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The Glucoamylase Multigene Family in *Saccharomyces cerevisiae* var. *diastaticus*: An Overview

Isak S. Pretorius and Marius G. Lambrechts

Department of Microbiology and Institute for Biotechnology, University of Stellenbosch, Stellenbosch 7600, South Africa.

Julius Marmur

Department of Biochemistry, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461.

Referee: Dr. James R. Mattoon, Biotechnology Center, University of Colorado, 1420 Austin Bluffs Pkwy., Colorado Springs, CO 80933-7150.

ABSTRACT: *Saccharomyces cerevisiae* has been used widely both as a model system for unraveling the biochemical, genetic, and molecular details of gene expression and the secretion process, and as a host for the production of heterologous proteins of biotechnological interest. The potential of starch as a renewable biological resource has stimulated research into amylolytic enzymes and the broadening of the substrate range of *S. cerevisiae*. The enzymatic hydrolysis of starch, consisting of linear (amylose) and branched glucose polymers (amylopectin), is catalyzed by α- and β-amylases, glucoamylases, and debranching enzymes, e.g., pullulanases. Starch utilization in the yeast *S. cerevisiae* var. *diastaticus* depends on the expression of the three unlinked genes, *STA1* (chr. IV), *STA2* (chr. II), and *STA3* (chr. XIV), each encoding one of the extracellular glycosylated glucoamylases isozymes GAI, GAII, or GAIII, respectively. The restriction endonuclease maps of *STA1*, *STA2*, and *STA3* are identical. These genes are absent in *S. cerevisiae*, but a related gene, *SGA1*, encoding an intracellular, sporulation-specific glucoamylase (SGA), is present. *SGA1* is homologous to the middle and 3′ regions of the *STA* genes, but lacks a 5′ sequence that encodes the domain for secretion of the extracellular glucoamylases. The *STA* genes are positively regulated by the presence of three *GAM* genes. In addition to positive regulation, the *STA* genes are regulated negatively at three levels. Whereas strains of *S. diastaticus* are capable of expressing the *STA* genes, most strains of *S. cerevisiae* contain *STA10*, whose presence represses the expression of the *STA* genes in an undefined manner. The *STA* genes are also repressed in diploid cells, presumably by the *MATa*/*MAT*α-encoded repressor. *STA* gene expression is reduced in liquid synthetic media, it is carbon catabolite repressed by glucose, and is inhibited in petite mutants.

KEY WORDS: glucoamylase, *Saccharomyces* var. *diastaticus*, starch, *STA* genes, amylolytic enzymes.

I. INTRODUCTION

In nature two widely distributed complex carbon sources, starch and cellulose, are the sources of energy for most forms of life. Starch is therefore an important renewable biological resource. This polymer, produced mainly in higher plants, is composed of two high-molecular-weight components, amylose and amylopectin. Amylose is mainly a linear polysaccharide formed by α-1,4-linked α-D-glucose residues and some α-1,6 branching points. Amylopectin is a highly branched tree-like structure in which linear chains of α-1,4-linked α-D-glucose residues are interlinked by α-1,6-glucosidic bonds.[1] The relative content of amylose and amylopectin varies with the source of starch. Amylose generally accounts for 20 to 25% of the starch weight and has an average chain length of 1000 glucose units. The chain profile of amylopectins usually has a bi-

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modal distribution with longer and shorter chains having average lengths of 40 to 60 and 11 to 25 D-glucosyl residues, respectively.[1] The proportion of α-1,6-branches is an important property of starch because amylolytic enzymes hydrolyze substrates, differing in extent of branching, with different specificities. There are seven classes of amylolytic enzymes of microbial origin (Table 1). The enzymatic degradation of starch to glucose (Figure 1) involves two processes, namely, liquefaction (by α- and β-amylases) and saccharification (by glucoamylases and debranching enzymes).[1,2] Amylolytic enzymes have been studied extensively in bacteria and fungi. Studies on bacterial α- and β-amylases have been carried out mostly with the genus *Bacillus* and have played a major role in the development of microbial enzymes for industrial applications.[3] Most work on debranching enzymes has been performed with the pullulanase of *Klebsiella pneumoniae*, the only pullulanase produced commercially.[4] Glucoamylases are rare in bacteria but have been found in several genera of fungi. These fungal enzymes have been studied in *Aspergillus* spp.,[5] *Rhizopus* spp.,[6] *Schwanniomyces* spp.,[7] *Lipomyces* spp.,[8] *Saccharomycopsis* spp.,[9,10] and *Saccharomyces* spp.[11,12]

The ever-increasing demand for the production of ethanol (as a fuel extender) and single cell protein (as food and feed supplements) from surplus starch-rich agricultural crops and industrial waste has focused on the potential of amylolytic yeasts for the one-step bioconversion of starch to ethanol and single cell protein (SCP) (for a review see Steyn and Pretorius[33]). Of the more than 500 yeast species that are currently recognized,[13] approximately 150 contain strains that are capable of using starch as carbon and energy sources. Amylolytic yeasts of genera other than *Saccharomyces* are generally not suitable for the production of ethanol and SCP. *S. cerevisiae* has a fast growth rate, a high ethanol tolerance, is an efficient ethanol producer,[14] consists of 48% high-quality protein, and has been associated for centuries with food and beverage production.[15-17] A logical step would be to introduce genes encoding amylolytic enzymes into industrial strains of *S. cerevisiae*. For that reason, heterologous amylase genes derived from various organisms have been cloned and expressed in *S. cerevisiae*. These include the α-amylase genes from mouse salivary glands,[18] mouse pancreas,[19] wheat,[20] *Bacillus amyloliquefaciens*,[21,22] and *Schwanniomyces occidentalis*[23] and the glucoamylase genes from *Aspergillus awamori*,[24,25] *Rhizopus oryzae*,[6] *Saccharomycopsis fibuligera*,[26] and *S. cerevisiae* var. *diastaticus*.[27-31] Kim et al.[32] transformed a hybrid strain of *S. cerevisiae* var. *diastaticus* with a plasmid containing a mouse α-amylase gene. This strain is capable of simultaneous secretion of glucoamylase and α-amylase. For the same purpose our laboratory introduced the α-amylase *(AMY)* gene of *B. amyloliquefaciens* and the glucoamylase *(STA2)* gene of *S. cerevisiae* var. *diastaticus* into *S. cerevisiae* strains.[33] This genetically engineered *S. cerevisiae* strain can convert soluble starch to fermentable sugars with an efficiency greater than 93%. Furthermore, our laboratory has cloned the pullulanase *(PUL)* gene of *K. pneumoniae*.[34] An *amylase cassette*, which will harbor an α-amylase, a glucoamylase and a pullulanase gene is presently being constructed. This *amylase cassette* will be stably introduced into industrial strains of *S. cerevisiae* to be used for the production of ethanol and SCP from starch.

Besides glucose, *S. cerevisiae* and closely related species can also utilize galactose, maltose, melibiose, sucrose, and starch as carbon and energy sources. The ability to ferment particular sugars has often been used as a taxonomic criterion. For example, the taxonomic difference between the brewing yeast species, *S. cerevisiae* (ale yeast) and *S. uvarum (carlsbergensis)* (lager yeast), was based previously on the ability of the latter to produce α-galactosidase (that hydrolyzes melibiose to galactose and glucose), whereas *S. cerevisiae* is deficient in this extracellular enzyme.[35] In the search for the cause of superattenuation (beer with a specific gravity much lower than the usual attenuation limit), Andrews and Gilliland[36] isolated a yeast strain that could convert wort dextrins to fermentable sugars and subsequently ferment the sugars produced. This diastatic (starch-degrading) yeast was assigned to a separate species, *S. diastaticus*.[35] *S. cerevisiae* and *S. diastaticus* are closely related and can interbreed efficiently to produce fertile progeny.[11] Recently, the species *S. cerevisiae* was taxonomically redefined, and both *S. uvarum*

TABLE 1
Starch-Degrading Enzymes Produced by Amylolytic Microorganisms

Amylolytic enzymes[a]		Specific substrate and end products of amylolysis[b]
α-Amylase (1,4-α-D-glucan glucanohydrolase)	EC 3.2.1.1	An extracellular endoenzyme that catalyzes the hydrolysis of the α-1,4-glucosidic linkages and is capable of bypassing α-1,6-linkages, producing poly- and oligosaccharide chains of varying length *(Bacillus amyloliquefaciens, Aspergillus oryzae)*
β-Amylase (1,4-α-D-glucan maltohydrolase)	EC 3.2.1.2	An extracellular exoenzyme that hydrolyzes alternate α-1,4-glucosidic linkages from the nonreducing end and incapable of bypassing α-1,6-glucosidic linkages to produce dextrin and the β-anomeric form of maltose *(Bacillus polymyxa, Clostridium thermosulfurogenes)*
γ-Amylase/glucoamylase/ amyloglucosidase (1,4-α-D-glucan glucanohydrolase)	EC 3.2.1.3	An extracellular exoacting enzyme that splits α-1,4 and in some cases α-1,6-glucosidic linkages and also some 1,3-glucosidic linkages from the nonreducing ends of α-glucans to yield β-D-glucose *(Aspergillus awamori, Saccharomyces cerevisiae* var. *diastaticus)*
Pullulanase (α-dextrin 6-glucanohydrolase)	EC 3.2.1.41	An extracellular debranching enzyme that hydrolyzes α-1,6-linkages of pullulan and other branched oligosaccharides to form maltotriose and dextrins, respectively *(Klebsiella pneumoniae, Bacillus stearothermophilus)*
Isoamylase (glycogen 6-glucanohydrolase)	EC 3.2.1.68	An extracellular debranching enzyme that hydrolyzes α-1,6-glucosidic linkages of amylopectin, glycogen various branched dextrins and oligosaccharides with no activity on pullulan *(Pseudomonas amyloderamose, Lipomyces kononenkoae)*
Cyclodextrin glycosyltransferase [1,4-α-D-glucan 4-α-D (1,4-α-D-glucano)-transferase]	EC 2.4.1.19	An extracellular enzyme that produces a series of nonreducing cyclodextrins (rings of 6, 7, and 8 glucose units) from starch, and other polysaccharides *(Bacillus macerans, Klebsiella pneumoniae)*
α-Glucosidase (α-D-glucoside glucohydrolase)	EC 3.2.1.20	An extracellular or intracellular enzyme that appears to hydrolyze short chain α-1,4 or α-1,6-linked saccharides arising from the action of other enzymes on starch *(Bacillus licheniformis, Schizosaccharomyces pombe)*

[a] Systematic names of amylolytic enzymes are listed in parentheses.
[b] Examples of amylolytic microorganisms are listed in parentheses.

