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The secretion of glucagon by transformed yeast strains

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Saccharomyces cerevisiae strains were transformed with plasmids coding for modified mating factor α_1 leader sequences followed by glucagon. Glucagon-containing peptides which were secreted into the fermentation broth were isolated and their amino acid sequences determined. The yeast strain transformed with the sequence coding for the complete mating factor α_1 leader sequence preceding the glucagon gene (MT556) secreted glucagon plus glucagon extended at its N-terminal by parts of the leader sequence. The yeast strain transformed with the sequence coding for a truncated mating factor α_1 leader sequence before the glucagon gene (MT615) secreted glucagon. These observations suggest that *S. cerevisiae* is a suitable vehicle for the efficient expression of plasmids coding for polypeptides similar to glucagon (e.g. VIP, secretin, GIP).

Recombinant glucagon secretion; (Yeast)

1. INTRODUCTION

The pancreatic hormone glucagon is synthetized by the α-cells of the islets of Langerhans. In, for example, the rat the peptide is derived from a 180 residue precursor molecule, preproglucagon, consisting of a twenty amino acid residue leader peptide and a 160 residue proglucagon [1]. In the pancreas, peptides formed from proglucagon by cleavages at Lys-Arg sequences include proglucagon 1–30 or GRPP [2] and proglucagon 33–61 or glucagon. In the yeast, *Saccharomyces cerevisiae*, enzymes within the secretory compartments [3,4] process the primary yeast mating factor α_1 (MFα1) gene product (prepeptide-Lys-Arg-(Glu-Ala)$_2$-αF-Lys-Arg-(Glu-Ala)$_3$-αF-(Lys-Arg-Glu-Ala-Asp-Ala-Glu-Ala-αF)$_2$ [5]) into the mature α-factor tridecapeptide [6].

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Abbreviations: MeCN, acetonitrile; GIP, gastric inhibitory peptide; VIP, vasoactive intestinal peptide; GLI, glucagon-like immunoreactivity

A series of genes for dibasic insulin precursors which were fused to a modified MFα1 leader sequence have recently been expressed in a yeast system [7]. The availability of this system led us to examine the expression of a synthetic glucagon gene fused to the MFα1 leader sequence. Two plasmids were constructed which contained a synthetic gene for glucagon placed after either the complete MFα1 leader sequence [5] (pMT544) or the leader sequence from which the portion coding for the last four residues (Glu-Ala-Glu-Ala) had been deleted (pMT612). *S. cerevisiae* was transformed with these plasmids. The glucagon-related peptides secreted by the transformants were isolated and sequenced.

2. MATERIALS AND METHODS

2.1. *Gene synthesis*

Oligonucleotides were synthesized on an automatic DNA synthesizer (model 380A, Applied Biosystems, Foster City, CA) using phosphoramidite chemistry on a silica support [8] or by semiautomatic column synthesis using the phos-

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photriester approach with di- and trinucleotide blocks and a polystyrene support [9].

A synthetic gene for glucagon was constructed from the oligonucleotides I–V indicated in fig.1. A 66-mer was obtained by ligation of I and II in the presence of III used as a 'splint'. Similarly a 73-mer was obtained by ligation of III, IV, and V using II as a splint. The 66-mer and the 73-mer, the 3′-ends of which are complementary over 39 bases, were annealed and the 3′-ends were filled in using Klenow polymerase and deoxyribonucleotide triphosphates resulting in a 100 base pair double-stranded DNA molecule (fig.1). The *Kpn*I-*Hin*dIII fragment of the synthetic gene was used for the construction of the expression plasmid pMT544.

