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The sexual inducer of *Volvox carteri*: purification, chemical characterization and identification of its gene

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The sexual inducer of *Volvox carteri f. nagariensis* is a glycoprotein and one of the most potent biological effector molecules known. It is synthesized by sperm cells and converts asexually growing males and females to the sexual pathway. Until now, large-scale production of the inducer was made impossible by an inherent biological 'switch' mechanism, the spontaneous self-induction of asexually growing males. Here we describe a method overcoming this problem for the first time. Large-scale production and purification allowed a detailed chemical characterization of the inducer with respect to partial amino acid sequences and sugar composition. Chemically synthesized oligodeoxynucleotides corresponding to derived amino acid sequences were used to screen a genomic gene bank of *V. carteri* HK 10. A positive clone (Ind-28) was shown to encode the inducer gene by subcloning and sequencing.

Key words: Volvox carteri/sexual inducer/glycoprotein/'switch' mechanism

Introduction

The sexual inducer of *Volvox carteri* is a glycoprotein synthesized and released by sexual males at about the time they release sperm packets (Starr, 1970; Starr and Jaenicke, 1974). The sperm cells were identified as being responsible for inducer synthesis (Gilles *et al.*, 1981). The *Volvox* inducer is one of the most potent biological effector molecules known: it exhibits full effectiveness in converting asexually growing males and females to the sexual pathway at $\sim 10^{-17}$ M (Starr, 1970; Gilles *et al.*, 1984).

For large-scale production of inducer it is necessary to grow the male strain asexually to a high population density and then continue with the synchronous induction of the sexual pathway. During the following generation all colonies would develop sperm packets which secrete the inducer. Unfortunately, up to now this experimental approach was hampered by an inherent biological 'switch' mechanism (Starr, 1972; Callahan and Huskey, 1980; Weisshaar *et al.*, 1984) which cause 'spontaneous' sexual males to appear in an otherwise asexually growing culture at a frequency of up to 2×10^{-4}. A single spontaneous sexual male produces sufficient inducer to trigger sexual development of the remaining *Volvox* suspension. This mechanism therefore prevents asexual growth to high population density, and this has previously allowed cultivation of males only in small volumes and at low population densities. This in turn was a main hindrance of the detailed biochemical characterization of the inducer.

In this paper we describe a cultivation method which overcomes these difficulties, allowing large-scale production of sexual inducer. Furthermore, we describe a purification procedure yielding homogeneous inducer and we report on the isolation of the inducer gene with the help of oligodeoxynucleotides which were derived from peptide sequences.

Results

Large-scale production of inducer

With a simple experimental trick, we were able to prevent spontaneous sexual induction thus allowing large-scale production of the inducer. Since there is experimental evidence for an inducer-harvesting and/or transporting system within the extracellular matrix (Gilles *et al.*, 1984; Wenzl and Sumper, 1986), we tried to destroy this system, and thereby to inhibit sexual induction, by mild proteolytic treatment of *Volvox* spheroids. In the experiment shown in Figure 1 increasing amounts of pronase were added to a *Volvox* population together with a sexual inducer 8 h before the onset of embryogenesis. After the end of embryogenesis the percentage of sexually induced daughter spheroids was determined by microscopic inspection. Pronase concentrations of 0.5-1.0 μg/ml were able to completely inhibit sexual induction and did not disturb growth or inactivate the inducer. Low protease levels in the culture medium would therefore not reduce the yield of inducer.

Remarkably, pronase-mediated inhibition of sexual development is only effective in the embryogenesis that follows the application of the protease. The resulting asexual daughters fully regain the ability to be sexually induced by the glycoprotein. However, repeated pronase treatment can inhibit sexual induction again. The highly negatively charged extracellular matrix probably binds the protease immediately after its application, restricting its action to the extracellular matrix of the mother spheroid. Repeated pronase treatment allows asexual growth of males to high population densities even in large culture vessels.

