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Coregulation of Starch Degradation and Dimorphism in the Yeast *Saccharomyces cerevisiae*

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ABSTRACT: *Saccharomyces cerevisiae*, the exemplar unicellular eukaryote, can only survive and proliferate in its natural habitats through constant adaptation within the constraints of a dynamic ecosystem. In every cell cycle of *S. cerevisiae*, there is a short period in the G_1 phase of the cell cycle where "sensing" transpires; if a sufficient amount of fermentable sugars is available, the cells will initiate another round of vegetative cell division. When fermentable sugars become limiting, the yeast can execute the diauxic shift, where it reprograms its metabolism to utilize nonfermentable carbon sources. *S. cerevisiae* can also initiate the developmental program of pseudohyphal formation and invasive growth response, when essential nutrients become limiting. *S. cerevisiae* shares this growth form-switching ability with important pathogens such as the human pathogen, *Candida albicans*, and the corn smut pathogen *Ustilago maydis*. The pseudohyphal growth response of *S. cerevisiae* has mainly been implicated as a means for the yeast to search for nutrients. An important observation made was that starch-degrading *S. cerevisiae* strains have the added ability to form pseudohyphae and grow invasively into a starch-containing medium. More significantly, it was also shown that the *STA1-3* genes encoding three glucoamylase isozymes responsible for starch hydrolysis in *S. cerevisiae* are coregulated with a gene, *MUC1*, essential for pseudohyphal and invasive growth. At least two putative transcriptional activators, Mss10p and Mss11p, are involved in this regulation. The Muc1p is a putative integral membrane-bound protein similar to mammalian mucin-like proteins that have been implicated in the ability of cancer cells to invade other tissues. This provided us with an excellent example of integrative control between nutrient sensing, signaling, and differential development.

KEY WORDS: nutrient sensing, signal transduction, pseudohyphal formation, invasive growth, glucoamylases.

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I. INTRODUCTION

The "domesticated workhorse" of the baking, brewing, and wine industries, *Saccharomyces cerevisiae*, is typically isolated from its natural environments as a diploid, homothallic organism. Yeasts are usually found in alliance with animals, man, plants, and fruits, in soil as well as in fresh and marine waters, where it mainly colonizes the gut of certain fish species (Andlit et al., 1995). Here, yeast is not only an ideal experimental eukaryote or industrial fermenter, but a member of the ecosystem that consequently has an impact on the environment in which it proliferates. *S. cerevisiae* has the ability to undergo a dimorphic switch from growth in a yeast form (ovoid cells) to growth in a filamentous pseudohyphal form (Gimeno et al., 1993). It shares this trait with important animal and plant pathogens such as *Candida albicans* (candidasis) (Barki et al., 1993; Bailey et al., 1996) and *Ustilago maydis* (corn smut) (Wösten et al., 1996), where a morphology switch to the hyphal form has been linked to the ability to cause disease. Because of its long association with human food and beverage products for human consumption, *S. cerevisiae* has always been looked upon as a harmless saprophyte. Occasional reports do exist of infections where *S. cerevisiae* is the causative agent, mostly in severely debilitated, traumatized, or immune-deficient patients (McCusker et al., 1994a,b). In comparison with the "normal" saprophytic *S. cerevisiae* strains, the virulent phenotype seems to impress distinct attributes on the infectious strains (McCusker et al., 1994b). The virulent *S. cerevisiae* strains all have the ability to form pseudohyphae and in addition proliferate at suboptimal, elevated temperatures (37°C, 39°C, and 42°C) (McCusker et al., 1994b), traits shared with pathogenic *C. albicans* strains. Although suspected as a virulence factor, the pseudohyphal and invasive growth phenomena have been linked to foraging for nutrients in *S. cerevisiae*, a hypothesis that complements our observation that starch-degrading *S. cerevisiae* strains form extensive networks of filaments when grown on complex polysaccharides.

A small group of *Saccharomyces* strains designated *S. cerevisiae* var. *diastaticus*, hydrolyze starch-polysaccharides by secreting glucoamylases. The study of these glucoamylases and the regulation of their encoding genes (*STA1, STA2, STA3*, and *SGA1*) has been the focus of our laboratory for several years. While studying the glucoamylase multigene family, we observed that starch-degrading strains of *S. cerevisiae* show a strong pseudohyphal growth pattern when grown on starch-containing media (Lambrechts et al., 1996a). Further analysis revealed that the induction of genes involved in the starch-degradation process is coregulated with the recently cloned *MUC1* gene shown to be critical for pseudohyphal differentiation in yeast. The *MUC1* gene encodes a mucin-like protein (Lambrechts et al., 1996a) and is regulated by a putative transcriptional activator of the *STA1-3* genes *MSS10* (Lambrechts et al., 1996b). In other words, such diverse activities as the metabolic breakdown of starch and the differential development pathway of pseudohyphal growth in yeast unexpectedly share some elements of regulation. This observed coregulation is discussed in more detail in further sections of this review.

In previous relevant reviews the phenomena of pseudohyphal differentiation in yeast (Gimeno et al., 1993), the utilization of polysaccharides by *S. cerevisiae* (Pretorius, 1997), the properties of yeast glucoamylases, the structure of the *STA1-3* and *SGA1* genes (Pretorius et al., 1991), as well as the regulation of the glucoamylase multigene family in *S. cerevisiae* (Lambrechts et al., 1994b) have been discussed thoroughly. Here we focus on three overriding subjects intertwined

into the sections to follow. We state the current status of the glucoamylase genes and their regulation as well as discuss the functions and mutual relationships of the recently cloned genes involved in glucoamylase expression and pseudohyphal growth. We also explore the implications of the recently observed coregulation of the glucoamylase genes and the cell differentiation pathway of pseudohyphal growth.

A. Starch Utilization in Yeast

Starch is an abundant plant material, and various living organisms have a complement of enzymes responsible for degradation of these energy stores. Of the approximately 600 yeast species that are currently recognized, 150 contain strains that are capable of using starch as carbon and energy sources (Pretorius, 1997). Some yeasts degrade starch with great efficacy such as *Endomyces fibuliger*, which expresses a raw starch-degrading glucoamylase in addition to an α-amylase with debranching activity. The glucoamylases from *S. cerevisiae* var. *diastaticus* are encoded by a multigene family (*STA1-3*) that release single glucose residues from the nonreducing end of the starch polymer, endowing the yeast with a rather poor ability to hydrolyze soluble starch. The initial interest in amylolytic enzymes was industrially based; the brewing and baking industries have a huge need for these enzymes in various processes. Fittingly then that the first starch-degrading *S. cerevisiae* strain was isolated from superattenuated beer by Andrews and Gilliland (1952). Since then the possibility to genetically engineer microorganisms became a reality, and "improved" starch-degrading yeasts were some of the first recombinant organisms constructed. To this end, our laboratory has cloned and co-expressed the *STA2* glucoamylase from *S. cerevisiae*, the *Bacillus amyloliquefaciens* α-amylase (*AMY1*), and the *Klebsiella pneumoniae* pullulanase (*PUL1*) in a recombinant *S. cerevisiae* strain capable of assimilating 99% of the starch provided in its growth medium (Janse and Pretorius, 1995). We have also expressed the raw starch-degrading amylases of *E. fibuliger* and *Lipomyces kononenkoae* in *S. cerevisiae* (Steyn and Pretorius, 1996). Phenotypically, starch-degrading *Saccharomyces* strains are easy to recognize because a halo with a characteristic precipitation ring appears around the colonies when grown on solid starch media. In addition, the cells form distinctive hyphal-like extensions, pseudohyphae, and grow invasively into the solid, starch-containing media.

B. Pseudohyphal Formation in Yeast

Pseudohyphal differentiation was initially ascribed to diploid *S. cerevisiae* cells under nitrogen stress (Gimeno et al., 1992). Pseudohyphal development is attributed to the ability of the yeast cell to switch from an axial to a polarized growth pattern. A pseudohypha is defined as a chain of elongated cells that arose through polarized budding and failed to detach from adjacent cells. Dickinson (1996) suggested that one needs to differentiate between a pseudohypha and a hyphal-like extension. He defines a pseudohypha as "a chain of regular-shaped, elongated cells in which polar budding predominates". A hyphal-like extension is "a structure originating from a bud which has undergone abnormal length extensions so as to exceed the length of the mother cell from which it arose, but without the isotopic swelling characteristic of the normal yeast cell cycle" (Dickinson, 1996). More recently, a pseudohyphal growth

pattern has also been described for haploid cells (Kron et al., 1994; Roberts and Fink, 1994; Lambrechts et al., 1996a). The phenomenon of forming elongated cells is probably an adaptation of *S. cerevisiae* strains to expand their periphery and forage for distant nutrients, whereas in other dimorphic fungi the filamentous stage is important in animal and plant pathogenic behavior. Having said that, increasing numbers of instances are reported where *S. cerevisiae* strains are implicated as pathogens in patients with immune deficiency. Studies of these virulent strains revealed that they differ from nonpathogenic strains through their ability to grow at elevated temperatures and their ability to form pseudohyphae (McCusker et al., 1994b).