2.2. *Expression plasmids*

The plasmid pMT544 (fig.2) is a YEP13-derived *E. coli-S. cerevisiae* shuttle plasmid where an expression cassette has been inserted in the *Bam*HI site. The expression cassette consists of a triose phosphate isomerase promotor fragment fused to the sequence encoding the MFα1 leader peptide [7] and followed by a synthetic adaptor (AGCTCACTCTCAAGGTAC/CTTGAGAGTG) joining the *Hin*dIII at position 263 [5] of the MFα1 leader to the *Kpn*I of the synthetic glucagon 4–29 gene. The adaptor also encodes glucagon 1–3. Transcription is terminated by means of the MFα1 terminator sequences: the *Hin*dIII of the synthetic glucagon gene is joined to the *Hin*dIII at position 451 of the MFα1 sequence [5] and the expression cassette is ended at a *Bam*HI approx. 0.8 kb downstream. The sequences encoding amino acids Glu-Ala-Glu-Ala immediately preceding the first amino acid of glucagon were removed by site-directed deletion mutagenesis (see below) to give the plasmid pKFN6. The expression unit from pKFN6 was transferred as a *Bam*HI-*Sph*I (partial) 2.1 kb fragment to plasmid CPOT [7] to give the plasmid pMT612 (fig.2).

The plasmid pMT544 was transformed into a *S. cerevisiae* (α, leu2, pep4–3) selecting for leucine protophy to give the transformant strain MT556. The plasmid pMT612 was transformed into a triose phosphate isomerase mutant of *S. cerevisiae* (a/α, Δtpi/Δtpi, pep4–3/pep4–3) selecting for growth on glucose to give the transformant strain MT615.

2.3. *Site-directed deletion mutagenesis*

The plasmid pMT544 was linearized by cutting in the unique *Xba*I site in the glucagon gene and then treated with *Exo*III nuclease under conditions such that approx. 250 nucleotides were removed from each 3′-end of the linearized plasmid [10]. A kinased 26-mer mutagenic deletion primer d(CTTTGGATAAAAGACACTCTCAAGGT) was annealed to the mutation site. A double-stranded circular DNA was made by filling in with Klenow polymerase and ligation with T_4 ligase thereby destroying the original *Xba*I site in the glucagon gene. After transformation of *E. coli* (MT172), mutants were identified by colony hybridization with the 5′-^{32}P-labelled deletion mutagenesis primer.

The destroyed *Xba*I site in the glucagon gene was then reconstructed by ligation of a 4.4 kb *Bam*HI-*Xho*I and a 0.9 kb *Bam*HI-*Kpn*I fragment (containing the glucagon (4–29) gene) from pMT544 with a 6.6 kb *Xho*I-*Kpn*I fragment from a plasmid with the 12 base pair deletion (see fig.2).

The ligation mixture was used to transform *E. coli* (MT172) and plasmids isolated from the transformants were screened for the presence of a *Xba*I site. The deletion was confirmed in four independent plasmids by DNA sequencing [11]. One plasmid, pKFN6, was selected for further use. The yeast strains were grown on YPD medium [7].

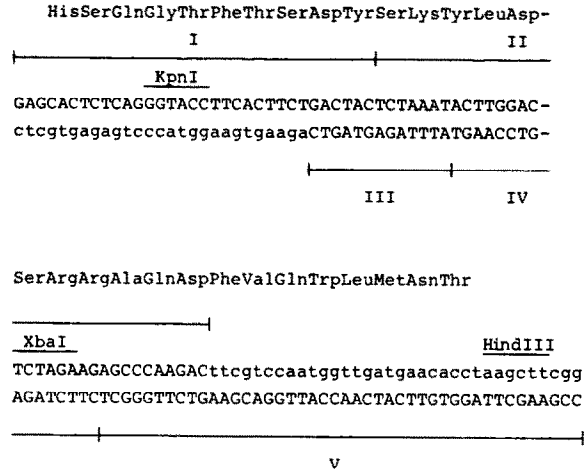

Fig.1. Synthetic gene coding for glucagon 1–29. Capital letters, chemical synthesis; small letters, enzymatic prolongation.

