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Glucoamylases G1 and G2 from *Aspergillus niger* are synthesized from two different but closely related mRNAs

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By the use of glucoamylase-specific synthetic oligodeoxyribonucleotides and molecular cloning of cDNA synthesized from *Aspergillus niger* total poly(A)$^+$ RNA, the primary structure of the glucoamylase G1 mRNA was determined. Glucoamylase G1 is synthesized as a precursor of 640 amino acid residues containing a putative signal peptide of 18 residues, a short propeptide of six residues and the 616 residues long mature enzyme. *In vitro* translations of mRNA and immunoprecipitations with glucoamylase-specific antisera showed that two glucoamylase polypeptides are synthesized. The larger form with an apparent mol. wt. of 71 000 corresponds to the precursor of glucoamylase G1, and the shorter form with an apparent mol. wt. of 61 000 corresponds to the precursor of glucoamylase G2. From the nucleotide sequencing data of several glucoamylase-specific cDNA recombinants it is shown that the G1 mRNA contains a 169 bp long intervening sequence that can be spliced out to generate a G2 mRNA. Only the 3′ part of the G1 mRNA is modified by this splicing event. This kind of differential mRNA processing to give different protein products from one primary transcript has previously only been demonstrated in higher eukaryotes.

Key words: cell-free translation/filamentous fungi/molecular cloning/differential mRNA splicing/oligodeoxynucleotide priming

Introduction

Glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) catalyses the release of glucose from the non-reducing ends of starch and related oligo- and polysaccharides (Reilly, 1979; Hiromi *et al.*, 1983). Mold glucoamylases are of great commercial importance in the production of glucose from starch and dextrins as well as in the brewing industry and in the manufacture of fuel alcohol and alcoholic beverages. Most fungal glucoamylases exist in multiple forms varying in size (Pazur *et al.*, 1971; Ueda, 1981). In general only the larger of these forms is able to adsorb to raw starch and to degrade this substrate (Ueda, 1981), while all of the forms have similar enzymic properties towards soluble polysaccharides and oligosaccharides (McCleary and Anderson, 1980; Svensson *et al.*, 1982). Partial amino acid sequence determination of the large form, G1, and the smaller one, G2, of glucoamylase from *Aspergillus niger* has shown that their polypeptide chains are very closely related and it was suggested that G1 is extended in the COOH-terminal region with a peptide fragment missing in G2 (Svensson *et al.*, 1982). The two forms also show immunological cross-reactivity (Lineback *et al.*, 1969). A similar structural relationship has been suggested for glucoamylases M_1 and M_2 from *A. saitoi* (Inokuchi *et al.*, 1982; Takahashi *et al.*, 1981). In contrast, multiple forms of glucoamylase from *Rhizopus* sp. differ in the NH_2-terminal region of the polypeptide chain (Takahashi *et al.*, 1982). The smaller forms of the glucoamylases were thought to arise perhaps by limited proteolysis. Thus *in vitro* treatment of glucoamylase from *A. awamori* var. *kawachi* with protease resulted in forms similar to those isolated from cultures of this mold strain (Yoshino and Hayashida, 1978; Hayashida and Yoshino, 1978). Here we describe the complete nucleotide sequence of the cDNA synthesized from glucoamylase G1 mRNA from *A. niger*, which fully agrees with the amino acid sequence published recently (Svensson *et al.*, 1983b). We present evidence that the smaller glucoamylase G2 can be synthesized from a shorter mRNA, derived as a splicing product from the G1 mRNA.

Results

Cell-free translation and immunoprecipitation

Total poly(A)-containing RNA from *A. niger* was translated in a rabbit reticulocyte lysate supplemented with L-[^{35}S]-

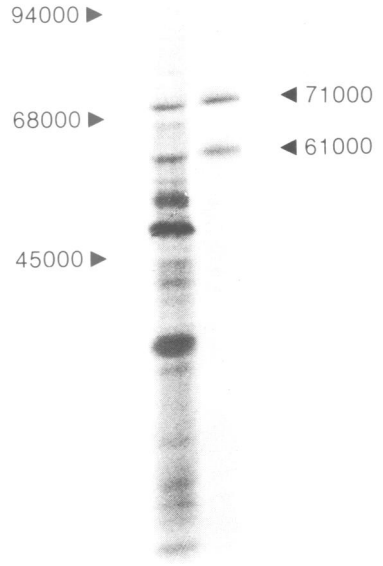

Figure 1. SDS-polyacrylamide gel electrophoresis, followed by autoradiography of *in vitro* translation products and their immunoprecipitates. Total mRNA extracted from *A. niger* was translated in a rabbit reticulocyte lysate with L-[^{35}S]methionine **(left lane)** and immunoprecipitated with rabbit antisera against *A. niger* glucoamylase G1 *(right lane)*. Two primary translation products of apparent mol. wt. 71 000 and 61 000 are precipitated. The position of mol. wt. standards are shown to the left.

