Schistosoma mansoni: Control of Female Fertility by the Male

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We have established an in vitro *culture system for adult schistosomes that allows monitoring gene expression for up to more than ten days. Comparing female worms that are paired with those that have been separated, we find distinct differences, clearly documenting an influence of the male in female gene expression. In perfect coincidence with classical observations that were based on histological techniques, we find that the male particularly regulates gene expression in those tissues that are characterized by cell proliferation, e.g. the vitellaria. From these results, we hypothesize that the key target for the inductive signal that is transferred from the male to the female during pairing is the activation of a growth factor that stimulates mitotic proliferation.*

Key words: *Schistosoma mansoni* - *in vitro* culture - gene expression - male - female interaction

The eggs of schistosomes are very remarkable as they are not only essential to continue the life cycle of the parasite, but - most important - they also are the causative agent of the disease. Therefore, the eggs must be in the focus of programs that control the disease, and it is the final goal of our research to understand the molecular mechanisms that regulate egg production.

We are focussed on a unique biological property of *Schistosoma* that is long known, but still not understood in its mechanisms. Adult schistosomes are closely paired with each other for their entire life. Egg formation occurs only in females that are connected with males by direct body contact. Interruption of this contact stops egg formation immediately while repairing of the two genders restores full fertility (Popiel & Basch 1984).

In this paper, we try to elucidate the role that the male plays in regulating female gene expression. We have constructed a hypothetical scheme on the mode of action of the male inductive signal that is transferred to the female. This hypothesis is supported by classical observations that were based on histological techniques.

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MATERIALS AND METHODS

S.mansoni were collected by perfusion from hamsters infected with worms of both sexes. Female worms were separated from males and immediately transferred into culture medium (Mercer & Chappell 1985). After varying numbers of days of *in vitro* culture, samples of 10 female worms were extracted for mRNA. They were homogenized for 3 x 10 sec on ice in 400 µl extraction buffer (LBS: 100 mM Tris/HCl pH 8.0, 500 mM LiCl, 10 mM EDTA, 5 mM DTT). SDS was added immediately to a final concentration of 1%, and the total volume was enlarged to 800 µl. After centrifugation at 14.000 g for 2 min, 200 µl (1 mg) of Dynabeads Oligo $(dT)_{25}$ solution (in LBS buffer with 1% SDS) was added to the supernatant, and the mRNA was allowed to bind to the beads for 5 min on ice. The bead-bound mRNA was magnetically concentrated for 30 sec and washed 3 times with washing buffer (10 mM Tris/HCl pH 7.5, 150 mM LiCl, 1 mM EDTA, 0.1% SDS). The mRNA was eluted with 10 µl of elution buffer (2 mM EDTA in DEPC-treated H_2O) at 65° C for 2 min. Northern blots have been performed using Hybond N (Amersham) membranes with mRNA (400 ng) fixed by UV crosslinking. Hybridization was carried out with ^{32}P labelled T7 transcripts.

RESULTS

Since genes involved in egg formation must be female specifically expressed genes, our first step involved the isolation of such genes. We have constructed a subtractive cDNA library highly enriched

in female specific transcripts (Fig. 1). The details of the procedure are published elsewhere (Michel et al. in prep.). Male cDNA, excised *in vivo* from recombinant λ-ZAP-vectors was prepared double stranded and used for *in vitro* transcription of biotinylated RNA. Alternatively, female cDNA in λ-ZAP-vectors was prepared single stranded. Biotinylated male RNA and female cDNA were hybridized in solution, and the hybrids were removed by streptavidine agarose. The cDNA fraction that remained unhybridized with male RNA was highly enriched for female specific transcripts and used to construct a library that yielded a complexity of about 2500 primary clones.

