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The sexual inducer of *Volvox carteri*

Its large-scale production and secretion by *Saccharomyces cerevisiae*

Elke Haas and Manfred Sumper

Lehrstuhl Biochemie I, Universität Regensburg, 8400 Regensburg, Germany

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The DNA sequence coding for the sexual inducer glycoprotein of *Volvox carteri* and its N-terminal signal peptide was placed under the control of the repressible acid phosphatase promoter of the yeast *Saccharomyces cerevisiae* in a yeast–*E. coli* shuttle vector. Yeast transformed by this construct synthesized and secreted into the culture medium biologically active inducer in amounts two to three orders of magnitude higher than observed in the *Volvox* system.

Sexual inducer (*Volvox carteri*); Heterologous expression (*Saccharomyces cerevisiae*); Secretion

1. INTRODUCTION

The sexual pheromone (sexual inducer) of the multicellular green flagellate *Volvox carteri* is a glycoprotein synthesized and released by sperm cells [1,2]. It is one of the most potent biological effector molecules known exhibiting full effectiveness at 6×10^{-17} M. A single sexual male produces enough inducer to convert many millions of asexually growing males and females to the sexual pathway.

Only recently the inducer was isolated in amounts sufficient for obtaining amino acid sequence data [3] and both the gene and the cDNA of the inducer were cloned [3,4]. However, isolation of the glycoprotein from *Volvox* cultures is still a difficult task and produces amounts hardly exceeding the range of 10–100 μg. This fact limits the possibility for structural studies of this fascinating molecule.

In this paper we report the heterologous expression of the inducer cDNA in yeast, producing a glycosylated polypeptide. The vector construct allows the secretion of biologically active inducer into the growth medium.

2. MATERIALS AND METHODS

2.1. *Strains*

The yeast strain AH22 (a, leu_{2-3}, leu_{2-112}, his_{4-519}, can1) was used for all transformation experiments [5]. All plasmid constructs were propagated in *E. coli* JM 109 [6].

2.2. *Plasmids*

cDNA of the sexual inducer was subcloned in pUC 8 and 18 [4].

Correspondence address: M. Sumper, Lehrstuhl Biochemie I, Universität Regensburg, D-8400 Regensburg, Germany.

Plasmid pAM82 [7] was a generous gift of A. Miyanohara (Osaka University).

2.3. *Exonuclease digestion*

DNA (5 μg) was dissolved in a volume of 25 μl, containing 20 mM Tris-HCl (pH 8.0), 12 mM $CaCl_2$, 12 mM $MgCl_2$, 600 mM NaCl and 1 mM EDTA. After the addition of Bal 31 exonuclease (0.05 U), aliquots were removed after appropriate times (100–200 s). Under these conditions, 30–100 bp were removed. The reaction was terminated by the addition of EGTA and treatment with phenol.

Protruding termini of DNA were removed with mung-bean nuclease: DNA (0.1 μg/μl) was dissolved in 50 mM sodium acetate (pH 5.0), 30 mM NaCl and 1 mM $ZnSO_4$ and 0.5 U/μg DNA mung-bean nuclease were added. Incubation was at 30°C for 30 min.

2.4. *Transformation of yeast cells*

Preparation of spheroplasts was performed as described by Oertel and Goulian [8], with some modifications: yeast was grown in YEPD (1% yeast extract, 2% peptone, 2% glucose), spheroplasts were cultivated in YEPDS (1 M sorbitol in addition) and 10 μg/ml zymolyase 100T (ICN) were used instead of glusulase. Transformation of the spheroplasts was performed as described by Hinnen et al. [9]. Leu^+ transformants were selected on 2% agar plates containing minimal medium (0.67% yeast nitrogen base, 2% glucose, 1 M sorbitol, histidine, uracil and adenine (50 μg/ml each)).

2.5. *Yeast cell growth and induction*

Recombinant yeast cells were grown at 30°C in YNBG (Difco) supplemented with histidine, adenine and uracil (50 μg/ml each). Cells were collected by centrifugation at a density of approximately 6×10^6 cells per ml, and resuspended in the same volume of phosphate-free minimal medium (per liter: 5 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4$, 0.1 g NaCl, 0.1 g $CaCl_2$, 0.6 g KOH, 0.3 g NaOH, 1 g succinic acid, 20 g glucose, 50 mg L-histidine, 50 mg uracil, 50 mg adenine, trace elements and vitamins according to [9]). Growth was continued for two more days.