methionine. After immunoprecipitation with rabbit antisera raised against purified *A. niger* glucoamylase G1, the products were analysed by SDS-polyacrylamide gel electrophoresis (Figure 1). Two proteins with apparent mol. wts. of 71 000 and 61 000 were precipitated with the antisera, indicating that glucoamylase G1 and G2 respectively are synthesized as these primary translation products, pre-G1 and pre-G2, in good agreement with the length of 616 amino acids for the G1 protein (Svensson *et al.*, 1983b) in its secreted form, and a close to 10 000-dalton shorter G2 protein as reported (Svensson *et al.*, 1982; Smiley *et al.*, 1971). There is immunological cross-reactivity between G1 and G2 (Lineback *et al.*, 1969). Pre-G1 and pre-G2 are the most abundant translation products among the larger proteins synthesized in this *in vitro* experiment.

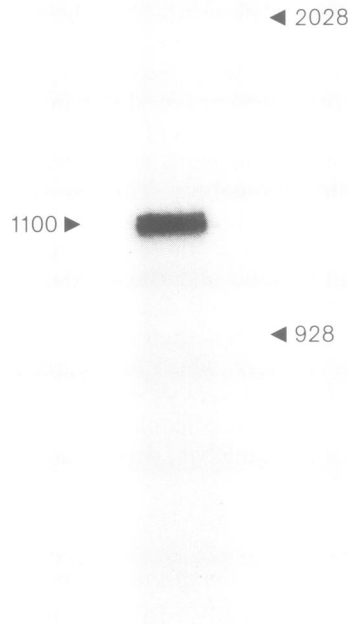

Fig. 2. Autoradiographic representation of a 3.5% polyacrylamide/urea gel of d(T-T-A/G-T-C-C-C-A-T/C-T-G-A/G-T-A)-primed glucoamylase cDNA. The cDNA was transcribed fom 5 μg of *A. niger* total mRNA, using 5′ end-labeled primer and unlabeled dNTPs. The 1100 bases long reverse transcript was shown by nucleotide sequencing to represent the 5′ end of glucoamylase mRNA. The nucleotide length of molecular markers are indicated on the right.

Identification and cloning of A. niger glucoamylase cDNA

Poly(A)-containing RNA isolated from *A. niger* cells growing at optimized conditions for glucoamylase production, was transcribed with reverse transcriptase, primed by the 5′-labeled tetradecamer oligodeoxynucleotide mixture d(T-T-A/G-T-C-C-C-A-T/C-T-G-A/G-T-A). One of these sequences is complementary to the glucoamylase mRNA in the region coding for Tyr-Gln-Trp-Asp-Lys (Svensson *et al.*, 1983b). Analysis of the cDNA products on a denaturing 3.5% polyacrylamide gel identified only one dominating cDNA component ~1100 bases long (Figure 2). This cDNA was electroeluted and sequenced by the chemical cleavage procedure (Maxam and Gilbert, 1980). 290 nucleotides were determined and found to be in complete agreement with the amino acid sequence of glucoamylase G1 from *A. niger* (Svensson *et al.*, 1983b). Although G1 and G2 seemed to be synthesized as two separate preproteins (see above) only one nucleotide sequence could be established for this central part of the mRNA coding for the amino acids 230–330 as numbered by Svensson *et al.* (1983b). cDNA clones primed by oligo(dT)$_{12-18}$ or the glucoamylase-specific tetradecamer mixture were constructed and screened by colony hybridization as described in Materials and methods. About 3% of the oligo(dT)-primed clones hybridized to the ^{32}P-labelled 1100 bases long glucoamylase cDNA. Among positive clones, only