The ability of fungi to vary their growth polarity enables them to produce cells of varying shape (and function) that include yeast-like (ellipsoidal), spores (spherical), and hyphae (elongated filaments). It is suspected that pathways controlling growth form transitions in fungi have been evolutionary conserved (Gavrias et al., 1996). Normal budding yeast cells exhibit a short period in the late G_1 phase of the cell cycle, where growth is hyperpolarized to allow bud emergence; budding in these instances is asynchronous (Pringle and Hartwell, 1981). In contrast, when pseudohyphae are formed, cells have a prolonged period of hyperpolarization extending well into the G_2 phase. In addition, the mother and daughter cells divide when the daughter cell is fully grown and budding is synchronized (Kron et al., 1994). How this dramatic change in cell cycle regulation is obtained is still obscure. Interestingly, yeast mutants that result in stabilization of the G_1 cyclins, Cln1p and Cln2p, exhibit prolonged polarized growth that eventuate in constitutive pseudohyphal formation. These mutants are defective in *GRR1*, encoding a protein implicated in several nutrient-linked signaling pathways (Barral et al., 1995). Although information on the signaling pathways resulting in this morphogenetic developmental switch in budding yeast is still fragmentary, a few putative role players have been implied. For one, a direct and/or indirect role of the Ras-cAMP pathway has been suggested (Gimeno et al., 1992; Mösch et al., 1996). Also, pseudohyphal growth in *S. cerevisiae* requires elements of the mitogen-activated protein (MAPK) cascade of the mating pheromone signal transduction pathway. Several of the *STE* genes (*STE7*, *STE11*, *STE12*, *STE20*) have also been implicated as role players (Liu et al., 1993). A host of other genes, including *PHD1-7* (Gimeno and Fink, 1994), *MUC1* (Lambrechts et al., 1996a), *TEC1* (Gavrias et al., 1996), *SOK2* (Ward et al., 1995), *MSS10* (Lambrechts et al., 1996a,b), and *MSS11* (Webber et al., 1997) are involved in pseudohyphal development. *PHD1* induces pseudohyphal growth when present in multiple copies as does *MUC1*. *TEC1* is a transcriptional activator, whereas *SOK2* encodes an inhibitor of pseudohyphal differentiation; it shares a high degree of similarity in its DNA-binding motif with that of Phd1p (Ward et al., 1995). *SOK2* plays a general role in the cAMP-dependent protein kinase A (PKA) signal transduction pathway, probably acting downstream of PKA, mediating regulation of genes important in growth and development (Ward et al., 1995). *MSS10* encodes a transcriptional activator that acts on the *STA1-3* genes as well as *MUC1*, a gene critical for pseudohyphal development. *MSS11* encodes a novel protein that exerts the same effect on the *STA1-3* and *MUC1* genes as does *MSS10* (Webber et al., 1997). Mutations in three other unlinked gene loci, *ELM1*, *ELM2*, and *ELM3*, cause constitutive pseudohyphal development. The intergenic relationship of these genes, their mechanism of regulation, and their connection to the MAPK cascade are still obscure and are discussed in the last section of this review, with specific reference to the role of the *MUC1* gene and its shared regulation with the glucoamylase

genes. Because *MUC1* is involved in pseudohyphae formation and invasive growth in *S. cerevisiae*, it seems reasonable to suspect that homologues or functional analogues of this gene are found in other pseudohyphal yeasts. This aspect could prove to be important if these putative homologues play a role in pathogenic yeasts' ability to become virulent by producing pseudohyphae.

II. THE STRUCTURE OF THE GLUCOAMYLASE AND MUCIN GENES AND THE PROPERTIES OF THEIR ENCODED PROTEINS

A. Evolution of the *STA1-3* and *SGA1* Glucoamylase Genes

In *S. cerevisiae* var. *diastaticus* (previously designated *S. diastaticus*), the ability to ferment starch is attributed to the presence of any one of the heavily glycosylated, secreted glucoamylase isozymes Sta1p, Sta2p, or Sta3p (also known as GAI, GAII, or GAIII) (Pretorius et al., 1991). Their encoding genes are three polymeric genes designated *STA1* (*DEX2, MAL5*), *STA2* (*DEX1*), and *STA3* (*DEX3*) (Erratt and Stewart, 1978; Yamashita and Fukui, 1983b, 1984a,b; Erratt and Nasim, 1986a; Pretorius et al., 1986a,b) of which only one is sufficient to ferment starch. While the *STA1-3* genes are only prevalent in *S. cerevisiae* var. *diastaticus* strains, an intracellular glucoamylase gene, *SGA1*, is found in virtually all strains of *S. cerevisiae*. The intracellular glucoamylase activity appears specifically in sporulating cells heterozygous for the mating type locus (*MAT*) (Yamashita and Fukui, 1985). Although *SGA1* encodes a glycogen-hydrolyzing glucoamylase (sporulation-specific glucoamylase, Sga1p or SGA), which is only expressed late in meiotic development, the gene is dispensable for sporulation (Kihara et al., 1991).

Cloning and sequencing of the *STA1*-3 and *SGA1* genes revealed extensive sequence similarities between them (Figure 1) (Yamashita and Fukui, 1983b; Yamashita et al., 1985b; Erratt and Nasim, 1986b; Pardo et al., 1986; Pretorius et al., 1986a,b; Yamashita et al., 1987; Lambrechts et al., 1991). DNA hybridization studies also showed that both starch-fermenting as well as starch nonfermenting strains carried DNA sequences (designated *S1* and *S2*) highly homologous to the 5′ regions of the extracellular glucoamylase genes (Yamashita et al., 1985a; Pretorius et al., 1986a). *S1* and *S2* were later partly cloned and sequenced by Yamashita et al. (1987), but no function could be ascribed to them at that stage.

The obvious sequence similarities of *S1* and *S2* with the 5′ regions of the *STA1-3* genes together with the homology of *SGA1* with the middle and 3′ regions of the *STA1-3* genes led to the proposal that the ancestral *STA* gene developed through gene fusions of these resident DNA sequences (Yamashita et al., 1987). It was postulated that *S2, S1*, and *SGA1* encode homologues to the hydrophobic leader peptide for protein secretion, the Thr/Ser-rich domain, which has a possible role in starch binding and catalytic activity, and the catalytic domain of the *STA1-3*-encoded glucoamylases, respectively. Mapping studies showed that the *STA1-3* genes were dispersed to different chromosomes (chromosomes IV, II, and XIV for *STA1, STA2*, and *STA3*, respectively) (Pretorius and Marmur, 1988; Bignell and Evans, 1990). The putative progenitors of the ancestral *STA* gene, *S1, S2*, and *SGA1*, all mapped to chromosome IX; *SGA1* is located on the left arm, 32 kb proximal of *HIS5*, whereas both *S1* and *S2* mapped to the right arm, next to each other and close to *DAL81* (Lambrechts et al., 1995). Unexpectedly, cloning and sequencing of DNA frag-

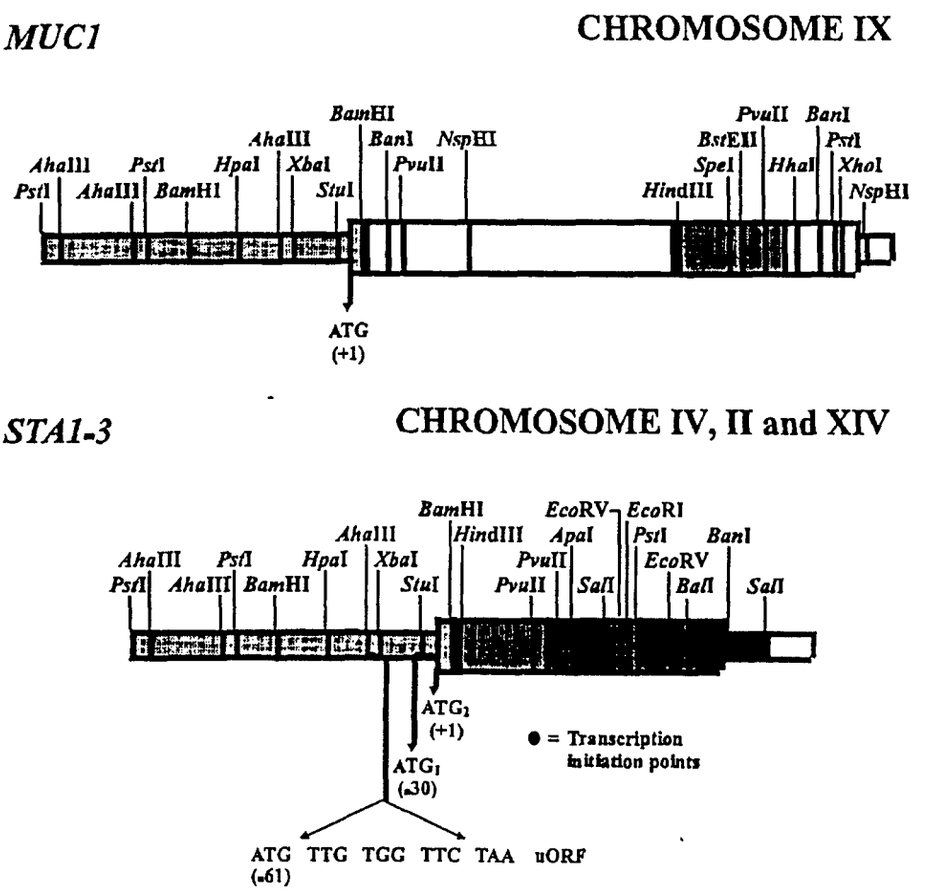

FIGURE 1. Homology between the *STA1-3*, *SGA1* and *MUC1* genes on the nucleotide level. *STA1-3* have identical restriction maps and nucleotide sequences. The *STA1-3* genes consist of an unique ORF of 2286 nt with two functional, in-frame ATG codons 30 nt apart. In relation to the second ATG codon, the transcriptional initiation sites are located at -13, -23, -26 and -100 (Lambrechts et al., 1991). The *SGA1* gene consists of an ORF that encodes 510 amino acids (Yamashita et al., 1987) and a putative AT-rich promoter (Pardo et al., 1988). The sequences spanning Phe-33 to Asn-510 in *SGA1* are virtually identical to the sequences located between Phe-290 to Asn-767 of the *STA1* and *STA2* genes (Yamashita et al., 1987). Sequence comparison between the *STA1-3* and *MUC1* genes showed an overall identity of 97% from nt -2547 to +96, consisting of the promoter areas and the first part of the ORFs of these genes. In addition, part of the Thr/Ser-rich tract (amino acids 869 to 1172) of Muc1p is also homologous to amino acids 31 to 286 of Sta1p, Sta2p and Sta3p except that the peptide from Val-84 to Thr-127 of the *STA1-3* encoded glucoamylases was duplicated in the Muc1p (Lambrechts et al., 1996a).

ments containing *S1* and *S2* revealed that they are part of one gene (Lambrechts et al., 1996a). After analysis, it became clear that the protein encoded by this *S1-S2* gene has extensive structural homology with mammalian membrane-bound mucins and was therefore designated *MUC1* (Lambrechts et al., 1996a). Sequence homology between *STA1-3* and *MUC1*, as well as the proposed role of *MUC1* in the evolution of the glucoamylase-encoding genes are depicted in Figures 1 and 2, respectively. The initial evolutionary fusion event of the progenitor sequences most likely was followed by the dispersal of the genes to their locations by telomeric rearrangements. The fact that the *STA2* gene (and most probably *STA1* and *STA3*) has a subtelomeric arrangement (*STA2* and the melibiase gene, *MEL1*, were reported to be idiomorphic and mapped to the end of the left arm of chromosome II; Lyness et al., 1993) confirms this notion.

B. Structure of the Glucoamylase and Mucin Genes

1. *The* STA1-*3 and* MUC1 *Genes*

Because the promoter area and the first 96 nucleotides (nt) of the *MUC1* open reading frame (ORF) are 97% homologous to that of the *STA1-3* genes (Figure 1), the following discussion of the *STA1-3* promoter automatically includes characteristics of the *MUC1* promoter; differences are pointed out.