2.6. *Purification of the pheromone*

Yeast cells were removed from the culture fluid by centrifugation. The growth medium was diluted with 9 volumes of water. Subsequently, the inducer was adsorbed to SP-sephadex and purified as previously described [3].

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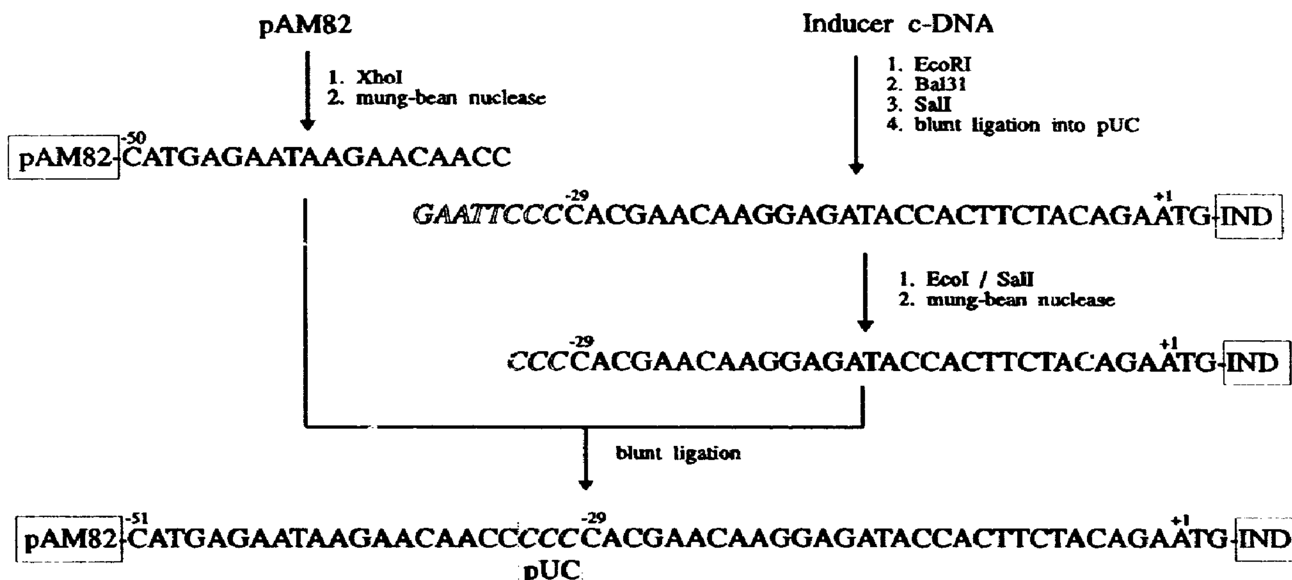

Fig. 1. Insertion of sexual inducer cDNA into expression vector pAM82. Plasmid pUC8 containing an inducer cDNA insert (at the *Sma*I-site) was opened by *Eco*RI and digested with Bal31 exonuclease. The trimmed cDNA insert was removed by digestion with *Sal*I and inserted into the *Sma*I-site of pUC8 by blunt-end ligation after treatment with mung-bean nuclease. The deletion end points were determined by sequence analysis. A plasmid containing a cDNA insert trimmed to position −29 which respect to the ATG initiation codon of the inducer signal peptide was selected and isolated by digestion with *Eco*RI and *Sal*I. The purified cDNA was treated with mung-bean exonuclease and inserted into pAM82 by blunt-end ligation. Only a portion of the sequence upstream the *Xho*I-cloning site of pAM82 is shown. Position −50 indicates the distance from the original ATG initiation codon of the phosphatase gene, used to construct expression vector pAM82.

