SITE AND TIME OF FORMATION OF THE SEX-INDUCING GLYCOPROTEIN IN *VOLVOX CARTERI*

Rainer GILLES, Claudia BITTNER and Lothar JAENICKE
Institut für Biochemie der Universität zu Köln, An der Bottmühle 2, 5000 Köln 1, FRG

Received 17 December 1980

1. Introduction

The colony-forming green alga *Volvox carteri* offers the opportunity to study sexual differentiation in a simple and well-controllable system. The presence (or absence) of an inducing hormone in the medium determines whether the algae grow sexually or asexually. Under the influence of this sex inducer the cleavage pattern of the reproductive cells (gonidia) is altered resulting in a sexual embryo which contains up to 64 gynogonidia (eggs) or 256 androgonidia (sperm-packets) instead of an asexual embryo containing up to 16 vegetative gonidia (details in [1]).

We have considerable knowledge about the biochemical nature of the sex inducer: it is a single-chain glycoprotein of 27 500 M_r [2]. The carbohydrate residues make up ~40% of the molecule, and the hormone interacts strongly with certain lectins, free and Sepharose-bound (unpublished). However, as far as the origin of the sex inducer is concerned, we only know that the hormone is exclusively secreted by the male strain, appearing in the medium when sperm-packets are released.

Here, we try to answer the following questions:

(i) Which type of cells: viz, somatic cells, androgonidia, sperm-packets; are responsible for the hormone production?
(ii) When and for how long is the sex inducer excreted?
(iii) Does the translation of the hormone's protein portion and/or its glycosylation immediately precede the release?

The evidence presented here indicates that the sperm-packets are the site of hormone production and release. The sex inducer is excreted for 10 h beginning with the disintegration of the male colony and the concomitant release of the sperm-packets. The amount of hormone produced by one single sperm-packet (64 sperm) is 1.6×10^{-13} g. The synthesis of the polypeptide chain takes place up to 35 h prior to secretion. Presumably the protein moiety is stored within the sperm-cells, and the glycosylation immediately precedes the hormone release. The mechanism of the secretion process, however, is still unknown.

Address correspondence to L. J.

2. Materials and methods

2.1. *Culture conditions*

Volvox carteri f. nagariensis, strain HK-10 (female) and 69-1b (male) were a gift from Dr R. C. Starr, Austin, TX. Axenic cultures were grown in *Volvox* medium [3] in an 8 h dark/16 h light (10 000 lux) cycle at 28°C.

2.2. *Fluorescence microscopy*

Concanavalin A (con A) (2.5 mg) or antiserum against highly purified sex inducer (prepared as in [4]) were incubated with 250 μg fluorescein isothiocyanate (FITC) in 0.5 M carbonate buffer (pH 9.5) for 1 h at 22°C. Unbound FITC was removed by filtration through Sephadex G-50 and dialysis against phosphate-buffered saline (PBS). For fluorescence microscopy cells were suspended with FITC–con A or FITC-antibodies (100 μg/ml) in *Volvox* medium for 1 h, then washed 3 times by centrifugation.

2.3. *Pulse labelling*

Well-synchronized colonies (100) were incubated with 0.25 μCi [^{14}C]arginine in 500 μl *Volvox* medium for 1 h at 28°C and 10 000 lux. The colonies were

washed 3 times with *Volvox* medium by centrifugation; unlabelled arginine (10^{-4} mol/l) was added and the colonies were further grown until the sperm-packets disintegrated. After centrifugation, the medium was tested for radioactivity in total trichloroacetic acid precipitable protein and in the sex inducer (by immunoaffinity chromatography).

2.4. *Immunoaffinity chromatography*

The rabbit anti-sex inducer antiserum [4] and normal rabbit serum (RS) were purified as in [5]. The immunoadsorbent preparation followed essentially the cyanogen bromide coupling procedure in [6]. About 1 ml wet vol. antiserum Sepharose (AS–Sepharose) or normal rabbit serum Sepharose (RS–Sepharose) was poured into a capillary pipet plugged with glass-wool.

The immunoaffinity chromatography was done by the following steps. (i) 200 μl 1% (w/v) bovine serum albumin (BSA) in PBS; (ii) 500 μl 1 mM arginine in PBS; (iii) 50 μl sample (0.1 vol. 10-fold concentrated PBS was added to the medium); (iv) 500 μl 1 mM arginine in PBS; (v) 1000 μl 1 mM arginine/1 M NaCl in PBS; (vi) 1000 μl 6 M urea. The column was regenerated with PBS.