The experimental protocol for large-scale growth of males is summarized in Table I. Using a 20-l glass fermenter, the pro-

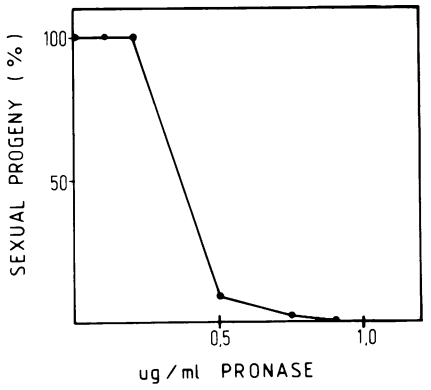

Fig. 1. Inhibition of sexual induction by pronase. Inducer (10 units/ml) was added to asexually growing *Volvox* suspensions 8 h before the onset of embryogenesis. At the same time, increasing amounts of pronase were added to the cultures. After the end of embryogenesis the percentage of sexual progeny was determined by microscopic observation.

Table I. Large-scale production of sperm-cell-containing spheroids

	Total number of spheroids	Culture vessel	Remarks
Inoculum	4×10^2	2 Fernbach flasks	Spheroids taken at the time of release
1st Generation	4×10^3	2 Fernbach flasks	Addition of 0.9 μg/ml pronase
2nd Generation	4×10^4	2 Fernbach flasks	Addition of 0.9 μg/ml pronase
3rd Generation	4×10^5	Transfer into 15-l fermenter	Addition of sexual inducer
4th Generation	4×10^6	as above	
Spermatogenesis	4×10^6	as above	

cedure results in the production of quantitatively induced male spheroids at a population density of ~250 000 colonies/l. It is essential for the success of this procedure that the *Volvox* culture develops synchronously, otherwise, the spheroids fail to be quantitatively induced in the final generation. The *Volvox*-free culture fluid serves as starting material for inducer purification.

Purification of the inducer

Starr and Jaenicke (1974) reported the first partial purification procedure for the inducer, and additional purification steps (Gilles, 1983; Wenzl *et al.*, 1984) were later shown to increase the specific activity of the inducer, indicating that the original procedure resulted in preparations containing <5% pure inducer. The following procedure, based mainly on h.p.l.c. purification steps, yields an inducer preparation, which appears homogeneous both in SDS–polyacrylamide gels and by analytical h.p.l.c.–chromatography (reversed phase C18).

The culture fluid was first passed over a column of QAE–Sephadex, which removes nearly 70% of all protein material. Chromatography on SP–Sephadex at pH 5 resulted in a further 10-fold purification of the inducer. The active fractions from the SP–Sephadex column were immediately concentrated and adjusted to 6 M urea, which efficiently prevents irreversible aggregation of the inducer, a problem which becomes increasingly serious with purer preparations (Gilles, 1983), without inactivating its biological activity. H.p.l.c.- or f.p.l.c.–ion exchange chromatography on Mono S columns increases the specific activity ~10-fold. Purification to homogeneity is achieved by h.p.l.c. on reversed phase RP-300 in 0.1% trifluoroacetic acid applying a linear gradient of acetonitrile. Inducer activity elutes at 43% acetonitrile in a single sharp peak (Figure 2). Table II gives a quantitative description of this purification procedure. The overall yield is remarkably high, >50% of the original inducer activity is usually recovered.

Characterization of the inducer

An additional h.p.l.c. on hydroxyapatite did not increase the specific activity of the inducer indicating its homogeneity. However, analysis of the inducer on SDS–polyacrylamide gels resulted in a pattern of 2–3 bands with apparent molecular masses around 31 kd (Figure 3). Since the inducer is a glycoprotein, different degrees of glycosylation could cause this apparent heterogeneity. Indeed, after deglycosylation with anhydrous HF, the inducer consists of a single polypeptide chain with an apparent molecular mass of 25 kd (Figure 3B, lane 2).

The composition of neutral sugars of the inducer was determined after acid hydrolysis by capillary gas chromatography of their alditol acetates. The amino sugar content was evaluated using an amino acid analyser. Table III gives the mean values obtained from independent preparations.

