0273-2289/94/4402—0187$04.60

Properties and Engineering of a Mutant *STA* Promoter of *Saccharomyces diastaticus*

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Received August 19, 1992; Accepted August 2, 1993

ABSTRACT

A new allelic variant of the *STA2* gene of *S. diastaticus*, designated as $STA2^K$, was cloned and characterized (*1;* accompanying paper). An application-oriented analysis of the promoter region of $STA2^K$ is described, with an emphasis on its peculiar structural feature: A 1.1-kb natural deletion located 189 nucleotides upstream of the translation start codon.

The strength of the $STA2^K$ promoter was found comparable to that of known strong constitutive yeast promoters (*ADH1, GAPDH*). Regulated glucoamylase expression was demonstrated by chimeric promoters, which were constructed by placing the $STA2^K$ promoter under the control of either the *PHO5* or *CYC1* upstream regulatory sequences. On high-copy-number vectors, induction of the UAS_{PHO5}-$STA2^K$ chimeric promoter by phosphate depletion resulted in a destructive overexpression of the secreted glucoamylase, which completely halted cell growth, and promoted cell decay. In contrast, UAS_{CYC1} was shown to mediate a fine-tuned regulation both by glucose concentration and, indirectly, by starch, the substrate for the glucoamylase to produce glucose.

Index Entries: Yeast glucoamylase; promoter, gene expression; *STA* genes; Upstream Regulatory Sequences; UAS; *PHO5; CYC1.*

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INTRODUCTION

Among the microbial enzymes capable of starch saccharification, the extracellular glucoamylase (1,4-α-D-glucanglocohydrolase, EC 3.2.1.3) isoenzymes of the diastatic strains of *Saccharomyes* cleave α-1,4-glucosidic linkages from the nonreducing end of dextrin chains. Three unlinked genes, *STA1*, *STA2*, and *STA3*, coding for isozymes of secreted glucoamylase (GA I, II, and III, respectively) have been identified in *S. diastaticus*. These genes have been cloned (*2–5*), and *STA1* and *STA2* have been sequenced (*6,7*). *STA1*, *STA2*, and *STA3* are localized on chromosomes IV, II, and XIV, respectively (*8*).

An extensive study of the *STA1* promoter was reported by Shima et al. (*9*). The position of internal deletions created within the *STA1* promoter was correlated with the levels of *STA1*-specific transcription, leading to the identification of two putative long Upstream Activation Sequences (UAS1, UAS2), positioned 1.8 kb and 1.2 kb upstream from the transcription start site. The *STA1* promoter was used to express the human 5-lipoxygenase gene in *S. cerevisiae* (*10*).

An allelic variant of *STA2*, designated as $STA2^K$ was previously cloned in our laboratory from a genomic library of a *S. diastaticus* strain CL9 exhibiting an unusually high level of secreted GA production (*1;* accompanying paper). Nucleotide sequencing revealed, besides a few alternations within the sequence encoding the catalytic domain, a large deletion in the promoter region of $STA2^K$. The importance of these nucleotide changes to the observed high level of GA expression and secretion by the *S. diastaticus* strain CL9 has yet to be elucidated.

Our previous study of the $STA2^K$ gene expression in *S. cerevisiae* (*1*) demonstrated the lack of its repression by glucose as well as, in **a**/α diploids, by the *MAT***a**/α gene product, indicating that at least some of the putative regulatory sequences might have been deleted from the mutant $STA2^K$ promoter.

In this paper we determine the extent to which the natural deletion in the $STA2^K$ promoter affects promoter functions. In the expression studies on the $STA2^K$ promoter, *S. cerevisiae* laboratory strains were used as heterologous hosts. A series of engineered variants of the $STA2^K$ promoter was constructed to render the GA expression regulatable by the media composition. The $STA2^K$ promoter was placed under the control of the phosphate-control promoter element (UAS_{PHO5}) of the repressible-inducible *PHO5* gene, or the upstream glucose-repressible regulatory sequence of the *CYC1* gene (UAS_{CYC1}). The results indicate that either for large-scale industrial production of recombinant glucoamylase, or for the construction of engineered glucoamylase-expressing brewing yeast strains, a nontight repression-induction system offers the most feasible strategy to maintain regulated expression of $STA2^K$.

MATERIALS AND METHODS

Strains, Vectors, and Media

Escherichia coli strain DH5αF′ (F′/*endA1 hsdR17* ($r_K^-m_K^+$) *supE44 thi-1 recA1 gyrA* (*Nal*^{r}) *relA1* Δ(*lacZYA-argF*)*U169* (*ϕ80dlac*Δ(*lacZ*)*M15*) (Bethesda Research Laboratories, Gaithersburg, MD) was used as host for all plasmid transformations experiments. Plasmid pGEM7Zf$^+$ was from Promega (Madison, WI), the yeast vector YCp50 (*11*) was from the American Type Culture Collection (Rockville, MD; ATCC No. 37419), and plasmid pLGΔ312 was a gift from Leonard Guarente (MIT, Cambridge, MA). Yeast vector YEp2B was provided by Dr. Rimantas Siekstelé (Institute of Enzymology, Vilnius, Lithuania).

Saccharomyces diastaticus strain CL9 was from our collection. *Saccharomyces cerevisiae* strain DBY-746 (*MAT***a** *his3-D1 leu2-3, 2-112 ura3-52 trp1-289a;* ATCC 44773) was used for all transformations with *STA2*^{K} expression vectors. Yeast strain GG100-14D (*MATa ura3 his3 trp1 pho5 pho3*) was kindly provided by Stephen Parent (Merck, Sharp and Dohme Research Laboratories, Rahway, NJ)

Yeast growth media are described in the accompanying paper (*1*).

