to serious physiological difficulties, and both arguing his functional explanations. Yet his basic idea is ingenious and rich in consequences; when modified in the light of what is known and assumed today about these processes, it organizes an array of divergent facts. It therefore deserves to be reclaimed.

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RESEARCH ARTICLE

Expression, Glycosylation, and Secretion of an *Aspergillus* Glucoamylase by *Saccharomyces cerevisiae*

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Glucoamylases (E.C. 3.2.1.3) catalyze the release of glucose from starch and related malto-oligosaccharides (*1*). *Aspergillus awamori* and the related species *A. niger* secrete two glycosylated forms of glucoamylase designated GAI and GAII (*2–4*). Both forms are encoded by a single gene, are immunologically related, and have the same amino terminal amino acid sequence (*4–7*). The two forms differ in the primary structure and length of their carboxyl terminal sequences, and recent evidence suggests that these differences arise as a consequence of differential RNA splicing (*8*). Both enzymes hydrolyze α(1,4)- and α(1,6)-glycosidic linkages, and the larger (GAI) form differs functionally from the smaller (GAII) form only by its capacity to degrade raw starch (*9*).

We have reported the molecular cloning and DNA sequence of the glucoamylase structural gene and a complementary DNA (cDNA) clone for GAI messenger RNA (mRNA) from *A. awamori* (*5, 6*). The primary structure of the glucoamylase gene and of cDNA clones for GAI and GAII from *A. niger* have also been reported (*7, 8*). Comparison of the primary structure of the glucoamylase gene with the cDNA clones has established that four intervening sequences are spliced from both the GAI and GAII mRNA's. A fifth intervening sequence is spliced from the GAII mRNA. Analyses of putative regulatory sequences within the 5′- and 3′-flanking regions, as well as within the intervening sequences, of the glucoamylase gene revealed striking similarities to consensus sequences defined for structural genes of *S. cerevisiae* (*6*). Despite these structural homologies, we report here that we were unable to detect either initiation or termination of transcription or proper splicing of the intervening sequences of the *A. awamori* glucoamylase gene in *S. cerevisiae*.

Construction of a yeast expression plasmid and modifications of the glucoamylase gene. A 15-kilobase (kb) Hind III fragment that includes the entire glucoamylase structural gene was introduced into laboratory strains of yeast on an autonomously replicating plasmid, YEp13 (*10*). These transformants had no detectable enzymatic activity, immunoreactive peptide, or glucoamylase mRNA sequences (*11*). Because glucoamylase transcripts were not detected in yeast with the native *Aspergillus* gene, a vector containing yeast regulatory signals was used for the expression of glucoamylase in yeast (Fig. 1). This expression vector (pAC1) contains an *Escherichia coli* origin of replication, the *bla* gene from pBR322, the yeast 2μ origin of replication, and a yeast *LEU*2 structural gene. These features of the plasmid permit autonomous replication and selection in *E. coli* and yeast. The vector also contains the promoter and termination regions of one of the yeast enolase

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genes, designated *ENO*1. The DNA sequence and RNA transcription initiation site of the *ENO*1 gene have been reported (*12, 13*); in addition, considerable information regarding the regulation of this promoter is known from deletion mapping (*14*) and from linker scanning studies (*15*). On the basis of these studies, it can be concluded that the *ENO*1 5′-flanking region present in pAC1 contains all of the elements that define the promoter. A single Hind III site located at the junction between the promoter and termination fragments (Fig. 1) can be used to introduce heterologous genes for expression in yeast.

Abstract. *A strain of* Saccharomyces cerevisiae *capable of simultaneous hydrolysis and fermentation of highly polymerized starch oligosaccharides was constructed. The* Aspergillus awamori *glucoamylase enzyme, form GAI, was expressed in* Saccharomyces cerevisiae *by means of the promoter and termination regions from a yeast enolase gene. Yeast transformed with plasmids containing an intron-free recombinant glucoamylase gene efficiently secreted glucoamylase into the medium, permitting growth of the transformants on starch as the sole carbon source. The natural leader sequence of the precursor of glucoamylase (preglucoamylase) was processed correctly by yeast, and the secreted enzyme was glycosylated through both* N- *and* O-*linkages at levels comparable to the native* Aspergillus *enzyme. The data provide evidence for the utility of yeast as an organism for the production, glycosylation, and secretion of heterologous proteins.*

The glucoamylase structural gene was modified for insertion into pAC1 by introducing Hind III restriction sites at the 5′ and 3′ ends of the gene (Fig. 2). A second series of modifications involved the excision of four intervening sequences from the glucoamylase gene.