For the elucidation of at least parts of the primary structure of the polypeptide it was necessary to first deglycosylate the inducer, because the intact glycoprotein is almost resistant to proteolysis. Incubation of the inducer in the presence of pronase (50 μg/ml) for 3 h does not significantly affect its biological activity. The deglycosylated polypeptide, however, is readily digested by trypsin, chymotrypsin or *S. aureus* protease. After each proteolytic digestion the resulting peptide mixture was separated by reversed phase C18 h.p.l.c. The elution patterns obtained for the trypsin and the chymotrypsin digests are shown in Figure 4. Well-separated peaks were directly submitted to protein sequence analysis and a number of different peptides were found to be pure: all sequence information derived is summarized in Table IV. An additional peptide which could be sequenced was obtained from a digest with *S. aureus* protease: Leu-Phe-Lys-Pro-Thr-Thr-Ile-Asn-Glu. This is the N-terminal extension of the peptide no. 12 (Table IV).

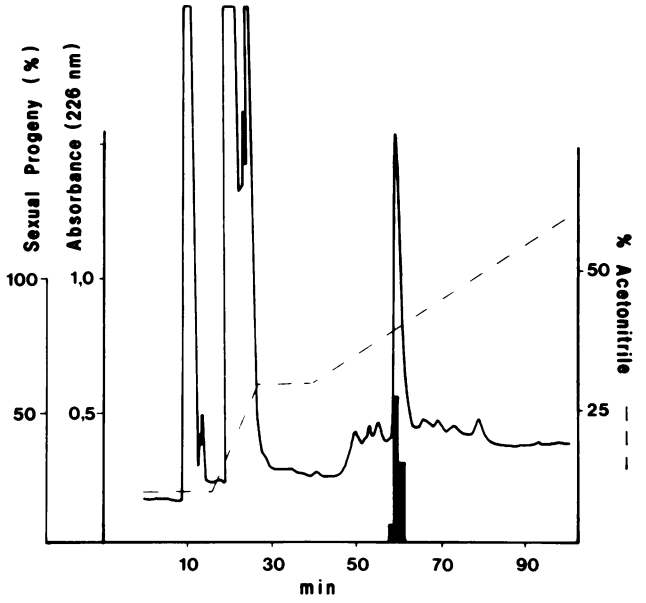

Fig. 2. Purification of the inducer by h.p.l.c. on a reversed-phase RP-300 column. Elution was performed with a gradient of acetonitrile in 0.1% TFA. All fractions were diluted 10^{12}-fold and assayed for biological activity (black bars) as described in Materials and methods.

Table II. Summary of purification of sexual inducer

Step	Volume (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg)
Culture medium	30 000	231	15.0×10^{11}	6.5×10^9
QAE–Sephadex	30 000	77	12.9×10^{11}	1.7×10^{10}
SP–Sephadex	400	7.3	11.6×10^{11}	1.6×10^{11}
Mono S	6	0.97	9.36×10^{11}	1.0×10^{12}
RP-300	3	0.1	9.0×10^{11}	9.0×10^{12}

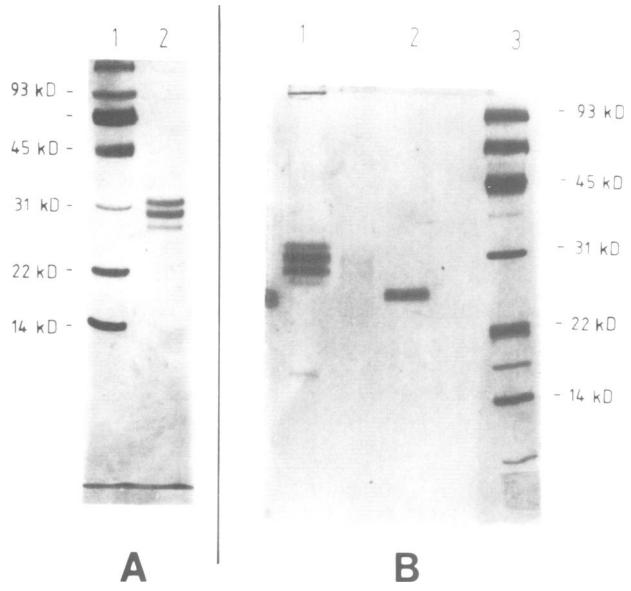

Fig. 3. SDS–polyacrylamide gel electrophoresis of purified inducer in its native **(A)** or deglycosylated state **(B)**. **(A) Lane 1,** mol. wt standard; **lane 2,** purified inducer; **(B), Lane 1,** purified inducer; **lane 2,** purified inducer after deglycosylation with anhydrous HF; **lane 3,** mol. wt standard. Proteins were visualized by the silver staining technique.

