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Expression of highly active sex-inducing pheromone of *Volvox carteri* f. *nagariensis* in a mammalian cell system

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A cDNA fragment coding for the sex-inducing glycoprotein of *Volvox carteri* f. *nagariensis* was expressed in a mammalian cell system (baby hamster kidney (BHK) cells). The transfection product exhibited a specific biological activity intermediate between the natural pheromone of the strains *Volvox carteri* f. *nagariensis* and *Volvox carteri* f. *weismannia*. Immunoblot analysis showed that the sex-inducing activity was expressed as a set of three iso-glycoproteins (35, 34 and 31 kDa).

Glycoprotein; Sexual inducer; Pheromone; BHK cell; *Volvox carteri*

1. INTRODUCTION

Sexual induction in different species of the higher Volvocales is controlled by pheromones. The *Volvox* strain studied best is the dioecious *Volvox carteri* f. *nagariensis*. The difference between sexual and vegetative spheroids results from a shift towards a later time point in the differentiating cleavage during embryogenesis as indicated by morphological observations [1].

It is not known how the inducer works. Its action takes considerable time: only the next generation will be induced when the pheromone is added to a freshly hatched culture. A 10^{-16} M solution is capable of completely inducing all algae of that subspecies present [2]. It is species specific: *V. carteri* inducer only induces *V. carteri* strains; however, the two forms of *V. carteri*, f. *nagariensis* (IPS) and f. *weismannia* (65-30(12)) do cross- induce, although at different concentrations [3].

From our previous studies it is known that the inducer is a glycoprotein, formed as a set of iso-inducers by the maturing male spheroids and excreted when the sperm packets disintegrate [4]. The protein moiety has a molecular mass of 21 kDa; it is *O*-glycosylated on threonine side chains by galactose, arabinose and xylose residues, and *N*-glycosylated at the 3 middlemost asparagine residues by short-branched oligosaccharides consisting of chitobiose, mannose and xylose giving the molecule a polar hydrophilicity [5]. The total molecular mass of the glycoprotein is between 24 and 27 kDa depending on the isoform. It seems that the primary structure of the inducers of both *V. carteri* strains is very close if not the same, whereas the glycosylation is different in quality, not in quantity [3]. Active pheromone from *Volvox carteri* has recently been expressed in *Saccharomyces cerevisiae* [6]. Accordingly, it seems to be highly mannosylated; specific activities, however, have not been measured.

Here we describe the successful expression in moderate yield of active *Volvox* inducer in mammalian cells. This protein was synthesized by BHK cells in three differently glycosylated isoforms exhibiting a specific activity close to that of the original pheromone produced by *Volvox carteri*.

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Abbreviations: BHK, baby hamster kidney; DMEM Dulbecco's Modified Eagle's Medium; FCS, fetal calf serum; G418, geneticin; PBE-94, polybuffer exchanger 94; PBS, phosphate-buffered saline.

2. EXPERIMENTAL

The culture conditions of *Volvox carteri* (f. *nagariensis*, strains HK10 and IPS-22, and f. *weismannia*, strain 65-30(12), all obtained by courtesy of Professor R.C. Starr from the Algal Culture Collection at the University of Texas), the microdetermination of protein, and the electrophoretic procedures were performed as described [1,4].

2.1. *Cloning of inducer cDNA*

A cDNA clone (1647 bp) coding for the sexual inducer of *Volvox carteri* f. *nagariensis* containing a single open reading frame of 624 bp was isolated from a λgt-10-cDNA-library prepared from IPS-22 mRNA by means of hybridization to an oligonucleotide mixture corresponding to a partial sequence of inducer protein and subcloned into the *Eco*RI restriction site of pUC119 (Gilles, unpublished results). From this, a 1565 bp cDNA fragment containing the coding region was cut out and inserted into the respective sites of the polylinker of the mammalian expression vector pMPSVEH (kindly provided by H.-J. Hauser [7], Fig. 1).