Recombinant DNA Techniques

Transformation of *E. coli* was carried out as described by Inoue et al. (*12*). For yeast transformations either lithium acetate (*13*), or electroporation (*14*) was used. Standard DNA manipulations were performed according to Sambrook et al. (*15*). Restriction endonucleases, DNA modification enzymes and MMLV reverse transcriptase were purchased from New England Biolabs, and used according to manufacturer's instructions. DNA sequence determination was carried out using the dideoxy chain-termination method of Sanger et al. (*16*), modified for plasmid sequencing (*17*). The Sequenase™ kit from US Biochemicals was used according to the manufacturer's instructions.

The Transcription Start Point(s) (tsp) in the *STA2*^{K} Promoter

Tsp was determined by mapping the 5′ termini of *STA2*^{K}-specific mRNA(s) by primer extension analysis (*21*). Total RNA was prepared from exponential yeast cultures (20 mL) of GA-producing *S. cerevisiae* transformants harboring the multicopy *STA2*^{K} expression plasmid YEp(STA)K6 (*1; see* accompanying paper) by resuspending the cells in 2 mL cold 50 m*M* Tris/HCl, pH 7.9, 1 m*M* EDTA, 0.1*M* NaCl, and vortexing with glass beads. The PolyATract™ mRNA Isolation Systems (Promega, Madison, WI) was used (according to the manufacturer's protocol) to isolate poly(A)$^+$ RNA.

The poly(A)$^+$ was hybridized to 65 ng of a 24-mer end-labeled oligo-DNA primer (5'CAAATAAGCGAGTAGAAATGGTCTTTG3') complementary to the 5'-terminal sequence (from bases #27-4) of the coding region of *STA2* (*7*). Reverse transcription was carried out at 42°C for 3 h. The sample containing the extension products complementary to the *STA2^K* mRNA was electrophoresed in a 6% sequencing polyacrylamide gel along with a DNA sequencing reaction with the same primer, and subsequently autoradiographed.

Northern-Blot Analysis of *STA2^K*-Specific mRNA

Poly(A)$^+$ RNA samples (0.2 μg) of each GA-expressing yeast were denatured in formamide, and size-fractioned by electrophoresis in 1.4% agarose containing 1.25% formaldehyde. The gels were blotted onto nylon filters (Zeta-Probe, Bio-Rad) and hybridized with a digoxigenin-dUTP-labeled DNA probe. The DNA probe was generated by using the large (Klenow) fragment of the *E. coli* DNA polymerase (*polI*K) on the 0.8-kb *Nhe*I-*Hin*dIII *STA2^K* promoter fragment in the presence of digoxigenin-dUTP. A digoxigenin-UTP-labeled fragment of the *HIS3* gene was used as a control probe to monitor a constitutively produced transcript. The Genius™ Nucleic Acid Detection Kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) was used to visualize the hybridization via an alkaline-phosphatase-coupled immune assay.

Assay for Acid Phosphatase (AP) Activity

1. Colony staining *in situ*. Replica-plated colonies expressing the *PHO5* gene (under the control of the cloned promoter sequences) appeared brown-red when overlayed with staining agar containing 1% Difco agar, 0.1*M* Na-acetate buffer (pH 4.0), 0.5 mg/mL α-naphthyl phosphate (Sigma) and 5 mg/mL Fast Blue RR Salt (Sigma) (*18,19*). 2. In vitro enzyme assay. Cultures (20 mL) of GG100-14D yeast transformants were grown to an absorbance at 600 nm of 1.8 in 0.67% Yeast Nitrogen Base (Difco) plus 2% dextrose, and, when appropriate, tryptophan (20 mg/mL) and histidine (10 mg/mL). Cells were harvested by centrifugation and resuspended in 2 mL of 10 m*M* Na-acetate buffer (pH 4). Cell extracts were prepared by homogenization with glass beads and clarified by centrifugation for 10 min at 18,000*g*. The protein concentration was determined in the clarified extract and 1 mL of this extract was added to 1 mL of AP-assay mixture consisting of 0.64 mg of *p*-nitrophenyl phosphate (Sigma) in 50 m*M* Na-acetate buffer, pH 4. The reaction was carried out at 35°C for 10 min, and stopped by the addition of 0.2 mL 50% trichloroacetic acid. Insoluble precipitate was removed by centrifugation (12,000*g*; 5 min), after which 2 mL of the supernatant fluid was added to 2 mL of saturated solution of Na_2CO_3 (*20*). Liberated *p*-nitrophenol was determined spec-

trophotometrically at 420 nm. One unit of AP enzyme activity was defined as the amount of enzyme that liberates 1 mmol of *p*-nitrophenol/mg protein/min.

Assay for Secreted Glucoamylase (GA)

Transformants expressing the cloned *STA2*^{K} gene were selected for their ability to hydrolyze starch, i.e., to form halos on plates of selective minimal medium, containing 2% starch and 0.5% dextrose, after incubation at 30°C for 4 d, followed by refrigeration at 4°C for 2 d. The in vitro assay for GA is described in the accompanying paper (*1*).

GenBank Accession Number

At GenBank the sequence of *STA2*^{K} can be accessed under M90490.

