Expression of Wheat γ-Gliadin in *Saccharomyces cerevisiae* from a Yeast *ADH1* Promoter

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A complete γ-gliadin coding region including the signal peptide was inserted into a yeast expression vector containing a yeast *ADH1* promoter and *CYC1* transcription terminator. Yeast transformed with this recombinant plasmid (pY2Gc) produced gliadin-coding RNA using the *ADH1* promoter and *CYC1* terminator signals. Immunological techniques showed that the RNA was translated into γ-gliadin in the yeast. It was not possible to determine whether the γ-gliadin signal peptide was removed, but a low level of γ gliadin was found in the medium.

Gliadins and glutenins are the storage proteins of wheat endosperm and contribute to the bread-making qualities of wheat flour. Gliadins, the prolamins of wheat, are encoded by a large multigene family and are classified as α/β, γ, or ω according to their electrophoretic mobility in acidic aluminum lactate gels (Woychik et al., 1961). These proteins are characterized by extractability in 70% ethanol (Osborne, 1924) and are difficult to separate into individual proteins. The protein sequences of several α/β- and γ-gliadins have been determined from the sequences of full-length cDNA (Kasarda et al., 1984; Bartels et al., 1986) and genomic (Rafalski et al., 1984; Sumner-Smith et al., 1985; Anderson et al., 1984; Reeves and Okita, 1987; Rafalski, 1986; Sugiyama et al., 1986) clones. They contain relatively high levels of glutamine and proline. Gliadins are synthesized in the endosperm during seed development (Mecham et al., 1981) with a signal peptide (Rafalski et al., 1984; Scheets et al., 1985) that is removed during posttranslational processing. The mature protein is packaged into membrane bound protein bodies (Graham et al., 1962). A source of an individual purified gliadin would permit studying structural features of these unusual proteins and properties important for bread making.

As a possible source of a single gliadin species, we have cloned a γ-gliadin gene into a yeast expression vector and tested for gliadin expression. Improvements in this expression system may represent a significant advance in approaches to studying proteins with the unusual properties characteristic of gliadins.

MATERIALS AND METHODS

Standard procedures (Maniatis et al., 1982) were used for bacterial growth, agarose gel electrophoresis, plasmid isolation, radiolabeling of DNA, colony hybridization, and DNA-modifying enzyme reactions.

Plasmid Construction. The replicative form (double-stranded DNA) of an M13 subclone of pW1020 (Scheets and Hedgcoth, 1988) containing the coding region for a γ-gliadin, Gam20HH, was digested with *Hpa*I and *Hind*III. After the *Hind*III overhang was filled, the 1028-bp coding fragment was isolated and blunt-end-ligated to the shuttle vector pYcDE-2 (provided by B. D. Hall, University of Washington), which had been cut with *Eco*RI, filled in, and dephosphorylated.

Transformation and Selection of Plasmids in *Escherichia coli*. *E. coli* DH5 was transformed (Hanahan, 1983), and recombinants were selected on plates containing ampicillin. Colonies were replated, and duplicate colonies were screened by colony hybridization using a ^{32}P-labeled probe for the coding sequence of pW1020. The orientation of inserts in positive colonies was determined from restriction digests of plasmid minipreps.

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Transformation and Selection in Yeast. DNA from plasmid minipreps of *E. coli* was used to transform *Saccharomyces cerevisiae* XP660-19D (MATα, *trpl*, *adel*, *his2*) by a variation (MacKay, 1983) of the procedure of Beggs (1978). Transformants were selected on agar plates lacking tryptophan. Characterization of plasmids in yeast was done by isolation of plasmid from yeast (MacKay, 1983), transformation into *E. coli* DH5, isolation of bacterial plasmid, and restriction enzyme mapping.

Isolation of Yeast RNA. Fifty-milliliter cultures of transformed yeast were grown to late log phase (4×10^7 cells/mL) in supplemented MV (MV is 0.67% Difco yeast nitrogen base without amino acids, 2% glucose; supplements are 20 μg/mL of adenine and 10 μg/mL of histidine). Total RNA was isolated with use of the method of Srepati and Warner (1978) except that NaCl replaced LiCl in all buffers. Plasmid DNA was removed by adjusting the solution to a volume of 3.6 mL containing 4 M guanidinium thiocyanate, 25 mM HEPES–KOH, pH 7.5, and 0.5% Sarkosyl, layering it over a 1.2-mL cushion of 5.7 M CsCl in 25 mM sodium acetate and 1 mM EDTA and centrifuging at 36 000 rpm for 15 h at 20 °C in an SW50 rotor (Chirgwin et al., 1979). The RNA pellets were dissolved in sterile deionized water and precipitated with 250 mM ammonium acetate and 2.5 vol of ethanol. The RNA was dissolved in sterile deionized water and stored at −70 °C.