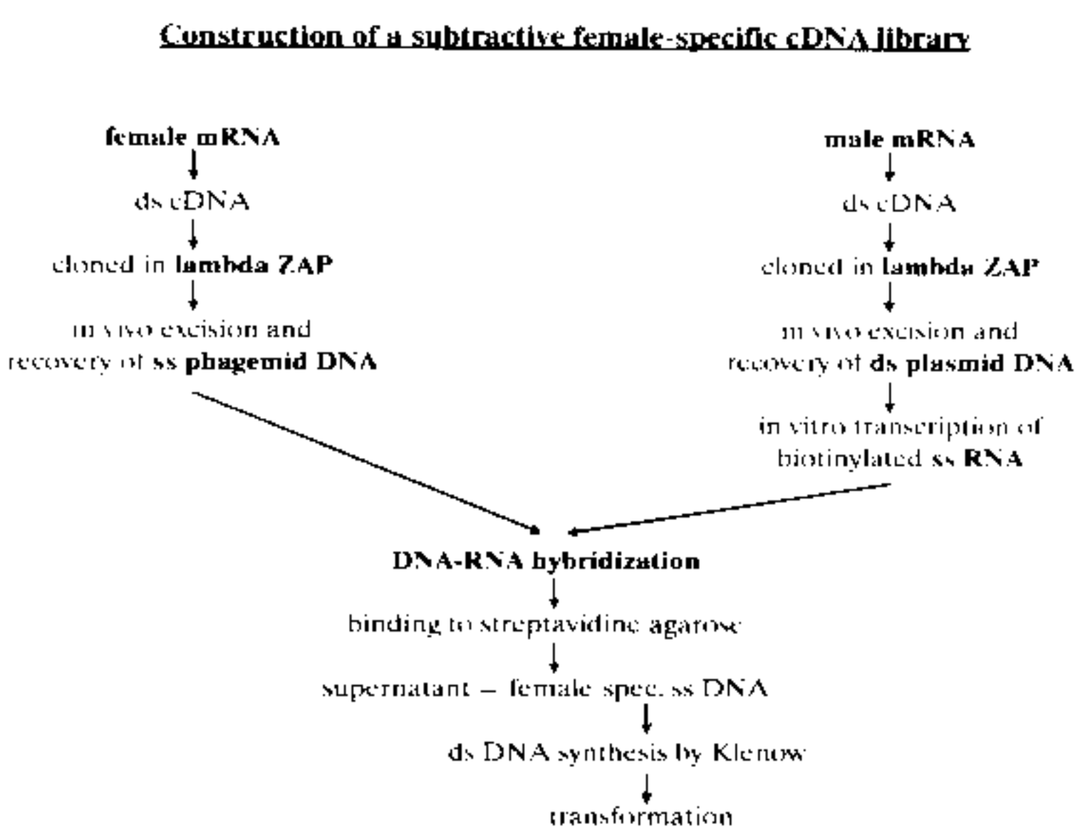

Fig. 1: strategy of construction of the female-specific library that was used to isolate the cDNA sequences described in this paper.

From this library, we have isolated and sequenced a couple of genes that have been shown on Northern blots to be female specifically expressed. Some of these sequences were identified to code for egg shell proteins. At the present, three different genes are known in *S.mansoni* that code for egg shell proteins: p14 (Bobek et al. 1986, Köster et al. 1988, Pena et al. 1990), p48 (Johnson et al. 1987), and p19 (Michel et al. in prep.). Each of these genes has been shown to be expressed selectively in the vitelline cells of mature, egg-laying females.

To study the regulation of the egg shell genes after separation of females from males, we have established an *in vitro* culture system that allows the observation of gene expression for at least 10 days. Quantification of mRNA on the Northern blots requires a reference gene whose expression is supposed not to be influenced by the male. For this purpose, we have chosen the gene for protein disulfide isomerase which is a house-keeping gene that is expressed in most cells of schistosomes (Finken et al. 1994). This gene shows only a slight decrease in expression during the first six days of culture of separated females, documenting the maintenance of almost native conditions for gene expression during the first days in our *in vitro* culture and demonstrating that PDI expression is independent from the presence of males.

Quite differently reacts the mRNA of the egg shell genes when the two sexes are separated. While paired females maintain egg shell gene expression *in vitro* for six or even ten days, although with decreasing intensity (Fig. 2), egg shell mRNA is drastically reduced after one day of separation already, and has totally disappeared after three days of separation (Fig. 3). If separated females are repaired *in vitro*, egg shell mRNA reappears (although very faintly) after five days (data not shown).

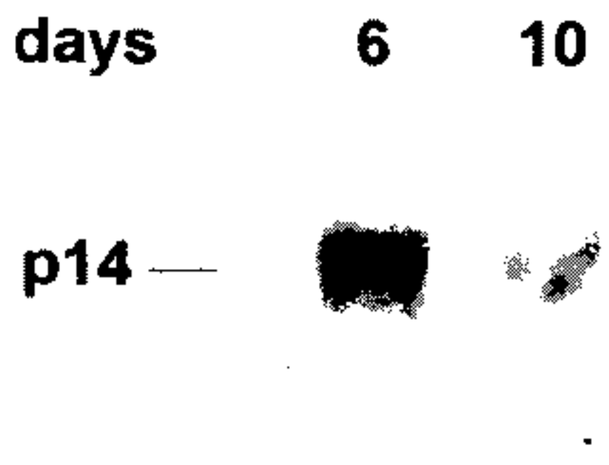

Fig. 2: Northern blots showing expression of the p14 egg shell gene in paired females after 6 and 10 days of *in vitro* culture. Expression is still clearly visible.