3. RESULTS AND DISCUSSION

The yeast–*E. coli* shuttle vector pAM82 contains a cloning site placed under the control of the repressible promoter of acid phosphatase from *Saccharomyces cerevisiae* [7]. The promoter fragment has its 3′-terminal end at position −33 with respect to the initiation codon ATG of the original acid phosphatase gene. At this position, a *Xho*I-linker had been joined as a cloning site. In order to match the relative distances of the original phosphatase gene, the 5′ non-coding region of the sexual inducer cDNA was trimmed by digestion with Bal31 exonuclease to position −29 with respect to the initiation codon ATG of the signal peptide. The cloning strategy outlined in Fig. 1 caused the insertion of three additional base pairs (CCC, derived from the *Sma*I-site of plasmid pUC8) and consequently restored the original positioning for the initiation codon ATG.

The recombinant plasmid propagated in *E. coli* JM 109 was transformed into a yeast recipient strain AH22 by standard transformation techniques, selecting for Leu^+ colonies. The Leu^+ cells were grown in liquid medium containing phosphate to a density of approximately 6×10^6 cells per ml and collected by centrifugation. Cells were resuspended in phosphate-free medium and incubated for a further two days. At appropriate times, the culture medium was tested for the presence of *Volvox* pheromone using the standard biological assay [1]. Maximum inducing activity was found after two days. Dilutions of the cell-free culture medium as high as 10^8–10^9 exerted 100% sexual induction in the standard biological assay. This corresponds to a pheromone concentration two to three orders of magnitude higher than observed in pheromone producing cultures of *Volvox* (male strain 69-1b). Remarkably, the signal peptide of the *Volvox* gene is correctly recognized in yeast and causes secretion of the pheromone into the culture medium. Therefore a purification protocol worked out previously [3] using *Volvox* culture fluids as the starting material could be applied with only minor modifications (section 2).

On SDS-polyacrylamide gels, the sexual pheromone isolated from *Volvox* culture fluids exhibits a pattern of 3 or more bands with apparent molecular masses around 31 kDa (Fig. 2, lane 1). Since the pheromone is a glycoprotein, different degrees of glycosylation cause this apparent heterogeneity. After chemical deglycosylation with anhydrous HF, the inducer consists of a single polypeptide chain with an M_r of 25 kDa (Fig. 2, lane 3). The pheromone produced in yeast migrates

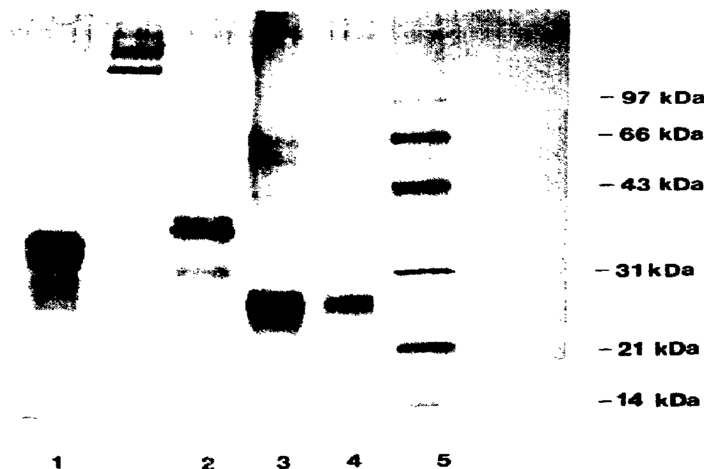

Fig. 2. Comparison by SDS-polyacrylamide gel electrophoresis of sexual inducer produced in *Volvox* and yeast. Lane 1, authentic *Volvox* inducer; lane 2, inducer synthesized in yeast; lane 3 and 4, as 1 and 2, after chemical deglycosylation with anhydrous HF; lane 5, mol.wt. standard.

significantly slower on SDS-polyacrylamide gels (Fig. 2, lane 2) corresponding to an M_r of 36 kDa. However, after deglycosylation, the polypeptides produced in *Volvox* and yeast are indistinguishable with respect to M_r (Fig. 2, lanes 3 and 4). It is well documented that the N-glycosylation system in yeast adds 50–150 mannose residues to the core saccharide structure [10]. Most probably this fact explains the difference in the M_r, since *Volvox* lacks this elongation system [11].

The efficient expression of biologically active pheromone in yeast allows the production of this molecule in amounts sufficient for most structural studies. Furthermore, all the techniques of site-directed mutagenesis are now applicable for functional studies.

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