Northern Blots. Ten micrograms of yeast total RNA in 40 mM Tris-Cl, pH 7.5, containing 6 mM $MgCl_2$ was digested with 2 units of DNase I (RNase-free, Pharmacia) for 30 min at 37 °C. The samples were adjusted to 12.5 mM methylmercuric hydroxide, 10% glycerol, 0.1% bromophenol blue, and 0.5× buffer E (1× = 50 mM boric acid, 5 mM sodium borate, 10 mM sodium sulfate) (Bailey and Davidson, 1976) and loaded onto a 1% agarose gel containing 5 mM methylmercuric hydroxide and 0.5× buffer E. Poly(A)$^+$RNA from wheat endosperm (750 ng) was similarly prepared and loaded onto the same gel. After electrophoresis in 0.5× buffer E, marker lanes were cut off, stained for 30 min in 0.5 M ammonium acetate containing 0.5 μg/mL ethidium bromide, and photographed in UV light. The remainder of the gel was blotted onto nitrocellulose (Thomas, 1983). After baking, the filter was heated for 5 min in a boiling solution of 20 mM Tris-Cl, pH 8, and 15 mM 2-mercaptoethanol. Blots were prehybridized in a solution of 0.75 M NaCl, 50 mM NaH_2PO_4, pH 7.4, 5 mM EDTA, 50% deionized formamide, 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% poly(vinylpyrrolidone), 200 μg/mL of denatured sheared salmon sperm DNA, and 0.1% SDA (sodium dodecyl sulfate) at 42 °C for 8 h and hybridized in a fresh volume of the same solution containing 10% dextran sulfate and denatured ^{32}P-labeled probe at 42 °C for 16–20 h. Blots were washed three times for 30 min at room temperature in 0.5× SSC (1× SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7) containing 0.1% SDS, once at 65 °C for 30 min in 0.2× SSC containing 0.1% SDS and for 15 min in 0.2× SSC containing 0.1% SDS.

Isolation of Gliadin from Culture Medium. Yeast cells were grown to late log phase (4×10^7 cell/mL) in 50 mL of supplemented MV. Cells were removed by centrifuging for 20 min at 20000*g* at 4 °C. The supernatant was stored at −70 °C until used. Thawed supernatant was lyophilized, and the residue was dissolved in a small volume of sterile deionized water. After dialysis against sterile deionized water (Popineau and Pineau, 1985), the solution was concentrated by lyophilization. The final residue

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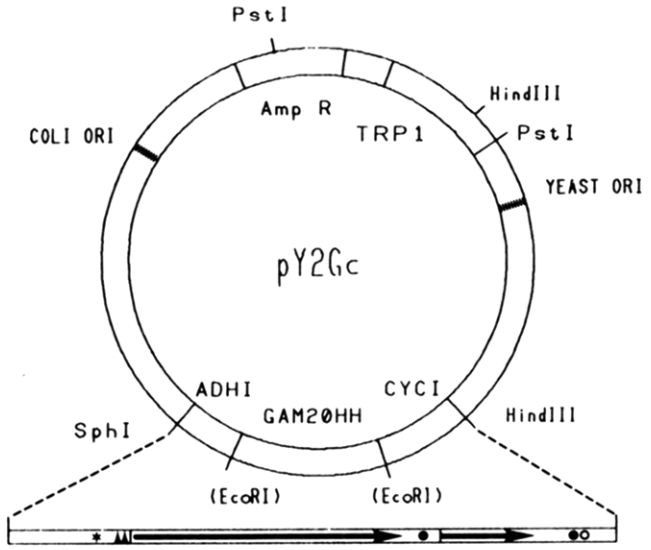

Figure 1. Structure of pY2Gc. A 1028-bp fragment containing the gliadin coding region was blunt-end-ligated into the unique *Eco*RI site of the yeast *E. coli* shuttle vector pYcDE-2. The *Eco*RI sites in parentheses denote the insertion point and were not regenerated in pY2Gc. An enlargement of the *ADH1* promoter, gliadin gene insert, and *CYC1* terminator indicate the location of the TATAAA box (*), mRNA start sites (▲), polyadenylation signals (●), and polyadenylation site (○). The coding region for the gliadin gene and partial coding region of the *CYC1* gene are shown as heavy arrows.