Egg shell genes are structural genes that become active during cell differentiation after mitotic divisions of stem cells. Besides this type of gene, we have also studied a developmentally quite distinct type of gene that is also female specifically expressed, however in a terminally differentiated tissue that is not characterized by continuous cell proliferation. The translation product of this gene contains many repeated N-glycosylation sites Asn-X-Ser/Thr and other sequence characteristics of a secretory protein of muco-protein type (Menrath et al. in prep.). This gene is expressed in the secretory wall cells of the vitelloduct close to its junction with the oviduct. First preliminary results with mRNA of isolated females on Northern blots show that the expression of this gene is also controlled by pairing with a male.

DISCUSSION

The presence of the female in the gynecophoric canal of the male is an absolute requirement for vitellar development, and (this needs not necessarily be the same causal event) for the maintenance of vitellar function. We favour the hypothesis that the male produces a signal of chemical nature that is transferred to the female where it stimulates gonad development and function.

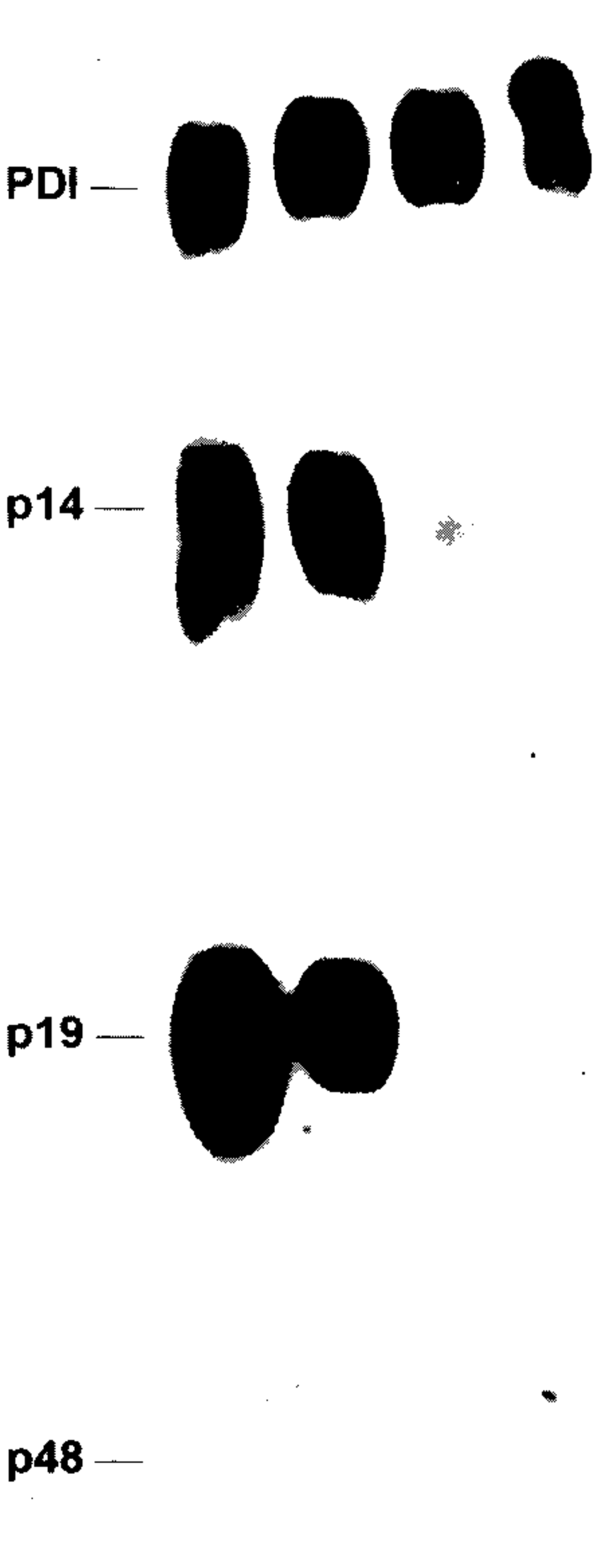

Fig. 3: expression of three egg shell protein genes (p14, p19, and p48) and the gene for protein disulfide isomerase (PDI) in females of *Schistosoma* separated from males and cultured *in vitro* for 1, 2, and 3 days. Poly(A)$^+$ RNA from 10 adult females was blotted and hybridized with transcripts of cloned cDNA's of the respective genes. Expression of egg shell genes is decreasing after one day of separation already, and has totally disappeared after three days of separation. PDI mRNA, which is not controlled by the male, shows only a slight decrease in expression during the first three days of culture of separated females.