RESULTS

Structural Features of the *STA2*^{K} Promoter

The sequence of the *STA2*^{K} upstream region is shown in the accompanying paper (*1*). It was determined using an upstream 834-bp *Xho*I-*Hin*dIII fragment of *STA2*^{K} cloned into pGEM7Zf$^+$ (the *STA2*^{K}-promoter plasmid was designated pSP681). When the promoter sequence of *STA2*^{K} was compared to those of the *STA1* and *STA2* genes (*6,7*), a large deletion of 1.1 kbp was revealed at −189 nucleotides upstream of the initiator ATG (*1*). The promoter sequence was subjected to computer analysis, using the program "EUKPROM" from PC/GENE (IntelliGenetics, Inc., Mountain View, CA). Eukaryotic promoter elements have been found at positions − 140 (CAAT box), −100 (TATA box), and −80 (Cap site) indicating that the 1.1-kb deletion did not involve core promoter sequences.

The location of the *transcription start point(s)* (*tsp*) was determined by primer extension using reverse transcriptase on poly(A)$^+$ RNA template (*21*) and a primer complementary to a 24-nucleotide fragment of the *STA2*^{K} coding sequence. We found that the *tsp* nucleotides in *STA2*^{K} were G at −26 and T at −23 (Fig. 1), which was in accordance with the finding of Lambrechts et al. (*7*) who have localized the two major *tsp-s* of the *STA2* gene at the same sites.

Assessment of the Promoter Strength Using *STA2*^{K} Promoter-*PHO5* Gene Fusions

The promoterless *PHO5* gene has been shown to be a convenient reporter gene to assess promoter strength in yeast (*19,22*). When fused with cloned promoters of *ADH1, GAPDH, TRP1* (the yeast genes encoding alcohol dehydrogenase 1, glyceraldehyde-3-phosphate dehydrogenase,

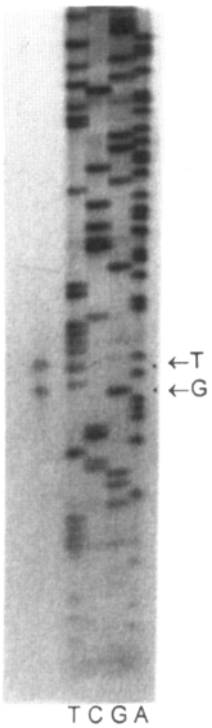

Fig. 1. *Transcription start point(s)* in the *STA2^K* promoter. Extension products poly(A)$^+$ RNA and yeast transformant harboring YEp(STA)K6 (*1;* accompanying paper) aligned with a sequencing ladder of the 5′-proximal promoter segment of *STA2^K*.

and *N*-(5′-phosphoribosyl)-anthranilate isomerase, respectively), the level of the acid phosphatase expression correlated with promoter strength, and/or with the presence of upstream activating sequences.

A cassette containing a *STA2^K* promoter-*PHO5* gene fusion, cloned into the single-copy centrometric yeast vector YCp50 (plasmid YCpPS2; Fig. 2), served as a tool to assess the strength of the *STA2^K* promoter in a *pho5$^-$ pho3$^-$* strain of *S. cerevisiae.* The constitutive yeast promoters *ADH1, GAPDH, TRP1,* fused to the promoterless *PHO5* gene (*19*), and cloned into YCp50, were used for reference.

Table 1 shows the results of the in vitro assay of the AP activity expressed in the GG100-14D transformants. Compared to the expression levels of the reporter gene (*PHO5*) directed by the reference promoters, the 681-bp version of the *STA2^K* promoter is a strong constitutive promoter capable of directing the expression of the *PHO5* reporter gene at a level slightly higher than the *ADH1* promoter, but lower than the *GAPDH* promoter.

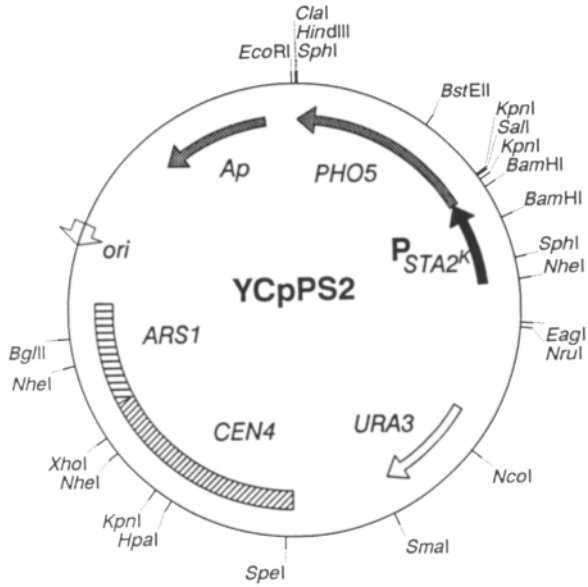

Fig. 2. Map of single-copy self-replicating plasmid YCpPS2 containing the *STA2*^{K}-promoter-*PHO5* gene fusion cassette. The *STA2*^{K} promoter ($\mathbf{P}_{STA2^K}$; a 0.6 kbp *Xho*I-*Stu*I fragment) was inserted upstream of the promoterless *PHO5* gene. The *STA2*^{K}-promoter-*PHO5* gene fusion cassette (*Xho*I-*Hin*dIII) was then inserted into YCp50, cleaved previously with *Sal*I and *Hin*dIII. The yeast GG100-14D (*MATα ura3 his3 trp1 pho5 pho3*) was transformed directly with the $\mathbf{P}_{STA2^K}$-PHO5-YCp50 ligation mixture, and the AP-expressing transformants were selected by staining colonies *in situ* using a diazo-coupled overlay technique (*18,19*). Plasmid YCpPS2 was isolated (as total DNA) from one of the AP-expressing yeast colonies, and was subsequently amplified in *E. coli* DH5αF′. The physical map of YCpPS2 was verified by restriction enzyme digestions.