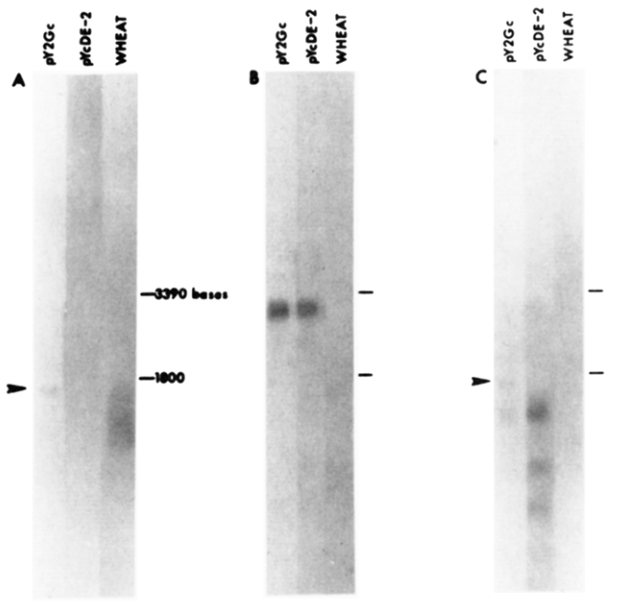

Figure 2. Autoradiograms of RNA blots hybridized with GamHH or vector-specific probes. Total RNA (10 μg) from yeast containing pY2Gc or pYcDE-2 was electrophoresed on a denaturing agarose gel with 0.75 μg of poly(A)$^+$RNA from wheat endosperm and transferred to nitrocellulose. The blot was hybridized with ^{32}P-labeled DNA from (A) Gam20HH, a subclone of pW1020 containing the gliadin coding region; (B) the 3.5-kb *Pst*I/*Eco*RI fragment of pYcDE-2 containing the *ADH1* promoter and pBR322 region; and (C) the 2.5-kb *Pst*I/*Eco*RI fragment of pYcDE-2 containing the *CYC1* terminator, 2-μm region, and yeast *ori*. Arrowheads indicate the gliadin coding mRNA. Yeast 26S RNA (3390 nucleotides) and 18S RNA (1800 nucleotides) were used as size markers.

was dissolved in sterile deionized water and stored at −20 °C.

Isolation of Total Protein from Yeast. About 10^8 yeast cells from the above culture were resuspended in 1.25 mL of sterile distilled water and transferred to a 1.5-mL conical tube. After being centrifuged for 1 min, the pellet was quick-frozen in dry ice/ethanol and stored at −70 °C (Corragio et al., 1986). The pellet was ground with an equal volume of fine glass beads in a prechilled mortar and pestle and transferred to a 1.5-mL tube. One hundred microliters of 2X SDS–sample buffer (0.25 M Tris–phosphate, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.07% pyronin Y) was added and the resultant mixture vortexed for 1 min. The sample was centrifuged for 1 min to remove insolubles.

Yeast Colony Immunoblot. For each yeast strain, 1 μL of sterile water containing 10–20 yeast cells was spotted onto a nitrocellulose filter placed on a plate of 2% agar in supplemented MV. After 3 days of growth at 30 °C, cells were lysed under alkaline conditions (Lyons and Nelson, 1984). The filter was rinsed with distilled water and incubated for 2 h with 13 mL of blocking buffer (2% hemoglobin, 10 mM Tris-Cl, pH 7.2, 50 mM NaCl, 2 mM EDTA, 0.1% NaI) (Tweten and Iandolo, 1981) at 37 °C. The solution was replaced with 13 mL of fresh blocking buffer containing 0.05% NP-40 and 10 μL of antigliadin serum (Calbiochem-Behring) and incubated for 5 h at 37 °C. The filter was washed five times for 5 min with washing buffer (1% NaCl, 50 mM Tris-Cl, pH 8, 0.05% NP-40) and then incubated for 9 h at 37 °C with 13 mL of blocking buffer containing 0.05% NP-40 and ^{125}I-labeled protein A (100 000 cpm/mL) (gift from V. Stout and J. Iandolo, Kansas State University). After the washing buffer step as described above, the damp filter was wrapped in plastic and autoradiographed.