(carlsbergensis) and *S. diastaticus* were included in *S. cerevisiae*.[13] For the purpose of this discussion, *S. cerevisiae* var. *diastaticus* is referred to as *S. diastaticus*.

The ability to ferment various sugars and their polymers depends on the genetic background of each particular species or strain and is governed by complex and interacting regulatory mechanisms, such as induction, catabolite-repression, and inactivation. Starch utilization in *S. diastaticus* depends on the expression of any one of the three unlinked genes, *STA1*, *STA2*, or *STA3*, each encoding one of the extracellular glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) isozymes GAI, GAII, or GAIII.[11,12,37,38] Although *S. cerevisiae* lacks extracellular glu-

coamylase activity, both *S. cerevisiae* and *S. diastaticus* contain the *SGA1* gene encoding a sporulation-specific, intracellular glucoamylase, SGA.[39] Another difference between *S. cerevisiae* and *S. diastaticus* is that most strains of *S. cerevisiae* carry the *STA10* gene whose protein product inhibits glucoamylase expression.[40]

In this review we concentrate on the properties of yeast glucoamylases and the structure and regulation of the *STA* and *SGA* genes, with particular emphasis on the work conducted in our own laboratory.

II. STRUCTURE OF THE GLUCOAMYLASE GENES

A. Genetic Characterization of the Glucoamylase Genes

Van der Walt[41] stated that "*S. diastaticus* may be considered to be derived from *S. cerevisiae* by the acquisition of glucoamylase activity." The original *S. diastaticus* strains were isolated in different parts of the world. The first strain with "dextrinase" activity was isolated by Andrews and Gilliland[36] in Dublin, Ireland. In 1960, Van Uden isolated a strain in Lisbon, Portugal, able to ferment starch.[11] Similar strains were isolated by Kleyn et al.[42] in Seattle, U.S., and by Takahashi[43] in Osaka, Japan. All of these strains were originally isolated from superattenuated beer. Soon after the isolation of these *S. diastaticus* strains, genetic studies on the ability to ferment starch were conducted in several laboratories.

In 1953, Gilliland[44] demonstrated that a complementary gene action exists between the *S* (amylase synthesis and *M* (maltase synthesis) genes. In 1956, Lindegren and Lindegren[45] reported that two genes were involved in starch fermentation, namely, the *DX* gene that controlled fermentation

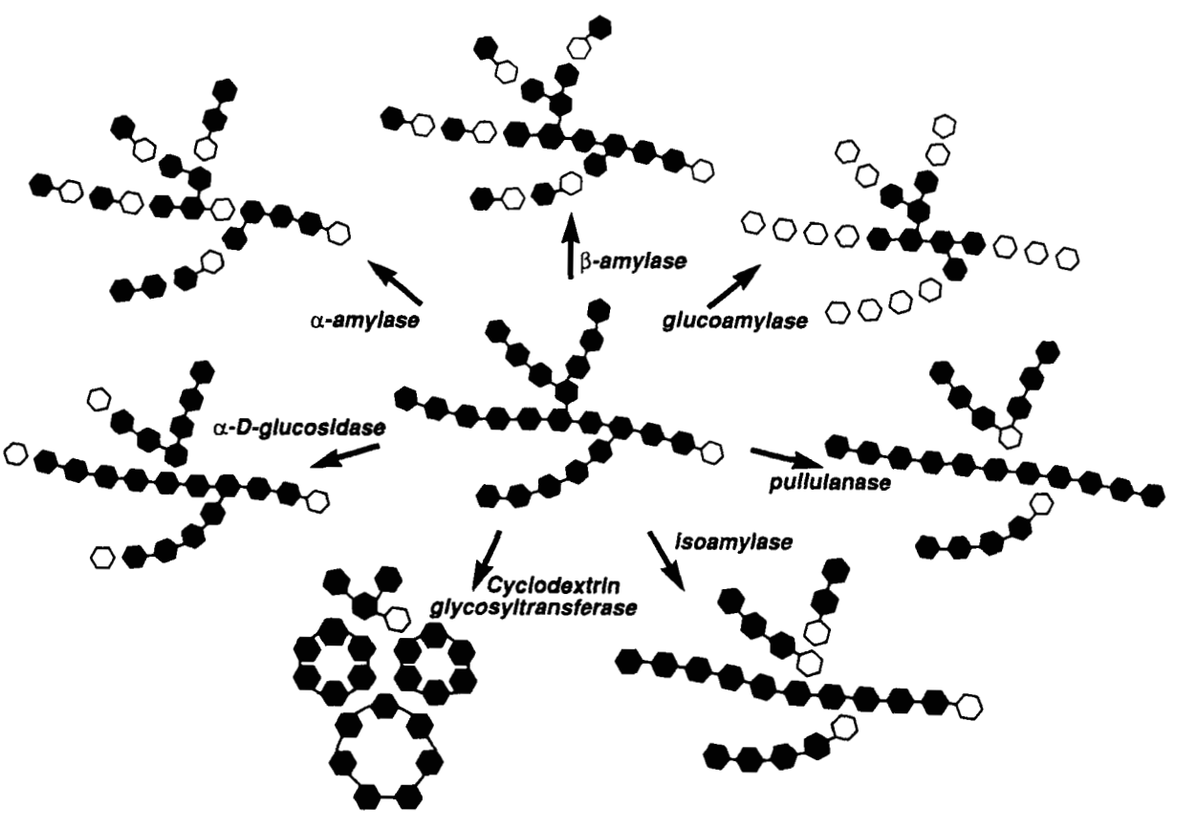

FIGURE 1. A schematic representation of the starch molecule and activity patterns of amylolytic enzymes. The hexagonals represent glucose units. Hydrolyzed linkages are indicated by disconnected, white hexagonals.[1]

of dextrin and glycogen, and the *ST* gene that controlled starch fermentation. It was also demonstrated that the *DX* (dextrinase synthesis) and *ST* (amylase synthesis) genes segregated independently, but that some irregular segregations occurred,[45] probably due to the presence of an inhibitor gene(s).[46] In 1966, Takahashi[43] identified a *STA* gene that was different from Gilliland's *S* gene with respect to the rate of starch fermentation. In the 1970s linkage analyses of genes responsible for starch fermentation in *S. diastaticus* were conducted in two laboratories. Tamaki[11] found that three polymorphic genes, *STA1*, *STA2*, and *STA3*, controlled starch fermentation in *S. diastaticus* and demonstrated that these *STA* genes were located in different linkage groups. Independently, Erratt and Stewart[12] studied dextrin utilization by *S. diastaticus*, designating the responsible gene *DEX1*. Erratt and Stewart[37,38] later described two other genes, *DEX2* and *DEX3*, that controlled glucoamylase production in *S. diastaticus*. To eliminate confusion in the designation of the *S. diastaticus* extracellular glucoamylase genes, we investigated allelism between the different *STA* (controlling starch hydrolysis), *DEX* (controlling dextrin hydrolysis), and *MAL5* (a gene once thought to control maltose metabolism) genes. Pretorius et al.[47] reported that *STA1* is allelic to both *DEX2* and *MAL5*, *STA2* is allelic to *DEX1*, and *STA3* is allelic to *DEX3*. Those results were confirmed by Erratt and Nasim.[48] Based on the fact that the glucoamylases secreted by *S. diastaticus* can hydrolyze both starch and partially degraded starch (dextrin) and on the wider use of the nomenclature *STA*, it was decided to retain *STA* as the designation for genes encoding extracellular glucoamylases in *S. diastaticus*.

Colonna and Magee[49] described an α-1,4-glucosidase activity present during meiosis in *S. cerevisiae*. This activity was absent in vegetative cells, but appeared coincidently with the appearance of glycogenolysis and of mature ascospores; it then increased progressively until sporulation was complete. Clancy et al.[39] designated the sporulation-specific α-glucosidase as sporulation amyloglucosidase (SAG) and reported that it was responsible for the rapid degradation of intracellular glycogen that follows the completion of meiosis in *S. cerevisiae*. Yamashita et al.[50] identified a "cryptic *sta* gene", designated Δ*sta*. Yamashita and Fukui[51] demonstrated that Δ*sta* (now known as *SGA1*) encoded an intracellular, sporulation-specific glucoamylase, SGA.

Currently, it is accepted that starch utilization in *S. diastaticus* depends on the presence of any one of a polymorphic family of *STA* genes. Strains carrying one of the unlinked genes, *STA1*, *STA2*, or *STA3*, produce extracellular glucoamylase isozymes named, respectively, GAI, GAII, and GAIII. The *SGA1* gene, present in both *S. cerevisiae* and *S. diastaticus*, encodes the sporulation-specific, intracellular glucoamylase, SGA.