In the course of our studies, we found that the intricate regulatory behavior of *STA2* (and by extension *STA1*, *STA3*, and *MUC1*) is the cause of a complex promoter containing several regulatory regions (Figure 3). These genes' promoters (2500 bp) are exceptionally long in comparison with the typical

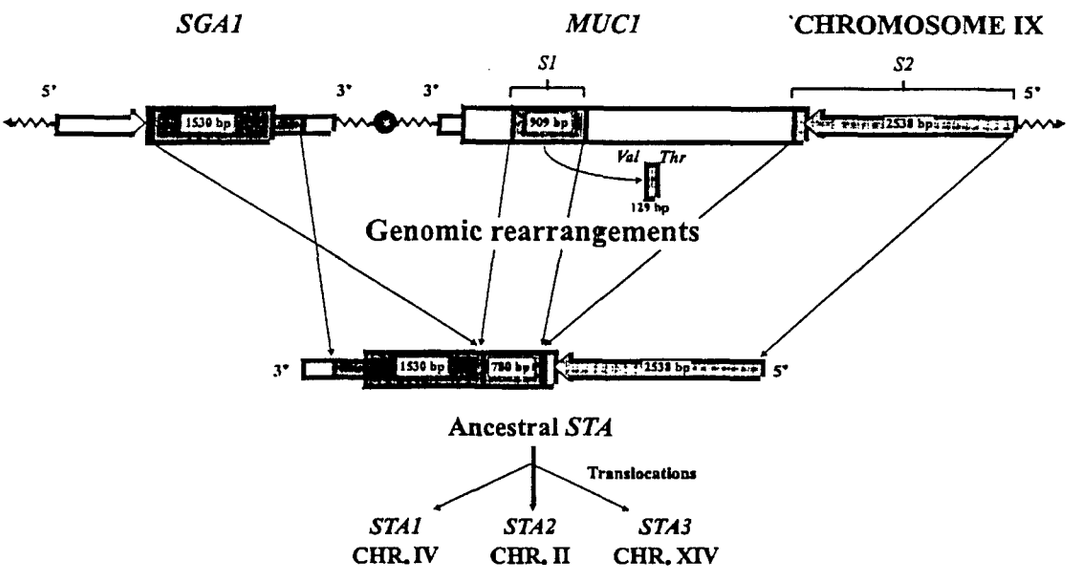

FIGURE 2. A gene-fusion model as a putative mechanism of evolution of the *STA1-3* genes also depicting the physical maps of *SGA1* and the progenitor sequences from *MUC1 (S1* and *S2).* The ancestral *STA* gene arose through separate fusion of *S2-S1* and *S1-SGA1* resulting in a configuration of *S2-S1-SGA1* after the direct repeat of Val-84 to Thr-127 was deleted (Yamashita et al., 1987). In this configuration the *SGA1*-encoded region is responsible for the catalytic action, whereas *S2-S1* provide the promoter, the secretion signal as well as a starch binding domain.

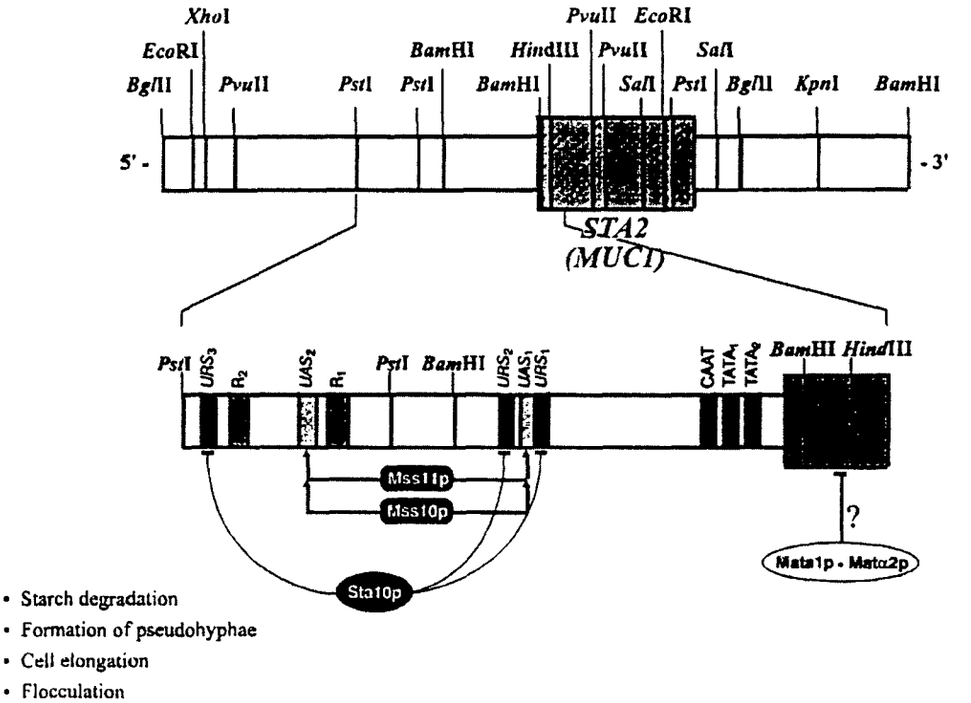

FIGURE 3. A model for the interaction of regulatory proteins of the glucoamylase system through the *cis*-acting regulatory sequences in the promoters of the *STA1-3* genes. Because the promoter regions of the *STA1-3* and *MUC1* genes are 97% homologous, the depicted scheme include *MUC1* by extension. The 2547 bp promoter contains two putative TATA sequences; $TATA_1$ between nt –100 to –94 (TATAAA) and $TATA_2$ between –70 to –75 (TATAAT). Two upstream activation sequences (UAS_1 from nt –1026 to –1390 and UAS_2 from –1815 to –1940) and two areas for down-regulation were identified between nt –1940 to –2457 (R_2) and –1390 to –1815 (R_1) possibly interacting with the global activators and repressors as described in Table 1. Three operator regions responsive to Sta10p repression were identified: URS_1 is located between –1026 and –1390, URS_2 between nt –1390 and –1650 and URS_3 upstream from nt –2457. Mat1p-Mat2p mediated repression either affects *STA1-3* expression downstream from the translational start site or indirectly.

yeast promoter (Lambrechts et al., 1994a). The 5′ region of *STA1-3* is one of the few examples where two AUG translation initiation codons are present in the same reading frame. Both proved to be functional translational initiation codons; however, the second AUG (although of comparable sequence context) seems to be the preferred site (Lambrechts et al., 1991). In addition, the positions of the major transcriptional initiation sites preclude the upstream AUG, confirming that the majority of the proteins initiate at AUG_2 (Figure 1). Some transcripts do, however, initiate further upstream to secure the first AUG as a functional translation initiator (Pardo et al., 1988; Lambrechts et al., 1991). Investigations into the functionality of this arrangement revealed that localization differences exist between proteins initiated at either of the two AUG codons. Mutant analysis showed that when proteins are initiated at the upstream AUG and thus have a N-terminal extension of 10 amino acids, secretion into the culture medium is more efficient (Vivier et al., 1997).

Evidence is mounting that these 10 amino acids function as an extra signal sequence in the secretion of glucoamylases. The presence of such a signal has been confirmed with the determination of the N-terminus of purified Sta2p. Two protein species were identified: a major fraction of the proteins started at amino acid +22 (AUG_2 being amino acid +1) and a minor fraction commenced at amino acid +1 (Vivier et al., 1997). Our hypothesis is that the signal with the downstream processing site is sufficient to deliver the proteins to the periplasmic space, after which secretion into the medium progresses undirected, driven by the saturation levels of the protein. When the additional signal sequence is present, however, secretion into the medium seems to progress via a more direct route. Consistent with these results, it was indicated that even when heterologous proteins are fused to both the downstream signal and the extra 10 amino acids, the proteins are efficiently secreted (Vanoni et al., 1994). One of the differences between the *STA1-3* genes and *MUC1* is that the *MUC1* ORF only contains ATG_2 (ATG_1 present in the *STA1-3* promoters is mutated to TTT in *MUC1*). It is tempting to speculate that the lack of the upstream ATG, and thus the extra secretion signal, plays a role in Muc1p being membrane-bound such as its mammalian structural homologue.

The primary sequence of *STA1-3* also revealed a short upstream ORF (uORF) of four amino acids present in the longer transcripts encoding Sta2p. In the well-studied *GCN4* system, the 5′ leader of *GCN4* (a positive regulator of general amino acid biosynthetic genes) mRNA is unusually long and contains four short uORFs. These sequences normally block translation of the *GCN4* mRNA. However, during amino acid starvation their inhibitory effect is overcome and translation of *GCN4* mRNA becomes more efficient (Hinnebusch, 1986; Miller and Hinnebusch, 1990). In this system, the suppression of ribosomal reinitiation at some of the uORFs forms the basis for *GCN4* control. To establish whether the uORF in the *STA1-3* promoter is in fact translated, a fusion was made between the *lacZ* gene and the ATG and the *STA2* uORF encoding the next three amino acids. Results showed that this ATG codon failed to initiate translation of the reporter gene's transcript. A process of "leaky" scanning must be employed by the preinitiation-complex to overlook the upstream AUG (probably due to an unfavorable context) and initiate at the *STA1-3* initiator codons (Vivier et al., 1997).

Two putative TATA elements are present in the native *STA1-3* promoter, $TATA_1$ conforming completely to the consensus sequence TATAAA and $TATA_2$ with the sequence TATAAT (Shima et al., 1989; Lambrechts et al., 1991). Shima et al. (1989) deleted the upstream TATA and concluded that although it had no effect on the total level of *STA1* mRNA, it enhanced proper transcriptional initiation. These deletions of the consensus sequence included removal of up to 23 surrounding base pairs and/or the insertion of palindromic sequences, which could greatly influence the inherent transcriptional activity. In contrast, studies with the *STA2* system clearly showed that $TATA_1$ is the functional TATA-element and removal of this sequence resulted in the complete obliteration of glucoamylase transcripts (Vivier and Pretorius, 1997).