GA Expression from Truncated Promoter Variants and from *UAS*$_{PHO5}$-*STA2*^{K} and *UAS*$_{CYC1}$-*STA2*^{K} Chimeric Cassettes

The physical map of the *STA2*^{K} promoter fragment is shown in Fig. 3. The restriction sites *Nhe*I, *Sph*I, *Sca*I, *Bsp*MI, and *Hin*cII were used to create truncated variants of the *STA2*^{K} promoter contained in the plasmid subclones pSP300, pSP357, pSP515, and pSP535, the numbers denoting the length of the promoter variants (300, 357, 515, and 535 *nt*, respectively). The promoter variants from these plasmids were excised by *Xho*I-*Hin*dIII digestion and used subsequently to replace the 681-bp *STA2*^{K} promoter. All *STA2*^{K}-expression cassettes (*Xho*I-*Bgl*II), differing from each other in promoter length, were then cloned into YCp50 between the unique *Sal*I and *Bam*HI sites. The resulting yeast plasmids referred to as

Table 1
AP Expression Directed by the *STA2*^{K}, *GAPDH*, *ADH1*, and *TRP1* Promoters (on YCp50-Based Vectors) in GG100-14D Yeast Transformants[a]

Promotor/*PHO5* fusion	AP enzyme activity, U[b]
Control[c]	0.11
STA2^K[d]	6.94
GAPDH[e]	8.55
ADH1[e]	6.73
TRP1[e]	1.88

[a]The results are mean values of three parallel experiments.
[b]See Materials and Methods section for the AP enzyme assay.
[c]For the control experiments, showing background AP expression, a G100-14D transformant harboring a promoterless *PHO5* gene cloned into YCp50 (between the *Sal*I and *Hin*dIII sites) was used.
[d]Plasmid YCpPS2 (Fig. 1). Sequence of the *STA2*^{K} promoter-*PHO5* gene fusion:

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          -40          -30
           |            |
P(STA2K)...A TATACTATGG TAGGggggatccaatg...PHO5
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[e]The fusion sequences between the *GAPDH*, *ADH1*, and *TRP1* promoters and the promoterless *PHO5* gene have been described by Zvonok et al. (1988).

YCpSP300, YCpSP357, and YCpSP515 were used to transform strain DBY 746 and successively tested for GA expression. The highest level of GA expression was observed in yeast recombinants transformed with YCpSP357 and YCpSP515 (Table 2).

Because our primary goal was to establish conditions for the regulation of *STA2*^{K} expression, known regulatory sequences (UAS_{PHO5} and UAS_{CYC1}) were cloned into pSP357 upstream of the 357-bp *STA2*^{K} promoter sequence to determine whether GA expression could be regulated by medium composition. UAS_{PHO5} is a 228-bp *Hin*cII-*Cla*I fragment of the repressible-inducible *PHO5* promoter shown to confer phosphate control over different yeast promoters (*23,24*). UAS_{CYC1} is a 176-bp *Sma*I-*Xho*I fragment of the plasmid pLGD-312 (*25*) carrying both UAS1 and UAS2 of the *CYC1* promoter (*26*). The *STA2*^{K} expression cassettes, along with UAS_{PHO5}, or UAS_{CYC1}, were transferred to YCp50 (between the *Sal*I and *Bam*HI sites; Fig. 4) to create single-copy yeast vectors YCpSP357/UAS_{PHO5}, YCpSP357/UAS_{CYC1}, respectively. They were also inserted into YEp2B (between the unique *Xho*I and *Bgl*II sites; Fig. 4) resulting in multicopy plasmids YEpSP357/UAS_{PHO5} and YEpSP357UAS_{CYC1}.

GA expression was monitored in DBY746 transformants harboring plasmids YCpSP357/UAS_{PHO5} or YCpSP357/UAS_{CYC1}. The cells were grown in uracil-free YNBCA medium in the presence of 1% KH_2PO_4, 2%

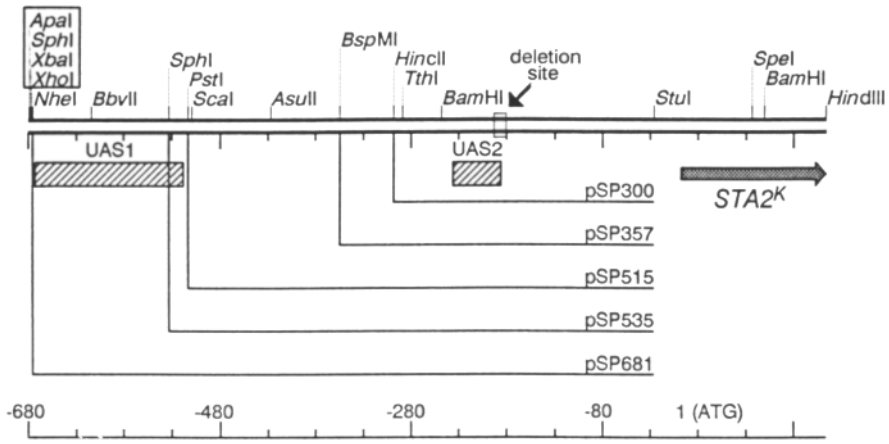

Fig. 3. Deletion variants of the *STA2*^{K} promoter. Shown is the physical map of the *STA2*^{K} promoter region indicating the restriction sites used to create truncated versions of the *STA2*^{K} promoter and also to insert heterologous UAS sequences (UAS_{PHO5}, UAS_{CYC1}). The series of pSP...plasmids were constructed from pSP681 by digestions with *Nhe*I and restriction enzymes *Pst*I, *Bsp*MI and *Hinc*II, respectively, followed by filling in and/or polishing the 5′ termini with *pol*IK, and religation of the blunt ends. The names of the resultant pSP... plasmids reflect the length of the truncated *STA2*^{K} promoter sequence, i.e., 300, 357, 515, 535, and 681 bps, respectively. The *Xho*I site, upstream of the promoter and the *Hin*dIII site (in the *STA2*^{K} coding sequence) were used to transfer the promoter variants to a *STA2*^{K} expression cassette (not shown), cleaved previously by *Xho*I and *Hin*dIII.