2.2. *Co-transfection of adherent BHK cells*

*Bgl*I-linearized pMPSVEH [7] containing the pheromone-encoding

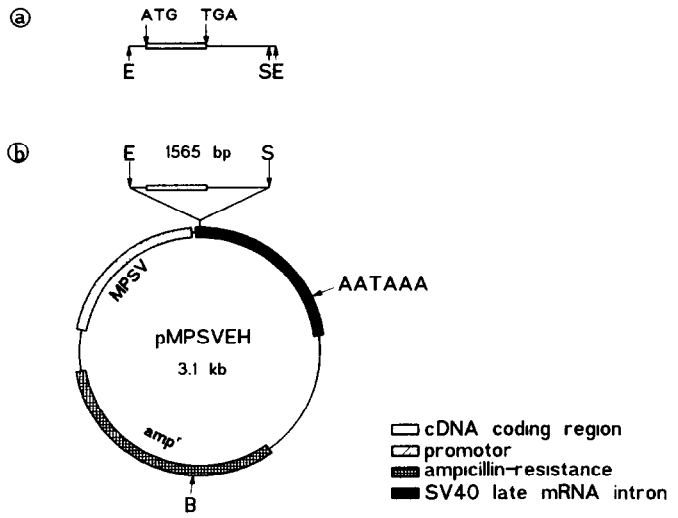

Fig. 1. Insertion of inducer-cDNA into pMPSVEH. A single *Sal*I endonuclease restriction site situated in the 3'-untranslated region of inducer encoding cDNA (1647 bp) inserted into the *Eco*RI site of a cloning vector (a) allowed to subclone a 1565 bp *Eco*RI/*Sal*I fragment containing the 624 bp coding region (ATG–TGA) in an oriented fashion into the mammalian expression vector pMPSVEH [7] (b). The polylinker of this vector is placed inside the SV40 19S late mRNA intron ensuring polyadenylation of the transcript produced under the control of the myeloproliferative sarcoma virus promotor MPSV. Endonuclease restriction sites used are indicated by symbols: B: *Bgl*I; E: *Eco*RI; S: *Sal*I. The maps are not drawn in scale.

cDNA (20 μg) was co-transfected with *Pvu*I-linearized pSV2neo (1 μg) [8]. Individual neomycin-resistant clones were selected by culturing the cells in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10^3 U/ml penicillin, 1 mg/ml streptomycin and 10% FCS in the presence of G418 (800 μg/ml; Gibco BRL). Pheromone-producing clones were identified by assaying the respective cell culture supernatants for inducing activity on freshly hatched spheroids (see below).

2.3. *Production of BHK-inducer*

One of the pheromone producing stable BHK transfectants was chosen to grow in mass culture and was subsequently maintained for 3 days in FCS-free DMEM supplemented with 10^3 U/ml penicillin and 1 mg/ml streptomycin. The supernatant containing the biological activity at ca $1/10^8$ dilution for 10 to 25% induction was used as starting material for all further experiments.

2.4. *Purification of the inducer produced by BHK cells*

One liter serum-free supernatant of BHK cells expressing the inducer gene was lyophilized to about 50 ml and dialysed extensively against 50 mM sodium citrate/sodium phosphate buffer, pH 3.6. Carboxymethyl-Sephadex and Poly-Buffer-Exchanger 94 (PBE 94) chromatographies for enrichment and purification of the pheromone have been described [5].

2.5. *Immunoblotting*

Immunoblotting was performed with aliquots containing purified inducer from BHK-cells. After SDS-PAGE (12% polyacrylamide minigel) proteins were transferred by semi-dry electroblotting (4°C; 5 mA/cm^2; 25 min) onto nitrocellulose sheets (Schleicher and Schuell). The blots were treated as described [4] except that they were developed with anti-rabbit-IgG-F_{ab}-fragment/alk. phosphatase conjugate (Sigma; 1:2000 in 0.1% BSA/PBS; 1.5 h at room temperature) using the bromochloroindolyl phosphate/nitro-blue tetrazolium substrate.

2.6. *Assay of inducer*

To 10 ml *Volvox* culture medium representing serial dilutions of the solutions to be assayed, 3 freshly hatched spheroids of *Volvox carteri* f. *nagariensis* (female strain HK10) were added and the tubes incubated at 28°C under a 16 h light (12,000 lx)/8 h dark regime for 2 days. Then, one drop of formaldehyd was added and the contents poured into a watch glass. The percentage of induced females (with eggs) was counted under a dissecting microscope at 24× magnification [1].