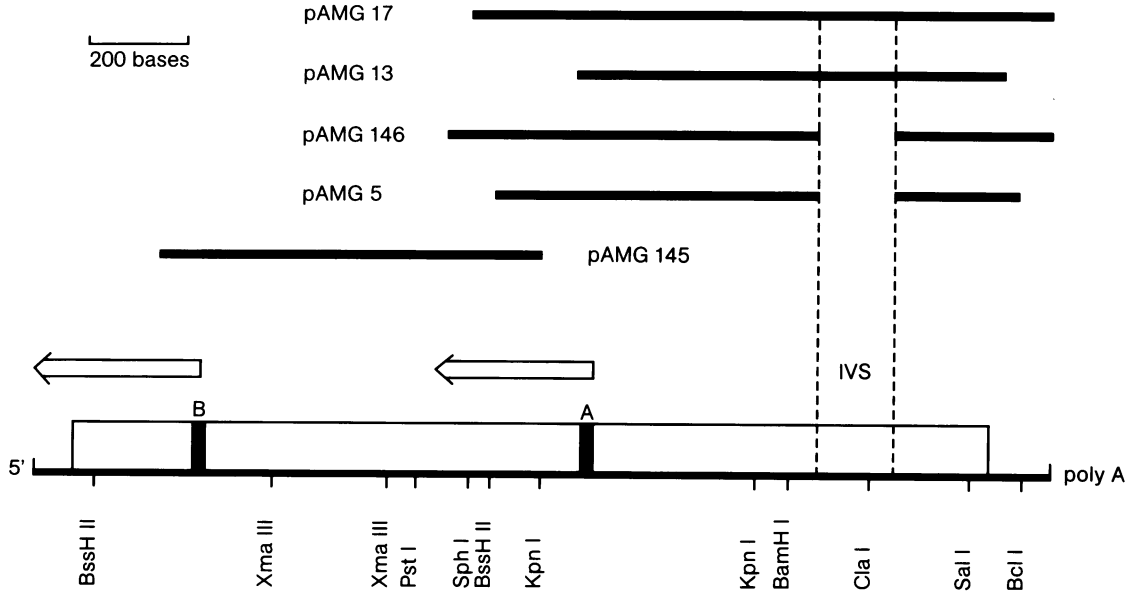

Fig. 3. Restriction endonuclease map of the inserts in four 3′-specific and one 5′-specific glucoamylase cDNA clone. IVS designates a 169 bases long intervening sequence (see text and Figure 6) found in the recombinants pAMG13 and pAMG17, but missing in the clones pAMG5 and pAMG146. Thick vertical blocks A and B show the positions of a tetradecamer mixture and a pentadecamer, respectively, used to determine part of the nucleotide sequence of the glucoamylase mRNA by direct cDNA sequencing as indicated by thick open arrows.