Expression of the *STA1-3* genes were previously shown to be regulated transcriptionally by both positive and negative factors. To identify the *cis*-acting elements responsible for transcriptional activation, as well as the transcriptional repressor effects of *STA10* and *MATa/MATα*, sequential and internal deletions were constructed in the promoter of *STA2*. The areas identified to be involved in glucoamylase expression are shown in Figure 3. Two upstream activating sequences (*UASs*), designated UAS_1 and UAS_2

were identified and confirmed as areas of *STA2* activation with fusions to the *CYC1 UAS*-less promoter employing the *LacZ* gene as a reporter (Lambrechts et al., 1994a). Again, compared with the general structure of promoters in *S. cerevisiae*, the regions are abnormally far upstream from the translational start codon. Two areas for down-regulation of glucoamylase expression have been identified, designated R_1 and R_2. Also, three repression-target (operator) regions responsive to *STA10* repression were identified: URS_1, URS_2, and URS_3. *STA10* repression on *STA2* was not alleviated when a deletion mutant of the GTACAAG motif, a putative Sta10p responsive motif suggested by Claros et al. (1992), was introduced into a *STA10* strain.

Expression of the *STA1-3* genes are suppressed in some but not all sporulation-competent diploids (Patel et al., 1990); repression has been reported to be regulated by the *MATa1/MATα2*-encoded repressor (Pretorius et al., 1986c; Dranginis, 1989). Although a putative consensus sequence for the site of repression by Mata1p-Matα2p was previously identified in the *STA1-3* promoter (Lambrechts et al., 1991), no change in glucoamylase expression could be detected with mutants containing various deletions in the promoter area (Lambrechts et al., 1994a). In fact, replacement of the *STA2* promoter with the modified alcohol dehydrogenase I (*ADH1*) promoter (Bennetzen and Hall, 1982) still resulted in diploid repression of *STA2*. Thus, the Mata1p-Matα2p-mediated repression either effects the *STA1-3* genes downstream from the translational start site or indirectly.

2. *The* SGA1 *Gene*

The intracellular glucoamylase gene is a member of the late class of sporulation-specific genes. Homology between *SGA1* and the *STA1-3* genes is shown in Figure 1. Transcription of *SGA1* is under both positive and negative control and is most likely conducted through the *cis*-acting elements found in the promoter area. The action of a 19-bp *UAS* was shown to be constitutive and not requiring heterozygosity at the *MAT* locus. A 49-bp negative regulating element *(NRE)* was also identified (Kihara et al., 1991). A model for the regulation of *SGA1* expression (Figure 4) through these sequences and the genes responsible for modulating these effects are discussed in the next section.

C. Protein Structure and Localization of the Glucoamylase and Mucin-Like Proteins

Although extensive sequence homology exists between the *STA1-3* and *SGA1* genes and between *STA1-3* and *MUC1*, the locations, functions, and properties of their encoded products are quite diverse. In essence, this means that genes involved in polysaccharide metabolism share considerable homology with a gene, *MUC1*, critical for the cellular differentiation pathway of pseudohyphal growth. The structure of these proteins might contain some hints explaining their diverse functions but shared regulation.

1. *The* STA1-3-*Encoded Glucoamylases*

The Sta1p, Sta2p, and Sta3p isozymes contain three characteristic regions, a hydrophobic leader peptide (HL), a Thr/Ser-rich tract (TS), and a catalytic domain (CD). These isozymes have been isolated by various groups and although conflicting reports exist concerning their sizes, subunit structure, pH and tem-

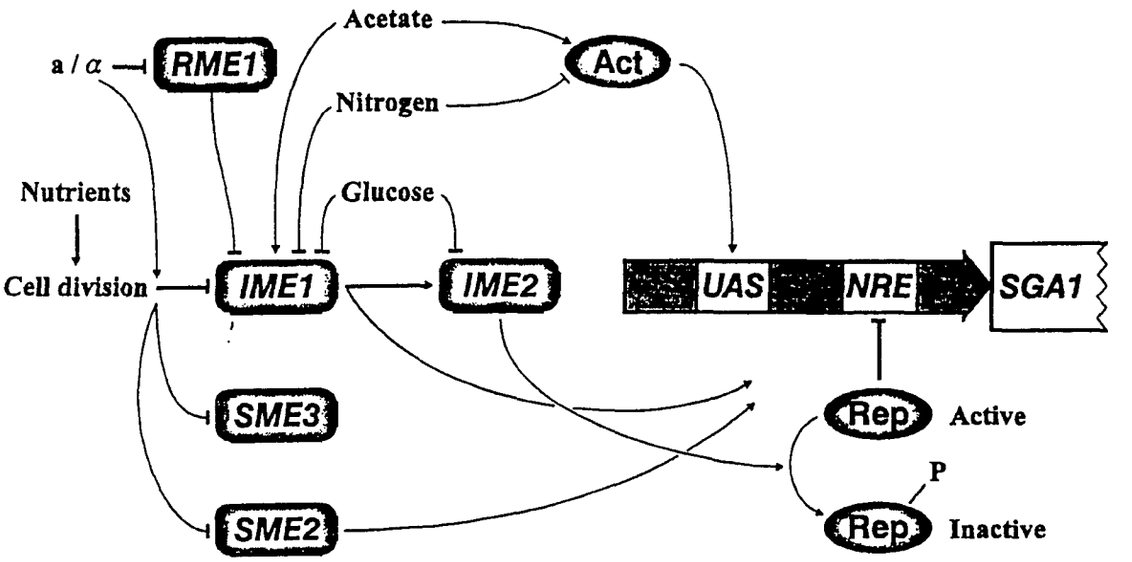

FIGURE 4. The regulatory cascade modulating the expression of *SGA1*. Regulation occurs via the putative presence of an activator and repressor protein acting on an *UAS* located between nt −233 and −224 and a negative regulating element (*NRE*, located between nt −183 and −135). *SGA1* is induced by the presence of the inducer genes for meiosis, *IME1* and *IME2*, whereas transcription of *SGA1* is regulated negatively by both nutritional signals and the gene product of a haploid-specific negative regulator gene for meiosis, *RME1*. During meiosis, the *IME1* gene product activates, the *IME2* product which causes derepression of *SGA1* via the *NRE* element (Kihara et al., 1991). Both *IME1* and *IME2* are negatively regulated by glucose and nitrogen and require acetate for full expression (Kawaguchi et al., 1992). *SME2* and *SME3* encodes positive regulators of transcription for *SGA1* and *IME1*, respectively. During conditions of nitrogen and carbon starvation, *SME3* is activated, which in turn activates *IME1*. The *IME1* gene product activates *IME2*, which in turn activates *SGA1*. The *SME2* gene product also activates *SGA1*.

perature optima, the heavily glycosylated nature of these proteins is uniformly accepted (Yamashita et al., 1984a,b; Kleinman et al., 1988). Deglycosylation studies showed that the carbohydrate fraction, consisting mainly of mannose residues, contributes approximately 80% to the molecular weight of the Sta2p (Modena et al., 1986). The current model of Sta2p suggests the presence of two identical subunits, whose average sizes are 300 kDa. Under denaturing conditions the native dimeric enzyme readily dissociates to monomers. Carbohydrates are linked to the protein via both *N*- and *O*-bonds; deglycosylation of the Sta2p yielded a protein of 56 kDa (Modena et al., 1986). The fact that glycosylation stabilizes many proteins is well documented and may explain why the *S. cerevisiae* glucoamylases are extremely stable and active over a broad pH and temperature range.

Given the identical restriction maps and nucleotide sequences of *STA1-3*, the differences in molecular weights and subunit structures reported for Sta1p, Sta2p, and Sta3p are unexpected. The inherent heterogeneity of glycoproteins, the different purification protocols, and differences in glycosylation patterns among different yeast strains can partially account for the variation in molecular weights among extracellular glucoamylase isozymes prepared in different laboratories (Modena et al., 1986).

N-terminal sequencing of the Sta1p protein yielded a major protein species starting

at amino acids +22 (if AUG_2 is considered to encode amino acid +1) (Yamashita et al., 1986). Significant amounts of secreted glucoamylase is located within the yeast periplasmic space. The yeast mating pheromone α factor (*MFα1*) secretion signal has been widely used to secrete heterologous gene products into the culture medium. However, replacement of the *STA2* secretion signal with that of *MFα1* did not result in an increase over wild-type levels of glucoamylase in the culture medium (D'Aguanno and Pretorius, 1994).

The heavily glycosylated nature of glucoamylases have prevented their three-dimensional study with X-ray crystallography. The structure of a partially deglycosylated, yet catalytically active, fragment of an *Aspergillus awamori* glucoamylase has been resolved to 2.2 Å resolution (Aleshin et al., 1992). They detected 13 α-helices of which 12 were in an α/α-barrel motif. An inner core of six mutually parallel α-helices are connected to each other through a peripheral set of six α-helices. The putative active site presumably is in the packaging void of the inner set of helices. The last 30 residues of the protein constitutes part of the putative starch-binding domain with several *O*-glycosylation domains and is in an extended conformation wrapped around the "waist" of the α/α-barrel (Aleshin et al., 1992). The fact that most glucoamylases share a similar structure, the TS-region of Sta2p is probably also in an extended and accessible conformation assisting in the starch-binding activity this region is suspected to have.

2. The SGA1-Encoded Sporulation-Specific Glucoamylase

Sga1p is translated from a 2.0-kb transcript (Pretorius et al., 1986c) and has an apparent size of 90 kDa (Pugh et al., 1989). The native protein is a dimer of 190 kDa, which dissociates during purification to yield active monomers of 90 kDa. Sga1p is glycosylated and located in the yeast vacuole, thus containing information to enter into the early stages of the secretory pathway.

3. The MUC1-Encoded Mucin-Like Protein

In general, mucins are best known to be the major component of mucus. Mucins can be structurally defined as large (typically > 200 kDa), viscous glycoproteins composed of approximately 75% carbohydrates linked via *O*-glycosidic bonds to Ser- or Thr-residues (Bansil et al., 1995). In mammals, both secreted- and membrane-bound forms of mucins were identified with characteristic repetitive sequences of many Ser- and Thr-residues in the protein backbone as a common feature. In addition, the mucin domains contain many Pro- and other helix-breaking amino acids that in conjunction with the numerous *O*-glycosylation sites result in rigid domains several hundred nanometers long that possibly extend beyond the glycocalyx (Hilkens et al., 1992). The best studied example of cell membrane-associated mucins is the episialin protein in humans; the structure of this protein can be used as a reference of this class of mucins. The protruding mucin-like domains of these proteins that extend beyond the glycocalyx of the cells strongly reduce cellular adhesion. In malignant tumors, polarization often becomes aberrant, which could lead to the abnormal expression of the mucin proteins on the entire cell surface, resulting in strongly reduced adhesion and immune recognition properties that may play an important role in invasion and spread of the tumor cells (Hilkens et al., 1992). The

S. cerevisiae MUC1-encoded mucin-like protein is structurally closely related to the episialin class of mucins and is critical for the invasive and pseudohyphal growth patterns observed in *S. cerevisiae* (Lambrechts et al., 1996a).

