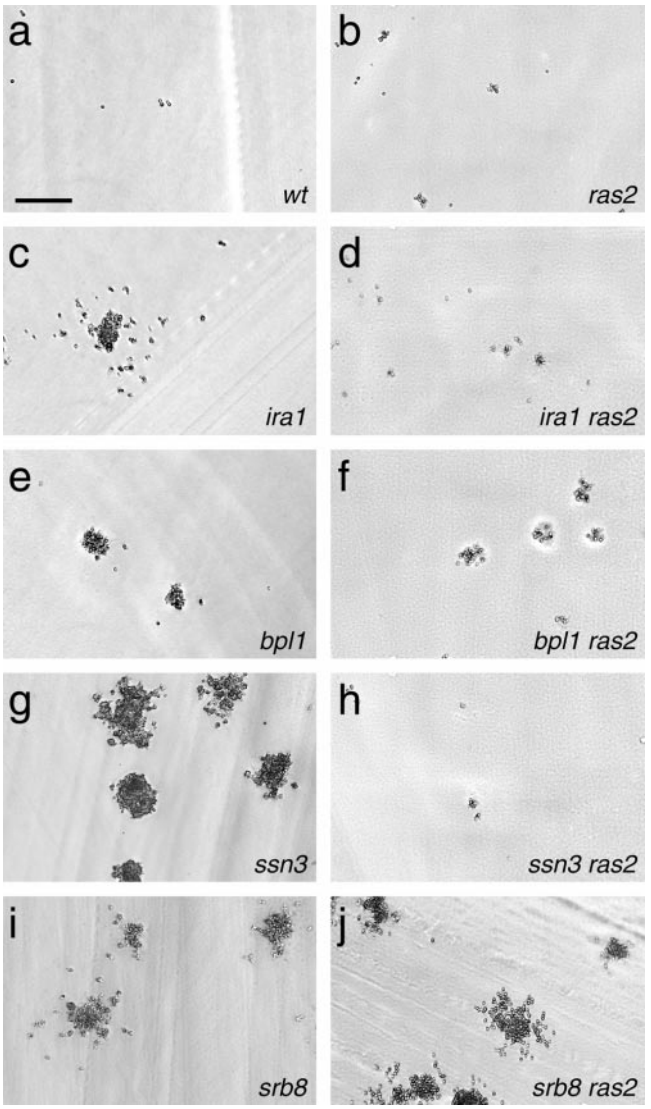

FIGURE 8.—Hyperinvasion of *dia* strains, except *ira1-100*, *bpl1-100*, and *ssn3-100*, does not require Ras2 activity. Haploid *dia* strains were streaked on YPD to obtain single cells. The plates were incubated at 21° for 96 hr. Cells that did not penetrate the agar were removed by rubbing the plate with a finger under running water. Representative colonies were imaged. *ira1-100*, *bpl1-100*, and *ssn3-100* invasion is abrogated by *RAS2* deletion but *srb8-100* invades via a *RAS2*-independent mechanism. Bar, 100 μm.

bud8 deletion confers proximal budding to each of these *dia* strains, except the *elm1* and *bem2* mutants. The loss of polar budding in the *dia bud8* strains is accompanied by suppression of the invasive phenotype, even in strains that induce *FLO11* expression (*e.g.*, *pgi1*, *gnd1*, *ydj1*, *aro7*, and *dia2*). Interestingly, polar budding in *elm1* seems to be independent of normal bud site selection processes, possibly due to septin localization and bud site misassembly at the bud tip (BOUQUIN *et al.* 2000).

Our work is distinct from previous studies (*e.g.*, ROBERTS *et al.* 1997; LO and DRANGINIS 1998; GALITSKI *et al.* 1999) that described wild-type haploid cells of the Σ1278b background as invasive and diploids as noninvasive. Consistent with our findings that polar bud site selection is sufficient to confer a *dia* phenotype, we find instead that wild-type diploids invade to a degree at least equivalent to many of our *dia* mutants. In turn, pseudodiploid strains and strains that express both mating type loci also invade significantly more than wild-type haploids. This discrepancy with the literature is likely due to differences in the protocol used to assay invasion and the definition of "invasive growth." We have used a combination of flowing water and manual force to sweep away cells that do not penetrate below the surface of the agar where other assays often rely on the flow of water alone to remove nonadherent cells from the agar plate. We have found that under running water, cell-cell and cell-agar adhesion are sufficient to permit haploid colonies to remain in place unless they are removed by rubbing the plate. Typically, no cells are observed within the agar below a haploid colony. By contrast, the surface cells of a diploid colony are