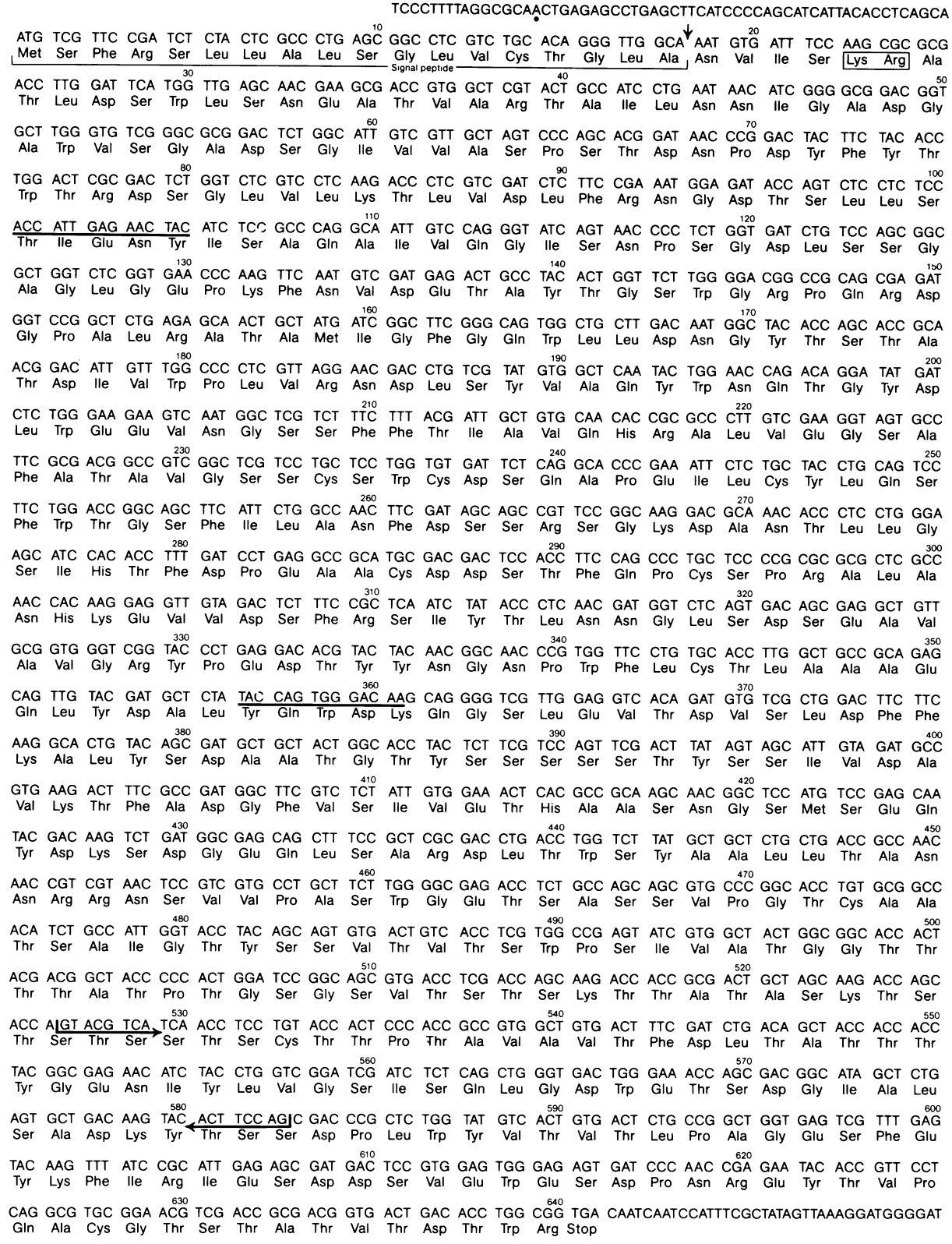

Fig. 4. Sequence of the *A. niger* glucoamylase G1 cDNA as determined from the insert of clones pAMG13, pAMG17, pAMG145 and from the 5′ end specific cDNA primed by the pentadecadeoxyribonucleotide (dGTAGTTCTCAATGGT). The amino acid sequence deduced from the cDNA sequence is numbered from the initiating methionine. In the protein sequence the putative signal peptide is underlined, and a potential signal peptidase site is indicated by an arrow. Processing by a dibasic specific peptidase at the boxed Lys-Arg (23 – 24) residues will generate the glucoamylase G1 as it is known in its principal secreted form. Thick horizontal arrows at codons Ser-527 and Ser-583 show the splice junctions of a 169 bases long intervening sequence. Removal of this region from the G1 mRNA generates a G2 mRNA (see text and Figure 6). The positions of the pentadecamer (101 – 105) and the tetradecamer mixture (357 – 361) used in cDNA priming and colony screening are indicated by thick underlinings. A dot under the second A in the sequence GCAACT in the 5′ end designated a second initiation point of the transcription.

those having the longest insert were selected for further analysis. Clones selected in this way from the oligo(dT)-primed library (pAMG5, pAMG13, pAMG17 and pAMG146) covered the 3′ half of the mRNA from poly(A) and ~1300 bases towards the 5′ end (Figure 3). A clone pAMG145 primed by the tetradecamer mixture covered most of the 5′ half of the mRNA from the site of priming to a position corresponding to amino acid Asn-69 (Figures 3 and 4). The insert of pAMG145 has ~200 bases in common with the four 3′ end clones mentioned above, but since it did not contain the mRNA sequence from the cap site to the codon Asn-69, this region was determined by sequencing a cDNA primed by the pentadecamer d(GTAGTTCTCAATGGT) complementary to the mRNA coding for Thr-Ile-Glu-Asn-Tyr in position 101 – 105 (Figure 4). From the nucleotide sequence determined from this cDNA, pAMG145 and from the two overlapping clones pAMG17 and pAMG13, the total sequence of the glucoamylase G1 mRNA could be established (Figure 4).

	U		C		A		G		
U	Phe	4	Ser	16	Tyr	6	Cys	3	U
		18		19		21		7	C
	Leu	0		4	Stop	0	Stop	1	A
		7		14		0	Trp	19	G
C	Leu	3	Pro	4	His	0	Arg	4	U
		17		10		4		8	C
		2		0	Gln	3		4	A
		19		8		14		2	G
A	Ile	12	Thr	20	Asn	6	Ser	11	U
		11		39		19		24	C
		1		5	Lys	0	Arg	1	A
	Met	3		10		13		1	G
G	Val	6	Ala	25	Asp	21	Gly	15	U
		15		19		23		22	C
		2		10	Glu	9		7	A
		19		11		17		3	G

Fig. 5. Codon utilization for the glucoamylase G1 gene from *A. niger*.