2.5. *Analytical procedures*

The bioassay for the sex inducer has been published [1]. Double diffusion was performed as in [7]. Radioactivity was measured in a liquid scintillation spectrometer using a scintillator solution as in [8].

3. Results

3.1. *Which cell type produces the sex inducer?*

When trying to distinguish the hormone-secreting cells in all stages of the developing male colony by fluorescence staining with FITC-antibodies against the hormone, only the sperm-packets are labelled (see fig.1). The somatic cells, the androgonidia (also while cleaving to sperm-packets) and the individual sperm-cells show no fluorescence at all. In the case of FITC-antibodies from RS, even the sperm-packets do not bind any dye. The staining pattern of the sperm-packets viewed from the top shows triangular spandrels in the interstices between the conical spermatozoids, and in the side view the anterior region bearing the flagella is strongly stained, and faint lines (which are poorly reproduced on the micrograph) run through

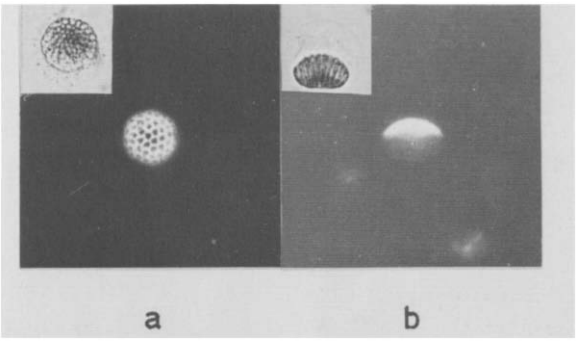

Fig.1. Sperm-packets stained with anti-inducer–FITC-antibodies, Fluorescence microscopy: (a) top view; (b) side view. In the upper left light-micrographs of sperm-packets in corresponding orientations are inserted.

the whole sperm-packet. The same pattern is formed when FITC–con A is used. This is consistent with the glycoproteinaceous nature of the sex inducer, which binds strongly to con A–Sepharose. FITC–con A binding is not so specific: The membrane of the somatic cells, the boundaries of the cell walls, the outlets of the flagella and the envelope which surrounds the freshly released sperm-packets were stained too [9].

We also succeeded in obtaining a sperm-packet preparation only weakly contaminated with dead somatic cells by repeated filtration through Nytal nylon nets (50, 30, 10 μm). These purified sperm-packets (fig.2 (a)); mature colonies which are ready to release the sperm-packets (b); young spheroids containing un-

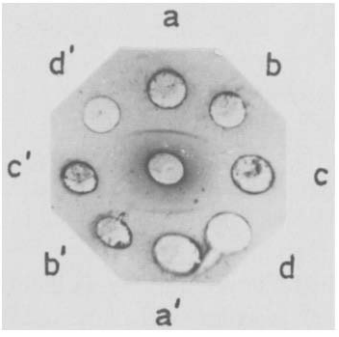

Fig.2. Double diffusion analysis of different developmental stages. The central hole was filled with 5 μl anti-inducer–antiserum: (a,a') sperm-packets; (b,b') colonies with mature sperm-packets; (c,c') spheroids with uncleaved androgonidia; (d,d') washing fluid (*Volvox* medium). Diffusion for 24 h at 28°C; gel was washed with PBS and water, dried and stained with Coomassie brilliant blue.

cleaved androgonidia (c) and the washing fluid of the preparations (d) were filled into the sample holes of double diffusion slides. A precipitation line is formed with antihormone–antibodies only in the first case (fig.2), which indicates that the sperm-packets excrete the sex inducer during the process of double diffusion.

3.2. *During which time is the sex inducer excreted?*

To answer this question the culture medium of an induced male colony was tested for biological activity. Because of the great sensitivity of the bioassay we are able to work with a single colony, thus eliminating the problem of synchrony. The hormone appears in the medium 9–11 h after completion of cell cleavages and formation of the sperm-packets. At this time the sperm-packets are ready for release, but the moment of release is not clearly determined. During the next 10 h there is an exponential increase (fig.3) of hormone concentration in the medium. After this period the sperm-packets begin to disintegrate into individual sperms and the inducer production stops. One male colony releases 6×10^{-11} g of sexual hormone, which corresponds to 5.5×10^{4} molecules/sperm cell. Fluorescence microscopy shows that the binding of the antibodies to the sperm-packets reflects exactly this interval of excretion of sex inducer, as indicated in fig.3.