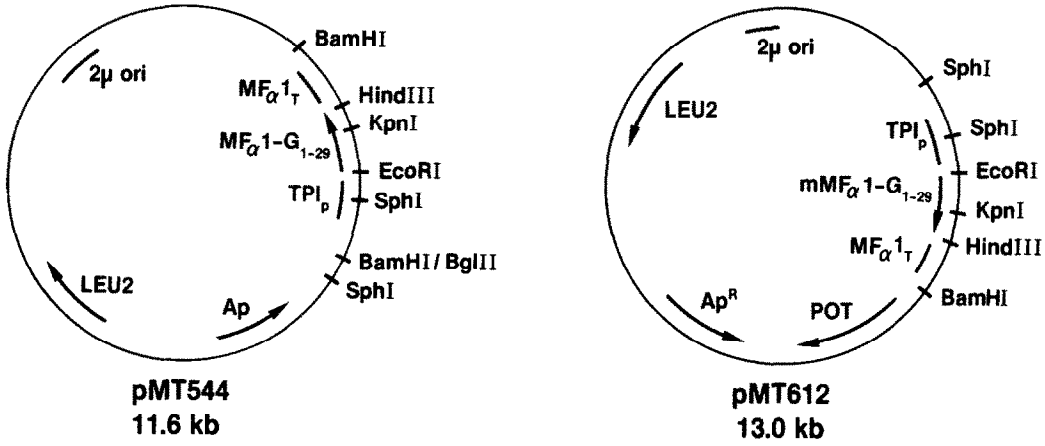

Fig.2. Yeast expression plasmids for glucagon.

2.4. *Analysis of products*

Glucagon-like immunoreactivities were assayed by radioimmunoassays using anti-glucagon sera K6248 (K6248 GLI) and K5563 (K5563 GLI) which recognize the N-terminal portion and C-terminal portion of glucagon, respectively [12]. Glucagon was identified and estimated by high pressure liquid chromatography (HPLC) by analyzing a sample with and without added glucagon. Peptides with significant GLI, or those which eluted on HPLC to the same position as glucagon, were isolated as described in section 3 and fully sequenced on a gas phase sequencer (model 470A, Applied Biosystems, Foster City, CA) as described [13].

3. RESULTS

3.1. *Expression of glucagon 1–29 in yeast strain MT556*

The supernatant from the fermentation of yeast strain MT556 contained 177 nM K6248 GLI. Ethanol (96%) was added to 800 ml of the supernatant to give a final concentration of 5% (v/v). The supernatant was concentrated on a Sep-Pak column (Waters). Two thirds of the Sep-Pak eluate, corresponding to 533 ml supernatant, was passed through an antiglucagon-Sepharose column (monoclonal antibody NOVO Glu 00102). The bound peptides were eluted at low pH and fractionated by HPLC on a 10 μm Waters μBondopak C-18 column (3.9 × 300 mm). The A and B buffers were 0.1% trifluoroacetic acid (TFA) in H_2O and 0.07% TFA in MeCN, respectively. The column was equilibrated with 25% B (flow: 1.5 ml/min) and the peptides were eluted with a linear gradient of MeCN (1%/min) and detected at 276 nm.

The peak containing K6248 GLI eluted in the same area as glucagon 1–29. This fraction was dried in vacuo, and the amino acid sequences of the constituent peptides determined. The expression products consisted of: Glu-Ala-Glu-Ala-Glucagon 1–29; Glu-Ala-Glucagon 1–29 and glucagon 1–29 in approximately equimolar amounts. The isolation of peptides with GLI secreted by MT556 is summarized in table 1.