Discussion

Structure of the glucoamylase G1 mRNA and its primary translation product

The 5′-untranslated end of the G1 mRNA determined from direct nucleotide sequencing of the pentadecamer-extended cDNA turned out to be heterogeneous in length, since two glucoamylase specific DNAs: 375 and 350 bases long were synthesized from the same priming site (data not shown). Southern blot analysis of chromosomal *A. niger* DNA and molecular cloning of glucoamylase specific fragments from the genome has shown that *A. niger* only contains one glucoamylase gene (Boel, Hansen and Fiil, in preparation). Thus this gene might contain two different transcription initiation sites. One reverse transcript contained a 5′-untranslated region of 59 nucleotides before the ATG codon (Figure 4), and the other transcript clearly had exactly the same sequence, but the second A in the sequence GCAACT was the first nucleotide in the primary transcript, leaving only 44 nucleotides in the mRNA upstream from the ATG codon. Multiplicity of initiation points has been observed for yeast genes such as alcohol dehydrogenase I (Bennetzen and Hall, 1982a), iso-1-cytochrome c (Faye *et al.*, 1981) and the tryptophan synthase gene (TRP5) (Zalkin and Yanofsky, 1982). From the intiation codon ATG to the stop codon TGA the G1 mRNA contains an open reading frame consisting of 1920 nucleotides and coding for a 640 amino acid residues long, glucoamylase precursor of mol. wt. 68 234, corresponding to the primary translation product with an apparent mol. wt. of 71 000 as determined from SDS

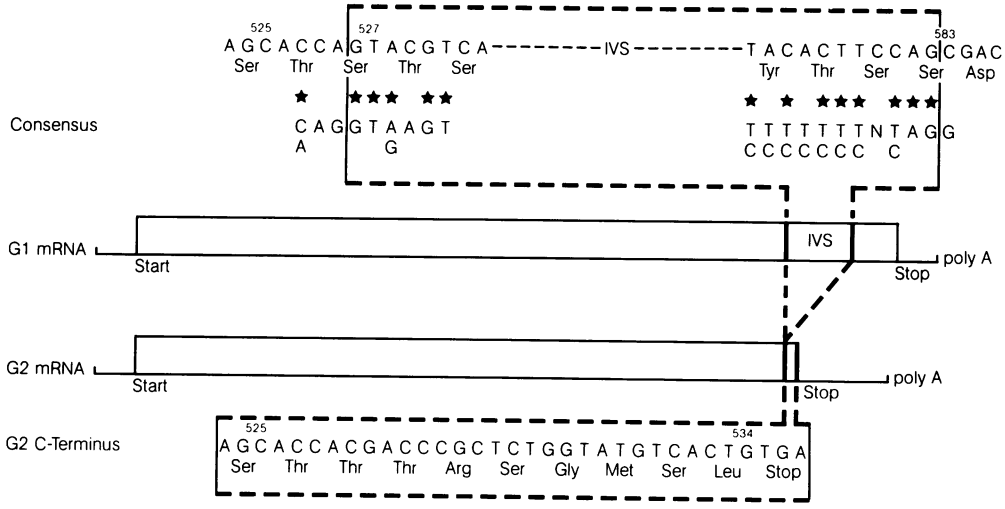

Fig. 6. Diagram of glucoamylase G1 and G2 mRNAs showing the 169 bases long intervening sequence (IVS) in the G1 mRNA between the two Ser-codons 527 and 583 as numbered in Figure 4. Asterisks indicate homology with the consensus sequence found at eukaryotic splice junctions. After splicing out the IVS from the glucoamylase G1 mRNA, a shorter mRNA identified by the inserts in the cDNA clones pAMG5 and pAMG146 (see text) is produced. The COOH-terminal part of this glucoamylase G2, as translated from the changed open reading frame after splicing, is shown at the bottom. From Ser-525 towards the NH_2 terminus, glucoamylase G2 would have an amino acid sequence identical to that of glucoamylase G1 (Figure 4).