Transformation of yeast and glucoamylase expression. The expression vector pAC1 and the derivative plasmids pGC21 and pGAC9 were used to transform yeast strain C468 (α, *leu*2-3, *leu*2-112, *his*3-11, *his*3-15, *mal*$^-$) by standard procedures (*16*) to leucine prototrophy. To detect clones that expressed and secreted glucoamylase, the transformants were transferred onto plates containing 2 percent starch and examined for clearing of turbidity. After 6 days of growth at 30°C, C468:pGAC9 transformants, but not C468:pAC1 or C468:pGC21 transformants, showed distinct zones of clearing around the growth area, indicating that they could hydrolyze starch (Fig. 3). In addition, C468:pGAC9 transformants had a marked growth advantage on starch compared to transformants containing pAC1 or pGC21. Because pGAC9 and pGC21 are identical except for the presence of introns 1 to 4 in pGC21, it is probable that the failure of C468:pGC21 to produce a zone of clearing was related to the inability of *S. cerevisiae* to splice the glucoamylase intervening sequences.

Characterization of the recombinant glucoamylase transcripts. Although C468:pGAC9 contains the fifth intron sequence, which is differentially recognized by *Aspergillus* (*7*), we can only detect production of the larger (GAI) form of the enzyme in *S. cerevisiae*. Polyadenylated [poly(A)$^+$] mRNA from C468:pAC1, C468:pGC21, and C468:pGAC9 transformants was analyzed for hybridization to a glucoamylase-specific probe (Fig. 4). The in vivo glucoamylase transcript from pGAC9 was a prominent 2.2-kb band. In contrast, the major transcript from pGC21 was approximately 200 nucleotides larger and, thus, appears not to have been processed since the total length of intron sequences in the glucoamylase gene is 249 nucleotides. While a small fraction of the pGC21 transcript comigrated with the pGAC9 transcript, C468:pGC21 transformants produced no detectable glucoamylase.

Hybridization experiments with a labeled *ENO*1 terminator probe indicated that both the pGC21 and pGAC9 in vivo transcripts terminated within the *ENO*1 terminator distal to the glucoamylase gene (*11*). Because these constructs include the known 3′-polyadenylation region of the glucoamylase gene, this result shows that the *Aspergillus* termination signal is not recognized in yeast.

Simultaneous hydrolysis and fermentation of starch by S. cerevisiae. In order to examine the relationship between glucoamylase expression and growth on starch as sole carbon source, we compared the abilities of C468:pAC1 and C468:pGAC9 to ferment starch in liquid cultures (Fig. 5). Transformants containing pAC1 did not ferment starch, whereas pGAC9 transformants used 95 percent of the starch in the medium. When exogenous glucoamylase was added to the culture medium, the two transformant strains fermented starch at identical rates. These data suggest that the ability of transformants containing pGAC9 to