Muc1p is translated from a 4.3-kb transcript and has a predicted molecular weight of 136 kDa. Extensive sequence and structural homologies exist between Muc1p and mammalian mucin proteins, as well as yeast agglutinin and flocculation proteins (Lambrechts et al., 1996a). A hydrophobicity blot of the predicted protein showed the N- and C-terminal regions to be hydrophobic with the central part hydrophilic. The putative signal peptide is in fact the sequence implicated for secretion of the glucoamylases to mostly the periplasmic space (Lambrechts et al., 1996a; Yamashita et al., 1986). Muc1p is extremely rich in Ser, Thr, and Pro, as is the case for mammalian mucins, and consists of several repetitive sequences spanning the central part of the molecule, a feature distinctive of membrane-bound mucins in mammals. In addition, the protein contains putative transmembrane domain(s) at its C-terminal. The predicted secondary structure shows that Muc1p is composed of consecutive β-sheets, coils, and extended regions with α-helices only at the N- and C-terminal regions. The central part of Muc1p is presumably a rigid, stem-like structure due to the predominance of Pro (a helix-breaking amino acid) in the central repeat units. The possible *O*-glycosylation of the copious Ser- and Thr-residues would add to the extended conformation as is the case for mammalian mucins. The Muc1p is in addition structurally related to the proteins encoded by the flocculation genes. In fact, the *MUC1* gene has been cloned recently by another group who designated it *FLO11* due to its role in flocculation in *S. cerevisiae* (Lo and Dranginis, 1996). The flocculation proteins are putatively rod-like in structure and one, the *FLO1*-encoded protein, was recently shown to be localized in the cell wall with the N-terminus exposed to the medium (Teunissen and Steensma, 1995), possibly interacting with neighboring cell wall mannoproteins. Based on the sequence data and the homology with the flocculation and mucin genes, Lambrechts et al. (1996a) predicted that Muc1p is located in the cell wall with the N-terminus exposed on the cell surface provided the protein spans the cell wall.

III. REGULATION OF THE GLUCOAMYLASE MULTIGENE FAMILY AND THE MUCIN GENE

A. Effect of Growth Media Composition

1. *The* STA1-3 *Genes*

The expression of the glucoamylase genes are modulated by carbon sources at the transcriptional level (Pretorius et al., 1986c; Kuchin et al., 1993). Suntsov et al. (1991) suggested three modes of expression for the *STA1-3* genes: repressed, basal, and induced. When cells are grown in rich media with nonlimiting concentrations of mono- or disaccharides, the expression of the *STA1-3* genes is repressed, whereas the basal expression level is exhibited when cells are grown in rich medium supplemented with glycerol and ethanol as sole carbon source. Cells grown in minimal media with starch as the sole carbon source were induced approximately sevenfold over the basal expression level. However, the induction leveled out during the logarithmic growth phase until it reached values similar to that of the basal mode. Suntsov et al. (1991) explained this by suggesting that the induction mode gave way to the basal mode through feedback

inhibition by glucoamylase that accumulates extracellularly. Lambrechts et al. (1994a) reported conflicting results while screening deletion mutants for *UAS* sequences. Their study included glucoamylase-assays of intracellular as well as extracellular fractions, whereas Suntsov et al. (1991) only assayed extracellular fractions. Lambrechts et al. (1994a) found no induction of glucoamylase synthesis when grown on starch medium, but more glucoamylase is secreted into the growth medium when grown on starch when compared with glycerol plus ethanol as carbon sources. In addition, they concluded that in starch medium the expression of the glucoamylases is under a different type of control (both positively and negatively) than in the case of glycerol plus ethanol medium. Clearly, the mechanism the cell uses to sense that starch is available in the media still remains to be elucidated. Also, the mechanism of the observed glucose repression and the relevant responsive sites in the *STA1-3* genes have not yet been identified and remains to be studied.

2. The SGA1 Gene

SGA1 is regulated negatively through nutritional signals that seem to exert their actions through the *UAS* element present in the *SGA1* gene promoter (Kihara et al., 1991). It also appears as if the *SGA1* gene is regulated directly through a nutritional signal. During conditions of nitrogen and carbon starvation, a positive regulator, *SME3*, is activated, which in turn activates the inducer genes for meiosis *IME1* and *IME2*, resulting in the activation of *SGA1*. Both *IME1* and *IME2* are negatively regulated by glucose and nitrogen, whereas acetate is required for their full expression (Kawaguchi et al., 1992). A model showing all the elements and their responses to nutritional signals is shown in Figure 4.

3. The MUC1 Gene

As mentioned before, it was observed that strains (both haploid and diploid cells) of *S. cerevisiae* capable of starch hydrolysis also displayed strong pseudohyphal and invasive growth patterns when grown on starch as carbon source (Lambrechts et al., 1996a). Both cell types form filaments by dividing and forming buds in a polarized pattern. The amount of cell elongation, as well as the number of cells penetrating the agar surface, were dependent on the carbon source, being maximal in maltotriose and starch-containing media. Pseudohyphal differentiation and invasive growth appears to be always associated with each other and in addition can be observed in starch-containing media even in an excess of nitrogen. This suggests that the differentiation process is caused by the limiting amount of the carbon source available to the cell at any given moment. Pseudohyphal differentiation is thus not only a response to nitrogen starvation but a general response to nutrient limitation (Blacketer et al., 1995; Lambrechts et al., 1996a). In agreement with this notion is the observed large hyphal-like extensions of haploid strains grown on media supplemented with the poor carbon source ethanol and leucine as the source of nitrogen (Dickinson, 1994). The relevance of this observation has been corroborated by the recent finding that fusel alcohols (especially isoamylalcohol) induce pseudohyphal growth and hyphal-like extensions. These fusel alcohols are the natural products of the catabolic breakdown of several amino acids (Dickinson, 1996). These variations in the induction of the dimorphic switch in relation to different nutrients (starch, fusel alcohols, limited nitrogen) indicate that the diverse genetic background of the strains tested is probably the key in these instances. Also, the formation of pseudohyphae is strictly dependent on the presence of oxygen (Wright et al., 1993).

B. Effect of Ploidy and Heterozygosity

1. *The* STA1-3 *Glucoamylase Genes*

The *MAT* locus regulates the expression of a large number of unlinked genes that determine cell type in yeast. Expression of *STA1-3* was shown to be repressed in some *MATa/MATα* diploids (Yamashita and Fukui, 1983a; Pretorius et al., 1986c). The repression effect in affected heterozygous diploids was shown to be at the transcriptional level by the Mata1p-Matα2p repressor (Pretorius et al., 1986c; Dranginis 1986, 1989). Studies of *STA1* diploids with mutations in *MAT* suggested differential expression of repressor genes in different media. The Mata1p-Matα2p repressor is responsible for the down-regulation of *STA1* transcription in glycerol and lactate medium, whereas both the Mata1p-Matα2p and the Mata2p cause repression at the posttranscriptional level of *STA1* diploids when grown on starch (Inui et al., 1989). Although the *STA1-3* promoter region contains a putative consensus sequence for mating-type control, as reported by Lambrechts et al. (1991), recently it was shown that this site is nonfunctional in diploid repression; removal of this site did not relieve the repression (Lambrechts et al., 1994a). Repression is thus mediated from either within the coding region or downstream from the translational stop codon or perhaps indirectly through the down-regulation of an activator protein (Lambrechts et al., 1994a). In fact, Vidal et al. (1991) showed that the *RPD1* gene is essential for transcriptional activation as well as repression of a complement of unrelated genes. The accurate repression of haploid-specific genes in diploid cells was also dependent on *RPD1*. The gene products of two other genes, *CYC8* and *TUP1*, were also necessary for cell-specific repression. These gene products were shown to be required for the Matα2p repression of α-specific genes as well as the Mata1p-Matα2p repression of haploid-specific genes (Mukai et al., 1991).

2. *The* SGA1 *Glucoamylase Gene*

Induction of *SGA1* is specific to sporulating *MATa/MATα* diploids and expression of *SGA1* is dependent on the product of *MAT a1* (Dranginis, 1989; Yamashita and Fukui, 1985). The expression of *SGA1* is further positively regulated on the transcriptional level by both the Mat1p and the Matα2p (Yamashita and Fukui, 1985).

3. *The* MUC1 *Gene*

When in the pseudohyphal growth pattern, yeast cells have a characteristic, elongated cell shape, modified cell separation properties, as well as a specific budding pattern. Recently, it was observed that under specific conditions, haploid yeast cells also have a similar pseudohyphal/invasive growth pattern (Kron et al., 1994; Roberts and Fink, 1994). The haploid cells also switch from an axial budding pattern to the pseudohyphal budding pattern. Although similar to the established diploid developmental program, the haploid cells form pseudohyphal filaments only on rich media and not when nitrogen is limiting. Furthermore, Roberts and Fink (1994) claimed that haploids form filaments only beneath the colony and have less elongated cells than in the case of diploids where the filaments also extend beyond the perimeter of the colony. Lambrechts et al. (1996a) observed strong invasive growth and pseudohyphal differentiation when haploid and diploid starch-

degrading *S. cerevisiae* strains were grown on starch as the sole carbon source. In their study, some of both the haploid and diploid strains had filaments extending beyond the perimeter of the colonies, with a considerable amount of cells being elongated. The cells on top of the agar surface had a yeast-like shape. It should be noted that these results were obtained in nitrogen-rich media; previously, pseudohyphal growth was mainly associated with nitrogen starvation. No differences were observed between haploids and diploids in their ability to grow invasively, or to form pseudohyphae, on all media tested. Their results show that the amount of cell elongation, as well as the number of cells penetrating the agar, was dependent on the carbon source as well as the yeast strain. This is in accordance with Blacketer et al. (1995), who proposed that pseudohyphal differentiation is a general response to nutrient limitation and not only a response to nitrogen limitation.

C. Effect of Repressor and Activator Genes

1. Global Activators/Repressors of the STA1-3 and SGA1 Genes

Gene products involved in the activation and repression of the *STA1-3* and *SGA1* genes were thoroughly discussed in a recent review by Lambrechts et al. (1994b). No gene(s) specifically involved in *STA1-3* regulation has been identified, although genes involved in global activation processes were shown to regulate expression of the *STA1-3* genes. To avoid unnecessary duplication, the identified genes and their functions are summarized in Table 1. Some of the known activators of the *STA1-3* genes include the products of *GAM1* (*SNF2, SWI2, HAF1, TYE3*), *GAM2* (*RPD1, SIN3, UME4, SDI1, CPE1*), *GAM3* (*ADR6, SWI1*), *HAF2* (not allelic to any of the *SNF* genes but behaving like *SNF2, SNF5*, and *SNF6*), *HAF3* (encoding a protein kinase and allelic to *SNF1, CAT1, CCR1*), and *HAF4* (*SNF5, SWI10, TYE4*) (Table 1). A body of evidence is mounting that suggests extensive interactions between pathways controlling many unrelated genes, such as the *STA, SUC*, and *HO* genes to mention only a few, and the global regulators are key to these interactions.