Table III. Sugar composition of sexual inducer

Sugar	Molar ratio
Mannose	6
Galactose	4
Arabinose	2
Xylose	4
Glucosamine	1

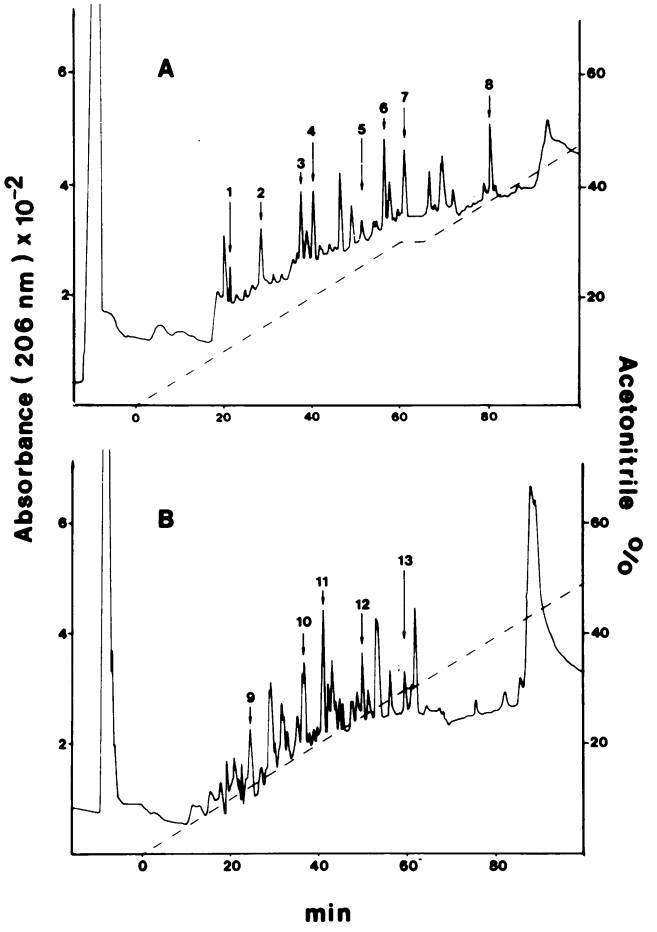

Fig. 4. Separation of tryptic **(A)** and chymotryptic **(B)** peptides from inducer preparations by h.p.l.c. Chromatography was performed on reversed-phase C18 columns using an acetonitrile gradient in 0.1% TFA. Amino acid sequence data could be obtained from the peptides marked with an arrow. Numbers refer to the peptides listed in Table IV.

Colony hybridization

From the protein sequence data the partial sequence Phe-Lys-Pro-Thr-Thr-Ile-Asn-Glu-Phe was selected for the synthesis of an oligodeoxynucleotide probe. Decisions regarding nucleotide assignment at redundant positions were guided by the codon usage data obtained from *Volvox* actin and tubulin genes (Mages *et al.*, 1987): the codon for phenylalanine is UUC with a probability >0.93, AAG is the only codon used for lysine. AAC (probability >0.91) and GAG (probability >0.95) were selected as codons for asparagine and glutamate, respectively. Isoleucine is only encoded by AUU or AUC. With these data the peptide was translated into the following oligodeoxynucleotide sequence:

5′ TTC AAG CCN ACN ACN AT$^{T}_{C}$ AAC GAG TTC 3′

Taking advantage of GT base-pairing, the candidate oligodeoxynucleotides were narrowed down to the following mixture, complementary to the above sequence:

3′ AAG TTC GG$^{G}_{T}$ TG$^{G}_{T}$ TG$^{G}_{T}$ TAG TTG CTC AAG 5′

The specificity of this 27-mer oligonucleotide mixture was tested with DNA from *V. carteri*. After digestion of the DNA with restriction endonuclease *SalI*, separation of the fragments on agarose and Southern transfer to nitrocellulose, the DNA fragments were probed with the ^{32}P-labelled oligodeoxynucleotide mixture. The probe specifically hybridized to an 8-kb fragment and was therefore used to screen a genomic *Volvox* gene bank in the vector EMBL 3 (kindly provided by Professor Schmitt, Regensburg). Fifty-four thousand recombinant phage clones (insert length ~17 kb) were screened. This number of phages assures representation of each *Volvox* gene at least once with a probability of 0.99. Two λ phage clones gave a strong hybridization signal. Purified DNA from clone Ind-28 was cleaved with *SalI*. A 2.7-kb fragment from the *Volvox* insert DNA strongly hybridized with the oligodeoxynucleotide mixture. This fragment was further subcloned into the pUC 18 vector and partially sequenced. Figure 5 shows the nucleotide sequence of this *SalI* fragment around the oligodeoxynucleotide sequence used for screening.

The nucleotide sequence immediately upstream of the probe codes for amino acid sequences which were found in the peptides derived from the sexual inducer polypeptide. This allows the firm conclusion that the cloned *Volvox* DNA fragment is part of the inducer gene. Remarkably, another series of the sequenced peptides is detected still further upstream. However, both of these coding regions are interrupted by a non-coding region, as indicated by the presence of stop codons within the reading frame. This indicates the existence of one or more intron sequences in the inducer gene. Potential exon–intron–exon boundaries can be detected in the nucleotide sequence (Figure 5). Assuming the correctness of this assignment, the nucleotide sequence shown encodes 125 amino acids of the sexual inducer.

Within a stretch of only 60 amino acid positions, a total of

Table IV. Sequences and N-terminal sequences of peptides obtained from sexual inducer by enzymatic digestions

Method	No.	Sequence
Trypsin	1	Thr Gln Ala X Gly Thr Arg
Trypsin	2	Leu Phe Pro Lys
Trypsin	3	Ser Ala Ala Thr Gln Leu Thr Ser Arg
Trypsin	4	Val Ser Val Tyr Ala Gln Ala Ala Gln Arg
Trypsin	5	Ala Gly Ala Pro . . .
Trypsin	6	Glu Leu Thr Pro Phe . . .
Trypsin	7	Phe Gln Ser Phe Leu Ser Pro . . .
Trypsin	8	Leu Ser Ala Phe Gly Val Leu Leu Ala Gly Ala Pro Val Ser X Met Thr Tyr Leu Thr Pro
Chymotrypsin	9	Thr Tyr
Chymotrypsin	10	Ser Ala Ala Thr Gln Leu
Chymotrypsin	11	Ser Lys Val Ser Val Tyr
Chymotrypsin	12	Lys Pro Thr Thr Ile Asn Glu Phe
Chymotrypsin	13	Val Ala Ala

five potential N-glycosylation sites [sequon Asn−X−Ser(Thr)] are found (boxed sequences in Figure 5). The peptide sequence data from peptides 1 and 8 (Table IV) prove glycosylation at the corresponding sequon sites. This high degree of glycosylation would explain the unusual chemical stability of the inducer.

Discussion

Sexual self-induction of asexually growing male spheroids was the main hindrance for large-scale production and detailed chemical analysis of the sexual inducer. The introduction of a controlled pronase treatment of *Volvox* spheroids at each stage preceding a new round of embryogenesis eliminated the problem. Thus no limitations remain for large-scale growth of male spheroids, which in turn produce sufficient sexual inducer to enable biochemical characterizations to be undertaken.