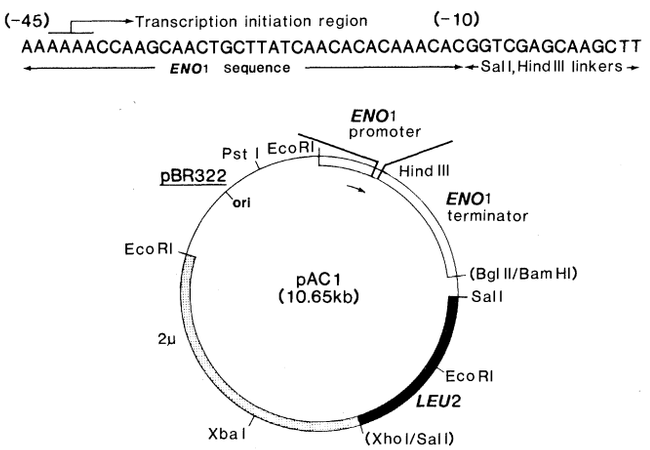


Fig. 1. Construction of yeast expression plasmid pAC1. Plasmid *peno*46 (*12*) containing the yeast *ENO*1 structural gene was digested to completion with Hind III. The resulting 14.6-kb fragment (extending from a Hind III site 130 bp downstream from the *ENO*1 initiation codon through the 11.2-kb pSF2124 plasmid vector and extending to a Hind III site at the termination codon of *ENO*1) was purified by agarose gel electrophoresis. This fragment (3 μg) was digested with 0.2 unit of *BAL*-31 (Bethesda Research Laboratories) in a 60-μl volume for 8 minutes at 30°C. After phenol extraction, the DNA was ligated (T4 DNA ligase) to phosphorylated Sal I linkers (Collaborative Research Inc.), cleaved with Sal I, ligated again in the presence of T4 DNA ligase, and used to transform *E. coli* RR101. Plasmid DNA was isolated from individual transformants and analyzed by digestion with Sal I and Xba I to determine the approximate size of each deletion. A deletion extending from the Hind III site within the coding region of *ENO*1 to a site 10 bp upstream from the translational initiation codon was identified by DNA sequence analysis and used for the construction of the *ENO*1 promoter region of pAC1. The Sal I site at −10 was adapted with Hind III linkers to regenerate a single Hind III site between the *ENO*1 promoter and terminator of pAC1. An expanded view of the DNA sequence surrounding the transcription initiation region of *ENO*1 is shown. The other components of pAC1 include: (solid line) pBR322 sequences; (stippled box) yeast 2μ sequences from the 3.7-kb Eco RI–Sal I fragment of pDB248 (*31*); (solid box) 2.2-kb Xho I–Sal I fragment of yeast *LEU*2 (*10*); (open box) *ENO*1 promoter-terminator sequences; the terminator is the natural Hind III–Bgl II fragment of *peno*46 (*12*).

ferment starch in the absence of exogenous glucoamylase was solely due to their ability to express and secrete functional glucoamylase. The pGAC9 transformants had an initial lag period in their ability to ferment starch that was followed by rapid fermentation. This initial lag period in fermentation may have been due to a time-dependent accumulation of glucoamylase in the medium. This hypothesis is supported by the observation that exogenously added glucoamylase abolished the lag period.

Recombinant glucoamylase is secreted into the extracellular medium. Localization of the secreted glucoamylase was determined immunologically by comparing the immunoreactive glucoamylase associated with the cells to the immunoreactive glucoamylase in the extracellular culture medium (Fig. 6). More than 90 percent of the glucoamylase was free in the medium rather than associated with the cells. This result is consistent with measurements of the glucoamylase activity in cell lysates (<0.1 unit per liter) when compared with the glucoamylase activity in the culture medium (25 units per liter). Most of the secreted glucoamylase migrated at a higher apparent molecular weight than did the cell-associated glucoamylase and was similar in mobility to that of the native glycosylated *Aspergillus* glucoamylase.

Purification and characterization of recombinant glucoamylase. To produce sufficient recombinant glucoamylase for biochemical characterization, we purified the glucoamylase from the culture medium of actively growing C468: pGAC9 by means of DEAE-Sepharose chromatography (Fig. 7). The peak fractions were approximately 90 percent pure as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE); thus 25 to 30 percent of the total supernatant protein was glucoamylase. The specific activity (25 to 50 units per milligram of protein) of the recombinant glucoamylase in these fractions was comparable to that of purified native *A. awamori* glucoamylase. The pooled glucoamylase material was subjected to NH_2-terminal amino acid sequence analysis. The identities of 30 residues were determined by automated techniques (*17*) and found to be identical to the NH_2-terminal residues of *A. awamori* glucoamylase. There is no evidence for heterogeneity at the NH_2 terminus of the secreted recombinant glucoamylase.

The native enzyme is glycosylated at serine and threonine residues via *O*-glycosidic linkages and *N*-glycosidic linkages to asparagine residues (*2–4*). Alkaline borohydride treatment (*18*) and subsequent amino acid composition analysis of recombinant glucoamylase indicated that 22 to 26 percent of the serine residues and 18 to 20 percent of the threonine residues are glycosylated. Approximately two more threonine residues and one more serine residue in the recombinant glucoamylase are glycosylated than are found in the native enzyme. When purified native and recombinant glucoamylase proteins were treated with endo-β-*N*-acetylglucosaminidase H and an-