The *SGA1* gene is positively regulated by two inducer genes for meiosis, *IME1* and *IME2*, and negatively regulated by *RME1*, a haploid-specific negative repressor of meiosis (Kihara et al., 1991). Two other gene products are involved: *SME2* and *SME3*, which encode positive regulators of transcription of *SGA1* and *IME1*, respectively (Kawaguchi et al., 1992). A model for the regulation of *SGA1* is shown in Figure 4 combining all the relevant genes, their stimuli, and modes of action.

2. The STA10 Repressor Gene

The presence of a gene(s) inhibiting glucoamylase expression in most *S. cerevisiae* strains was determined by various groups and was designated *STA10* (Tamaki, 1968; Polaina and Wiggs, 1983). Since then several unsuccessful efforts have been made to clone this gene. Expression of the *STA1-3* genes were shown to be negatively affected by *STA10* at the transcriptional level (Pardo et al., 1986; Pretorius et al., 1986c), whereas *SGA1* expression was reported not to be influenced by the presence of *STA10* (Pugh and Clancy, 1990). One unconfirmed hypothesis is that the *STA10* effect results from the interaction between the two unlinked genes, *IST1* and *IST2* (Park and Mattoon, 1987). If *STA10*

Table 1
Regulators of the Glucoamylase-Encoding Genes and Their Functions

Gene	Synonym	Encoding Protein	Function	Ref.
		The glucoamylase multigene family		
STA1	*DEX2, MAL5*	Extracellular Sta1p	Hydrolyze soluble starch to glucose	Yamashita and Fukui, 1983a
STA2	*DEX1*	Extracellular Sta2p	Hydrolyze soluble starch to glucose	Pretorius et al., 1986a
STA3	*DEX3*	Extracellular Sta3p	Hydrolyze soluble starch to glucose	Yamashita and Fukui, 1983a
SGA1	*Δsta*	Intracellular Sga1p	Sporulation specific; converts glycogen to glucose	Erratt and Nasim, 1986b; Pardo et al., 1986; Yamashita et al.,1987
S1 and *S2*	Shown to be part of the *MUC1* gene	Membrane-associated Muc1p	Invasive growth; formation of pseudohyphae; cell aggregation	Lambrechts et al., 1996a
		Positive regulators of *STA1-3* and *MUC1*		
GAM1	*SNF2, SWI2, HAF1, TYE3*		Global activation	Yamashita and Fukui, 1984a
GAM2	*RPD1, SIN3, UME4, SDI1, CPE1*		Global activation	Okimoto et al., 1989
GAM3	*ADR6, SWI1*		Global activation	Okimoto et al., 1989
HAF2			*STA1-3* activation	Kuchin et al., 1993
HAF3	*SNF1, CAT1, CCR1*		Derepression of *STA1-3*	
HAF4	*SNF5, SWI10, TYE4*		Global activation	Kuchin et al., 1993
MSS10	*PHD2, FUP1, MSN1*		Multicopy suppressor of *STA10;* activation of *STA1-3* and *MUC1*	Lambrechts et al., 1996b
MSS11			Multicopy suppressor of *STA10;* activation of *STA1-3* and *MUC1*	Webber et al., 1997
		Negative regulators of *STA1-3*		
MATa1/ MATα2		Mata1p-Mat2p repressor	Represses *STA1-3* expression	Pretorius et al., 1986c; Dranginis, 1989
STA10	*IST1* and *IST2, INH1, STA°, SGL1*		Represses *STA1-3* expression	Not cloned
		Regulators of *SGA1*		
IME1			Induces meiosis	Kihara et al., 1991
IME2		Kinase	Induces meiosis	Kihara et al., 1991
RME1		Haploid specific gene	Inhibits meiosis	Kihara et al., 1991
SME2			Positive transcriptional regulator of *SGA1*	Kawaguchi et al., 1992
SME3			Regulator of *IME1*	Kawaguchi et al., 1992

does consist of more than one gene, this would explain the difficulties that have been encountered in attempting to clone *STA10*. Pretorius et al. (1986c) showed that the repression effect of *STA10* is overcome by *STA2* when present in multiple copies. Further studies in our laboratory confirmed that *STA10* repression is mediated primarily from within the upstream *STA2* regulatory region; replacement of the *STA2* promoter with the (*ADH1*) promoter relieved the repression effect of Sta10p (Lambrechts et al., 1994a). As described in a previous section of this review, three operator regions responsive to Sta10p repression (URS_{1-3}) were identified in the *STA2* regulatory region.

3. *The* MSS10 *and* MSS11 *Multicopy Suppressor Genes*

To investigate the role of the *STA10*-encoded repressor, we cloned a suppressor gene of *STA10*, designated *MSS10* (multicopy suppressor of *STA10*), that overcomes *STA10* repression of glucoamylase synthesis when present on a multicopy plasmid (Lambrechts et al., 1996b). *MSS10* is located on chromosome XV and is allelic to *FUP1* (an enhancer of iron-limited growth; Eide and Guarente, 1992), *PHD2* (an inducer of pseudohyphal growth in diploid yeast cells; Gimeno and Fink, 1994), and *MSN1* (an activator of invertase expression; Estruch and Carlson, 1990). Expression of glucoamylases is increased substantially on the transcriptional level when *MSS10* is overexpressed in haploid (*STA2 sta10* and *STA2 STA10*) and diploid (*STA2/sta2 sta10/sta10*) strains when grown in media containing starch. *MSS10* disrupted strains (Δ*mss10*) are unable to grow on media containing starch as carbon source (Lambrechts et al., 1996b).

An important observation made was that the haploid and diploid transformants containing multiple copies of *MSS10* showed enhanced pseudohyphal differentiation and invasive growth (Lambrechts et al., 1996a). These phenomena were exhibited even in strains without the presence of functional *STA1-3* genes. The *STA1-3* genes are thus not involved in pseudohyphal differentiation, suggesting that *MSS10* activates other gene(s) involved in pseudohyphal differentiation. One such gene is the cloned *MUC1* gene, shown to be critical for pseudohyphal differentiation and specifically invasive growth (Lambrechts et al., 1996a). As mentioned previously, the promoters of the *STA2* and *MUC1* genes are highly homologous. As expected, the presence of multiple copies of the *MSS10* gene also activates the transcription of the *MUC1* gene. To ascertain whether Mss10p regulates pseudohyphal differentiation and invasive growth through *MUC1*, Lambrechts et al. (1996a) examined the effect of multiple copies of *MSS10* in a *MUC1* deletion strain. The deletion mutant (Δ*muc1*) showed virtually no invasive growth even after prolonged incubation times, suggesting that the Mss10p regulates pseudohyphal differentiation by activating *MUC1* as well as other gene(s). Furthermore, an intact copy of *MUC1* is necessary for pseudohyphal differentiation and invasive growth. One other phenotype observed in overexpressing *MSS10* is cell aggregation (flocculation). In starch-degrading strains, multiple copies of *MSS10* resulted in vigorous cell aggregation compared with the moderately flocculating wild-type strain, whereas a deletion of *MSS10* resulted in reduced flocculation. In an effort to determine the role (if any) of the Muc1p in flocculation, it was shown that deletion of *MUC1* abolished flocculation, but that overexpression of the *MUC1* gene did not enhance flocculation further.

Another multicopy suppressor of *STA10* (designated *MSS11*) has been cloned recently

and characterized in our laboratory (Webber et al., 1997). Although slightly less pronounced, the effect of *MSS11* is the same as that of *MSS10* in enhancing the expression of the *STA1-3* genes and inducing pseudohyphal growth. The protein is, however, not homologous to Mss10p or any other cloned gene (Webber et al., 1997). The *MSS11* gene encodes a 758 amino acids protein and contains regions where up to 30 Asn or Gln residues are repeated. These may represent the activation domains of the protein as these repeated regions are often found in transcriptional activator proteins. Interestingly, unlike *MSS10*, *MSS11* does not have any influence on flocculation. Preliminary results on the relationship between the Mss10p and the Mss11p were obtained with epistasis studies. Results suggest that the Mss11p is essential for Mss10p action, because deletion of *MSS11* abolishes the activation phenotype characteristic for the Mss10p. Conversely, when *MSS10* is deleted, the Mss11p still activates transcription of *STA1-3* and *MUC1* (Webber et al., 1997). From this one can speculate that the Mss11p is positioned below the Mss10p in a signal transduction pathway. Further studies will confirm or disprove this hypothesis and also shed light on DNA-protein and/or protein-protein interactions involved in this transcriptional activation processes.

IV. THE LINK BETWEEN NUTRITIONAL SIGNALING AND DEVELOPMENTAL PATHWAYS

As for any living creature, the old adage: "Adapt or die" holds true for *S. cerevisiae* as well. Metabolically, this facultative anaerobic organism has the ability to select from its environment those food sources that will enable it to have the best possible chance of surviving. It will always utilize hexose sugars, such as glucose and fructose, first. Also, a large number of genes responsible for utilization of alternative carbon sources are turned off in the presence of glucose, a phenomenon known as glucose repression in *S. cerevisiae* (Ronne, 1995). When all fermentable sugars have been utilized, the cells can adapt their metabolism in the diauxic shift to start utilizing nonfermentable carbon sources, including ethanol, lactate, and acetate, that have accumulated during previous fermentative growth. When those are depleted, the cells sense starvation and shut down their cellular and metabolic activities after entry into a survival period called stationary phase (G_0 phase of the cell cycle). Important to note here is the distinction between nutrient limitation and nutrient starvation. When starved for an essential nutrient, the cells become extremely stress tolerant and enter into stationary phase. Nutrient limitation, however, causes a metabolic change in the cells. This most probably transpires through a specific signaling pathway that results in activation of a different set of genes to utilize the available nutrients.

A. Nutrient Sensing and Signaling in Yeast

The best-studied example of nutrient signaling in *S. cerevisiae* is that of glucose-induced signal transduction, which includes the main glucose repression pathway (otherwise known as the Snf1p kinase pathway), the extracellular glucose sensing pathway (utilizing Snf3p and Rgt2p), the Ras-adenylate cyclase pathway and the fermentable growth medium (FGM) pathway. Although the information on nutrient sensing and signaling is by no means complete, the aforementioned pathways are briefly discussed here.