3.3. *During which time is the sex inducer synthesized?*

The fact that *Volvox* spheroids presumably take up arginine by a specific carrier [10] was used to follow protein and inducer synthesis by pulse labelling. Fig.3 shows at which time proteins released into the medium are synthesized. We are also able to determine specifically the synthesis of the sex inducer, because it can be purified from the media in a single step by immunoaffinity chromatography. It is evident from fig.3 that nearly all of the sex inducer is translated prior to its release. Protein synthesis is stopped during cell cleavages possibly by diversion of energy into other activities, but the messenger RNA for the inducer protein seems to survive that interval. The background

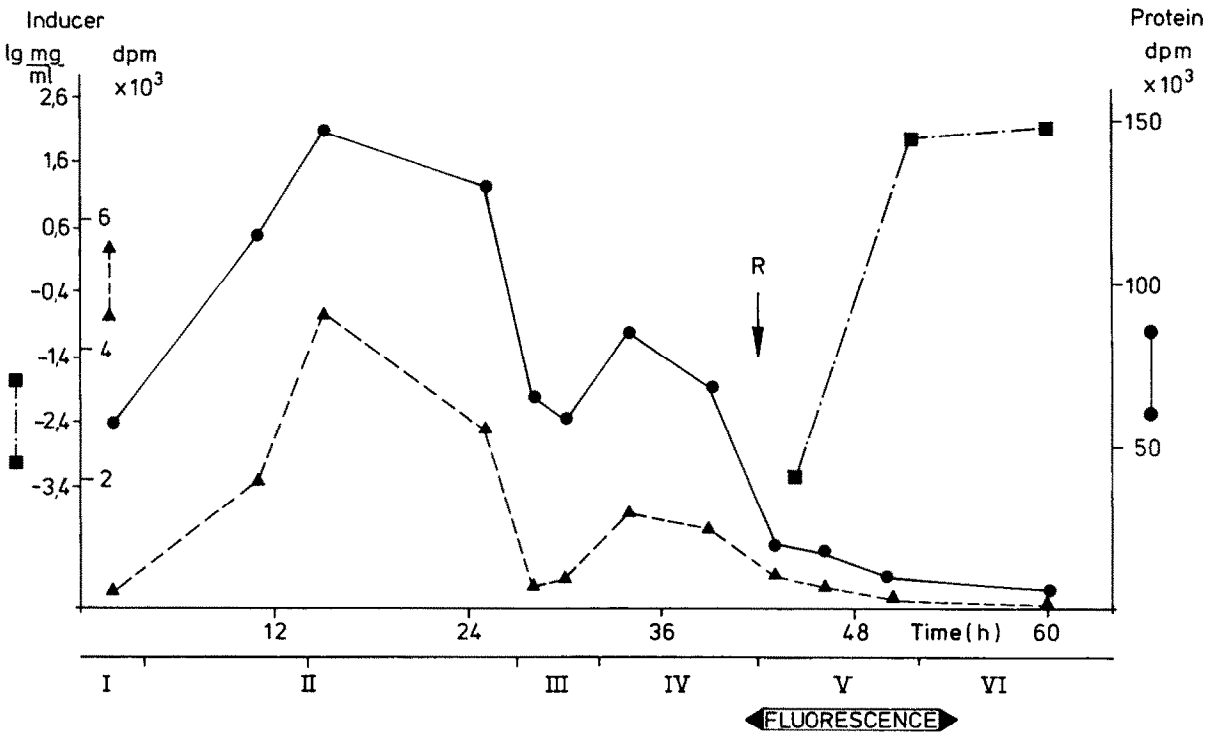

Fig.3. Pulse labelling experiment. The radioactivity in trichloroacetic acid-insoluble proteins (•) and in the sex inducer (▲) is plotted against the time at which the pulse (1 h) begins; log inducer concentration in the medium is determined by the bioassay (■); (R) release of sperm-packets. Developmental stages: (I) cleavage of gonidia; (II) maturation of spheroids; (III) cleavage of androgonidia; (IV) maturation of sperm-packets; (V) free-swimming sperm-packets; (VI) individual sperm. For details of the experimental procedure see section 2.

incorporation of labelled amino acid is due to the activity of somatic cells which are lysed when the whole colony disintegrates.