The inducer gene is silent in both females and asexual males. Expression of the inducer gene can be affected by either of three different modes: first, in asexual males, the gene is switched on by a spontaneous event taking place with a probability of 2×10^{-4} or, secondly, the gene is specifically turned on only in male strains by the presence of the gene product, the sexual inducer (Starr, 1972; Callahan and Huskey, 1980; Weisshaar *et al.*, 1984). In both these cases inducer synthesis is localized within the sperm cell. Recently, a third mode of activation was discovered: after heat shock conditions, somatic cells of both the female and the male strain are triggered to synthesize and to release the sexual inducer which then redirects development of the reproductive cells to egg-bearing or sperm-cell-bearing spheroids, respectively (Kirk and Kirk, 1986). The molecular mechanisms activating the inducer gene in a temporally and spatially controlled manner are unknown, but gene rearrangement may be involved, as is the case in the mating type switch in yeast (Kushner *et al.*, 1979; Nasmyth, 1983).The cloned inducer gene used as a probe in Southern blots of genomic DNA from asexual and sexual males will allow us to prove or disprove this hypothesis.

The inducer gene was cloned using a *Volvox* gene bank constructed from DNA of asexually grown females, containing a silent inducer gene. Sequencing of parts of this gene clearly shows the existence of introns. Therefore, the complete evaluation of the primary structure of the inducer protein requires additional information obtained either from mRNA sequencing or from a sperm-cell-derived cDNA bank. Both experimental approaches are currently in progress.

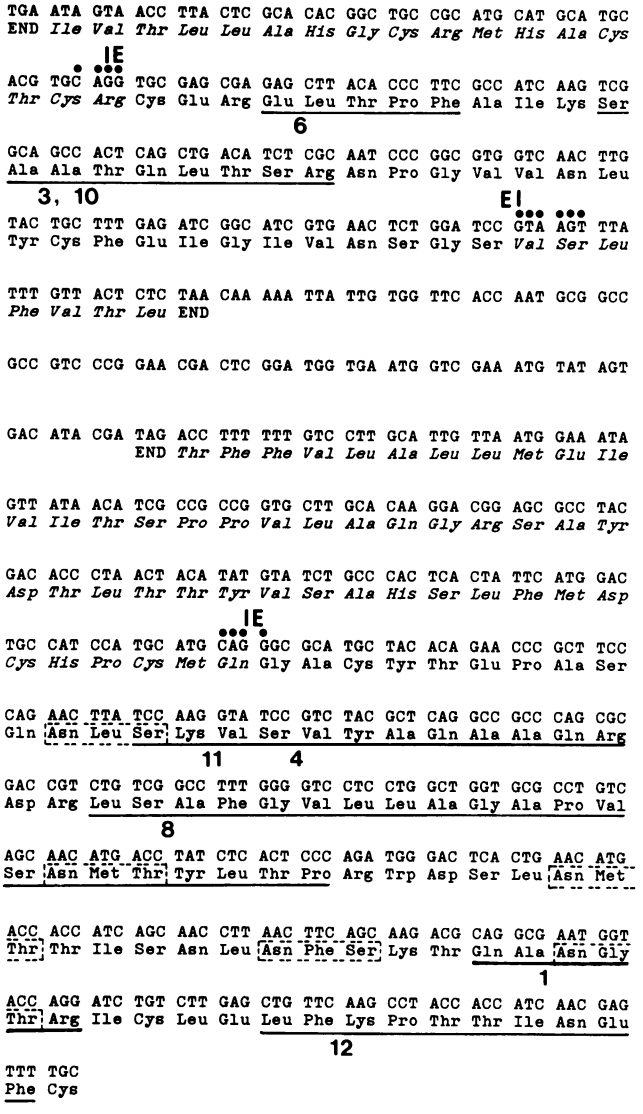

Fig. 5. Nucleotide sequence of clone Ind-28 DNA at the position of the oligonucleotide sequence used for screening the genomic library of *Volvox*. Amino acid sequences confirmed by chemical sequencing of peptides are underlined, numbers refer to the peptides listed in Table IV. Potential *N*-glycosylation sites are boxed. IE: Potential intron−exon boundary; EI: potential exon−intron boundary. Remarkably, each of these boundaries is 3′ adjacent to a palindromic sequence.