3. RESULTS AND DISCUSSION

BHK-cells transfected with the expression vector pMPSVEH containing the sequence coding for the *Volvox* sexual inducer were cultured for three days in DMEM free of FCS. The supernatant of this culture was analysed for inducing activity by bioassay. On average, biological activity of 10% induced algae was found at a dilution of 10^8 (expressed as 10% 10^8 in Table I) demonstrating that active pheromone can be expressed also in a mammalian cell system that, untransfected, does not exhibit any inducing activity. We did not distinguish if the inducing activity was solely due to pheromone actively secreted by the BHK cells or also to pheromone set free after decay of the cells or to a mixture of both. Centrifuged cell lysates, however, had only low inducer potency. The BHK cell culture supernatant was about two orders of magnitude less active than that of desintegrated male *Volvox carteri* f. *nagariensis* (strain IPS) but 100 times more active than supernatants from *Volvox carteri* f. *weismannia* males (strain 65–30(12)) (Table I). The inducer from BHK cell culture supernatant was purified according to the procedure established for the *Volvox carteri* pheromone proper [5]. The specific activity of the pheromone produced by BHK-cells was finally increased 300-fold (30%/10^{10}). This is intermediate between the specific activities of the IPS- (25%/10^{12}) and 65-30(12)-pheromone (20%/10^8), respectively. The yield of purified pheromone was rather low: whereas the f. *nagariensis* strain excreted on

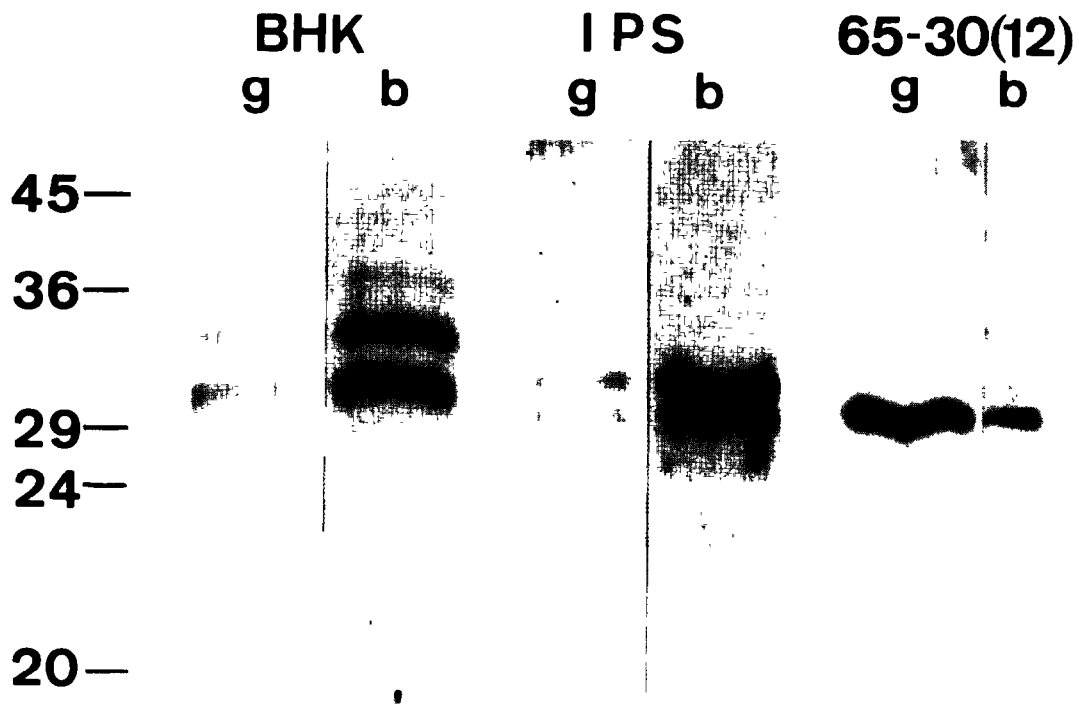

Fig. 2. SDS-polyacrylamide gel (g) and Western blot (b) of pheromones purified from BHK cells, *Volvox carteri* f. *nagariensis* (IPS) and *Volvox carteri* f. *weismannia* (65-30(12)). Parallel samples were separated by SDS-PAGE (12% minigels) and either silver-stained directly (g) or immunostained (b). BHK, 2.5 μg PBE 94-purified inducer; IPS, 1 μg HPLC-purified inducer; 65-30(12), 2 μg HPLC-purified inducer.

average 60 μg/ml medium, and the f. *weismannia* strain 100–150 μg/l medium, the BHK-cells produced only about 20 μg/l. On the other hand, this proves that the mammalian BHK system expresses a pheromone of comparatively high specific activity.