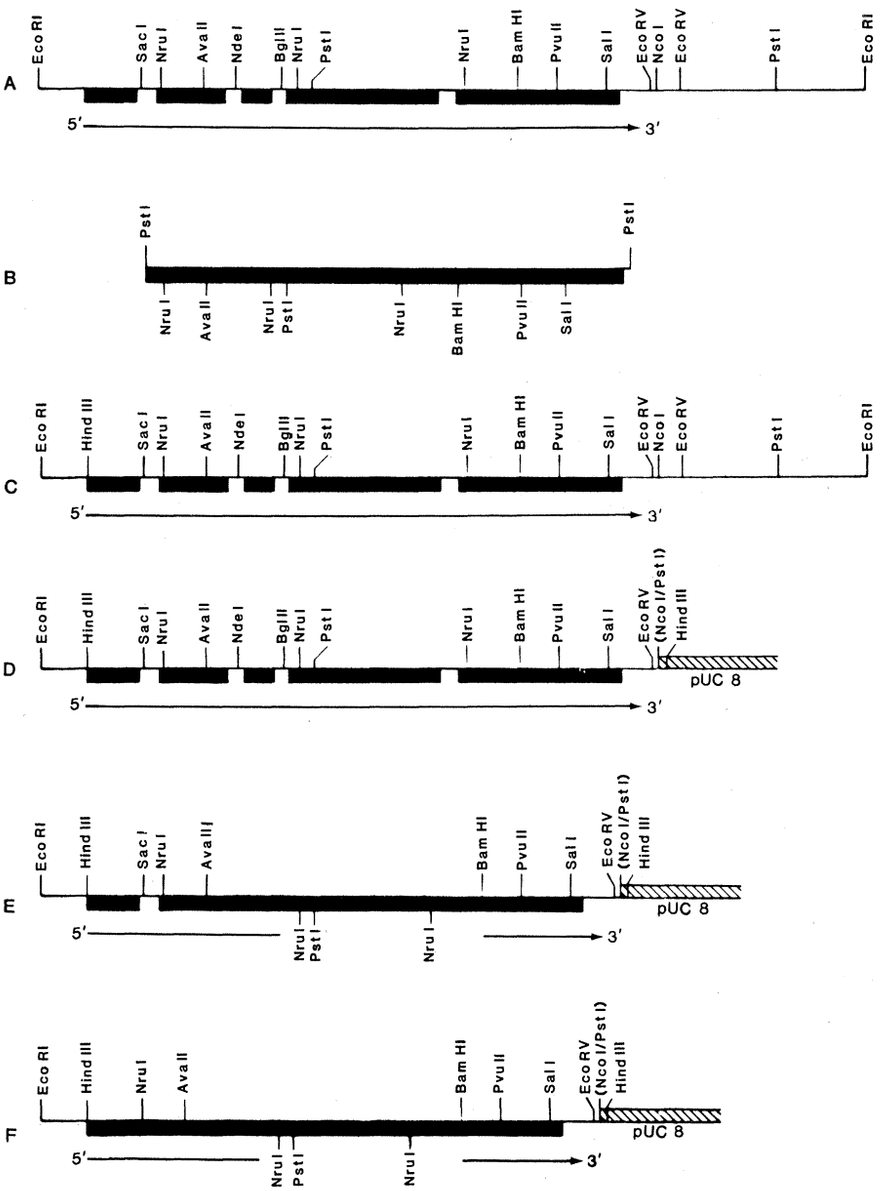


Fig. 2. Modifications of the glucoamylase gene for insertion into the Hind III site of pAC1 and deletion of introns 1 to 4. The 3.4-kb Eco RI fragment containing the glucoamylase structural gene (A) and the 2-kb partial cDNA, p24A2 (B), encoding the GAI mRNA sequence have been described (*6*). The Hind III site in (C), at the 5′ end of the glucoamylase structural gene, was added by primer-directed mutagenesis of (A) 32 bp upstream of the glucoamylase initiation codon. A genomic fragment of (C) was constructed by cleavage with Nco I, repair with DNA polymerase (Klenow), and cleavage with Eco RI. This fragment was cloned into the polylinker site of pUC8 [which had been trimmed by means of cleavage with Pst I followed by repair with DNA polymerase (Klenow) and cleavage with Eco RI] adjacent to the Hind III site. This resulted in the Hind III site in (D) at the 3′ end of the glucoamylase structural gene. Introns 2, 3, and 4 of the glucoamylase structural gene were eliminated in (E) by an Ava II–Bam HI fragment exchange with the cDNA clone shown in (B). Intron 1 was removed in (F) by site-directed mutagenesis (*32*) and a 29-bp oligonucleotide was used to span the intron region. The primer, which had homology to a 15-bp sequence upstream and 14 bp downstream of the intron, had the sequence 5′-CGGATAACCCGGACT ↓ ACTTCTACACCTGG-3′ where the ↓ indicates the position of the intron. The Hind III cassette of (D) was inserted into pAC1 at the single Hind III site, generating an intron-containing glucoamylase gene fusion to *ENO*1, designated pGC21. Likewise, the Hind III cassette of (D) was ligated into pAC1, generating an intron-deleted gene fusion, pGAC9.

alyzed by SDS-PAGE, an equivalent small mobility shift occurred for both the native and recombinant enzyme (Fig. 8). This result suggests that *N*-glycosidic linkages are present on the recombinant enzyme and that the extent of modification is similar to that of native glucoamylase. Although the apparent molecular weight of recombinant glucoamylase estimated by SDS-PAGE was slightly larger than that of the native enzyme, these results indicate that the total extent of glycosylation of recombinant glucoamylase is similar to that of native *Aspergillus* GAI glucoamylase.