Materials and methods

Culture conditions

Volvox carteri f. nagariensis, strains HK 10 (female) and 69-lb (male) were from the Culture Collection of Algae at the University of Texas at Austin (R.C.Starr). Synchronous cultures were grown in *Volvox* medium (Provasoli and Pintner, 1959) at 28°C in an 8-h dark/16-h light (10 000 lux) cycle (Starr and Jaenicke, 1974).

Assay of inducer

The assay of inducer was carried out as described by Starr (1970). One unit of inducer is defined as that amount which is required to produce 50% sexual progeny in 1 ml *Volvox* suspension.

Large-scale growth of strain 69-lb

A Fernbach flask containing 800 ml *Volvox* medium was inoculated with 200 spheroids of the male strain 69-lb. Two flasks are required as inoculum for the 20-l glass fermenter. Immediately after the release of the daughter spheroids from the first and second generation, 0.9 μg/ml pronase (stock solution 100 mg/ml, preincubated at 56°C for 15 min) was added to the culture fluid. The spheroids resulting from the third generation (~250 000/l) were transferred to a 20-l glass fermenter (Bioengeneering, type L 1523) containing 15 l *Volvox* medium. 10^6 units of sexual inducer were added and cultivation was continued under permanent illumination at 13 000 lux. The culture was aerated with 2% CO_2. The final generation produces ~4 × 10^6 sperm-cell-bearing spheroids and at the time of disintegration of sperm cell packets the culture fluid was collected by filtration over glass wool.

Purification of sexual inducer

Fifteen litres culture fluid were passed over a QAE−Sephadex column (600 ml bed volume) equilibrated in *Volvox* medium. Inducer fails to adsorb to QAE−Sephadex and appears in the flow-through.

This flow-through was adjusted to pH 5 with acetic acid. Under gentle stirring 150 ml of SP−Sephadex was added and stirring was continued for 12 h at 4°C. After sedimentation of the SP−Sephadex, the supernatant was removed and discarded, and the SP−Sephadex was collected in a glass column and washed with 5 mM sodium acetate pH 5. Elution of inducer was achieved with 100 mM sodium acetate pH 5.0. After addition of 3.6 g urea, the eluate (~400 ml) was immediately concentrated to 40 ml in a rotary evaporator. The concentrated solution was extensively dialysed against 6 M urea and inducer was further purified using a Mono S cation-exchange column (Pharmacia) and h.p.l.c. system. The inducer solution was loaded using a 50 ml Superloop (Pharmacia). Elution was carried out using a linear 0−50 mM gradient of NaCl in 5 mM sodium acetate and 2 M urea, applied over a 60-min period with a flow rate of 1 ml/min. Active fractions were adjusted to 0.1% trifluoroacetic acid and 10% acetonitrile and directly applied to a reversed phase C18 RP-300 column (Kontron). A linear 10−30% gradient of acetonitrile in 0.1% trifluoroacetic acid was applied over 10 min, then the acetonitrile concentration was raised to 60% over a period of 60 min at a flow rate of 1 ml/min. Inducer eluted at 43% acetonitrile and the active fractions were immediately neutralized with pyridine. Salt-free inducer solutions were obtained by gel permeation chromatography on Sephadex G 25 fine.

Deglycosylation of inducer

Deglycosylation was achieved with condensed HF according to Mort and Lamport (1977), with the modification described by Wieland *et al.* (1983).

Sugar analysis

Inducer was hydrolysed in 40% trifluoroacetic acid at 100°C for 4 h. Neutral sugars were analyzed by g.l.c. as their alditol acetates (Laine *et al.*, 1972) on a Durabond 1701 capillary column (30 m, ICT Laboratories, Frankfurt). Amino sugars were analysed using an automated amino acid analyser (LC 5000, Biotronic, FRG) after hydrolysis of the samples at 100°C for various times.