gels (Figure 1). This protein contains an NH_2-terminal peptide of 24 amino acid residues ending in Lys-Arg at position 23 – 24 (Figure 4), indicating that this amino-terminal peptide is cleaved from the G1 glucoamylase, which, in its secreted form, starts with the amino acids Ala-Thr-Leu-Asp-Ser-Trp (Svensson *et al.*, 1983b). The origin of G1 molecules missing the NH_2-terminal tripeptide Ala-Thr-Leu (Svensson *et al.*, 1983a) and beginning with Asp-Ser-Trp is not known. The NH_2-terminal peptide shows the characteristics of a signal peptide, and a typical signal peptidase cleavage site would be between residues 18 and 19 (Ala-Asn) according to von Heijne (1983). This leaves the hexapeptide Asn-Val-Ile-Ser-Lys-Arg as a short propeptide to be processed by a trypsin-like protease. The sequence of the following 616 residues in the G1 glucoamylase is in total agreement with the results of Svensson *et al.* (1983b). In its 3′ end the G1 mRNA has an untranslated region of 124 nucleotides before the poly(A) tail. This region does not contain a poly(A) addition signal AATAAA found in most mRNAs from higher eukaryotes.

Glucoamylase G1 mRNA codon usage

The codon usage in the *A. niger* glucoamylase G1 mRNA is tabulated in Figure 5. 57 out of the 61 possible coding triplets are being used, this is quite unlike the extreme codon bias seen in *S. cerevisiae* (Bennetzen and Hall, 1982b) and in *S. pombe* (Russell and Hall, 1983). The codon usage is very similar to that of weakly expressed *Escherichia coli* and animal cell genes (Grantham *et al.*, 1980). Perhaps more interestingly a close resemblance to the codon utilization in the exo-cellobiohydrolase I gene from *Trichoderma reesei* (Shoemaker *et al.*, 1983) is noted. Therefore filamentous fungi seem to show the same bias in codon selection when expressing an abundant protein.

Relationship between the glucoamylase G1 and G2 mRNAs

The nucleotide sequence of the two independently isolated glucoamylase G1 clones, pAMG13 and pAMG17, showed no discrepancies, but the inserts of pAMG5 and pAMG146 (Figure 3) were both quite different from the inserts in pAMG13 and pAMG17 since both recombinants showed a deletion of the same 169 nucleotides coding for the amino acid residues Ser-527 to Ser-583 (Figures 4 and 6). Since the two recombinants pAMG5 and pAMG146 did not have exactly the same length of the insert, they were clearly independent isolates from the oligo(dT)-primed library. Their nucleotide sequence showed no difference from the two glucoamylase G1 clones mentioned above except for the 169-bp deletion. We therefore think that cloning artefacts have not occurred. The inserts in the clones pAMG5 and pAMG146 thus seem to represent the reverse transcription of a glucoamylase-specific mRNA different from the G1-type. As indicated in Figure 6, this mRNA is probably derived from the G1 mRNA by further processing of the primary transcript from the glucoamylase gene. Clearly the 169-bp deletion is flanked by sequences in good agreement with the consensus found at eukaryotic splice sites (Mount, 1982). The deletion of this intervening sequence from the G1 mRNA changes the open reading frame to give a mRNA coding for a glucoamylase with the COOH-terminal octa-peptide Thr-Thr-Arg-Ser-Gly-Met-Ser-Leu-STOP (Figure 6) added to the Thr found at position 526 in glucoamylase G1 (Figure 4). The glucoamylase precursor encoded by this spliced mRNA would be 534 residues long and have a mol. wt. of 56 668, in other words 11 566 daltons less than the precursor of glucoamylase G1. It corresponds to the protein with an apparent mol. wt. of 61 000 as determined from the SDS gels. Since Svensson *et al.* (1982) reported glucoamylase G2 to have Leu or Ser as the COOH-terminal residue as determined by carboxypeptidase Y, and since the difference in mol. wt. between G1 and G2 is close to 10 000, we propose that the glucoamylase synthesized from the spliced mRNA represented by pAMG5 and pAMG146 could be glucoamylase G2 (Figure 6). This is supported by the *in vitro* translation experiment (Figure 1) showing that two glucoamylase-specific primary translation products with a difference in apparent mol. wt. as determined from the SDS-gel of ~10 kd are synthesized from *A. niger* poly(A)$^+$ RNA.