B. Physical Characterization of the Glucoamylase Genes

To provide more information on the structure and polymorphism of the *STA* genes and on the mechanism of their evolution, as well as to extend the understanding of the regulation of glucoamylase production (synthesis and secretion), glucoamylase genes from *S. diastaticus* were cloned in at least five laboratories. The *STA1* and *STA3* genes, coding for GAI and GAIII, respectively, were initially cloned from two strains provided by Tamaki.[31,50] The *DEX1* gene coding for AMG (amyloglucosidase) was cloned from a British beer strain.[28] Our laboratory cloned the *STA2* gene, encoding GAII, as an 8.3 kb *Bgl*II fragment from a strain supplied by Tamaki.[30] The latter fragment complemented a *sta*^{0} strain (a Sta$^-$ *sta10* segregant from a cross between a *STA1* and a *STA3* strain), and we have confirmed that the cloned fragment carried the *STA2* gene by one-step integrative gene-disruption and gene-fusion experiments.[30] The *SGA1* gene coding for the intracellular, sporulation-specific glucoamylase was also cloned.[27,29,52] The restriction endonuclease maps of *STA1*, *STA2*, *STA3*, and *DEX1* are identical (Figure 2). The *SGA1* gene is homologous to the middle and 3′ regions of the *STA* genes, but lacks a 5′ sequence that encodes the domain required for secretion of the extracellular glucoamylase (Figure 2).

Pretorius et al.[47] used subcloned *STA2* se-

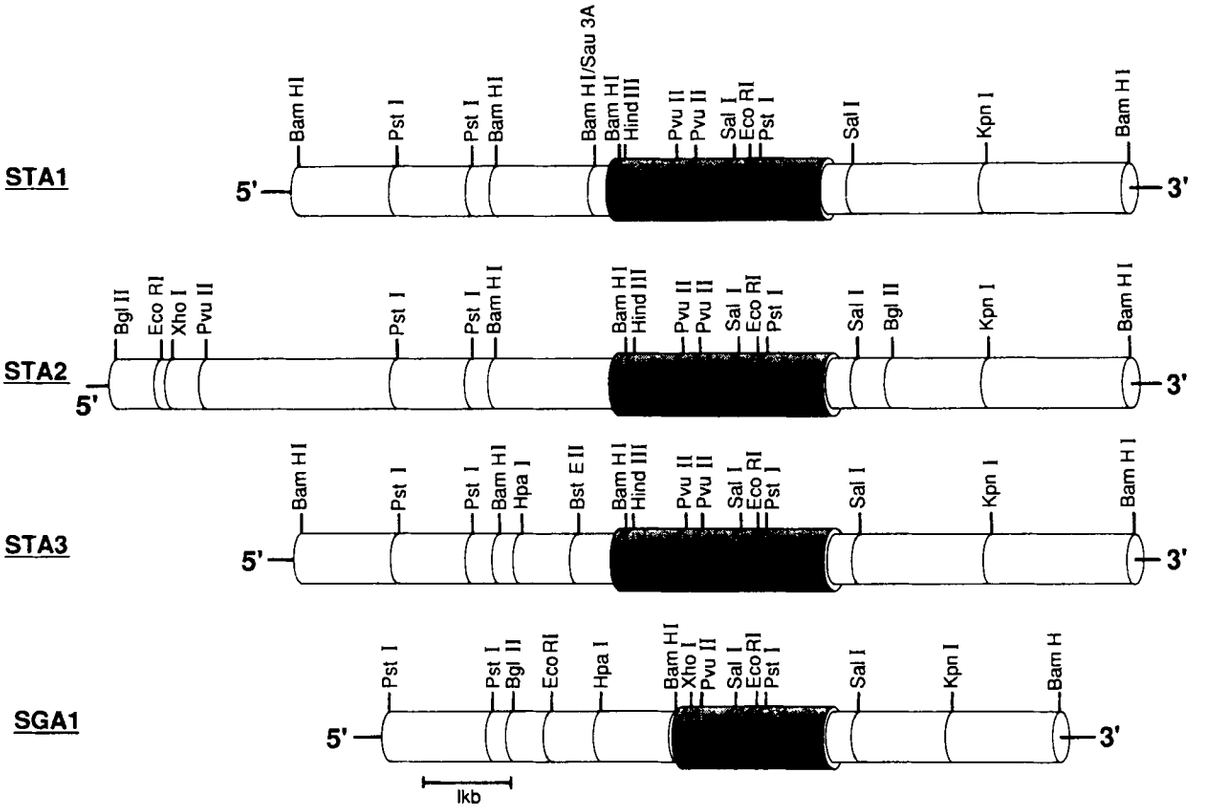

FIGURE 2. Restriction endonuclease maps of the *STA* and *SGA* genes from *Saccharomyces*. The structural genes encoding the glucoamylases are highlighted. The restriction maps of *STA1*, *STA2*, and *STA3* are identical.[30,31] The middle and 3′ regions of *SGA1* are identical to the corresponding regions of the *STA* genes.[29,50]

quences as hybridization probes to investigate the physical structure of the family of *STA* genes in the genomes of different *Saccharomyces* strains. Sta^+ strains, each carrying a single genetically defined *STA* gene, were crossed with a Sta^- strain and the segregation behavior of the functional locus (i.e., Sta^+) and sequences homologous to the cloned *STA2* glucoamylase structural gene at that locus were analyzed. Southern blot analyses of the meiotic products allowed the assignment of *STA1*, *STA2*, *STA3*, and *SGA1* to unique 11 kb *Ava*I, 19 kb *Hin*dIII, 11 kb *Xho*I, and 9 kb *Ava*I restriction fragments, respectively. Consistent with the finding of Yamashita et al.,[50] we have shown that two additional sequences, S1 and S2, exist in various *Saccharomyces* strains that exhibit homology to the 5′ region of a *STA2* gene probe.[47] Electrophoretic mapping was used to assign *STA1* to chromosome IV, *STA2* to chromosome II, *STA3* to chromosome XIV, and *SGA1* to chromosome IX.[90] By replacing an internal region of *SGA1* with a DNA fragment containing the *LEU2* gene, Pugh and Clancy[61] used a disrupted allele of *SGA1* to map it with respect to other markers. The *SGA1* gene was located on the left arm of chromosome IX, close to *lys11* and approximately 23 *cM* from *his5*. On the basis of their meiotic mapping data, Patel et al.[133] have speculated that the *STA2* gene may be located on the right arm of chromosome II or distal to *SUC3*, but is not part of a cluster of sugar fermentation *(SUC3, MAL3, MGL2)* markers.

As a further step in understanding the expression and evolution of the *STA* genes, the nucleotide sequences of *STA1*,[53] *STA2*,[54] *SGA1*, S1, and S2[52] were determined. Yamashita et al.[53] reported a unique open reading frame (ORF) of 2334 nucleotides (nt) for *STA1*, encoding a protein of 778 amino acid residues. The sequence revealed two ATG codons within 30 nucleotides from each

other. Yamashita et al.[53] concluded that nucleotide 1 is A of the first ATG in the ORF for the following reasons: the ATG is the first ATG codon encountered in the single major ORF of the *STA1* gene; the sequence 5′-TATAC-3′, immediately upstream from the initiation codon of the *ADH1* gene, is also found in the corresponding region of the *STA1* gene; and like most yeast genes that have been sequenced, *STA1* contains an A residue three nucleotides before the initiation codon. The latter two arguments also pertain to the second ATG starting at nt +34. However, according to Yamashita et al.[52] the amino-terminal residue of the *STA1* gene product was expected to be the second methionine in the ORF. Pardo et al.[29] stated that the amino-terminal residue of the *STA2* gene product is the second methionine in the ORF. This ATG codon is preceded by a CACACT sequence highly homologous to the consensus T/AAC/AACA (which precedes the ATG initiator codon of most yeast mRNAs) and is similar to that (CACAAT) of the *HMLa2* and *MATa2* genes.[55] The sequence 5′-TATAAA-3′ (position −67 to −62) of the *STA1* gene is in good agreement with the "Hogness-box" structure (5′-TATAAATA-3′) that functions as part of the eukaryotic RNA polymerase recognition site.[53] Relative to the second putative ATG translation initiation codon, this TATA-box is located at nt −100 to nt −105. There is also another possible TATA-box at nt −75 to nt −70.[55] Thus, *STA1* and *STA2* each have two putative TATA-boxes. Yamashita et al.[53] have noted that the nucleotide sequence, position −59 to −39 (position −92 to −72 if the ATG translation initiation codon is considered to be the second ATG in the *STA1* ORF), of the *STA1* gene is highly isologous to one of the two 5′-capping sites of the *ADH1* mRNA; it seems likely that the *STA1* mRNA starts within this conserved region. It is known that the sequences TC(G/A)A and PuPuPyPuPu account for more than half of the known yeast transcription initiation sites.[56] Therefore, the mRNA initiation site of *STA1* and *STA2* (relative to the second ATG in the ORF) could be the following: nt −20,[29] nt −30,[57] nt −180, nt −190, nt −220, nt −230, nt −250, nt −265, nt −270, nt −375, nt −415, nt −485, nt −495.[58] The *STA1* gene contains a mRNA-splicing signal sequence (5′-TACTAACT-3′) beginning at position +837, where *STA1* mRNA might be spliced if a frame shift does not occur; otherwise, translational stop codons would be encountered frequently.

SGA1 has a unique ORF of 510 amino acid residues.[52,55] Other putative ATG initiator triplets are present in the same reading frame at nt −405 and −204; however, the presence of nonsense codons at positions −399, −186, −51, −48, and −45 indicate that they cannot function as the starting codons for the *SGA1* gene product. No other significantly long ORF is present in the other two reading frames. The long AT-rich region from nt −496 to −546 could promote transcription if it is recognized as a constitutive promoter by RNA polymerase II. The sequence from Phe-33 to Asn-510 of the putative *SGA1* glucoamylase is almost identical to that from Phe-290 to Asn 767 of the *STA1* glucoamylase, in which there are present two silent substitutions and 14 replacement substitutions. In the 3′-flanking region (+1 to +327), substitutions of two nucleotides, an insertion of an A-A dimer, and a deletion of two nucleotides were observed.[52,55]

Yamashita et al.[52] compared the protein sequence deduced from the nucleotide sequence of S1, S2, *SGA1* to that of *STA1*. S1 encodes a protein of 570 residues, of which the sequence from Ser-109 to Val-411 matches the sequence from Ser-31 to Val-289 of the *STA1* glucoamylase, except that the sequence from Val-84 to Thr-127 of the *STA1* glucoamylase is duplicated in S1. In S1 there are 12 silent substitutions and 14 replacement substitutions. S1 is composed of six-unit sequences that are repeated two to nine times. S2 contains an ORF of 242 residues, of which the amino-terminal peptide of 32 amino acids is identical to the corresponding region of the *STA1* glucoamylase. In that region, two silent substitutions occur. In the flanking region compared (up to −191), 11 nucleotides are exchanged and two nucleotides are deleted.