Western Blotting. Protein samples were adjusted to 1× SDS–sample buffer and heated for 2 min in boiling water before electrophoresing on a 15% SDS–polyacrylamide gel (Laemmli, 1970) and electroblotting onto nitrocellulose (Burnette, 1981) in a buffer containing 20 mM Tris, 180 mM glycine, 0.05% SDS, and 30% methanol. After the blot was rinsed for 30 s in sterile deionized water, the blot was blocked and tested for the presence of gliadin as described for the colony immunoblot.

RESULTS

The 1028 by *Hpa*I/*Hind*III fragment containing the coding region of the γ-gliadin genomic clone pW1020 was cloned into the *Eco*RI site of the vector pYcDE-2 (Figure 1). The fragment consists of 14 bp of 5′ noncoding sequence and 909 bp of coding region followed by 107 bp of 3′ noncoding region, which contains a polyadenylation signal. The vector pYcDE-2 has about 400 bp of the *ADH1* promoter joined by an *Eco*RI site to 323 bp of *CYC1* incomplete coding region followed by about 275 bp of 3′ noncoding sequence, which contains a yeast transcription terminator. pYcDE-2 also contains a *TRP1* selectable marker and 2-μm plasmid origin of replication for yeast and the ampicillin resistance gene and pBR322 replication origin for selection and replication, respectively, in *E. coli* (B. D. Hall, personal communication).

A plasmid with the correct coding orientation, pY2Gc, was obtained. The distance from the TATAAA box in the *ADH1* promoter to the gliadin translation start site in pY2Gc is 7 bp farther than for the normal *ADH1* gene (Bennetzen and Hall, 1982a).

A *trp1*$^-$ strain of yeast was transformed with the recombinant plasmids and pYcDE-2 and grown on selective media. Clones from both plasmid types were isolated.

RNA was isolated from cultures grown to late log phase for both plasmid-containing strains. A Northern blot of total RNA from the yeast strains and poly(A)$^+$RNA from developing wheat endosperm was hybridized with a radioactive probe containing the coding region (referred to here as Gam20HH) of pW1020 and autoradiographed. An autoradiographic band of 1660 nt (nucleotides) was seen in pY2Gc RNA (Figure 2A). The transcript was 100–150 nt longer than the distance from the *ADH1* transcription start site to the *CYC1* termination site, which suggests the RNA was polyadenylated. No bands were seen in pYcDE-2 RNA. Poly(A)$^+$RNA from wheat showed a wide band of RNA of the size expected for the class of γ-gliadin mRNAs (Rafalski et al., 1984).

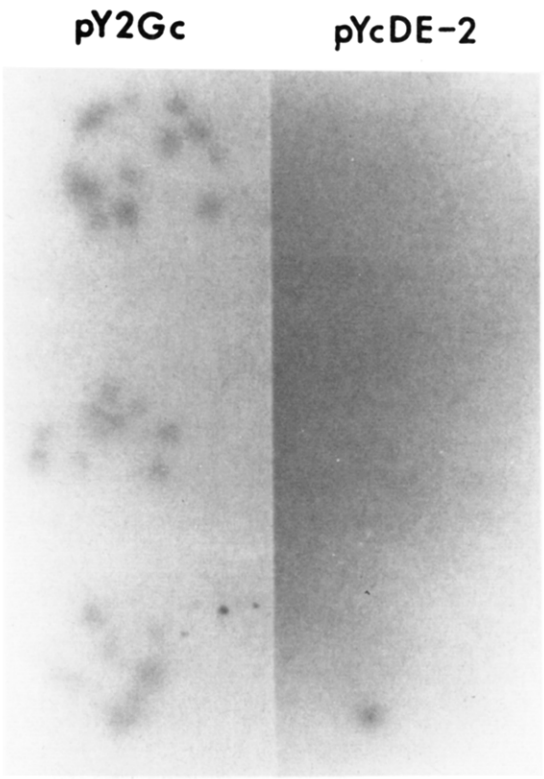

Figure 3. Autoradiogram of a colony immunoblot of yeast containing pY2Gc or pYcDE-2. Yeasts containing pY2Gc or pYcDE-2 were plated on nitrocellulose and grown on supplemented MV agar plates until 1-mm colonies were formed. After the colonies were lysed, γ-gliadin was detected by treating the filter with antigliadin serum and ^{125}I-labeled protein A.