1. The Snf1p Kinase Glucose Repression Pathway

This "general" glucose repression pathway is triggered by glucose and related easily fermented sugars; a prerequisite of this pathway is that glucose must be phosphorylated (Thevelein, 1994). The *HXK2* gene encoding hexokinase is primarily responsible for this phosphorylation event in glucose-grown cells (Entian and Schüller, 1997) and has thus become known as one of the central genes in the glucose repression pathway. The primary target for the glucose repression pathway seems to be the Snf1p (Cat1p) kinase, which is a required component for derepression of glucose-repressed genes (Thevelein, 1994; Lesage et al., 1996; Entian and Schüller, 1997). Snf1p (Cat1p) physically interacts with Snf4p (Cat3p) to form an active Snf1p/Snf4p (Cat1p/Cat3p) protein kinase when in the phosphorylated state (Celenza et al., 1989; Entian and Schüller, 1997). A downstream target of the glucose repression pathway is the Mig1p-Ssn6p-Tup1p transcription complex that controls genes involved in mitochondrial respiration, gluconeogenesis, the glyoxylate cycle, and the metabolism of alternative substrates (Thevelein, 1994; Lesage et al., 1996). Although genetic evidence has implicated Snf1p in alleviating transcriptional repression by the Mig1p-Ssn6p-Tup1p complex (Schüller and Entian, 1991; Treitel and Carlson, 1995), recent evidence showed that Snf1p kinase also enhances the expression of a transcriptional activator, *SIP4* (Lesage et al., 1996). Sip4p is differentially phosphorylated in a glucose-dependent manner by the Snf1p kinase (Lesage et al., 1996). Apart from the Snf1p and other proteins (such as Sip1p, Sip2p, and Gal83p) that form large complexes, several other genes are also involved in the glucose repression pathway but fall outside the scope of this review (for recent reviews see Thevelein, 1994; Ronne, 1995; Entian and Schüller, 1997). Snf1 kinase exhibits homology to the mammalian AMP-activated protein kinase, and, although Snf1p itself does not appear to respond directly to AMP, it has been suggested that AMP/ATP ratios in the cells might be regulated by glucose metabolism and that these adenine nucleotides might serve as second messengers *in vivo* to regulate Snf1 kinase activity (Wilson et al., 1996).

2. The Snf3/Rgt2 Glucose Sensor

While the Snf1 kinase pathway appears to respond to signals derived from glucose metabolism, another signaling pathway has been uncovered that is capable of monitoring extracellular glucose levels by means of transporter homologues that function as glucose receptors (Özcan et al., 1996). Previous studies identified Snf3p as a putative high-affinity glucose transporter which is required for low glucose induction (Özcan and Johnston, 1995); it is highly similar to other glucose transporters except for a long C-terminal extension predicted to be in the cytoplasm (Bisson et al., 1993). *RGT2* encodes another homologue, but it appears to be involved in only high-glucose induction (Marshall-Carlson et al., 1991; Özcan et al., 1996). Experiments employing the same dominant mutation in *RGT2* and *SNF3* (a highly conserved Arg residue predicted to be in one of the cytoplasmic loops, is mutated to Lys) indicate that these two receptors act as glucose sensors and their unusual predicted cytoplasmic C-termini are suspected to be signaling domains (Özcan et al., 1996). This dominant mutation differentiates between the signaling functions of Snf3p and Rgt2p and their glucose transport functions and strongly suggests Snf3p and Rgt2p function as low-glucose and high-glucose sensors, respectively (Özcan et al., 1996). It

seems that they signal via Grr1p, an early glucose signaling component (Flick and Johnston, 1991), but other components in this pathway remain to be defined.

2. The Ras-cAMP Signaling Pathway

This pathway has been identified in a search for yeast homologues of the mammalian *ras* genes (Tatchell, 1986; Wigler et al., 1988; Broach and Deschenes, 1990; Thevelein, 1991). In *S. cerevisiae* the *RAS1* and *RAS2* genes encode small G-proteins that are active when in the GTP-bound state. The concentration of cAMP in the yeast cell is controlled by the products of the *RAS1* and *RAS2* genes (Thevelein, 1992). The Ras proteins are key components in signaling pathways that regulate proliferation and differentiation in eukaryotes (Mösch et al., 1996). Activation of *RAS* in *S. cerevisiae* (by the *CDC25* gene product) leads to elevated levels of intracellular cAMP that in turn activates protein kinase A (cAMP-dependent protein kinase holoenzyme comprising a catalytic and regulatory subunit) indirectly by binding to its regulatory subunit, encoded by *BCY1* (Toda et al., 1987; Thevelein 1994). The catalytic subunits (encoded by *TPK1-3*) of the activated protein kinase A phosphorylate a host of targets, including proteins involved in the shift from gluconeogenic to fermentative growth, the hydrolysis of storage carbohydrates, stress tolerance, and growth control (Thevelein, 1994). When glucose or other easily fermentable sugars are present in the growth medium, yeast cells have high concentrations of cAMP (De Winde et al., 1997). Conversely, low cAMP levels reflect nutrient limitation and poor growth conditions. Also, nutrient-starved cells arrest at the same point in the cell-cycle as cAMP-depleted cells (Pringle and Hartwell, 1981). This leads to the notion that cAMP acts as a second messenger for nutrient availability in yeast. Rapidly fermentable sugars, such as glucose, fructose, and mannose, together with intracellular acidification, are the prime candidates as triggers of the Ras-adenylate cyclase pathway in yeast (Thevelein, 1994).

3. The FGM Signaling Pathway

It has been proposed that protein kinase A activity can, in addition, be regulated by an independent signaling pathway called the FGM pathway (Thevelein, 1994; De Winde et al., 1997). Activation of this largely undefined pathway seems to be dependent on the availability of all essential nutrients and not only glucose. In addition, the second messenger, cAMP, has no role to play in the FGM pathway because it contacts the catalytic subunits of protein kinase A directly (Hiremburegama et al., 1992). Ongoing work will hopefully clarify the mechanism cells use to sense such a variety of nutrients (glucose and all other nutrients required for growth in this instance) and yet transduce a specified signal causing a highly focused metabolic effect (Boles et al., 1997).

B. Integrative Control between Nutritional Signaling and Cell Morphology

Pseudohyphal and invasive growth are modulated by nutrient limitation (Gimeno et al., 1992; Blacketer et al., 1995; Dickinson, 1996; Lambrechts et al., 1996a). The perception of these limiting nutrients and transduction of the signal received is obviously of key importance here. In *S. cerevisiae*, the nutritional status of the cell is monitored with-

in a narrow window in the G_1 phase of the cell cycle (Pringle and Hartwell, 1981). If nutrients are limited, the decision to execute the diauxic shift is made during a fleeting delay in G_1 followed by metabolic reprogramming. It is also in G_1 that pseudohyphal formation is initiated because of a changed budding pattern and a prolonged period of hyperpolarized growth. All of this hints at several shared control mechanisms between nutritional signaling and cellular differentiation.

One excellent example of such an integrated control system was uncovered in starch-hydrolyzing strains of *S. cerevisiae*. The crucial observation made was that starch-degrading yeast strains have the ability to grow invasively and form pseudohyphae (Lambrechts et al., 1996a). Previously, the formation of pseudohypae has been studied mainly in response to the same stimulus, namely, nitrogen limitation. However, by using the starch-degrading yeast strains as genetic background, the cloned *MUC1* gene and the transcriptional activator *MSS10*, Lambrechts et al. (1996a) showed that pseudohyphae can form without any nitrogen starvation. This is in agreement with the notion that the pseudohyphal and invasive growth response is a general response to nutrient limitation (Blacketer et al., 1995; Dickenson, 1996). *MUC1* encodes a putative membrane-anchored protein that also shares structural homologies with mammalian mucins. We proposed that in addition to a general role in pseudohyphal differentiation, Muc1p may play a specific role in the response to the presence of polysaccharides (Lambrechts et al., 1996a). The *STA1-3* genes have a Thr/Ser-rich sequence similar to those found in other fungal glucoamylases; this sequence is important for starch binding in fungi and probably also in diastatic strains of *S. cerevisiae*. It therefore might be significant that one of the Thr/Ser-rich repeats of Muc1p is identical to the Thr/Ser-rich sequence found in the *STA1-3*-encoded glucoamylases (Lambrechts et al., 1996a). This sequence could give *S. cerevisiae* the ability to adhere to polysaccharide-rich environments via Muc1p. In its natural milieu it would be of the utmost importance for the yeast to effectively colonize and invade a food source, adhesion being the first step. Penetration of a polysaccharide substrate would then become possible by the polarized secretion of the STA1-3-encoded glucoamylases toward the growing tip of the pseudohypha. This hypothesis allows the *STA1-3* genes and the *MUC1* gene to be functionally linked and explains their observed coregulation (Lambrechts et al., 1996a).

The obvious question remains as to how this putative integration between signal transduction pathways operating in nutrient signaling and morphogenesis is accomplished.

4. The Pseudohyphal/Invasive Growth Response Signaling Pathway

The pseudohyphal growth pattern requires many of the components of the pheromone response pathway (Liu et al., 1993; Roberts and Fink, 1994). In addition, the Ras-cAMP pathway of *S. cerevisiae* has been implicated in the responses to both nutrient limitation and pseudohyphal development (Gimeno et al., 1992; Mösch et al., 1996).