Proteolytic digests

Deglycosylated inducer (30−60 μg protein) was digested with 1 μg trypsin (TPCK-treated) in 0.1 M *N*-ethyl-morphlinoacetate pH 7.5 containing 0.2 mM $CaCl_2$. Digestion was performed for 3 h at 37°C and after the addition of a further 1 μg trypsin incubation was continued overnight. Digestion with chymotrypsin (TLCK-treated) was performed as described for trypsin.

Peptide separation

Peptides were chromatographed on a LiChrosorb reversed-phase C18 column (Merck). A linear 0−60% gradient of acetonitrile in 0.1% trifluoroacetic acid was applied over a period of 120 min at a flow rate of 1 ml/min.

Peptide sequencing

Sequence analysis of peptides was performed on an automated gas-phase amino acid sequencer (Applied Biosystems) and the PTH derivatives were detected by reversed-phase h.p.l.c. as described by Lottspeich (1985).

Screening of the λ-EMBL 3 genomic library

Hybridization of recombinant plaques was done essentially as described by Maniatis *et al.*, 1982, but using a hybridization incubator (Bachhofer, Reutlingen). Oligonucleotides used as hybridization probes were labelled with [γ-^{32}P]ATP and polynucleotide kinase to a specific radioactivity of 5 × 10^6 c.p.m./pmol.

DNA sequencing

DNA sequencing was performed by dideoxynucleotide chain termination (Sanger *et al.*, 1977; Hattori and Sakaki, 1986) using ^{35}S-labelled α-thio-dATP and synthetic oligodeoxynucleotides as primers. Subclones of DNA in the vector pUC 18 were prepared as described (Hattori and Sakaki, 1986). The nucleotide sequence shown in Figure 5 was sequenced in both directions.

Synthetic oligodeoxynucleotides

Oligodeoxynucleotides were synthesized on an Applied Biosystems DNA synthesizer.

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References

Callahan,A.M. and Huskey,R.J. (1980) *Dev. Biol.*, **80**, 419−435.
Gilles,R. (1983) Dissertation, Universität Köln.
Gilles,R., Bittner,C. and Jaenicke,L. (1981) *FEBS Lett.*, **124**, 57−61.
Gilles,R., Gilles,C. and Jaenicke,L. (1984) *Z. Naturforschung*, **39c**, 584−592.
Hattori,M. and Sakaki,Y. (1986) *Anal. Biochem.*, **152**, 232−238.
Kirk,D.L. and Kirk,M.M. (1986) *Science*, **231**, 51−54.
Kushner,P.J., Blair,L.C. and Herskowitz,J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 5264−5268.
Laine,R.A., Esselmann,W.J. and Sweeley,C.C. (1972) *Methods Enzymol.*, **28**, 159−167.
Lottspeich,F. (1985) *J. Chromatogr.*, **326**, 321−327.
Mages,W., Salbaum,M., Harper,J. and Schmitt,R. (1987) *Nucleic Acids Res.*, in press.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York.
Mort,A.J. and Lamport,D.T.A. (1977) *Anal. Biochem.*, **82**, 289−309.
Nasmyth,K.A. (1983) *Nature*, **302**, 670−676.
Provasoli,L. and Pintner,I.J. (1959) In Tyron,C.A. and Hartmann,R.T. (eds), *The Ecology of Algae*. Special Publications No. 2, Pymatuning Laboratory of Field Biology, pp. 84−96.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463−5467.
Starr,R.C. (1970) *Dev. Biol.*, suppl. **4**, 59−100.
Starr,R.C. (1972) *Soc. Bot. Fr. Mem.*, 175−182.
Starr,R.C. and Jaenicke,L. (1974) *Proc. Natl. Acad. Sci. USA*, **71**, 1050−1054.
Weisshaar,B., Gilles,R., Moka,R. and Jaenicke,L. (1984) *Z. Naturforschung*, **39c**, 1159−1162.
Wenzl,S., Thym,D. and Sumper,M. (1984) *EMBO J.*, **3**, 739−744.
Wenzl,S. and Sumper,M. (1986) *Dev. Biol.*, **115**, 119−128.
Wieland,F., Heitzer,R. and Schaefer,W. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 5470−5474.

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