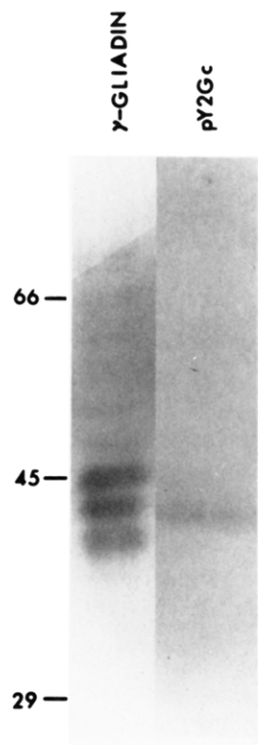

Figure 4. Autoradiogram of a Western blot of γ-gliadin from yeast and wheat. Samples of total protein from pY2Gc-containing yeast and partially purified γ-gliadin from wheat were separated on a 15% SDS–polyacrylamide gel and electroblotted onto nitrocellulose. Gliadins were detected by treating the filter with antigliadin serum and ^{125}I-labeled protein A. The sizes of protein markers are indicated in kDa.

The Northern blot was stripped and reprobed with a 2.5 kb *Pst*I/*Eco*RI fragment (*CYC1* probe) of pYcDE-2 containing the *CYC1* terminator and 2-μm origin of replication and then probed with the 3.5 kb *Pst*I/*Eco*RI fragment (*ADH1* probe) of pYcDE-2 containing the *ADH1* promoter and pBR322 origin of replication. In the pY2Gc RNA the *CYC1* probe and the *ADH1* probe hybridized to transcripts of the same size as the transcripts seen with Gam20HH probe (Figure 2B,C). Additional bands of vector origin were seen with both vector probes in all the transformed yeast RNA samples. Neither vector probe hybridized to wheat endosperm mRNA.

The blot (Figure 2) showed that transcription began at the *ADH1* transcription start site and terminated at the *CYC1* termination site. No termination occurred near the wheat polyadenylation signal.

To determine whether gliadin protein was expressed, a colony immunoblot was made from yeast containing pYcDE-2 and pY2Gc. After the blot was reacted with antigliadin serum followed by ^{125}I-labeled protein A, autoradiography showed that yeast containing pY2Gc produced detectable γ-gliadin protein (Figure 3). There was no signal for yeast containing pYcDE-2.

Total protein from pY2Gc-containing yeast cells grown in minimal selective media was electrophoresed on an SDS–polyacrylamide gel with partially purified γ-gliadins, electroblotted onto nitrocellulose, and reacted with antigliadin serum and ^{125}I-labeled protein A. A single band comigrating with the middle band of partially purified marker γ-gliadin was seen (Figure 4). The yeast cells containing pY2Gc had 40–80 ng of gliadin/mL of late log culture (4×10^7 cells/mL).

The presence or absence of the signal peptide cannot be determined from this experiment. The mature γ-gliadin encoded by pW1020 has a molecular weight of 32.1 kDa, which is in the middle of the range of mature molecular weights determined from cDNA and genomic sequence data (30.8–35 kDa). However, molecular weights estimated for gliadins on SDS–polyacrylamide gels (38.5–45 kDa from Figure 4) are higher than molecular weights determined from sequence data. This complicates estimating whether the signal peptide (2150 Da) is present or not.

Because γ-gliadins are synthesized with a signal peptide used to transport them across a membrane during packaging into protein bodies, we were interested to see whether yeast could use this signal to secrete the gliadin. Protein from the supernatant of the same culture of pY2Gc yeast used to identify internal gliadin was concentrated and purified by lyophilization and dialysis. The amount of gliadin in the final solution was estimated by spotting a sample onto nitrocellulose alongside a range of known quantities of gliadin and reacting the samples with antigliadin serum and ^{125}I-labeled protein A. The transformed yeast secreted 0.5–1.5 ng of gliadin/mL of culture (data not shown).

DISCUSSION

We have cloned a γ-gliadin gene into a yeast expression plasmid and detected products at the RNA and protein level. Zein, a prolamin from corn, has been transformed into yeast on an integrating vector (Langridge et al., 1984) and on plasmids (Corragio et al., 1986; Compagno et al., 1987). RNA transcripts were found in both systems, but zein was detected only in the plasmid-transformed yeast. An α-gliadin gene cloned into a yeast expression plasmid under control of the *CYC1* promoter expressed mRNA and protein (Neill et al., 1987).