3.2. *Expression of glucagon 1–29 in yeast strain MT615*

Analytical HPLC profiles of the supernatant

Table 1

Purification of GLI-peptides from yeast strain MT556 supernatant

Step	Volume (ml)	GLI (antibody K-6248) (nmol)	Yield (%)
Supernatant	800	142	100
Sep-Pak concentrate	2	111	78
Antiglucagon column	0.2	29	20
HPLC	2.0	11	8

from MT615 (upper panel) and the same sample after the addition of glucagon (lower panel) are shown in fig.3. A peptide peak (R_T 20.24 min) was increased by the addition of glucagon and it was assumed that the peak represented glucagon. In two successive batches of MT615 supernatant the glucagon was 250 and 960 nM, as estimated by HPLC. The GLI contents were 1156 and 3708 nM measured with antiserum K6248, and 557 and 2145 nM measured with antiserum K5563. Analytical HPLC of the MT615 supernatant separated the GLI into three fractions: the first eluted at a low concentration of MeCN and contained essentially only K4268 GLI; the second eluted just in front of glucagon and contained approximately equimolar amounts of K4268 and K5563 GLI's; the third contained equimolar amounts of K4268 and K5563 GLI's and co-eluted with glucagon.

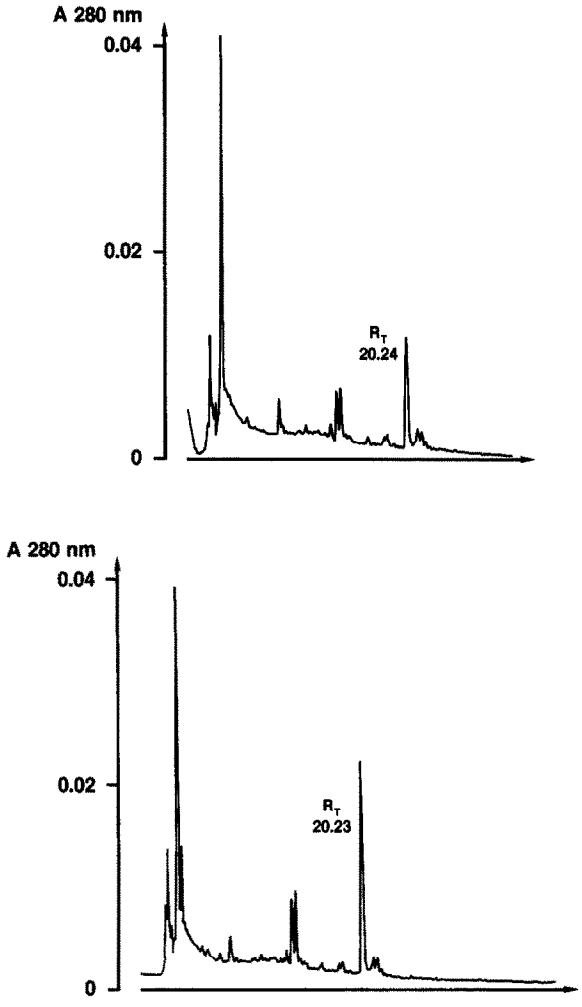

Fig.3. The identification of glucagon by HPLC in the supernatant from yeast transformant MT615. 25 μl of supernatant (upper panel) or 25 μl supernatant plus 3 μg glucagon (lower panel) were analyzed on a 4.6 × 250 mm column of Nucleosil 5 μm C-18 equilibrated at 1 ml/min with 25% (v/v) MeCN in 100 mM ammonium formate (pH 3.5). The column was eluted after 5 min with a gradient of MeCN in ammonium formate (25–40% during 35 min).

Glucagon was isolated from MT615 supernatant as follows. 2 g of dry SP Sephadex C-25 were added to 200 ml supernatant (pH adjusted to 4.3) and the suspension stirred for 20 min at room temperature. The SP Sephadex was recovered on a filter, washed with 50 ml of 25 mM ammonium formate (pH 3.5), and the bound peptides eluted with 0.5 M ammonium formate (pH 8.4). Glucagon was isolated from this eluate by reverse-phase HPLC (fig.4). The peptide was fully sequenced (average respective yield, 93.3%) and shown to be glucagon. The isolation is summarized in table 2.