Table 2
Expression of GA Under the Control
of Four Different *STA2*^{K} Promoter Variants[a]

Plasmid/promoter:	YCpSP681	YCpSP515	YCpSP357	YCpSP300	none
Secreted GA: U[b]	8.11	9.18	9.63	6.22	0.19

[a]In *S. cerevisiae* strain DBY746 host (*MAT***a** *his3-D1 leu2-3,2-112 ura3-52 trp1-289a*).
[b]Average of two independent measurements performed during midexponential growth phase in uracil-free YNBCA medium (20 h after inoculation); for each GA assay the culture media was diluted to contain an equal number of cells (5×10^8/mL).

glucose to a density (A_{600}) of 1.0. The presence of phosphate or glucose in the medium was inhibitory to the expression of *STA2*^{K} by repressing the activity of the 357-bp promoter either via the *cis*-acting UAS_{PHO5} (repressed by phosphate; Table 3) or UAS_{CYC1} (repressed by glucose), respectively.

Table 3 demonstrates, that when the cells containing YCpSP357/UAS_{PHO5}, or YEpSP357/UAS_{PHO5}, were transferred to a medium lacking phosphate, and grown for an additional 20 h, the extent of the induction of GA expression upon derepression of UAS_{PHO5} was approx 120-fold on both

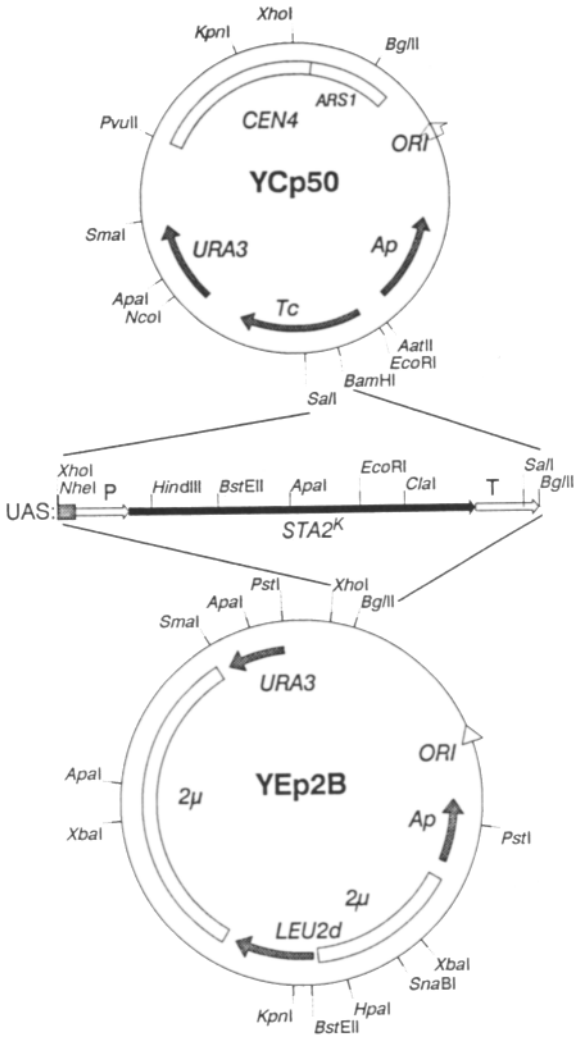

Fig. 4. Generalized map of the YCp50-based (single-copy) and YEp2B-based (multicopy) expression vectors YCpSP357UAS$_{PHO5}$, YCpSP357/UAS$_{CYC1}$, YEpSP357/UAS$_{PHO5}$, and YEpSP357/UAS$_{CYC1}$ vectors carrying *STA2*^{K} expression cassettes placed under the control of UAS$_{CYC1}$, and UAS$_{PHO5}$ (*see text* for details).

YCp50 is a self-replicating CEN-ARS vector (*11*); YEp2B is a multicopy vector containing the entire 2 μm plasmid, pBR322 sequences, *URA3*, and *LEU2d* selection markers.

the single-copy (YCp...), and the 2μ-based multicopy (YEp...) vectors under conditions of Ura$^+$ selection. The increased GA production was also accompanied by a lower growth rate of the cultures. When transformants harboring YEpSP357/UAS$_{PHO5}$ were transferred to leucine-free medium to increase the vector's copy number by using *LEU2d* as a selection marker (*27*), the cells exhibited normal growth only in phosphate-containing

Table 3
Expression of $STA2^K$ Directed by the 357-bp Promoter
Placed Under the Control of UAS_{PHO5} on Single-Copy, and Multicopy Vectors[a]

Plasmid	none	YCpSP357UAS$_{PHO5}$	YEpSP357UAS$_{PHO5}$	
Selection marker	—	*URA3*	*URA3*	*LEU2d*
GA activity[b]				
Repressed (by high PO_4)[c]	0.11	0.09	0.16	0.14
Induced (by PO_4 depletion)[c]	0.08	12.89	18.90	(1.66)[d]
Growth rate of induced culture (divisions × h^{-1})	0.39	0.22	0.16	<0.02[d]

[a]Assessment of expression levels was based on GA production by DBY746 yeast recombinants harboring $STA2^K$ expression plasmids.