In this communication, we describe how glucoamylase G2 from *A. niger* can be synthesized from a mRNA derived by splicing of the glucoamylase G1 mRNA. Differential splicing of cellular mRNAs has previously been described for an immunoglobulin (Early *et al.*, 1980), fibrinogen (Crabtree and Kant, 1982), calcitonin (Rosenfeld *et al.*, 1983) and fibronectin (Schwartzbauer *et al.*, 1983; Kornblihtt *et al.*, 1984), but these examples describe variations in the expression mechanism in mammals. To our knowledge the present experiments are the first to describe differential mRNA processing in lower eukaryotes. That the two mRNAs could be transcription products from two glucoamylase genes are excluded by Southern blot analysis and molecular cloning of the chromosomal glucoamylase specific regions (Boel, Hansen and Fiil, in preparation). The possible advantages in expressing two different glucoamylases are not known, but judged from the *in vitro* translations experiment (Figure 1), the two enzymes seem to be expressed in close to equimolar amounts. The internal conserved sequence TACTAAC observed by Pikielny *et al.* (1983) and others in intervening sequences of yeast chromsomal protein coding genes, is not found in the 169 nucleotides long intervening sequence of the glucoamylase G1 mRNA. This intervening sequence therefore must be spliced out by a mechanism different from the one suggested for introns in primary yeast chromosomal transcripts. It is important to note that the consensus for eukaryotic splice junctions (Mount, 1982) is followed very strictly by the 169-bp intervening sequence of the G1 mRNA.

The present findings of course do not rule out the possibility that glucoamylase G1 can be processed by *A. niger* proteases to give protein products that are secreted and are enzymatically active. These products might even be very similar to the glucoamylase G2 described in this work, but clearly differential splicing of glucoamylase mRNA contributes to the generation of multiple enzyme forms.

Materials and methods

Enzymes and reagents

DNA polymerase I 'Klenow fragment' (EC 2.7.7.7; from *E. coli*), polynucleotide kinase (ATP: 5′-dephosphopolynucleotide 5′-phosphotransferase, EC 2.7.1.78; from *E. coli* strain B infected with phage T4) and $(dT)_{12-18}$ were purchased from P-L Biochemicals (Milwaukee, WI). Reverse transcriptase (RNA-dependent DNA polymerase, EC 2.7.7.49; from avian myeloblastosis virus) was purchased from Life Sciences (St. Petersburg, FL). Terminal deoxynucleotidyl transferase (EC 2.7.7.31) was from Bethesda Research Labortories (Gaithersburg, MD). Restriction endonucleases were from New England BioLabs (Beverly, MA). RNasin (human placenta ribonuclease inhibitor) was from Biotec (Madison, WI). Nuclease S1 (EC 3.1.30.1; from *A. oryzae* was purchased from Sigma (St. Louis, MO). Oligo(dT)-cellulose was type T-2 from Collaborative Research (Waltham, MA). DEAE-cellulose was DE52 from Whatman. [α-^{32}P]dCTP (3200 Ci/mmol; 1 Ci = 3.7 x 10^{10} Bq), [α-^{32}P]dATP (3200 Ci/mmol) and [γ-^{32}P]ATP (7500 Ci/mmol) were ob-

tained from New England Nuclear. L-[^{35}S]methionine (600 Ci/mmol) and nuclease treated, messenger-dependent rabbit reticulocyte lysate were purchased from Amersham.

Preparation of mRNA

A. niger cells growing at conditions optimized for glucoamylase production were pelleted at 10 000 *g* and 4°C for 10 min. The pelleted cells were frozen in liquid nitrogen and stored at −80°C. To extract the RNA, 5 g of frozen cells was pulverized under liquid nitrogen in a mortar. Quartz was added to disrupt the cell wall. The resulting powder was suspended in 60 ml of a buffer containing 5 M guanidinium rhodanide, 50 mM Tris-HCl at pH 7.5, 10 mM EDTA, 5% (v/v) 2-mercaptoethanol, 4% (w/v) N-lauroylsarcosine, and 15% (w/v) CsCl. A few strokes with an Ultra Turrax homogenizer was used to decrease the viscosity of the suspension by shortening the length of chromosomal DNA from the cells. After pelleting insoluble material at 10 000 *g* at 4°C for 15 min, total RNA was isolated from the supernatant as described (Chirgwin *et al.*, 1979) by pelleting through a 5.7 M CsCl, 100 mM EDTA cushion in a SW41 Beckman rotor run at 37 000 r.p.m. for 18 h at room temperature. Poly(A)-containing RNA was obtained by two cycles of chromatography on oligo(dT)-cellulose (Aviv and Leder, 1972).