Based on the sequence analyses of *STA1*, *SGA1*, S1, and S2, Yamashita et al.[52] postulated that an ancestral *STA* gene was generated by two fusion events (S2-S1 and S1-*SGA*) and a deletion of one copy of the direct repeats, Val-84 to Thr-127, in S1 (Figure 3). It is also possible that S1 contained a single copy of the sequence Val-84 to Thr-127 when the fusion of S2, S1, and *SGA1*

occurred and that duplication of the sequence occurred recently in *S. cerevisiae*. According to Yamashita et al.[52] S1 may have functioned to join S2 and *SGA1* that encode the signal peptide for protein secretion and intracellular glucoamylase, respectively. Alternatively, it is possible that *STA1* was fragmented into S2, S1, and *SGA1*. The fusion model is more likely for the following reasons. (1) If *STA1* were an ancestral gene for S2, S1, and *SGA1*, several *Saccharomyces* species would contain *STA1*, since the DNA rearrangement is likely to have occurred very recently; alternatively, if S2, S1, and *SGA1* were precursors for *STA1*, it is reasonable that only *S. diastaticus* should contain *STA1*. Of the 33 *Saccharomyces* species examined by Yamashita et al.,[52] 10 were found to contain DNA sequences homologous with *SGA1* that could be progenitor candidates for *STA1*; however, as described above, only *S. diastaticus* secretes glucoamylase and can ferment starch. (2) Gene disruption experiments indicated that S1, S2, and *SGA1* are not essential for vegetative growth, sexual mating, or meiosis and sporulation.[51] It is reasonable to assume that *STA1* was generated from components S1, S2, and *SGA1* that had become nonessential, rather than nonessential genes evolving recently by disruption of *STA1*.

The nucleotide sequences around the junctions (S2-S1a and S1b-*SGA1*) and the corresponding sequences of *STA1* were compared by Yamashita et al.[52] Five short homologous blocks were found bracketing the junctions. It is possible that those homologous sequences played a role in the mechanism of gene fusion. Another structural feature is the existence of the sequence GTACCAAC at both junctions. Both sequences are located nine nucleotides upstream from the

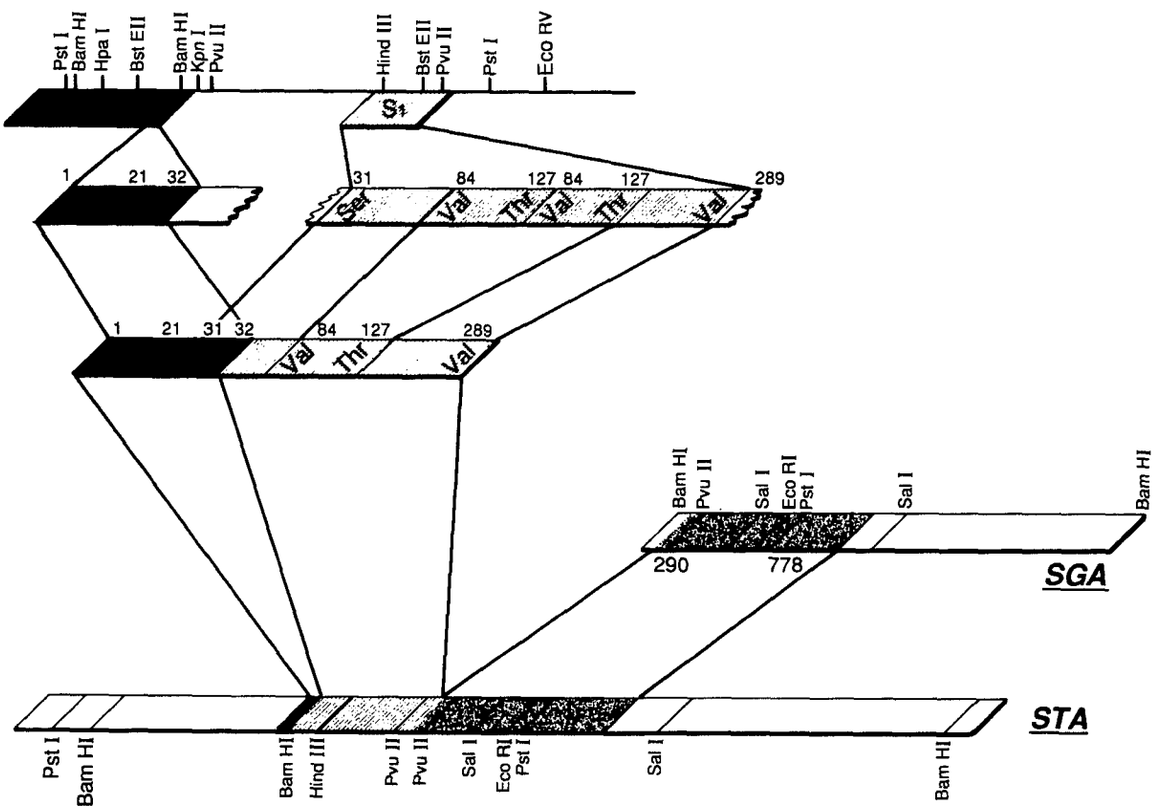

FIGURE 3. A diagrammatic representation of a gene-fusion model as a possible mechanism underlying the evolution of the *STA* genes and the physical maps of S1, S2, *SGA1* and the *STA* gene. The ancestral *STA* gene was supposedly generated by two fusion steps (S2-S1 and S1-*SGA1*) and a deletion of one copy of the direct repeats, Val-84 to Thr-127.[52]

putative junctions and could have played a role together with homologous blocks, in the gene-fusion model.[52]

III. PROPERTIES OF YEAST GLUCOAMYLASES

A. Biochemical and Structural Properties of Glucoamylases

In 1833, Payen and Persoz prepared an alcohol precipitate of malt extract that contained thermolabile, active material that separated the insoluble components of starch grains from the soluble dextrins. This work represents the first attempt to purify an enzyme that they named *diastase* (Greek, *to separate*).[59] In the late 1800s, Hans and Edward Buchner ground yeast cells with quartz and kieselguhr, pressed a cell-free liquid from this paste by means of a hydraulic press, and found that the liquid still fermented sugar. The active agent, an enzyme (Greek, *in yeast*), was named *zymase*.[59]

The actions of the seven classes of amylolytic enzymes of microbial origin can be divided into two categories. Endoamylases split linkages in a random fashion within the starch molecule. Exoamylases hydrolyze starch progressively from the nonreducing end generating low-molecular-weight end products. Another division can be made depending on the linkages hydrolyzed (Table 1). Glucoamylase (1,4-α-D-glucan glucanohydrolase EC 3.2.1.3) is defined as an exo-acting carbohydrase that splits α-glucans, yielding β-D-glucose from the nonreducing end of the substrate.[1] It was found that the glucoamylases produced by *S. diastaticus* are in fact exoglucosidases that cleave single glucose units from the nonreducing ends of starch-like substrates.[60] The rate of hydrolysis of nonreducing α-1,4 glycosidic linkages by GAII[60] and SGA[61] was shown to increase with decreasing molecular weight of the substrate, reaching a maximum with maltotriose. The α-1,6 glycosidic bonds were hydrolyzed at greatly reduced rates, isomaltose and isomaltotriose being cleaved at 0.6 and 1.4% rate of maltose, respectively.[60] A marked difference in the rate of hydrolysis of α-1,6 bonds was observed depending on whether the neighboring bond in the sequence is α-1,4 or α-1,6. Modena et al.[60] reported that the α-1,6 bond in panose was hydrolyzed eight times more rapidly by GAII than in isomaltotriose. The pH and temperature optima further define the properties of the glucoamylases from *S. diastaticus*. Yamashita and Fukui[31] reported an optimum pH of 5.3 and an optimum temperature of 50°C for GAII. The *DEX1* encoded glucoamylase was reported to have an optimum pH of 5.0 and an optimum temperature of 50°C,[28] whereas the pH and temperature otpima of GAII were found to be 5.1 and 63°C, respectively. Tucker et al.[62] reported pH and temperature optima for GAII of 5.5 and 60°C, respectively. The SGA enzyme was also found to be active over a broad range of pH and buffers, with an optimum of approximately pH 5.5 in sodium citrate buffer.[61]

There are multiple forms of glucoamylase that differ in their substrate preference. Glucoamylase is produced by many fungi (e.g., *Aspergillus, Mucor, Neurospora, Rhizopus, Trichoderma*), yeasts (e.g., *Endomycopsis, Lipomyces, Saccharomyces, Schwanniomyces*) but only by a few bacteria (e.g., *Bacillus stearothermophilus, Clostridium thermohydrosulfuricum, Flavobacterium* sp., *Halobacterium sodomense*). Many of the fungal glucoamylases are produced on an industrial scale. The number of glucoamylases per strain ranges between one and five. Several mechanisms have been presented to describe their synthesis and modification. Almost all of the fungal glucoamylases are glycoproteins and variations in kind and amount of carbohydrate could give rise to some of the observed multiple forms.[63] The pH of the culture medium was found to determine the pH stability of *A. awamori* var. *fumeus* glucoamylase.[64] *A. awamori* var. *kawachi* produces three different forms of glucoamylase in different media.[65] In the presence of zinc, glucoamylase was found to be degraded to smaller forms by proteases. The sizes of these glucoamylases are 90, 83, and 57 kDa, respectively.[66] Fiedurek et al.[67] reported that glucoamylase activities in *Aspergillus niger* mutants are directly proportional to the rates at which they are proteolyzed. Boel et al.[68] demonstrated that different forms of *A. niger* glucoamylase resulted from alternatively spliced mRNA. Amino-terminal amino acids of *Rhizopus* spp. glucoamyl-

ases are different[69] due to proteolytic modification.[70] *Aspergillus awamori*,[71] *A. niger*,[68] *R. oryzae*,[72] and *S. fibuligera*[26,131] each have a single locus for glucoamylase and these genes have been cloned, sequenced, and expressed in *S. cerevisiae*. Based on these reports, it is clear that various forms of glucoamylase are due to a variety of mechanisms, including mRNA modifications, limited proteolysis, and variation in the carbohydrate content.