[b]The data are mean values of two parallel experiments. The GA assay and enzyme unit defintion are described in the Materials and Methods section.

[c]GA assay was carried out 20 h after the cell cultures (20 mL) had been transferred to and grown in YNBCA medium containing 1% (wt/vol) KH_2PO_4 (to maintain conditions to repress UAS_{PHO5}), or, in a parallel culture, in YNBCA lacking phosphate (to induce promoter via UAS_{PHO5}).

[d]For recombinants harboring YEpSP357UAS$_{PHO5}$ no further cell growth was observed after transferring the cells to leucine-free YNB medium lacking phosphate. After 20 h in inducing medium, plating and colony counting indicated an approx 24% cell death, compared to the initial culture (data not shown).

medium, i.e., under conditions inhibiting the synthesis of GA via the UAS_{PHO5}. Transfer of the cells to a medium lacking phosphate resulted in a destructive overexpression of $STA2^K$, a complete cessation of cell growth, and promotion of cell death.

UAS_{CYC1}, under the conditions used in our experiments, affected $STA2^K$ expression differently. This is owing, first, to the lower induction levels. The presence of glucose in the medium was definitely inhibitory to $STA2^K$ expression, and the starch medium (lacking glucose) was presumed to provide the conditions required to induce GA synthesis via derepression of UAS_{CYC1}. As shown in Table 4, however, only moderate induction levels of GA expression were observed. Cells carrying either YCpSP357/UAS_{CYC1} or YEpSP357/UAS_{CYC1} produced about 2.5 units of GA under the growth and assay conditions used, regardless of the marker gene used to select for YEpSP357/UAS_{CYC1}-containing transformants.

Northern Hybridization of $STA2^K$ -Specific mRNA Transcripts

To see whether GA-specific activities and transcription from the promoter variants could be correlated, the steady-state levels of $STA2^K$ mRNA in two promoter-deletion variants (SP681 and SP357, Fig. 3), and of transcripts synthesized from SP357 under the control of UAS_{PHO5} and UAS_{CYC1}, at repressing and inducing conditions, were studied. Poly(A)$^+$ RNA was prepared from transformants grown under various conditions

Table 4
Expression of *STA2*^{K} Directed by the 357-bp Promoter Placed Under the Control of UAS$_{CYC1}$ on Single-Copy, and Multicopy Vectors[a]

Plasmid Selection marker	none —	YCpSP357UAS$_{CYC1}$ *URA3*	YEpSP357UAS$_{CYC1}$ *URA3*	 *LEU2d*
GA activity[b]				
Repressed, by glucose[c]	0.12	0.66	0.35	0.54
Induced, no glucose[c]	0.20	2.31	2.47	2.93
Glucose accumulation YNBCAS medium (mg × mL^{-1})[d]	nd[e]	8.0	11.4	10.1
Growth rate of induced culture (divisions × h^{-1})	0.39	0.28	0.22	0.18

[a] and [b] See Table 3.

[c] GA assay was carried out 20 h after the cell cultures (20 mL) had been transferred to either YNBCA medium or leucine-free YNB containing 2% glucose. In order to maintain repression of UAS$_{CYC1}$ the cultures were periodically (every 6 h) resuspended in fresh media containing 2% glucose. In parallel cultures glucose was replaced by 2% Lintner starch (to induce UAS$_{CYC1}$).

[d] The glucose concentration was measured in the supernatant fluid 20 h after the culture was transferred to starch medium.

[e] nd = not detected.

(*see* Tables 3 and 4, and Fig. 5). Equivalent amounts (~0.2 μg) of poly(A)$^+$ RNA preparations were size-fractionated and analyzed by Northern-blotting and hybridization simultaneously against labeled *STA2*^{K} and *HIS3* probes. As shown in Fig. 5, the semiquantitative assessment by Northern hybridization revealed a strong correlation between the GA activities (Tables 3 and 4) and corresponding transcript levels. The most dramatic increases in the transcript levels were observed with induced UAS$_{CYC1}$, on both the YCp... and YEp... plasmids (lanes C through F). The *HIS3* probe, used as an internal control, revealed a constitutively produced transcript of about 700 nucleotides long. It may be noted that induction of YEpSP357/UAS$_{PHO5}$ under Leu+ selection (i.e., forced high-copy-number-) conditions resulted in the appearance of degraded mRNA products.

Compared to the 357-bp promoter (lane B), the UAS$_{CYC1}$-driven variant (lanes I–N; Fig. 5) showed a lower transcript level, indicating inhibition of *STA2*^{K} expression by UAS$_{CYC1}$ in the presence of glucose. As in the case of the GA activities the selection conditions (reflected in the plasmid copy number) did not have a significant impact.

DISCUSSION

An analysis of the promoter region of a new allelic variant of the *S. diastaticus STA2* gene (designated as *STA2*^{K}) has been carried out. Because

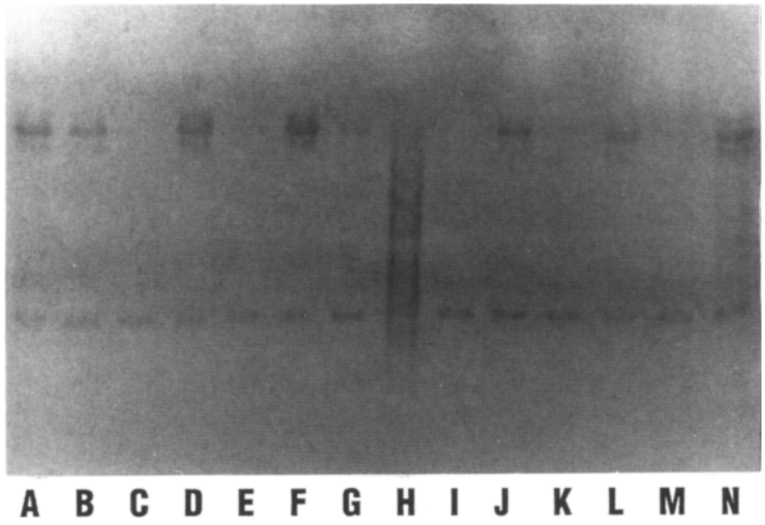

Fig. 5. Northern-blot hybridization of *STA2*^{K}-specific mRNA transcribed in *S. cerevisiae* transformants under the control of different variants of the 357-bp promoter.