Characterization of the glucoamylase leader sequence. Based on the DNA sequence of the glucoamylase structural gene, the first 29 amino acids of preglucoamylase are:

```
                5                  10
MetSerPheArgSerLeuLeuAlaLeuSerGlyLeuVal-
   15                  20
CysThrGlyLeuAla+AsnValIleSerLysArg-
25
AlaThrLeuAspSer
```

Comparison of this leader sequence with other prokaryotic and eukaryotic signal sequences (*19–21*) suggested that the primary signal peptidase cleavage site should occur after Ala at position 18 as indicated by the arrow. However, both mature glucoamylase from *Aspergillus* (*4, 6*) and the recombinant enzyme isolated from yeast culture supernatants began with Ala-Thr-Leu at residues 25 to 27. We speculated that cleavage after Lys-Arg at residues 23 and 24 is a secondary processing step involving the trypsin-like endopeptidase encoded by the *KEX2* gene of yeast, which cleaves similar Lys-Arg residues in the α-factor mating-hormone maturation process (*22*). In order to test this hypothesis, we transformed a yeast strain deficient in this endopeptidase (*kex2*) with the glucoamylase expression vector, pGAC9. The glucoamylase produced by the transformants was purified from the culture medium and subjected to NH_2-terminal amino acid sequence analysis. The NH_2-terminal sequence now began after Ala at position 18; these data support our conclusion that NH_2-terminal processing of glucoamylase involves both a signal peptidase cleavage step and an endopeptidase cleavage step. The function of the endopeptidase cleavage is unclear; the glucoamylase produced in the *kex2* strain was secreted to the culture medium at levels comparable to the C468:pGAC9 transformants, and the specific activity of the glucoamylase was not affected by the six additional NH_2-terminal amino acids.

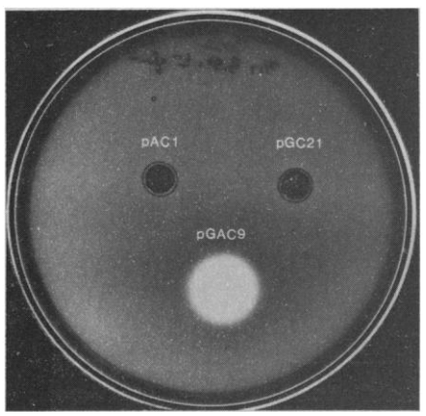
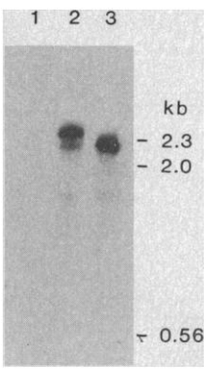


Fig. 3. Starch-clearing plate assay for glucoamylase expression. The indicated strains were transferred to yeast minimal salts medium supplemented with histidine (40 μg/ml) and with 2 percent crude starch as the sole carbon source. After 6 days incubation at 30°C, the plate was stained with iodine vapor. Clear zones indicate starch hydrolysis.