In contrast, *S. diastaticus* has three unlinked genes, *STA1*, *STA2*, and *STA3*, encoding extracellular glucoamylase isozymes, GAI, GAII, and GAIII, respectively.[11,12,37,38] Conflicting results regarding the molecular weight, in both native and deglycosylated forms, and the subunit structure of GAI, GAII, and GAIII, have been reported. Tamaki[73] reported sedimentation constants and sizes of 4.25 S and 68 kDa for GAI, 4.58 S and 84 kDa for GAII, and 4.38 S and 79 kDa for GAIII.

Purified GAI from *S. diastaticus* strain 5106-9A carrying *STA1* was found to be heterogeneous in size, ranging from approximately 80 to 66 kDa; it consisted of two nonidentical subunits, H and Y.[74] The size of subunit H was heterogeneous and was determined to be approximately 68, 59, and 53 kDa by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Similarly, the size of the hydrophobic subunit Y was determined as 14 kDa. The deglycosylated forms of subunits H and Y were reported to have sizes of 41 and 3.4 kDa, respectively, suggesting that the heterogeneity of GAI was due to glycosyl moieties of subunit H.[74] Purified GAI from *S. diastaticus* strain YIY2-12D and from *Schizosaccharomyces pombe*, HM123 transformed with a plasmid carrying *STA1* was reported to be monomeric with sizes of 250 and 180 kDa for the glycosylated and deglycosylated forms, respectively.[75,76] This result suggests that 70% of the molecular weight might be contributed by carbohydrate, consisting mostly of mannose. Further structural analysis revealed that N-linked glycosides accounted for 70 kDa, most of which are localized at the catalytic domain, and O-linked glycosides for 100 kDa. The diversity of molecular structures of the glucoamylases encoded by *STA1* was attributed to different extents of proteolysis and N-linked glycosylation reactions.[76] If translation starts at the first ATG, the 778-amino-acid protein encoded by the *STA1* gene yields a calculated size of 83.7 kDa[53] that is two-fold larger than that reported for the purified GAI: the combined sizes of the protein moieties of subunits H and Y is 44.4 kDa.[74] However, Yamashita et al.[52] regard the second ATG in the *STA1* ORF to be the initiating codon. This reconsideration implies that *STA1* codes for a 767-amino-acid protein[52] instead of a 778-amino-acid protein.[53] Tucker et al.[62] reported values for native GAII and its subunits of 306 and 186 kDa, respectively.

A size of 150 kDa was reported for GAII isolated from a strain carrying *DEX1*.[28] Modena et al.[60] purified the GAII encoded by the *STA2* gene to near homogeneity and reported that GAII consisted of two identical subunits whose average size is 300 kDa. Under denaturing conditions, the native dimeric enzyme readily dissociated to monomers. Enzymatic deglycosylation of the denatured enzyme gave rise to intermediate, partially glycosylated forms and to a completely deglycosylated protein of 56 kDa. Kleinman et al.[77] reported a native glucoamylase tetramer whose size is 250 kDa. Gel filtration indicated the existence of oligomers of larger size, whereas denaturation dissociates the native enzyme to monomers of 70 kDa. Consistent with our findings, Kleinman et al.[77] found that 80% of the enzyme consists of carbohydrate and that mannose accounts for 94% of the carbohydrate content. Given the identical restriction maps of *STA1*, *STA2*, and *STA3* and the identical nucleotide sequences reported for *STA1*[53] and *STA2*,[54] the different molecular weights and subunit structures reported for GAI, GAII, and GAIII are rather confusing. The inherent heterogeneity of glycoproteins, the different purification protocols and differences in glycosylation patterns among different yeast strains, can partially account for the differences in molecular weight among extracellular glucoamylase isozymes prepared in various laboratories.[60]

RNA blotting and protein immunoblotting revealed a 2.0-kb mRNA and 90-kDa protein that were coinduced in sporulating *sta°* diploids.[78] Consistent with these results, Pugh et al.[79] reported that the purified SGA protein sedimented with an apparent size of 90 kDa. Unpurified SGA

was found to sediment with an apparent size of 190 kDa. This behavior is consistent with the proposal that the native SGA molecule is a dimer and that dissociation occurs during purification to yield active monomers. Four bands detected on SDS-polyacrylamide gel profiles of partially purified SGA were shown to migrate at positions corresponding to sizes of 79, 75, 72, and 69 kDa.[79] The fastest-moving protein band corresponds to the predicted size of the 510-amino-acid SGA protein (68 kDa) based on its nucleotide sequence.[52] One possible interpretation of the banding pattern could be that SGA is glycosylated heterogeneously. Alternatively, the various peptides could represent partially processed forms or proteolytic degradation products of the intact SGA protein.[79]

B. Localization and Secretion of Glucoamylases

The DNA sequence analysis of *STA1*[53] showed that a putative GAI precursor consists of three characteristic regions, namely, a hydrophobic leader peptide sequence (HL), a threonine- and serine-rich tract (TS), and a catalytic domain (CD). If the first ATG in the *STA1* ORF is considered as the initiation codon, then the length of the leader is 32 amino acid residues.[53,80] The HL peptide was shown to act as signal peptide and has significant homology to that of the *Bacillus subtilis* α-amylase precursor.[53] When the signal sequence encoding the extended peptide of 32 amino acids was fused to a structural gene for *Escherichia coli* β-lactamase, Yamashita et al.[81] showed that yeast cells containing these hybrid plasmids secreted active β-lactamase. This experiment was repeated by Vanoni et al.[82] who reported secretion of *E. coli* β-galactosidase in yeast by using the corresponding promoter and signal sequences of *STA2*. Up to 76% of the *STA2* directed β-galactosidase activity was detected in the culture medium. This result indicates that other structural determinants, present in the sequence of mature glucoamylase, are required for targeting a protein to the medium, possibly through interaction with specific receptors.

It is generally believed that hybrid proteins constructed from hydrophobic leader sequences and naturally intracellular proteins such as the *E. coli* β-galactosidase are not secreted but become crowded in the endoplasmic reticulum (ER). This is likely due to certain sequences of intracellular proteins that interfere with the transport process.[83] The structural features of the TS tract (ca. 320 amino acids) are common to a family of secreted or transmembrane proteins such as red cell membrane proteins,[84] small intestinal brush border glycosidases,[85] the receptor for interleukin-2,[86] and virus surface glycoproteins.[87,88] It was reported that a FUS1-LacZ hybrid protein, carrying the amino-terminal half of the FUS1 protein that contains the TS tract and is required for sexual fusion of *S. cerevisiae* cells, can be transported to the cell surface.[89] To investigate the role of the TS tract in glucoamylase secretion, Yamashita[80] constructed and introduced into yeast a series of internal deletions of *STA1* and chimeric genes that encode various lengths of the GAI amino-terminal peptide and a constant carboxy-terminal peptide of either SGA or β-galactosidase and examined the secretory nature of their gene products. The altered GAI proteins without the TS tract were found not to be secreted, while the hybrid β-galactosidase proteins carrying TS were transported to the cell envelope. Based on these results, Yamashita[80] proposed two possible roles for the TS tract of GAI, namely, (1) the TS tract is important and sufficient for protein transport from the ER through the Golgi apparatus to the cell envelope and (2) the TS tract, together with HL, can stimulate protein translocation into the lumen of the ER. There is at present no clear evidence that TS recognizes a novel secretory pathway specific to TS or that TS masks the secretory defect of reporter proteins such as β-galactosidase. It also remains to be studied whether the threonine and serine residues in the TS tract really serve as a signal for transport. Yamashita's[80] data for transport of the deleted or fused proteins imply that some specific amino acid residues (encompassing amino acids 250 to 300 or 61 to 182) are required for transport. It is, however, equally likely that there is a minimum size for an effective TS region.[80] Ishiguro et al.[132] has identified a dominant mutation, *SSD1*, that suppresses deletion mutations in the secretory signal sequences of GA1. Yeast cells harboring the mutation, secreted hybrid β-

galactosidase proteins carrying either the HL region or the TS tract.

Nearly 55% of the amino acid residues of the TS tract are threonine and serine, whose amino acid side chains have hydroxyl groups.[53] Yamashita[80] hypothesized that this hydrophilic domain projects out from the globular fold of the protein and forms an unfolded tertiary structure that is not rigid. This hypothesis excludes the possibility of novel secretory pathways specific to the TS tract and suggests that the peculiar structure of the TS tract plays a role in secretion of glucoamylase. The observation that endogenous proteases readily cleave the TS tract from the catalytic domain[74] supports this hypothesis.