Total poly(A)$^+$ RNA was prepared from 20-mL cultures of DBY746 recombinants grown under conditions described in Tables 3 and 4. Denatured poly(A)$^+$ RNA (0.2 μg) of each recombinant was electrophoresed in each track, followed by blotting onto nylon filters. The *STA2*^{K}-specific transcript (upper band, corresponding to a transcript length of ≈2.4 kb) was detected by hybridization with a digoxigenin-dUTP-labeled DNA probe in the presence of a similarly labeled *HIS3* probe used as internal control (lower band corresponding to a 0.7 kb transcript; for technical details *see* Materials and Methods.

Lane designations:

A: *STA2*^{K} transcript synthesized from the 681-bp promoter (on plasmid YCpSP681);
B: Same from 357-bp promoter (plasmid YCpSP357);
C: YCpSP357/UAS$_{PHO5}$, repressed in high-phosphate medium (YNBCA-uracil);
D: YCpSP357/UAS$_{PHO5}$, induced in phosphate-depleted medium (YNBCA-uracil);
E: YEpSP357/UAS$_{PHO5}$, repressed (as above);
F: YEpSP357/UAS$_{PHO5}$, induced (as above);
G and H: Same as E and F, respectively, but from cultures grown in leucine-free YNB medium;
I: YCpSP357UAS$_{CYC1}$, repressed (YNBCA-uracil, 2% glucose);
J: YCpSP357UAS$_{CYC1}$, induced (YNBCA-uracil, 2% starch);
K: YEpSP357UAS$_{CYC1}$, repressed (YNBCA-uracil, 2% glucose);
L: YEpSP357UAS$_{CYC1}$, induced (YNBCA-uracil, 2% starch);
M and N: Same as K and L, respectively, but from cultures grown in leucine-free YNB medium.

of a natural deletion, the sequence of the *STA2*^{K} promoter is lacking most of the UAS2 sequence described by Shima et al. (*9*) for the promoter of *STA1* (*see* Fig. 3). UAS1, on the other hand, was found to be located much closer to the core promoter sequence than that of *STA1*. The location of the *transcription start point(s)* (*tsp*) apparently was not influenced by the deletion: we found that the *tsp* nucleotides in *STA2*^{K} were the same as reported for other *STA* genes (*7*): G at -26 and T at -23 (Fig. 1).

When tested with the promoterless *PHO5* reporter gene, the *STA2*^{K} promoter appears to be a strong, constitutive promoter (Table 1). It should be noted, that our original report (*19*) described the application of *PHO5* as a reporter gene in yeast promoter studies using high-copy-number yeast vectors. We have recently found that assessment of the promoter strength using *PHO5* yields more reliable results when the *PHO5* expression cassette is cloned into single-copy yeast vectors, such as YCp50 (G. Bajszar, unpublished observations). Expression levels from a single-copy plasmid vector do not depend upon gene dosage, so that distortions caused by *PHO5* overexpression (*28*), blockage of the secretory pathway, and/or inhibition of cell growth, are less likely to occur. We have observed that none of the *STA2*^{K} promoter variants (SP681, SP515, SP357, SP300; Fig. 3) was affected by the presence or absence of glucose and/or starch in the growth medium (data not shown). It has been shown, on the other hand, that the expression of *STA1* and *STA2* genes is repressed in the presence of glucose (*29*); although no glucose-control element has yet been defined in the upstream promoter sequences of any of the *STA* genes. *STA* expression is inhibited by *STA10* (*3,30*), also by the product of the *MAT***a**/α constitution in diploid cells (*31*), but most of these regulatory mechanisms remain to be elucidated. The target site for the *MAT***a**/α protein, whose consensus sequence has been identified by Miller et al. (*32*) as TCRTGTNNWNANNTACATCA, is present in the promoter of *STA2* (*7*). We have also discovered that the *STA2* promoter also contains the Heat-Shock Factor binding sequence TTCTAGAA (*see* ref. *33* for review). Because these sequence elements are contained in the 1.1-kb deletion, they do not function in the *STA2*^{K} promoter. As to the putative glucose-control sites, they have either been deleted in the construction of the truncated promoter variants, or they may also be located within the 1.1-kb promoter fragment, present in other *STA* genes, but absent from *STA2*^{K}.

The regulation of the *STA2*^{K} gene expression by medium composition could be accomplished by using heterologous upstream regulatory sequences. Interestingly, the orientation of UAS_{PHO5} and of UAS_{CYC1} sequences inserted upstream of the truncated *STA2*^{K} promoter variants has not been determined. Their effect on the *STA2*^{K} promoter activity was tested only empirically, i.e., only those yeast transformants were selected for GA enzyme assays that had been previously shown to be capable of starch degradation, i.e., halo formation on starch-containing plates. It

should be mentioned, however, that any possible impact of the so called "pBR322 enhancer sequence" (*22*) on the expression of *STA2*^{K} could be excluded as this short segment of the tetracycline-resistance gene in YCp50, was replaced when the *STA2*^{K} expression cassettes were inserted between the *Sal*I and *Bam*HI sites of YCp50.