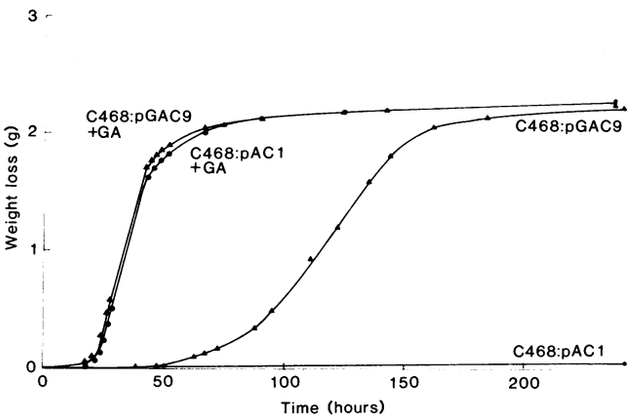


Fig. 4 (left). Analysis of in vivo glucoamylase transcripts. Total RNA was extracted from logarithmically growing cultures as described (*33*) except that cells were first treated with Zymolyase (Miles). Poly(A)$^+$-RNA was isolated by poly(U)-Sepharose chromatography (Pharmacia). Samples (2.3 μg) of poly(A)$^+$-RNA were fractionated by 1.4 percent agarose methylmercury gel electrophoresis (*34*), the RNA was transferred from the gel to a GeneScreen (NEN) filter and hybridized with a ^{32}P-labeled glucoamylase-specific probe. Lane 1, C468:pAC1; lane 2, C468:pGC21; lane 3, C468:pGAC9. Size markers, Hind III digest of bacteriophage λ DNA. Fig. 5 (right). Fermentation of starch by recombinant yeast strains. Strains C468:pGAC9 and C468:pAC1 were grown in yeast minimal salts medium containing histidine (40 μg/ml) and no leucine, with 10 percent washed Maltrin M150 (Grain Processing Corporation, Muscatine, Iowa) as the sole carbon source. Maltrin M150 is hydrolyzed corn starch with an average polymer size of 13 to 17 glucose molecules. Low molecular weight oligosaccharides were removed from the Maltrin M150 by washing with ethanol (70 percent by volume). The cultures were grown with and without the addition of glucoamylase (Sigma) at 1 unit/ml. Strains were grown in 50 ml of media in 125-ml Erlenmeyer flasks fitted with closures that minimized the flow of oxygen into the flasks. Cultures were incubated at 30°C and agitated at a speed of 200 rev/min. Fermentation was measured by weight loss due to CO_2 release; the CO_2 produced was directly related to glucose produced and fermented to ethyl alcohol. Weight loss due to evaporation was less than 0.05 g. (▲), C468:pGAC9; (●), C468:pAC1.

Conclusions. This article describes the construction of a yeast strain capable of complete hydrolysis and simultaneous fermentation of starch because of the expression of an *Aspergillus* glucoamylase, GAI. The yeast *S. diastaticus* is distinguished from *S. cerevisiae* primarily by its ability to ferment starch. This capability is due to three unlinked genes (*STA*1, *STA*2, and *STA*3) coding for yeast glucoamylases with α(1,4)-glycosidic activity (*23*). *Saccharomyces diastaticus*, however, is not known to produce extracellular enzymes capable of the complete hydrolysis of starch or related oligosaccharides. The extracellular glucoamylases from *A. awamori*, in contrast, can attack α(1,4)- and α(1,6)-glycosidic bonds and, thus, efficiently hydrolyze highly polymerized starch to glucose. A strain of the fermentation yeast *S. cerevisiae*, engineered to express one of the *Aspergillus* hydrolases, could shorten or eliminate separate hydrolysis steps in current industrial processes, thereby achieving significant process improvements.

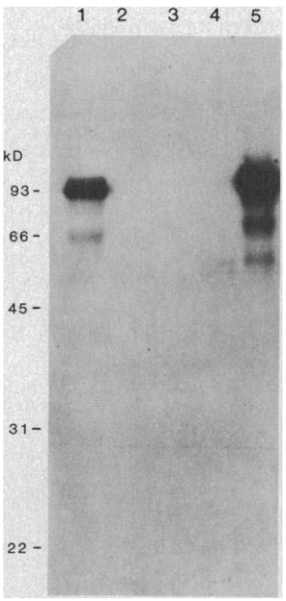
The putative control sequences of the *Aspergillus* gene for mRNA transcription and processing do not function in *S. cerevisiae*. Although there is consider-

able homology between regions of the 5′ flanking sequences of the *Aspergillus* glucoamylase gene and efficient yeast promoters, including a TATA (T, thymine; A, adenine) box (*24*), an upstream CAAT (C, cytosine) sequence (*25*), and a pyrimidine-rich transcription initiation region (*26*), we were unable to detect glucoamylase transcripts from the *Aspergillus* gene in yeast. While introns of higher eukaryotes are usually spliced in other higher eukaryotes, yeast appears to be more stringent about sequence specificity for splicing (*27*). Each of the intervening sequences within the glucoamylase gene contained a consensus sequence, beginning with T or A and followed by PuCT PuAC (Pu, purine), which may be homologous in function to the yeast consensus sequence TACTAAC, which is required for mRNA splicing in yeast (*26*). As shown here, however, *S. cerevisiae* either inefficiently or incorrectly spliced the glucoamylase intervening sequences (or both). Finally, we found that the 3′-transcription termination region of the glucoamylase gene was not recognized in yeast. This finding suggests that the consensus sequences TAGT (G, guanine) and TATGT, which have been postulated to be involved in polyadenylation in yeast (*28*), and which are repeated in the 3′-flanking region of the glucoamylase gene (between nucleotides 62 and 80 with respect to the stop codon for glucoamylase), are not by themselves sufficient for transcription termination in yeast.