3.4. *When is the sex inducer glycosylated?*

As demonstrated above, there is a delay between the synthesis of the hormone's polypeptide chain and the excretion of the glycoprotein. If the sex inducer is stored within the cells in a dissociable form, we should be able to release it by disruption of the cells. However, no biological active sex inducer could be set free after sonication and Triton X-100 treatment. We therefore assume that the hormone is stored in an inactive form. Some observations indicate that the carbohydrate portion of the sex inducer is necessary for the biological activity: Periodate oxidation and degradation by certain glycosidases render the molecule inactive. These results suggest that the sex inducer is stored as a non-glycosylated and therefore inactive polypeptide chain.

To obtain further evidence of this we tried to stop hormone secretion by addition of metabolic inhibitors to the culture medium. As demonstrated in table 1 glycosylation (blocked by tunicamycin), protein synthesis (blocked by cycloheximide) and energy supply (blocked by sodium azide) are indispensable during hormone secretion. Probably, the requirement for protein synthesis is not due to the hormone synthesis which is almost finished at this time but to the translation of proteins (enzymes) involved in glycosylation and secretion of the sex inducer. The effect of tunicamycin which is an inhibitor of *N*-glycosylation [11] supports the possibility that the sex inducer is glycosylated during the period of excretion; and in addition, that the carbohydrate moiety is needed for hormone activity.

4. Discussion

The maturing sperm-packets are identified as the site of hormone secretion by immunological methods. Therefore the possibility of unspecific binding or cross-reactivity must be carefully examined. However, neither immunoelectrophoresis, nor immunoprecipitation, nor the radioimmunoassay presented in [4] indicate the existence of such interferences.

The protein synthesis (at least for secreted proteins) has two interesting features:

(i) It is strongly suppressed during the cell divisions, which is reasonable in physiological terms;
(ii) It is low in the swimming sperm-packets which have only limited energy supply.

The synthesis of the sex inducer follows the general pattern of biosynthetic activity, which means translation takes place up to 35 h prior to secretion and is nearly completed when the sperm-packets are released, resulting in an average translation rate of 2 s/molecule.

The glycosylation of the sex inducer seems to be a crucial point for both excretion and biological activity.

Table 1
Percent inhibtion

Stage	4 h after cleavage of androgonidia	During release of sperm-packets	6 h after release of sperm-packets
Cycloheximide (1 μg/ml)	100	100	90
Tunicamycin (1 μg/ml)	99	90	50
Sodium azide (10 mM)	100	100	70

At the specified stages inhibitors were added to 1 male colony in 1 ml *Volvox* medium, and incubated under culture conditions until the sperm-packets disintegrated. The hormone concentration in the medium was tested immediately after addition of the inhibitor and at the end of the experiment by the bioassay. Controls without inhibitors were also tested. Typical results are given in this table. If added before completion of the androgonidia cleavages, each inhibitor blocks absolutely the development of the algae

The obvious temporal correspondence between glycosylation and excretion suggests a causal correlation. However, our experiments up to now are insufficient for an explanation of the excretion mechanism. The function of the carbohydrate moiety, which is likely to be essential for hormone action, is under current investigation.

Acknowledgement

This work was sponsored by the Deutsche Forschungsgemeinschaft through Sonderforschungsbereich 74 and Fonds der chemischen Industrie.

References

[1] Starr, R. C. (1970) Dev. Biol. Suppl. 4, 59–100.
[2] Starr, R. C. and Jaenicke, L. (1974) Proc. Natl. Acad. Sci. USA 71, 1050–1054.
[3] Provasoli, L. and Pintner, I. J. (1959) in: The Ecology of Algae (Tryon, C. A. and Hartman, R. T. eds) pp. 84–96, spec. publ. no. 2, Pymatuning Lab. Field Biol., University of Pittsburgh, PA.
[4] Gilles, R., Bittner, C., Cramer, M., Mierau, R. and Jaenicke, L. (1980) FEBS Lett. 116, 102–106.
[5] Livingston, D. M. (1974) Methods Enzymol. 34, 723–731.
[6] Wilchek, M., Bocchini, V., Becker, M. and Givol, D. (1971) Biochemistry 10, 2828–2834.
[7] Kelus, A. S. and Weiß, S. (1971) Nature 265, 156–158.
[8] Bray, G. A. (1960) Anal. Biochem. 1, 279–285.
[9] Kurn, N. and Sela, B. (1979) FEBS Lett. 104, 249–252.
[10] Kirk, M. M. and Kirk, D. L. (1978) Plant Physiol. 61, 549–555.
[11] Takatsuki, A. and Tamura, G. (1971) J. Antibiot. 24, 232–238.