Our results with UAS$_{CYC1}$ suggest that when the production of GA is subjected to feedback regulation by glucose via the glucose-repressible heterologous UAS, the levels of repression and derepression in starch medium did not depend significantly on the copy number of the yeast vector. A possible interpretation of this phenomenon is suggested by the observation that the glucose level in the starch medium is maintained at a nearly constant low level of 8–11 μg/mL irrespective of gene dosage (Table 4). This may reflect the development of a steady state in which the GA activity that produces glucose from starch is limited by feedback repression of *STA2*^{K} gene expression via the *cis*-acting glucose-control element UAS$_{CYC1}$. As confirmed by Northern-blot experiments (Fig. 5), this type of feed-back regulation of gene expression is realized at the transcriptional level, and is reflected in the synthesis rate, and/or steady-state levels of *STA2*^{K}-specific mRNA. The gene dosage, as a function of the copy number of the self-replicating yeast plasmid showed, to our surprise, little, if any, impact. It should be noted, that the labeled DNA probe used in the Northern-blot hybridizations had no sequence homology with the *SGA1* gene that encodes a sporulation-specific intracellular GA in both *S. diastaticus* and *S. cerevisiae* (*34*). Thus, detection of any unforeseen *SGA1*-specific transcripts could be entirely excluded.

When phosphate-control element UAS$_{PHO5}$ was used to regulate the expression of *STA2*^{K}, the accumulation of glucose in the culture medium had no inhibitory effect on the promoter activity, which, under the conditions of derepression, resulted in the synthesis of extremely high levels of *STA2*^{K}-specific mRNA molecules. When, on the other hand, the UAS$_{PHO5}$-*STA2*^{K} expression cassette was cloned on a 2μ-based vector, only selection for Ura+ transformants was permissible. Using multicopy yeast vectors carrying the 2μ-replication origin, or the entire 2-μm plasmid and selection markers *URA3* and *LEU2d* (as in YEp2B, Fig. 4), the copy number of the plasmid can be predetermined, i.e., the transformants may produce a low plasmid copy number in uracil-free media, whereas using the promotorless *LEU2d* as a selection marker, the recombinants are forced to maintain a high copy-number of the expression vector so as to supply adequate leucine for growth (*27*). When transformants containing this marker on plasmid YEpSP357UAS$_{PHO5}$ are maintained on leucine-deficient medium (Table 3, left column), growth is arrested. This is apparently caused by the blockage of the secretory pathway from the induced overproduction of the extracellular GA accompanying increased plasmid copy number. Overproduction of secreted proteins was either detrimental to the host cells (*28*) or resulted in the establishment of an "equilibrium"

of several parameters, such as cell growth, average plasmid copy number, and plasmid stability (*35*). Apparently, the combined effect of factors, i.e., induction of secreted GA production via UAS_{PHO5}, along with the forced high copy number of the expression plasmid create conditions exceeding the capacity of the yeast recombinants to establish the physiological "equilibrium." It would be of particular interest, therefore, to see whether mutants, or different host cells (e.g., brewing yeast) could be found that could overcome these limitations.

The impact of the strong $STA2^K$ promoter in maintaining the high GA expression in its homologous host (*S. diastaticus*) has yet to be determined. The strain CL9 has also another, presumably active, *STA1* locus (*36*). The mutant $STA2^K$ gene, and its strong promoter, on the other hand, may find their applications in constructing new amylolytic yeast strains, as well as expression systems for the commericial production of heterologous proteins.

SUMMARY

A new allelic variant of the *STA2* gene of *S. diastaticus*, designated as $STA2^K$, was cloned and characterized (*1;* accompanying paper). We describe here an application-oriented analysis of the promoter region of $STA2^K$, with an emphasis on its peculair sructural features: a 1.1-kb natural deletion located 189 nucleotides upstream of the translation start codon.

The strength of the $STA2^K$ promoter was assessed using a promoterless *PHO5* as a reporter gene, and it was found comparable to that of known strong constitutive yeast promoters (*ADH1, GAPHD*). A series of upstream delection of the $STA2^K$ promoter were created yielding nonregulated, constitutive promoter variants of different promoter strength.

Regulated glucoamylase expression was demonstrated by chimeric promoters, which were constructed by placing the $STA2^K$ promoter under the control of either the *PHO5* or *CYC1* upstream regulatory sequences. The expression of the secreted $STA2^K$ glucoamylase, controlled by the engineered promoter variants, was monitored in *S. cerevisiae* hosts using self-replicating single-copy and multicopy yeast vectors. On high-copy-number vectors, induction of the UAS_{PHO5}-$STA2^K$ chimeric promoter by phosphate depletion resulted in a destructive overexpression of the secreted glucoamylase, which completely halted cell growth, and promoted cell decay. In contrast, UAS_{CYC1} was shown to mediate a fine-tuned regulation both by glucose concentration and, indirectly, by starch, the substrate for the glucoamylase to produce glucose.

ACKNOWLEDGMENTS

The authors are grateful to Jennifer Morin and Donald Druin for their valuable and diligent technical assistance. We thank L. Guarente and S. Parent for providing us with some plasmids and strains used in this work. Thanks are owing to Finn Knudsen (Coors Brewing Co.) for the helpful discussions and support, and to Sandra Berry-Low for the critical reading of the manuscript. This work was supported in part by Coors Brewing Co., Golden, CO, and by a grant (to G. B.) from the Colorado Institute for Research in Biotechnology (CIRB).

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