It is evident that the TS tract of yeast glucoamylase can direct intracellular soluble proteins, which are not secreted with the aid of the HL peptide alone, to the cell envelope. The TS region is thus likely to be essential in the evolution of the extracellular glucoamylases (GAI, GAII, and GAIII) from the intracellular SGA. This assumption is supported by the observation that SGA is glycosylated and located in the yeast vacuole, indicating that SGA contains the information for entry into the early stages of the secretory pathway.[79]

It was also shown that the glycosylation-secretion pathway of the yeast cell could become saturated when GAII was produced above wild-type levels.[58] The *STA2* gene was cloned and expressed under the control of the strong, inducible UAS_{GAL} promoter. Immunoblots of secreted and cell-associated GAII showed that the protein synthesized by the transformed yeast strain (containing the UAS_{GAL}-*STA2* plasmid construct) was much more heterogeneous in size than the enzyme synthesized by the untransformed yeast strain (containing only the chromosomal copy of the *STA2* gene). Furthermore, less extracellular glucoamylase activity was detected in the transformant. Vanoni et al.[58] speculated that glycosylation could be a major rate-limiting step for efficient secretion of the heavily glycosylated GAII protein.

IV. REGULATION OF THE GLUCOAMYLASE GENES

A. Distribution and Relationship of Glucoamylase Synthesis to Growth Phase and Sporulation

It was found that the synthesis of extracellular glucoamylase (GAII) in haploid yeast strains is continuous throughout the growth phase, and follows a typical growth-associated production pattern. The fraction of secreted GAII increases during the exponential growth phase, reaching a maximum of 50% of the total glucoamylase synthesized during the early stationary phase.[78] By contrast, intracellular glucoamylase, SGA, is synthesized only during the sporulation phase of the life cycle. *SGA1* is one of a group of "late" sporulation-specific genes whose transcripts appear at the time of meiosis I and at no other stage in the life cycle of standard haploid or diploid laboratory strains of *Saccharomyces*.[39,91] The significance of *SGA1* in sporulation is unclear. Diploid cells homozygous for insertionally disrupted *SGA1* did not degrade intracellular glycogen but were able to undergo sporulation.[51]

B. Effect of Growth Medium Composition on Glucoamylase Expression

The process of decoding genes, synthesizing appropriate amounts of gene products and secretion of extracellular enzymes are complex, with regulation occurring at one or more of the various steps along the pathway.[56] We have analyzed physiological and genetic factors affecting glucoamylase production and have found that regulation of glucoamylase expression takes place primarily at the level of transcription.[78]

Different sizes and numbers of *STA* and *SGA* transcripts have been reported in the literature. A 2.0-kb *SGA1* transcript and a 2.7-kb *STA1* transcript were reported by Yamashita and Fukui[51]

and Yamashita et al.,[92] respectively. Pardo et al.[29] reported that the *STA1* gene produced a 2.85-kb transcript and that transcription of the *SGA1* gene was initiated from two different sites, yielding two transcripts of 1.95 and 2.40 kb. Pardo et al.[29] also found a 5.40-kb RNA species that shared homology with the *STA2* region that encodes the export domain of the external glucoamylase. Vanoni et al.[58] also detected a 5.0-kb RNA species on RNA blots probed with *STA2* DNA. The function of this 5.0 to 5.4-kb RNA species is not known. Erratt and Nasim[27] reported that two RNA species, measuring 2.1 and 1.5 kb, were found in both vegetative and sporulating cultures of *S. diastaticus*, whereas one 1.5-kb transcript was present only in RNA isolated from sporulating cultures of *S. cerevisiae*. Pretorius et al.[78] reported 2.5-kb transcripts for the *STA1*, *STA2*, and *STA3* genes, and a 2.0-kb transcript for the *SGA1* gene. The results of Pretorius et al.[78] were later confirmed by Dranginis.[93]

When the effect of growth medium on the expression of the *STA* genes was investigated, it was found that the carbon and nitrogen sources modulate glucoamylase expression at the transcriptional level.[78] No evidence for starch induction of *STA* gene expression could be detected in *Saccharomyces* strains grown on complex media. This is in contrast to a 200-fold increase of glucoamylase mRNA in *A. awamori* grown on starch compared to a noninducing carbon source such as xylose.[71] Glucoamylase production and *STA2* transcription in *S. diastaticus* strains were found to be carbon catabolite repressed by glucose to a relatively small extent; the degree of repression depending on the strain.[78] This finding was later confirmed by Dranginis[93] with *STA1* strains. Those results suggest that other, as yet unidentified, genes play a role in controlling glucoamylase expression.[94,95] It is surprising that *S. diastaticus* strains synthesize and secrete significant amounts of glucoamylase in complex media with glucose as the sole carbon source, since glucose brings about carbon catabolite repression of a variety of yeast enzymes and permeases involved in sugar catabolism. These include invertase,[96,97] alcohol dehydrogenase,[98] maltase and maltose permease,[99,100] and α-galactosidase.[101] As quoted by Erratt and Nasim,[102] Searle (1982) reported that in haploid cultures of *S. diastaticus* containing the *CDX1* gene, glucoamylase synthesis was constitutive, but the enzyme was not secreted into the growth medium. This suggests that *CDX1* regulates glucoamylase at the level of secretion from the cell wall, rather than at the levels of transcription or enzyme synthesis. Enzymes involved in the metabolism of starch and related compounds are known to be subject to catabolite repression in several yeast genera, and mutants derepressed for the synthesis of the starch degrading enzyme, α-amylase, have been isolated from *Schwanniomyces*.[103] Glucoamylase synthesis is carbon catabolite repressed by glucose in *Neurospora crassa*,[104] whereas *A. awamori* produces significant amounts of glucoamylase when grown in glucose-containing media.[71] Maximal levels of glucoamylase activity in *S. diastaticus* are reached in cells grown in complex medium supplemented with glycerol plus ethanol, starch or Maltrin M365 (a mixture of maltooligosaccharides, Grain Processing Corp., Muscatine, Iowa). When sugars such as galactose, maltose, raffinose, and sucrose served as carbon sources, they all supported glucoamylase synthesis, although at reduced levels.[78] Batch cultures of *S. diastaticus* grown in synthetic media do not produce detectable amounts of *STA* mRNA and extracellular glucoamylase. However, halo formation, reflecting extracellular glucoamylase by Sta^+ strains growing on synthetic agar plates containing starch, was similar to that formed on complex agar plates. Glucoamylase activity in batch cultures can be restored by addition of yeast extract, peptone, or Maltrin to the synthetic culture medium.[78] Similar results have also been reported for fructose 1,6-bisphosphatase and the inducible alcohol dehydrogenase II.[105] Respiratory deficient (*rho°* or petite) mutants containing *STA1*, *STA2*, and *STA3* do not produce glucoamylase except in complex media with su-

crose as carbon source and in synthetic media with Maltrin as carbon source.[78] The involvement of mitochondrial functions in membrane and surface phenomena (e.g., flocculation), and in the metabolism of several fermentable carbon sources, e.g., galactose and maltose, has been reported previously.[106-110] Although the biochemical basis of this nuclear-mitochondrial interaction has not been elucidated, the role of specific nuclear genes has been clearly established. It has been reported that a nuclear gene, *GEP1*, unlinked to *STA2*, determines glucoamylase expression in petites and that *GEP1* appears to interact with a glucoamylase-repressor gene, termed *SGL1*.[133]

The accumulation of anomalous *STA* mRNA species, differing principally at their 5′ ends and saturation of step(s) in the secretory pathway appear to be among the major factors limiting glucoamylase expression in synthetic media.[58] Vanoni et al.[58] showed that Sta$^+$ cells grown in synthetic media produced a large amount of the 2.8 kb *STA* transcript and that the quantities of the transcript decreased after the cells were transferred to rich medium. A predominant 2.5-kb transcript in Sta$^+$ cells grown in rich medium was previously reported by Pretorius et al.[78] Thus, growth medium composition may play a major role in the production of *STA* mRNA of various sizes. *S. cerevisiae* is quite flexible with respect to the use of TATA boxes and in the choice of transcription initiation sites.[56] The longer *STA* mRNA species originate from differential transcription initiation from a site(s) about 300 bp upstream of the major initiation sites. Regulated differential transcription initiation from different promoter elements has been reported previously in *Saccharomyces* and may result in mRNA with altered translatability.[111] Transcription initiation from the upstream site would result in a *STA* mRNA species with a very long untranslated 5′ sequence. This is unusual for yeast mRNAs, but some precedents exist. One example is the very long leader sequence of the *GCN4* gene encoding the regulatory protein GCN4.[112-114] It has multiple AUGs starting from the 5′ end of the mRNA, but the fourth one is the site of translation initiation. It was reported that when the *STA* gene dosage was increased, fairly high levels of *STA* mRNA accumulated without a concomitant increase in enzyme activity.[58,78] Extracellular glucoamylase is heavily glycosylated,[60] but incomplete glycosylation of the protein was reported when the enzyme was expressed beyond wild-type levels.[58] Thus, the extent of glycosylation might be a major rate-limiting step in the secretion of heavily glycosylated proteins such as extracellular glucoamylase, but might be less important as the number of glycosylation sites decreases.

C. Effect of the *STA10* Gene on Glucoamylase Expression

An early report by Gilliland[44] presented some evidence for the existence of a gene(s) inhibiting glucoamylase expression. Gilliland analyzed tetrads derived from crosses between *Saccharomyces chevalieri* and *S. diastaticus* for Mendelian inheritance of the ability to ferment dextrins and observed irregular segregation patterns. Similar aberrant meiotic segregation of the Sta$^+$ phenotype was documented by Tamaki.[115] Tamaki[46] suggested that an inhibitor gene(s) for starch fermentation might be responsible for the reported non-Mendelian segregation patterns. Polaina and Wiggs[40] analyzed crosses between *S. diastaticus* and laboratory strains of *S. cerevisiae* and identified a gene, *STA10*, that inhibited the expression of the amylolytic capability in a dominant fashion. These experiments were repeated by Yamashita and Fukui[95] who concluded that *S. cerevisiae* carries two inhibitory genes against glucoamylase, namely, *INH1* and *sta°*. Park and Mattoon[116] postulated that suppression of glucoamylase expression resulted from interaction of two, unlinked genes, *IST1* and *IST2*. It was found that neither *IST1* nor *IST2* acted singly on *STA1* to eliminate glucoamylase secretion, although each gene caused a decrease in production of extracellular glucoamylase. Based on these data, it is still not possible to elucidate the mechanism by which *STA10* acts to suppress glucoamylase synthesis. Patel et al.[133] detected a glucoamylase-repressor gene termed *SGL1*, but the relationship between it and *STA10*, *INH1*, *IST1*, and *IST2* remains to be determined.