Cell-free translation and immunoprecipitation

Total poly(A)-containing RNA from *A. niger* was translated in a rabbit reticulocyte lysate with L-[^{35}S]methionine as described by the supplier. After incubation for 60 min at 30°C, 1/4 of the reaction mixture was removed and frozen for later SDS-gel electrophoresis. Before immunoprecipitation, 1 vol of a buffer containing 500 mM Tris-HCl pH 7.5, 1% Triton X-100 and 1 mg/ml bovine serum albumin was added to the translation products. Fractionated rabbit glucoamylase G1 antisera was added to a final concentration of 1 μg IgG/25 μl lysate and incubated overnight at 4°C. To precipitate the antigen/antibody complex formalin-fixed *Staphylococcus aureus* cells were added (Cullen and Schwartz, 1976), and incubated on ice for 30 min with occasional vortex stirring. The cells were pelleted in an Eppendorf centrifuge for 2 min and suspended and washed at 4°C in the following solutions: (A) 50 mM Tris-HCl pH 7.5, 1% Triton X-100, 1 M NaCl, (B) 50 mM Tris-HCl pH 7.5, 0.1% SDS, 0.5 M LiCl, (C) 50 mM Tris-HCl pH 7.5. Before loading on a 10% SDS-polyacrylamide gel, the samples were boiled for 4 min in an SDS sample buffer and electrophoresed according to Laemmli (1970).

Oligonucleotide synthesis

A mixture of 8 tetradecamers, d(T-T-A/G-T-C-C-C-A-T/C-T-G-A/G-T-A), one of which is complementary to glucoamylase mRNA in the central region coding for Tyr-Gln-Trp-Asp-Lys (Svensson *et al.*, 1983b), and a pentadecamer d(GTAGTTCTCAATGGT) complementary to the mRNA in the NH_2-terminal part coding for Thr-Ile-Glu-Asn-Tyr were synthesized by the triester method on a 1% cross-linked polystyrene support (Ito *et al.*, 1982). The polymer was packed in a short column, and solvents and reagents were delivered semi-automatically by means of an h.p.l.c. pump and a control module. The oligonucleotides were purified by polyacrylamide gel electrophoresis and by h.p.l.c. on a LiChrosorb (Merck) RP18 column (Fritz *et al.*, 1978).

Oligodeoxynucleotide-primed cDNA synthesis

The tetradecamer mixture and the pentadecamer were labeled at the 5′ end with T4 polynucleotide kinase and used for priming in cDNA synthesis reactions with total *A. niger* mRNA as template as described (Boel *et al.*, 1983). The resulting cDNA products were analysed on polyacrylamide/7 M urea gels.

Construction and cloning of double-stranded cDNA

Oligo(dT)- and tetradecamer-primed cDNA libraries synthesized from total *A. niger* mRNA were constructed on pBR327 in a hsdR$^-$,M$^+$ derivative of *E. coli* MC1000 (Casadaban and Cohen, 1980) as described (Boel *et al.*, 1983).

Colony hybridizations

Bacterial colonies containing recombinant plasmids were transferred to Whatman 540 filter paper, lysed, and immobilized according to the procedure of Gergen *et al.* (1979). Hybridization with ^{32}P-labeled glucoamylase-specific single-stranded tetradecamer-extended cDNA was carried out at 68°C overnight in 900 mM NaCl, 90 mM Tris-HCl, pH 8.3, 0.1% SDS, and 1 mM EDTA with 0.2% each of bovine serum albumin, Ficoll and polyvinylpyrrolidone. The filters were washed in 1 x SSC [1 x SSC is 0.150 M NaCl and 0.015 M sodium citrate (pH 7)] at 50°C, dried and autoradiographed, using Agfa-Gevaert Curix RP2 X-ray film for 24 h with a Dupont Cronex Lightning Plus AH intensifier screen at −70°C. Hybridization with the ^{32}P-labeled (6.3 x 10^6 c.p.m./pmol, 2 pmol/9 cm filter) glucoamylase-specific tetradecamer-mixture was as above, except that hybridization was at 41°C for 2 h, and washing was at 35°C in 6 x SSC with four changes of 15 min each.

Plasmid DNA isolation and DNA sequence analysis

DNA was isolated according to the procedure of Birnboim and Doly (1979). The nucleotide sequence of 5′ end-labeled primer extended glucoamylase cDNA or 3′ end-labeled DNA restriction fragments from recombinant plasmids were determined by the chemical cleavage procedure of Maxam and Gilbert (1980).

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