For the reasons stated, it was necessary to fuse a yeast transcription unit to an intron-free glucoamylase gene in order to express the heterologous glucoamylase in yeast. The choice of the *ENO*1 promoter was based on its high level of expression in yeast grown on glucose as carbon source. We have also found that *ENO*1 transcription is not inhibited by the production of ethanol during fermentation (*11*). Thus, unlike the case of most hydrolases, glucoamylase expression from the *ENO*1 promoter is stimulated rather than repressed by the glucose produced during starch hydrolysis and fermentation.

Secretion from yeast is generally confined to the periplasmic space or the cell wall, and very few yeast enzymes are found free in the medium (*29, 30*). Here, we found that more than 90 percent of the recombinant glucoamylase could be recovered in the culture medium. Another feature of the secreted glucoamylase is the presence of *N*- and *O*-linked sugars that quantitatively resemble the native enzyme glycosylation pattern.

Constructs lacking the signal peptide do not secrete glucoamylase (*11*). The unusual leader sequence of the glucoamylase gene contained a primary cleavage site that was separated by six residues (ending in Lys-Arg) from the NH_2 termi-



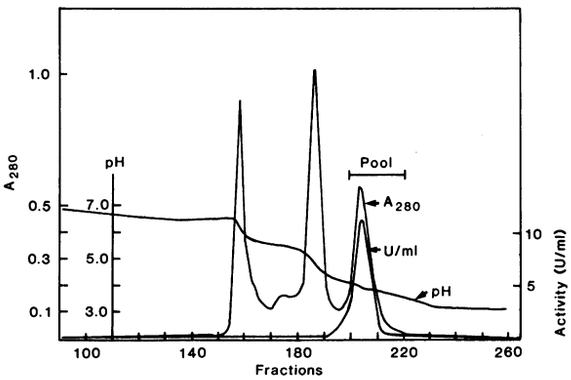


Fig. 6 (left). Localization of recombinant glucoamylase. The indicated recombinant yeast cultures were grown to mid-log phase (an A_{680} of 1.2) in yeast minimal salts medium (histidine, 40 μg/ml; no leucine, 2 percent glucose). Cells were sedimented in a microfuge from 250 μl of the culture, treated with Zymolyase (Miles), and lysed with water. Protein was concentrated from cell pellets or supernatants by precipitation with two volumes of ethyl alcohol. The precipitated fractions were subjected to SDS-PAGE in the presence of 7.5*M* urea. Proteins were electrophoretically transferred from unstained gels to nitrocellulose and incubated with rabbit antibody to native, purified glucoamylase form I (*6*). The presence of glucoamylase-specific polypeptide was detected after incubation of the filter with ^{125}I-labeled staphylococcal protein A and autoradiographic exposure. Lane 1, purified *A. awamori* GAI; lanes 2 and 3, precipitate and supernatant fractions from C468:pAC1 transformants; lanes 4 and 5, precipitate and supernatant fraction from C468:pGAC9 transformants, respectively. Fig. 7 (right). Production and purification of recombinant glucoamylase. C468:pGAC9 was grown in a 10-liter fermentation vessel containing minimal salts medium (in the absence of leucine). The batches of culture were fermented aerobically with a continuous glucose feed to an A_{680} of 30; the final cell density was approximately 10 g (dry weight) per liter. The supernatant was concentrated 15-fold by means of an Amicon Hollow Fiber Concentration Unit, adjusted to 50 m*M* phosphate, *p*H 7.5, applied to a DEAE-Sepharose (CL-6B) column, and eluted with a *p*H (7.5 to 3.0) gradient. Eighty percent of the glucoamylase activity applied to the column was recovered in fractions eluting between *p*H 4.5 and *p*H 4.3. Samples were analyzed for glucoamylase activity by means of the peroxidase-glucose oxidase (PGO)/*o*-dianisidine (ODAD) assay (Sigma Kit 510), which detects the glucose released from soluble starch. One glucoamylase unit was defined as the amount of glucoamylase that released 1 μmol of glucose per minute from washed soluble starch at 37°C.