In haploid strains carrying both *STA2* and *STA10* genes, the level of *STA2* mRNA and total

extracellular glucoamylase activity was found to be less than 5% of that found in *STA2* haploids.[78] In diploid strains, homozygous for the mating-type, carrying both *STA2/sta2* and *STA10/sta10*, a less dramatic inhibition of *STA2* expression (expressed both as mRNA and glucoamylase enzyme activity levels) was found, suggesting a gene dosage effect for the *STA10* gene. The repressive effect by the *STA10* gene was partially overcome when the *STA2* gene was present on a multicopy plasmid.[78] One interpretation of this effect is that the *STA10* gene encodes a protein that represses glucoamylase synthesis, but whose intracellular concentration is not high enough to fully control the expression of multicopy *STA2* genes. It is also possible that *STA10* might be the inactive allelic form of a positive activator of glucoamylase expression rather than a repressor. However, if *STA2* is transcribed at low levels in the absence of any functional positive activator, additional basal transcription of the *STA2* gene on a multicopy plasmid would result in increased levels of both *STA2* mRNA and its encoded glucoamylase activity. These data indicate that *STA10* affects glucoamylase synthesis mainly at the level of *STA2* mRNA accumulation, although minor effects in secretion cannot be ruled out at present.[78] Inui et al.[117] concluded from S1 mapping and RNA blot analyses that the *INH1* gene repressed the expression of *STA1* at transcriptional level when cells were grown in a rich medium with glycerol and lactate as carbon sources. When the cells were cultured in a rich medium containing starch as a carbon source, control of *STA1* expression was found to be at the posttranscriptional level. From their glucoamylase-immunoprecipitation experiments, Inui et al.[117] concluded that *INH1* prevented translation of *STA1* transcripts or destabilized GAI when the cells were cultured in the presence of starch. It was also found that multicopies of *STA1* did not overcome the inhibitory effect of *INH1*.[117]

Pardo et al.[29] reported that the *STA1*, *STA2*, *STA3*, and *SGA1* genes were found to be negatively coregulated at the transcriptional level by *STA10*. Given the divergence at the 5′ ends of *STA1*, *STA2*, and *STA3* compared to *SGA1*, it would be expected that these genes contain short, homologous regions that would account for their coregulation by *STA10*. In support of their proposal, Pardo et al.[55] identified four homologous sequences (homologies I, II, III, and IV) in the 5′ regions of *STA2* and *SGA1*. The homologous regions, I, II, and IV are repeated in *SGA1*, but are only present as single copies in *STA2*. Homology III, consisting of 9 nt, is present as a direct repeat in both genes. Each repeat has bilateral symmetry (PuCATTTAPyG) with the central T as the axis. In both the *SGA1* and *STA2* genes one of the direct repeats is located next to the TATA boxes and, therefore, is a good candidate to be the site of regulation by *STA10*. Indeed the DNA region downstream from the *Bst*EII site (Figure 2) of the *STA2* gene maintains both promoter activity and repression capacity by *STA10*. Since the sequences of *STA1* and *STA2* are identical in their promoter regions, this argument also applies to *STA1*. By contrast, Pugh and Clancy[61] found approximately equal levels of SGA from a high-copy construct introduced into *STA10* and *sta10* (haploid and diploid) strains. They concluded that *SGA1* is not regulated by *STA10*.

D. Effect of the Mating-Type Configuration on Glucoamylase Expression

Yeast developmental pathways are regulated by combinations of gene regulatory proteins specified by the mating-type locus (*MAT*). *MAT* consists of two alleles, *MATa* and *MATα*. *MATa* and *MATα* encode *MATa1* and *MATa2* and *MATα1* and *MATα2*, respectively. The *MAT* regulates the expression of a large number of unlinked genes that determine cell type in yeast. Different sets of genes are expressed in the three specialized cell types of *S. cerevisiae* (see References 118, 134 and 135 for primary references). The α-specific genes are expressed only in the *a*-haploid cell type, and the α-specific genes are only expressed in the α-haploid cell type. The haploid-specific genes (e.g., the *STA* genes) are transcribed in both *a*- and α-cells, but not in the *a*/α-diploid cell type. The *a*/α-diploid cell type can undergo meiosis and sporulation, a pathway absent in the *a*- and α-haploid cell types (Figure 4). The proteins encoded by *MATa1*, *MATα1*, and *MATα2* have been found to regulate the expression of genes that define cell type. *MATa2* produces a transcript whose function re-

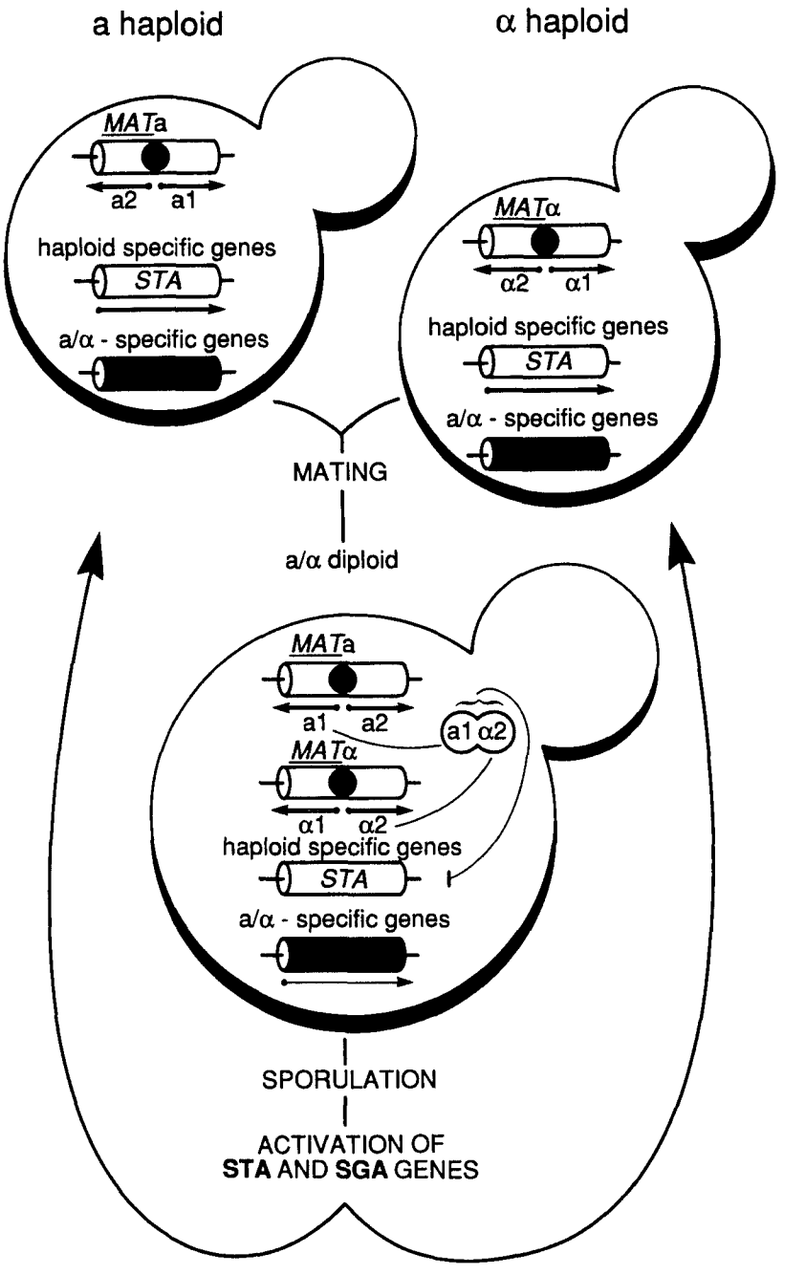

FIGURE 4. The yeast life cycle, illustrating *MAT* control of the mating-type of a cell as well as of haploid-specific genes such as the *STA* genes. The *STA* genes are expressed in *MATa* and *MATα* haploid cells but not in *MATa/MATα* diploids. RNA transcripts and protein products are indicated by arrows and circles, respectively. A curved line with a terminal bar indicates inhibition of gene expression.[134]

mains unknown.[118] In combination with the a1 product of *MATa*, the α2 product of *MATα* binds to distinctive sites in the upstream regions of haploid-specific genes (*HO*,[119] *RME1*,[120] and TY*1* elements[121]), causing repression.[135]