The pheromone response pathway is essential for haploid yeast cells of the mating type a or α to mate with each other. It is the best-studied example of a mitogen-activated protein kinase (MAPK) signal transduction pathway in yeast and basically consists of a receptor and a series of protein kinases that require phosphorylation to be activated (Herskowitz, 1995) (see Table 2 for a list of the components and their functions). Ste20p is most likely the link between the receptor and the kinase cascade that consists of Ste11p, Ste7p and Fus3p/Kss1p. Ste5p seems to be a scaffold protein for association of the three

TABLE 2
Pheromone Response and Invasive Growth Pathways: Components and Their Functions

Gene	Encoded Protein	Function	Pathway(s)
STE2	α-factor receptor	Binds α-factor in a-cells	Pheromone
STE3	α-factor receptor	Binds α-factor in α-cells	Pheromone
STE4	β-subunit of heterotrimeric G-protein	Stimulate activation of protein kinases through Ste20p	Pheromone
STE18	γ-subunit of G-protein	Stimulate activation of protein kinases through Ste20p	Pheromone
STE20	Kinase	Link between receptor and protein kinases	Pheromone and invasive growth
Cdc42	Rho-family protein	Regulate Ste20p/MAPK module for pheromone signaling	Invasive growth
Ras2	G-protein	Stimulates pseudohyphal growth	Invasive growth
STE11	MEKK	Phosphorylates and activates *STE7*	Pheromone and invasive growth
STE7	MEK (MAPK kinase)	Phosphorylates and activates the MAPKs, *FUS3, KSS1*	Pheromone and invasive growth
FUS3, KSS1	MAPK	Phorphorylates and activates *STE12*	Pheromone
STE12	Transcription factor	Activates numerous genes involved in pheromone response pathway as well as invasive growth response	Pheromone and invasive growth
STE5	Scaffold protein	Forming a complex with the MAPKs, MEK and MEKK	Pheromone

protein kinases (Herskowitz, 1995), of which the last phosphorylate, thereby activating the transcription factor Ste12p. Mutations in *STE20, STE11, STE7*, or *STE12* block pseudohyphal formation, cell elongation, and agar penetration of cells (Liu et al., 1993; Roberts and Fink, 1994). Mutations in the pheromone receptor do not influence the formation of pseudohyphae, suggesting that other molecules receive and transduce the signal that triggers filamentous growth (Liu et al., 1993). Ste20p is, however, essential for pseudohyphal development, and recent studies showed that this protein is in a complex with Cdc42p, which is necessary for cell polarity (Mösch et al., 1996). Also, mutational analysis indicated that the Fus3p and Kss1p are not required for the invasive behavior but, rather, modulate it. One possibility is that the pseudohyphal and invasive pathway uses a novel MAPK and in addition also a pathway-specific Ste5p analogue to transduce a signal (Mösch et al., 1996). The shared components involved in the pheromone response and invasive growth pathways are depicted in Figure 5.

How can the transcription factor, Ste12p, modulate two different sets of output signals to result in the two very different pathways of mating and pseudohyphal formation? This is presumably achieved by regulating the expression of genes specific to each developmental program by having at least two functional states. Differential phosphorylation patterns

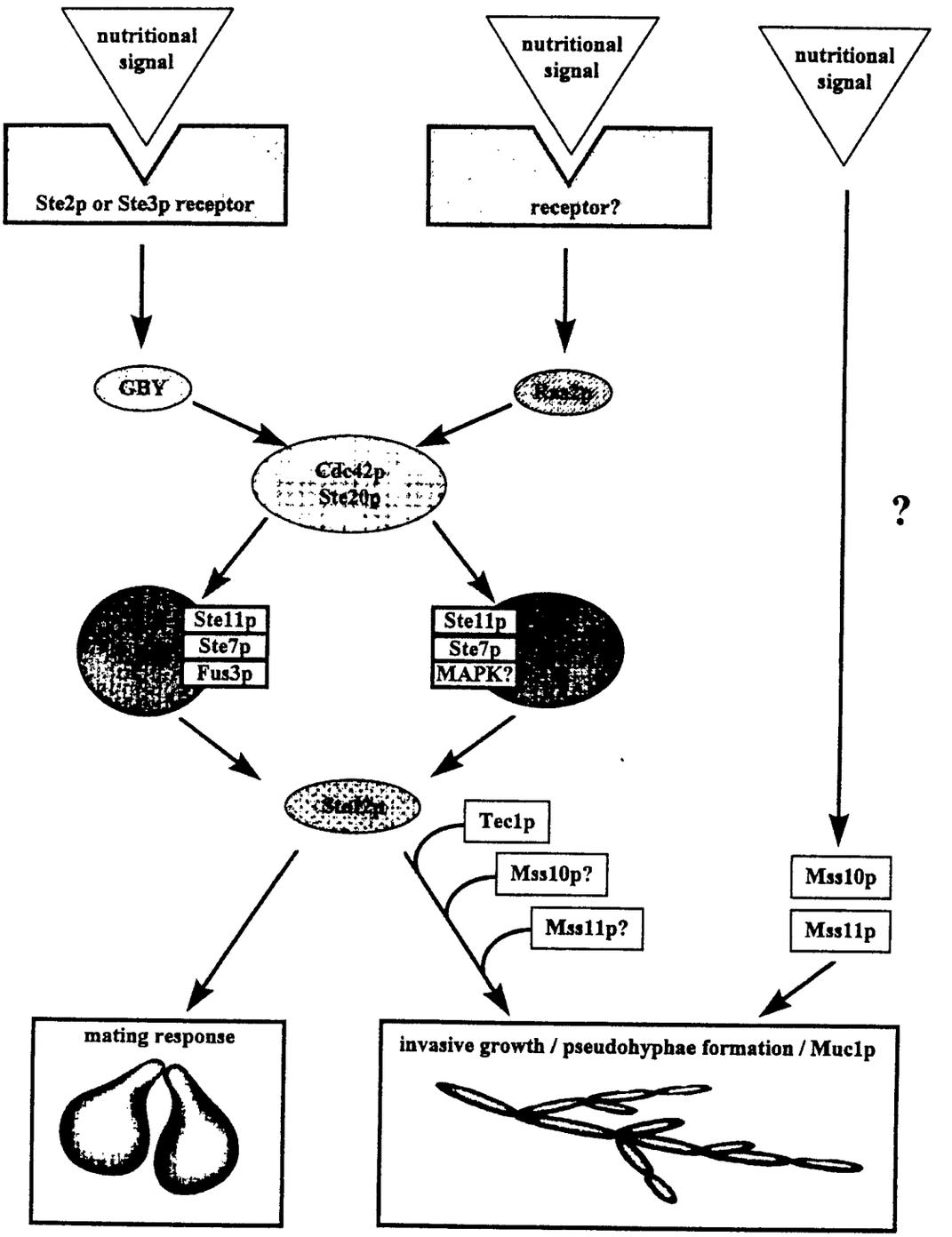

FIGURE 5. Shared and novel components involved in the pheromone- and pseudohyphal/invasive growth response pathways. The pseudohyphal/invasive growth responses use the Ste20p, Ste11p, Ste7p, and Ste12p, but not the Ste5p. Analogues of the Ste5p and Fus3p may be present in the pseudohyphal/invasive growth response. The transcription factors Mss10p (Lambrechts et al., 1996a,b) and Mss11p (Webber et al., 1997) may associate with the Ste12p to cause activation of genes specific for the invasive growth response in the pseudohyphal response pathway or act in a separate but parallel pathway. The mucin-like Muc1p, may act as a receptor of sort by evaluating the food status of the yeast by binding (or not binding) to a polysaccharide substrate.

have been proposed as a possible means to switch between the different functional states of Ste12p (Roberts and Fink, 1994). Alternatively or additionally, Ste12p has been thought to have binding affinity for another set of activators, such as the recently identified Tec1p, shown to be required for pseudohyphal growth, but not mating (Gavrias et al., 1996). In fact, recent evidence showed that this specificity of the signaling pathway is brought about by cooperative binding of Ste12p and Tec1p to enhancer elements aptly called filamentation and invasive response elements (FREs) (Madhani and Fink, 1997). Ste12p is known to bind to pheromone response elements (PREs) that have a consensus sequence of TGAAACA (Dolan and Kirkman, 1989). Tec1p again contains the TEA/ATTS consensus sequences (TCS). These presumptive binding sites occur together to form FREs; several of these FREs have been identified in genes required for the formation of pseudohyphae and the invasive growth response (Madhani and Fink, 1997). Mobility shift analysis of FREs and purified Tec1p and Ste12p clearly showed a cooperative binding effect of these two proteins to the FRE (Madhani and Fink, 1997). Also, the transcriptional activator Tec1p contains a FRE element in its promoter, suggesting autoregulation of this protein (Madhani and Fink, 1997). Interestingly, the promoters of the *STA1-3* and *MUC1* genes also contain sequences corresponding to the FREs (unpublished result).

The transcription factors encoded by *MSS10* (Lambrechts et al., 1996b) and *MSS11* (Webber et al., 1997) may also conduct their regulation of pseudohyphal development and invasive growth through association with Ste12p to promote transcription of the necessary genes for these phenomena or alternatively exist in a separate but parallel signal transduction pathway (Figure 5). Interestingly, most genes found to impact on pseudohyphal development are involved in signal transduction. Examples include Grr1p that forms part of several nutrient-induced signaling pathways and is also involved in transducing signals from the recently identified Rgt2/Snf3 glucose sensors. Mutations in *GRR1* cause pseudohyphal formation to be switched on continuously (Barral et al., 1995). The *ELM1-4* genes were also shown to cause constitutive filamentation and invasive growth response when mutated (Blacketer et al., 1993, 1994). Of particular interest is the fact that *ELM1* encodes a novel protein kinase homologue and seems to regulate the same cellular function as *CDC55*, which functions as a component of protein phosphatase 2A. A *cdc55* mutant exhibits the same pseudohyphal phenotype as an *elm1* mutant in response to nitrogen limitation. These results suggest that phosphorylation plays a role in the switch to filamentation and invasive growth response (Blacketer et al., 1993, 1994). In an effort to clarify the signal transduction pathway utilized for filamentation and invasive growth, a comprehensive genetic screen employing transposon mutagenesis was used that yielded 16 genes (*CDC39*, *STE12*, *TEC1*, *WHI3*, *NAB1*, *DBR1*, *CDC55*, *SRV2*, *TPM1*, *SPA2*, *BNI1*, *DFG5*, *DFG9*, *DFG10*, *BUD8*, and *DFG16*) (Mösch and Fink, 1997). These genes could be grouped into four different classes for signal transduction, bud site selection, cell morphogenesis, and invasive growth. Of key importance here is the notion that invasion is genetically distinct from pseudohyphal formation (Mösch and Fink, 1997). Epistasis analysis indicated some targets in the pseudohyphal signal transduction pathway of these identified genes; this analysis further suggested the presence of an invasive specific signaling pathway. The latter seems to be a target of the pseudohyphal formation pathway and includes a novel gene *DFG16* (Mösch and Fink, 1997). Mutations in *STE12*, *STE20*, *STE11*, and *STE7* were previously shown to cause defects in invasion, indicating the putative invasion path-

way to be a downstream target of Ste12p. The alternative, namely, the possible presence of a separate but parallel invasion pathway, should not be dismissed at this point in time.

No receptor specific for pseudohyphal growth has yet been identified, but the signal seems to be in nutrient limitation. It is possible though that different limitations (i.e., carbon or nitrogen limitation) utilize different sensors to perceive these signals. Further research will clarify the interrelationship between pseudohyphal development and the invasive growth response and the sensors and signal transduction pathways they employ to cause their typical morphologies.

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