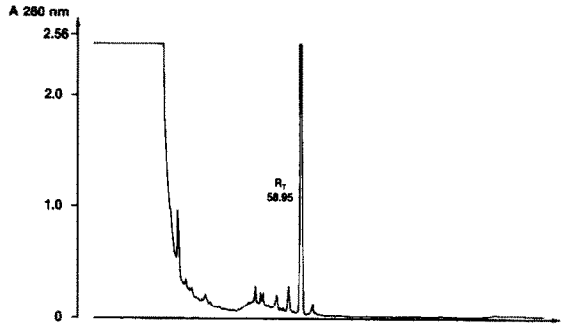

Fig.4. The isolation of glucagon from the SP Sephadex eluate prepared from the supernatant of yeast transformant MT615. 18 ml of eluate were pumped at 1 ml/min onto a 4.6 × 250 mm column of Nucleosil 5 μm C-18 previously equilibrated at 1 ml/min with 25% (v/v) MeCN in 100 mM ammonium formate (pH 3.5). The column was washed with 25% (v/v) MeCN in ammonium formate for a further 20 min. The peptides were then eluted with a gradient of MeCN in ammonium formate (25–30% during 5 min then isocratic for a further 40 min) starting at the left hand end of the UV trace. The effluent corresponding to the UV peak at R_T 58.95 was collected.

Table 2

Recovery of glucagon from yeast strain MT615 supernatant

Step	Volume (ml)	Glucagon[a] (nmol)	Yield (%)
Supernatant	200	192	100
Eluate from SP-Sephadex	18	117	61
HPLC	1	118	61

[a] As determined by HPLC

4. DISCUSSION

Expression and secretion of significant amounts of glucagon-containing peptides was obtained by transforming *S. cerevisiae* with plasmids coding for the sequences: (a) MFα1 leader-glucagon (pMT544); (b) MFα1 leader (minus the sequence coding for the C-terminal tetrapeptide)-glucagon (pMT612).

The pMT544 transformant MT556 secreted glucagon (33%) plus two peptides consisting of either Glu-Ala-Glu-Ala or Glu-Ala from the MFα1 leader sequence attached to the N-terminal residue of glucagon. In previous studies using the entire MFα1 leader sequence for the expression of insulin precursors [7] or of epidermal growth factor [14] much lower percentages of products correctly processed at the N-terminal were obtained. One reason for this discrepancy could be that the structure of glucagon is such that the Glu-Ala-Glu-Ala sequence was more accessible to processing enzymes than was the case in the expression of epidermal growth factor and insulin precursors.

The pMT612 transformant MT615 secreted glucagon as well as unidentified peptides which reacted only with K6248. These peptides could have been formed by limited cleavage of the glucagon sequence (perhaps at the Arg-Arg in glucagon 17–18) so that sequences containing only the epitope glucagon 11–15 were produced as well as intact glucagon 1–29. From previous studies it is known that the Arg-Arg and Lys-Arg sequences of proinsulin are cleaved rather rapidly, whereas other dibasic sequences such as Lys-Arg in the disulphide bridge linked insulin precursor B-chain-Lys-Arg-A-chain are not cleaved [7]. These results, together with the present findings, indicate that dibasic sequences are extensively cleaved by the yeast enzyme system only when flanked by hydrophilic spacer peptides. The reason for the yeast enzyme system not processing at the Arg-17–Arg-18 sequence of glucagon might be that this sequence is not flanked by hydrophilic spacer peptide sequences.

It is concluded that *S. cerevisiae* transformed with a plasmid (pMT612) containing a synthetic glucagon gene produces and secretes significant amounts of glucagon. Plasmids similar to pMT612 could prove a suitable means for expressing other peptides structurally related to glucagon (e.g. secretin, VIP and GIP) in yeast.

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