SDS-polyacrylamide gel electrophoresis of the purified BHK pheromone shows a pattern of 3 bands with apparent molecular masses of 35, 34 and 31 kDa (Fig. 2), conspicuously similar to the original iso-pheromones produced from *Volvox carteri* f. *nagariensis* (32, 30 and 27 kDa) whereas the inducer from *Volvox carteri* f. *weismannia* is homogeneous with 30 kDa. (The differ-

Table I

Purification of inducer produced by BHK cells, *Volvox carteri* f. *nagariensis*, IPS, and *Volvox carteri* f. *weismannia*, 65-30(12)

	BHK				IPS				65-30(12)			
	V (ml)	P (mg)	act.	sp. act.	V (ml)	P (mg)	act.	sp. act.	V (ml)	P (mg)	act.	sp. act.
CE	900	27	27%	10%	10,000	400	40%	10%	8,000	350	68%	24%
			10^9	10^8			10^{12}	10^{10}			10^8	10^6
CM	40	0.98	12%	12%	55	37	33%	90%	50	10	40%	40%
			10^9	10^9			10^{12}	10^{10}			10^8	10^7
CF	8	0.02	64%	32%	15	0.6	15%	25%	10	1.2	25%	21%
			10^8	10^{10}			10^{12}	10^{12}			10^8	10^8

For bioassay the protein solutions were diluted with culture medium as indicated and incubated with three freshly hatched female spheroids of *Volvox carteri* f. *nagariensis*, HK10. After two generations the percentage of induced progeny was counted.

CE, crude extract; CM, CM-Sephadex-dialysate; CF, PBE 94-eluate; V, total volume (ml); P, total protein (mg); act, total activity; sp. act, specific activity. The specific activity is given as the biological activity/mg protein (percentage of sexual test algae at the indicated dilution containing sexual and vegetative forms simultaneously)

ences in apparent molecular mass indicated of the pheromones to values published previously are due to the use of a different gel system.)

In order to confirm the identity of the protein purified from BHK cell culture supernatant with original inducer; we performed Western blots of the pheromones using a rabbit antiserum raised against the deglycosylated core protein of the f. *nagariensis* inducer, the specificity of which was demonstrated earlier [3,5]. As seen from Fig. 2, all three bands cross-reacted and thus were pheromone bands. From precedent [5] it is highly probable that the three cross-reacting proteins represent differently glycosylated isoforms of the inducer. The protein itself has a molecular mass of about 23 kDa.

The isoforms of the f. *nagariensis* pheromone differ in *O*-glycosylation, not in the composition of *N*-linked oligosaccharide chains which are essential for biological activity [5]. The inducer proteins of two strains of *Volvox carteri* f. *weismannia* showed quantitative difference in the composition of both the *O*- and the *N*-glycans relative to *Volvox carteri* f. *nagariensis* [3]. The consequences are different biological activity and the reduced immunological cross-reactivity [3]. From gel-migration patterns of purified protein it seems clear that the glycosylation state of the mammalian protein, too, differs from that of the original *Volvox* pheromone.

Taken together these results suggest a correlation between the glycosylation state and the biological activity of the pheromone modulating either the pheromone receptor binding or the activation of second messenger cascades [9].

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REFERENCES

[1] Starr, R.C. and Jaenicke, L. (1974) Proc. Natl. Acad. Sci. USA 71, 1050–1054.
[2] Gilles, R., Gilles, C. and Jaenicke, L. (1984) Z. Naturforsch. 39, 584–592.
[3] Al-Hasani, H. and Jaenicke, L. (1992) Sex Plant Reprod. 5, 8–12.
[4] Balshüsemann, D. and Jaenicke, L. (1990a) FEBS Lett. 264, 56–58.
[5] Balshüsemann, D. and Jaenicke, L. (1990b) Eur. J. Biochem. 192, 231–237.
[6] Haas, E. and Sumper, M. (1991) FEBS Lett. 294, 282–284.
[7] Artelt, P., Morelle, C., Ausmeier, M., Fitzek, M. and Hauser, H. (1988) Gene 68, 213–219.
[8] Southern, P.J. and Berg, P. (1982) J. Mol. Appl. Genet. 1, 327–341.
[9] Renwick, A. and Wiggins, P. (1992) FEBS Lett. 297, 1–3.