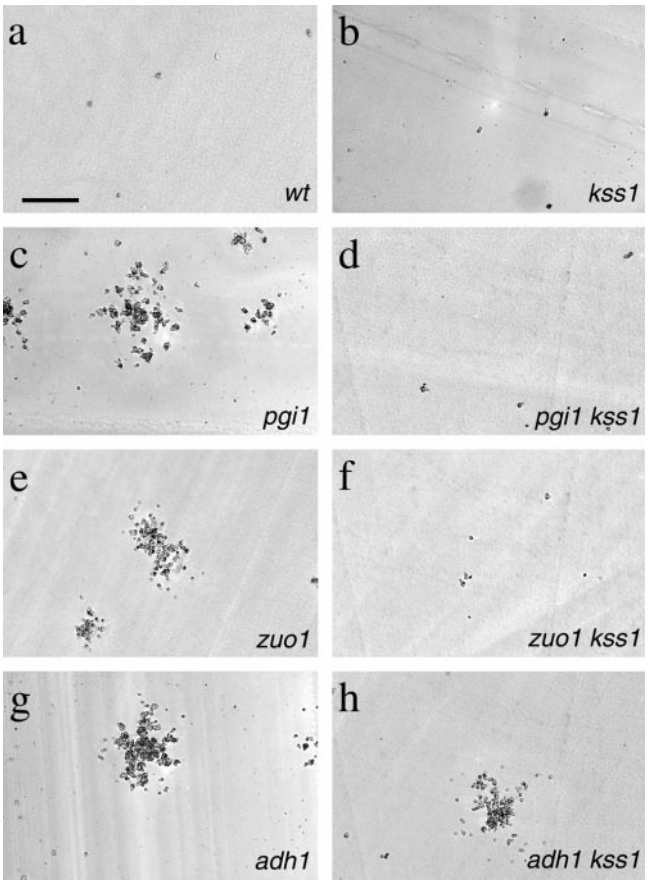

FIGURE 9.—Some *dia* strains require Kss1 activity for hyperinvasion of agar. Haploid *dia* strains were streaked on YPD to obtain single cells. The plates were incubated at 21° for 96 hr. Cells that did not penetrate the agar were removed by rubbing the plate with a finger under running water. Representative colonies were imaged. *pgi1-100* and *zuo1-100* require Kss1 to enhance agar invasion but *adh1-100* does not. Bar, 100 μm.

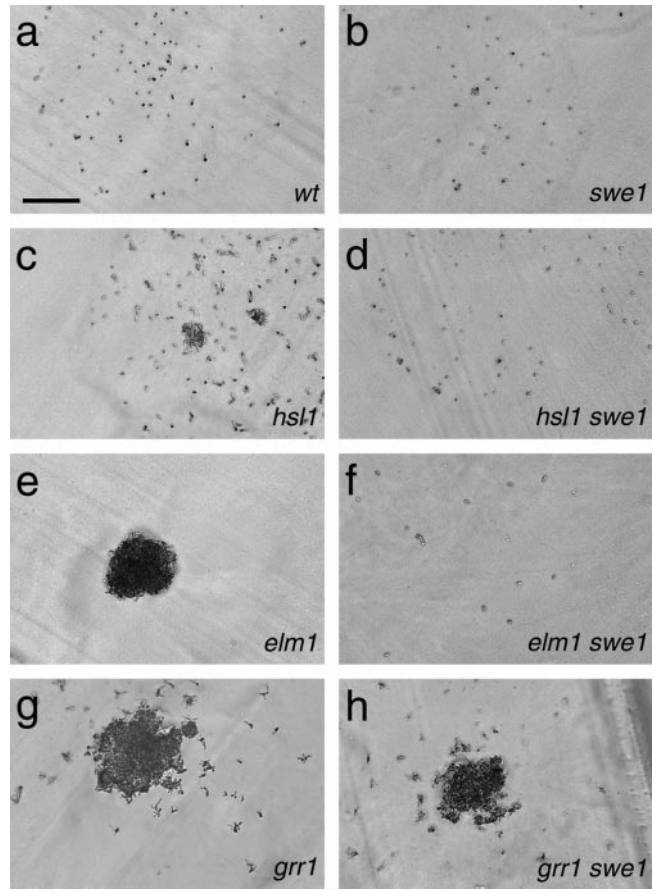

FIGURE 10.—*elm1* and *hsl7* mutant strains invade in a *SWE1*-dependent manner. Haploid *dia* strains were streaked on YPD to obtain single cells. The plates were incubated at 21° for 96 hr. Cells that did not penetrate the agar were removed by rubbing the plate with a finger under running water. Representative colonies were imaged. *pgi1-100* and *zuo1-100* require Kss1 to enhance agar invasion but *adh1-100* does not. Bar, 100 μm.

readily washed from the plate by simple rinsing but a core of the diploid colony that has penetrated the agar surface typically remains. We suggest that agar invasion measured by this method is a more reliable and specific measure of capacity for invasive growth as opposed to cell-cell adhesion.