Expression of *STA1*, *STA3*,[123] and *STA2*[78] wa shown to be repressed in *MATa/MATα* diploids Glucoamylase is secreted by both *MATa* an *MATα* haploid cells but not by *MATa/MATα* dip loid cells. Yamashita et al.[92] isolated a mutan

relieved from the mating-type control of *STA1* gene expression. Tetrad analysis indicated that the mutation was closely linked to *MATa* and could be complemented by a *MATa2* gene. Based on these data and RNA blot analysis, Yamashita et al.[92] proposed a role for the a2 product of *MATa* in the mating-type repression of *STA1* at a posttransciptional level. Pretorius et al.[78] found that both *STA1* mRNA and GAII activity were drastically and coordinately inhibited in *MATa/MATα* diploids. Both effects were partially overcome when the *STA2* gene was present on a multicopy plasmid. Consistent with the results of Pretorius et al.,[78] but in conflict with the results of Yamashita et al.,[92] Dranginis[93] reported that *MATa/MATα* diploid-specific repression of glucoamylase secretion can be accounted for by a decrease in *STA1* mRNA levels. Since diploid cells, which do not secrete glucoamylase, cannot grow well in liquid starch medium, Yamashita et al.[92] were forced to culture their diploid strains for 7 d in a medium containing starch as the sole carbon source. Dranginis[93] suggested that amylolytic mutants were selected during these protracted culturing conditions, explaining the *STA1* mRNA seen in diploid cells by Yamashita et al.[92] In every medium tested by Dranginis et al.,[93] *MATa/MATα* diploid-specific repression of *STA1* occurred at the transcriptional level. Strain differences cannot account for the discrepancy between the data of Dranginis[93] and Yamashita et al.,[92] since they have used the same strains. Dranginis[93] further showed that a yeast strain with a complete deletion of *MATa2* was unaltered in mating-type regulation of *STA1*, whereas deletion of *MATa1* sequences completely abolished diploid-specific repression. From these results it can be concluded that *STA1* and *STA2* (and by extrapolation *STA3*) are haploid-specific genes that are transcriptionally regulated in diploids like other known haploid-specific genes, by the a1-α2 repressor. Inui et al.[117] analyzed diploid cells carrying *mat* mutations and suggested that different sets of mating-type genes were required for the repression of *STA1* depending both on the copy number of *STA1* and culture conditions. When cells were grown in a medium with glycerol and lactate as carbon sources, *MATa1/MATα2* was required for the repression of *STA1* transcription, regardless of the copy number of *STA1*. However, when cells were grown in the presence of starch, they found that *MATa1*, *MATα2*, as well as *MATa2* were required for *STA1* repression at the posttranscriptional level. Inui et al.[117] also reported that two copies of *MATα2* inhibited *STA1* expression in a starch medium. Based on their finding that multicopies of *STA1* suppressed the inhibitory effect of *MATa/MATα* but not that of *INH1*, Inui et al.[117] suggested that the mechanism for inhibition by *MATa/MATα* is different from that by *INH1*.

Pretorius et al.[78] have shown that *STA2* mRNA (2.5 kb) and GAII (300 kDa) as well as *SGA1* mRNA (2.0 kb) and SGA (90 kDa) were coproduced in sporulating *STA2/STA2* diploids. Yamashita and Fukui[51] have reported the dependence of *SGA1* expression on *MATa1*, consistent with what is known about the sporulation-specific, intracellular glucoamylase.[39,49] Expression of *SGA1* was found to be positively regulated at the level of transcription by both *MATa1* and *MATα2*.[51] Despite the divergence at the 5′ ends of the *STA1*, *STA2*, and *STA3* genes vs. the *SGA1* gene, it has been proposed that all four glucoamylase genes are under common control by the *MAT* locus.[29,55] As mentioned earlier, Yamashita et al.[52] suggested that fusion of resident genes S2, S1, and *SGA1* in *S. cerevisiae* gave rise to an ancestral *STA* gene. To determine whether the sporulation induction of *SGA1* and the *STA1*, *STA2*, and *STA3* genes was conserved despite the divergence in the nucleotide sequence of the 5′-flanking regions of the *STA* and *SGA1* genes, Dranginis[93] investigated the induction mechanism(s) involved. It was found that *SGA1* was induced later in sporulation than *STA1*[93] and *STA2*.[78] It was also shown that the a1 product of *MATa* is required for induction of *SGA1* but not for expression of *STA1*.[93] Dranginis has demonstrated that *SGA1* and *STA1* represent two classes of genes that are induced during sporulation of yeast cells.[39,124] *SGA1* induction is specific to sporulating *MATa/MATα* diploids, whereas *STA1* is also induced in *MATa/MATa* or *MATα/MATα* diploids that are asporogenic in sporulating medium.[93] Based on the difference in the relative timing of induction of *STA1* and *SGA1* during sporulation, and the difference in their dependence of *MATa1*, Dranginis[93] concluded that distinct mechanisms are involved in

the induction during sporulation of *STA1* and *SGA1*. Pugh and Clancy[61] reported that expression of *SGA1* depended on the function of the *MAT* products to support sporulation and not on the formation of haploid progeny ascospores or on the composition of the *MAT* locus per se. It was concluded that the *STA* genes acquired regulation by *MAT* as well as by *STA10* by genomic rearrangements that led to their generation and that this regulation is distinct from that of the ancestral *SGA* gene.[61]

E. Effect of the *GAM* Genes on Glucoamylase Expression

Several mutations preventing expression of the *STA1* gene in *S. diastaticus* have been identified and have been ascribed to four complementation groups. Yamashita and Fukui[125] mapped the *amy1* (now known as *sta1*) mutation group in the *STA1* structural gene. The *amy2* (now known as *gam1*) mutation was found to prevent both glucoamylase production and flocculation.[125] Okimoto et al.[126] designated two other complementation groups, *gam2* and *gam3*. The *gam1* and *gam2* mutants were found to be pleiotropically defective in the use of nonfermentable carbon sources, and consequently did not undergo meiosis and sporulation. No pleiotrophy was detected with the *gam3* mutant. Okimoto et al.[126] also claimed that the deficiency in starch utilization of *gam1* and *gam2* mutants was not due directly to their petite traits, because petite (presumably *rho*°) derivatives of a *STA1* strain were able to ferment starch. RNA blot analysis revealed that *GAM1*,[117] *GAM2*, and *GAM3*[126] are required for transcription of *STA1*, regardless of culture conditions. One class of yeast transcriptional activator proteins, e.g., GAL4, GCN4, and HAP1, that bind to upstream promoter elements contains both DNA-binding domains and transcriptional activation domains.[127] These transcriptional activation domains are relatively acidic and are thought to interact with the transcriptional machinery such as RNA polymerase II and TATA binding proteins. A second class of transcription activation has been proposed for the protein kinase encoded by *SNF1*.[127] The molecular mechanism(s) underlying transcriptional activation of *STA1* by *GAM1*, *GAM2*, and *GAM3* remains to be elucidated. Okimoto et al.[126] speculated that the gene products of *GAM1*, *GAM2*, and *GAM3* might interact with upstream regulatory elements of the *STA* genes.

F. Effect of Upstream Activating Sequences on Glucoamylase Expression

A number of transcriptional control elements have been identified previously in eukaryotic promoters.[127] One element, the TATA-box, found in the 5′-flanking regions of virtually all eukaryotic genes, has been shown to be required for setting the site of transcription initiation[128,129] and, in some cases,[130] maintaining levels of transcription. A second element, the upstream activation sequence (UAS), has been found in the 5′-noncoding regions far upstream from the TATA-box. Deletion of these UAS elements greatly reduces levels of transcription.[127] The precise role of these elements and molecular mechanisms for transcriptional activation have yet to be elucidated.

A number of *cis*-acting upstream regulatory elements affecting yeast gene expression have been defined by analyzing the phenotypes of a set of deletion mutations. Generally, deletions up to a certain point retain the phenotype essentially indistinguishable from the wild-type, whereas deletions beyond a critical nucleotide sequence significantly reduce expression below the normal level. Shima et al.[57] used internal deletion analysis of the promoter region of *STA1* to identify control elements involved in efficient expression of the gene. Two upstream activating sequences, *UAS1* and *UAS2*, were found to be required for controlling the expression of the *STA1* gene. RNA blot analysis revealed that *UAS2* was subdivided into *UAS2-1* and *UAS2-2*. *UAS1* and *UAS2-2* determined the level of transcription, while *UAS2-1* did not. However, *UAS2-1* was necessary for producing translatable *STA1* mRNA (probably for initiating transcription at normal sites). *UAS1* and *UAS2* are positioned 1.8 and 1.2 kb upstream from the mRNA start sites, respectively. Both *UAS1* and *UAS2* contain short inverted repeat, AT-rich or T-rich sequences.[57] Whether these sequences of the *STA1* gene have any significance

in terms of direct interaction with regulatory proteins (e.g., gene products of *STA10*, *GAM1*, *GAM2*, *GAM3*, *MATa/MATα*) remains to be investigated.

V. PERSPECTIVES

Due to its ability to hydrolyze and ferment starch, studies on the budding yeast *S. diastaticus* have attracted considerable interest. In this review we have summarized the structural features of the three unlinked genes, *STA1*, *STA2*, and *STA3* of the *STA* multigene family and their respective encoded extracellular glucoamylase isozymes GAI, GAII, and GAIII. These genes were compared to the *SGA1* gene and its encoded sporulation-specific, intracellular glucoamylase SGA. We have focused on the current knowledge concerning the regulatory features of the *STA* gene system.

The regulation of expression of the *STA* genes is interesting, but not well understood. Their expression is regulated at various levels:

1. The *STA* genes are haploid specific, but the mechanism of mating-type control remains to be clarified. The upstream regions of the *STA* genes contain canonical sequences that have been shown to be sites of repression by the mating-type *MATa1-MATα2* protein in other systems.
2. The *STA10* gene inhibits the expression of *STA1*, *STA2*, and *STA3* at the level of transcription. Cloning the *STA10* gene, purification of its encoded protein and generation of mutants insensitive to *STA10* repression would allow one to study its mode of action.
3. It is unclear why *STA* gene expression is reduced in liquid synthetic media is subject to carbon catabolite repression and why the *STA* genes are poorly expressed in petite mutants. There are conflicting results as to whether the glucoamylases are induced by starch.
4. The secretion of glucoamylases into the culture medium provides a model system by which to study the factors involved in secretion as well as having a practical advantage in the construction of secretion vectors.

The availability of the cloned and sequenced *STA1*, *STA2*, and *SGA1* genes will allow an analysis of the *cis*-acting regulatory sequences. This should lead to interesting insights into starch metabolism in yeast and the potential role of amylolytic strains of *S. cerevisiae* in industrial fermentations.

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