Several different yeast promoters have been used to express plant gene products in yeast (Corragio et al., 1986;

Rothstein et al., 1984; Edenset al., 1984; Cramer et al., 1987). The *ADH1* gene produces fairly large quantities of mRNA (1–2% of poly(A)$^+$RNA) and 1% or more of total protein when cells are grown in the presence of glucose. If yeast are transferred to a medium with ethanol as a carbon source or allowed to reach late log or stationary phase, the cells produce less *ADH1* RNA. A shortened *ADH1* promoter (400 bp as in pYcDE-2) produces RNA even when ethanol is the carbon source (Beier and Young, 1982).

The *ADH1* promoter has been used to express several mammalian genes with results varying from 1–2% of total protein expressed as interferon (Hitzeman et al., 1981) to no detectable expression of rat growth hormone (Hitzeman et al., 1981). The low level of gliadin expression in pY2Gc cells may be due to any of several factors. The transcribed mRNA contains wheat encoded sequences both 5′ and 3′ to the protein-coding region. Kniskern et al. (1986) found that production of hepatitis B virus core antigen under control of the *GAP* promoter in yeast was increased by removing nonyeast DNA from the 5′ and 3′ flanking regions.

Inadequate amounts of proline and glutamine may have been available for translation of γ-gliadin mRNA. The protein is 17% proline and 30% glutamine. The predominant codons for phenylalanine, serine, and valine in the γ-gliadin mRNA are seldom found in yeast (Bennetzen and Hall, 1982b). The majority of the proline and glutamine codons used in the gliadin are the preferred codons in yeast, but 7.6% of the gliadin is encoded as CCC (Pro) and 6% is encoded as CAG (Gln), both of which are rare in yeast (Bennetzen and Hall, 1982a). The glutamine tRNA that recognizes CAG is probably the product of a single gene (Weiss and Friedberg, 1986). A limited supply of this and other minor isoacceptor tRNAs could lead to a low level of production of γ-gliadin protein. Conversely, very high levels (40% of cellular protein) of hepatitis B virus core antigen have been obtained in yeast (Kniskern et al., 1986) although the pattern of codon usage was unlike the biased pattern of highly expressed yeast genes (Bennetzen and Hall, 1982a).

The media and growth conditions of the yeast may have affected the expression level. Compagno et al. (1987) found that synthesis of a zein with no signal peptide was low during exponential growth in minimal media but increased during early log phase. Wheat α-amylase is secreted in greater amounts from yeast grown on rich media than from yeast grown on minimal media, although the yeast synthesized the same amount of α-amylase (Rothstein et al., 1987).

The γ-gliadin may be degraded at a rapid rate because the γ-gliadin signal peptide may allow the protein to begin passage through the yeast secretion process but leave it vulnerable to protease digestion at some stage.

The plant proteins wheat α-amylase (Rothstein et al., 1984, 1987), thaumatin (Edens et al., 1984), phaseolin (Cramer et al., 1987), and α-gliadin (Neill et al., 1987) have all been expressed in yeast. All four proteins had their own signal peptides removed by the yeast, and wheat α-amylase was secreted into the medium (Rothstein et al., 1984, 1987). Phaseolin, which is glycosylated in French bean, was also glycosylated in yeast (Cramer et al., 1987). A zein synthesized with its signal peptide had the signal peptide removed but was also degraded into three or four peptides (Corragio et al., 1986).

A small amount of γ-gliadin is secreted from pY2Gc-containing yeast. There is a change in solubility of the secreted gliadin compared to wheat gliadin. Altered solubility properties may result from interaction with other medium constituents, or the secreted gliadin may be glycosylated although gliadin is not glycosylated in wheat.

Several mammalian proteins have been secreted after transforming yeast with hybrid genes containing the coding region for the signal peptide of α factor (Ernst, 1986; Green et al., 1986) or killer toxin (Baldari et al., 1987) (yeast proteins secreted into the media) attached to mammalian coding regions. Efficient secretion of large amounts of γ-gliadin may require this type of signal peptide replacement.

This study demonstrates that heterologous proteins with unusual properties and characteristics can be synthesized in yeast and provides possible areas to explore to improve expression.

ACKNOWLEDGMENT

We are indebted to Dr. Thomas Manney for his guidance in culturing and transforming yeast. This work was supported in part by the Kansas Agricultural Experiment Station (Publication No. 89-72J) and by USDA Grant 86-CRCR-1-2144.

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Received for review August 26, 1988. Revised manuscript received December 28, 1988. Accepted January 10, 1989.