Despite the key role of bud site selection in invasive growth, we find that, like enhanced *FLO11* expression, elongated cell morphology is sufficient to induce agar invasion. Mutations in at least two *DIA* genes, *GRR1* and *CDC53*, confer elongated cell morphology without affecting axial bud site selection. Grr1 interacts with Skp1, a component of the E3 ubiquitin-ligating enzyme complex along with Cdc53 and Cdc4 (PATTON *et al.* 1998). Presumably, mutations in this complex stabilize enhancers of polarized growth. Candidates include G_1 cyclins Cln1 and Cln2, mitotic inhibitors Sic1 and Swe1, and the Cdc42 effector Gic2 (LI and JOHNSTON 1997; JAQUENOUD *et al.* 1998; KAISER *et al.* 1998). In addition, Grr1 represses glucose-repressed genes such as *HXT* glucose transporters (LI and JOHNSTON 1997; GANCEDO 1998) and possibly other genes that inhibit invasive growth.

Signaling mechanisms that repress invasive growth: Not all the *dia* mutants participate directly in limiting *FLO11* expression, bud polarity, or cell elongation. Our screen also identified negative regulators of the signaling pathways that control these outputs. The three stress response genes, *HSC82*, *ZUO1*, and *YDJ1*, can be linked to the STE MAP kinase pathway by their dependence on intact *KSS1* for their effects. *HSC82* encodes the constitutively expressed isoform of the heat-shock protein Hsp90. Hsp90 can induce signaling through Ste11 (LOUVION *et al.* 1998), which in turn activates *FLO11* transcription. Ydj1 has a synthetic lethal interaction with Hsp90 (KIMURA *et al.* 1995). The cAMP-dependent protein kinase pathway has been implicated in suppressing certain stress response pathways, leading to invasive growth (STANHILL *et al.* 1999). Mutations in stress response genes may heighten this effect. Also, hyperos-

motic shock can induce filamentation and invasion in a Kss1-dependent manner if the high-osmolarity glycerol response pathway is compromised (Davenport *et al.* 1999).

An independent group of mutants includes the RNA Pol II subunits *SSN3*, *SSN8*, *SRB8*, *CSE2*, and *MED1*. Mutations in these subunits also release transcriptional repression of glucose-repressible genes (Balciunas and Ronne 1995; Balcuinas *et al.* 1999), providing a possible link between carbon metabolism and invasive growth. This complex may repress cell invasion in high-glucose conditions but allow invasion during glucose starvation. The glycolytic enzymes *PGI1*, *GND1*, and *ADH1* also provide a link between carbon regulation and cell invasion. Perhaps an accumulation of metabolic intermediates is responsible for the hyperinvasion. Indeed, certain nonmetabolizable alcohols can induce cell elongation and polarized budding (Lorenz *et al.* 2000a). Alcohol-induced filamentation requires an intact STE MAP kinase cascade, as does invasion due to mutations in *PGI1* and *GND1*, suggesting a link between such a metabolite and STE pathway activation rather than glucose repression *per se*.

Several *DIA* genes that likely participate in signaling have no clear links to known pathways regulating invasive growth. *GTR1*, *MED1*, and *TAF25* enhance cell invasion without altering bud site selection, cell morphology, flocculation, or *FLO11* expression. As yet unknown factors beyond elongation, polar budding, or cell adhesion may contribute to agar invasion in these mutants. One potential target is enzymatic degradation of the agar, allowing cells to penetrate without significantly increasing the force they generate. The coregulation of the *FLO11* adhesin and the *PGU1* pectinase (Madhani *et al.* 1999) as well as the *STA1*, *2*, and *3* glycoamylases (Gagiano *et al.* 1999b) provides a paradigm for coordinated regulation of cell adhesion and matrix degradation, suggesting that agar degrading activities may indeed be targets of invasive growth signaling pathways.

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