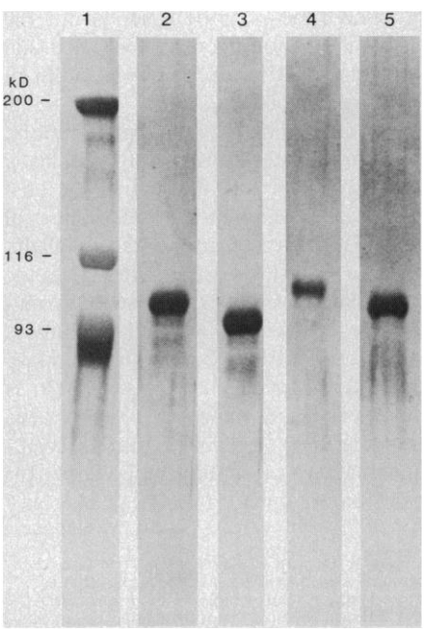


Fig. 8. Endo-β-*N*-acetylglucosaminidase H (Endo H) digestion of native and recombinant glucoamylase. Endo H cleaves the di-*N*-acetylchitobiose unit that connects *N*-linked oligosaccharide to polypeptide chains (*35*). Purified samples (10 μg each) of *A. awamori* and recombinant glucoamylase were subjected to Endo H treatment following boiling in SDS (*36*). The samples were equilibrated in 50 m*M* tris-HCl (*p*H 6.8) and analyzed by SDS-PAGE in the presence of 7.5*M* urea. Protein was visualized after Coomassie blue staining. Lane 1, molecular weight standards (Bio-Rad); lanes 2 and 3, *A. awamori* glucoamylase untreated and treated with Endo H; lanes 4 and 5, recombinant glucoamylase untreated and treated with Endo H, respectively.

nus of mature glucoamylase. Because both the primary signal peptidase cleveage and the secondary trypsin-like processing occur efficiently for pre-pro-glucoamylase, it may be possible to use the glucoamylase signal sequence to direct secretion of other foreign proteins from yeast.

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RESEARCH ARTICLE

Reversal of Oncogenesis by the Expression of a Major Histocompatibility Complex Class I Gene

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The immune system is involved not only in defense against infections but also against "spontaneously derived" aberrant cells (*1*). This latter immune function appears to be essential for the removal of autonomous cell variants that presumably arise frequently in multicellular organisms (*2*).

The development of malignant tumors, therefore, represents not only neoplastic transformation but the failure of host resistance to eliminate certain abnormal cells. Transformation of a cell is insufficient to ensure its escape from immune surveillance. Cells transformed in culture very often do not induce tumors when transplanted back into immunocompetent syngeneic hosts (*3*). It is those properties of certain tumor cells allowing them to resist immune recognition that are ultimately responsible for their tumorigenicity (*4*).

The major histocompatibility complex class I (H-2) antigens (designated K, D, and L in mice) are indispensable for the presentation of cells bearing "foreign" antigens to the cytotoxic T lymphocytes (*5*). The finding that certain malignant tumors, including teratocarcinomas (*6*), eccrine porocarcinomas (*7*), and cervical carcinomas (*8*), have markedly reduced or nondetectable levels of class I antigens at the cell surface (in contrast to their normal cellular counterparts) suggests a possible mechanism for their escape from immune surveillance. In support of this hypothesis is the recent finding that cells transformed by the highly oncogenic strain of human adenovirus (Ad12), in contrast to the nononcogenic strain (Ad5), also express reduced levels of class I antigens on their surfaces (*9, 10*).

This observation with Ad12 provides an experimental system for demonstrating that the absent or reduced expression of class I antigens is directly responsible for oncogenicity. We now show that transfection of a functional class I gene into a highly tumorigenic Ad12-transformed cell line that expresses no detectable class I surface antigens resulted in its complete loss of oncogenicity. This finding indicates one possible mechanism for the escape of certain tumors from immune surveillance and suggests future therapeutic approaches for the reversal of certain malignancies.

Expression of genes encoding class I antigens in Ad12-transformed mouse cell lines. Since it was not clear whether the suppression of class I antigens by Ad12 occurred only in rat cells and since at least one other study did not substantiate this finding (*11*), we sought to confirm and extend the observation. Two Ad12-transformed cell lines, designated C57AT1 and C3AT1 (*12*), established by transformation of embryonic cell cultures derived from C57BL/6 and C3H mouse strains, respectively, were selected.

Polyadenylated [poly(A)$^+$] RNA was extracted from these two cell lines and was compared to a preparation from nontransformed BALB/c3T3 cells by Northern blot analysis (Fig. 1). To determine the relative amounts of RNA in the three samples, the RNA blot was first hybridized with a ^{32}P-labeled genomic actin probe (Fig. 1, panel A). All three cell lines expressed comparable levels of an RNA transcript (~20*S*) characteristic of actin messenger RNA. Subsequent hybridization of the same RNA

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