To understand the nature of the inductive signal from the male, an elegant experiment of Popiel and Basch, published already 10 years ago (1984), allows far-reaching conclusions. These authors have shown that isolated dissected segments of males were perfectly able to induce vitellar development; this occurred, however, only in those limited parts of the female body that were directly in contact with the male segments. From these observations, one firstly can conclude that some specialized organs of the male (e.g. testes or brain ganglia) cannot be producers of the male inductive signal. Instead, the signal appears to be generated at several positions along the male body. Secondly, it appears that the way of signal transduction in the female is probably not via the nervous system; otherwise a longitudinal transport through the female body would be expected.

Some additional classical observations, made about 20 years ago by David Erasmus (1973) became important for our present concept. Erasmus studied vitellar development in schistosomes in the presence or absence of males. He subdivided the vitelline cells into four classes of ascending development. Class 1 are young cells that appear to be undifferentiated since they do not contain granules, platelets, or vacuoles with stored molecules for embryonic development. Class 2 to 4 are growing vitelline cells rich in endoplasmic reticulum and Golgi complexes that progressively accumulate carbohydrates, lipids, and huge amounts of egg shell proteins. DNA synthesis and mitotic divisions do occur only in class 1 cells (Den Hollander & Erasmus 1985).

This suggests that class 1 cells are proliferating stem cells that undergo mitoses whereas class 2 to 4 cells are terminally differentiated cells that complete a developmental program. Most important for our present concept are the observations of Erasmus that virgin females do contain only undifferentiated class 1 cells in their vitellaria (Erasmus 1973). When they become paired, these cells start mitoses and produce differentiated class 2 to 4 cells which are not present in virgin females. When paired females become separated from the male, class 1 cells stop DNA synthesis and cell division, but class 2 to 4 cells continue to complete their developmental program.

Our results on Northern blots correlate with these morphological observations. Structural genes expressed during the period of terminal differentiation of the vitelline cells (class 2 to 4 cells) (Köster et al. 1988), such as egg shell genes, are not immediately blocked after separation of the two genders. After one and two days, however, their expression is reduced more and more, and has disappeared after three days, whereas paired females maintain a high level of expression. Since the half-life time of mRNA is expected to be only a few hours, we believe that the observed decrease in mRNA level does not result from degradation of long-lived message, but from a retrogression of

newly synthesized mRNA molecules. The retrogression in expression after separation appears to be a consequence of the consumption of class 2 to 4 cells due to egg formation, while new cells are not provided anymore from the germinative layer of class 1 cells.

From these observations we have constructed a hypothetical scheme of the signal transduction factors that act in the female in consequence of the male inductive signal (Fig. 4). The direct target genes for the signal appear not to be the structural genes in the differentiated vitelline cells. Instead, the signal appears to activate a growth factor that acts as a mitogen stimulating the stem cells of the vitellaria to undergo mitoses and subsequent unequal divisions. Cells destined to undergo differentiation are believed to be equipped with a set of transcription factors that, once activated, warrant a coordinated program of gene expression without the need for a continued stimulus by the male.

In other organs, however, the male inductive signal appears not only to stimulate cell proliferation. Female specifically expressed genes in these tissues are also controlled by the male signal. A gene coding for a mucin-like protein that is active in the secretory wall cells of the vitelloduct close to its junction with the oviduct becomes repressed when the male is separated from the female. This gene appears to be an example for a structural target gene regulated by the male directly in a terminally differentiated tissue.

It is obviously very difficult in *Schistosoma* to find the molecular targets for the male signal, since mutants of genes regulating development as in mammals or *Drosophila* are not available in schistosomes. It will be a long way before it will be possible to study the genes in schistosomes that regulate cell division and differentiation. Some molecules involved in signal transduction, however, have already been identified in *S.mansoni*. The sequences for adenylate kinase (Cao et al. 1992), for an epidermal growth factor receptor which is a receptor tyrosine kinase (Shoemaker et al. 1992) and for the highly conserved α subunit of a G protein that mediates signals between serotonin receptors and adenylate cyclase (Iltzsch et al. 1992) have been determined. An opioid receptor with a presumptive role in signal transduction (Duvaux-Miret et al. 1993) and the activity of a protein kinase C have been found at the surface or in the tegument of schistosomes, respectively (Wiest et al. 1992a), and two genes for Ca-binding proteins have been cloned and sequenced (Ram et al. 1989) (Michel et al. in prep.). The generation of the second messengers inositol phosphate and diacylglycerol by phospholipase C which is activated by a G protein has been suggested to play a role in specific gene regulation in schistosomes (Espinoza et al. 1991, Wiest et al. 1992c).

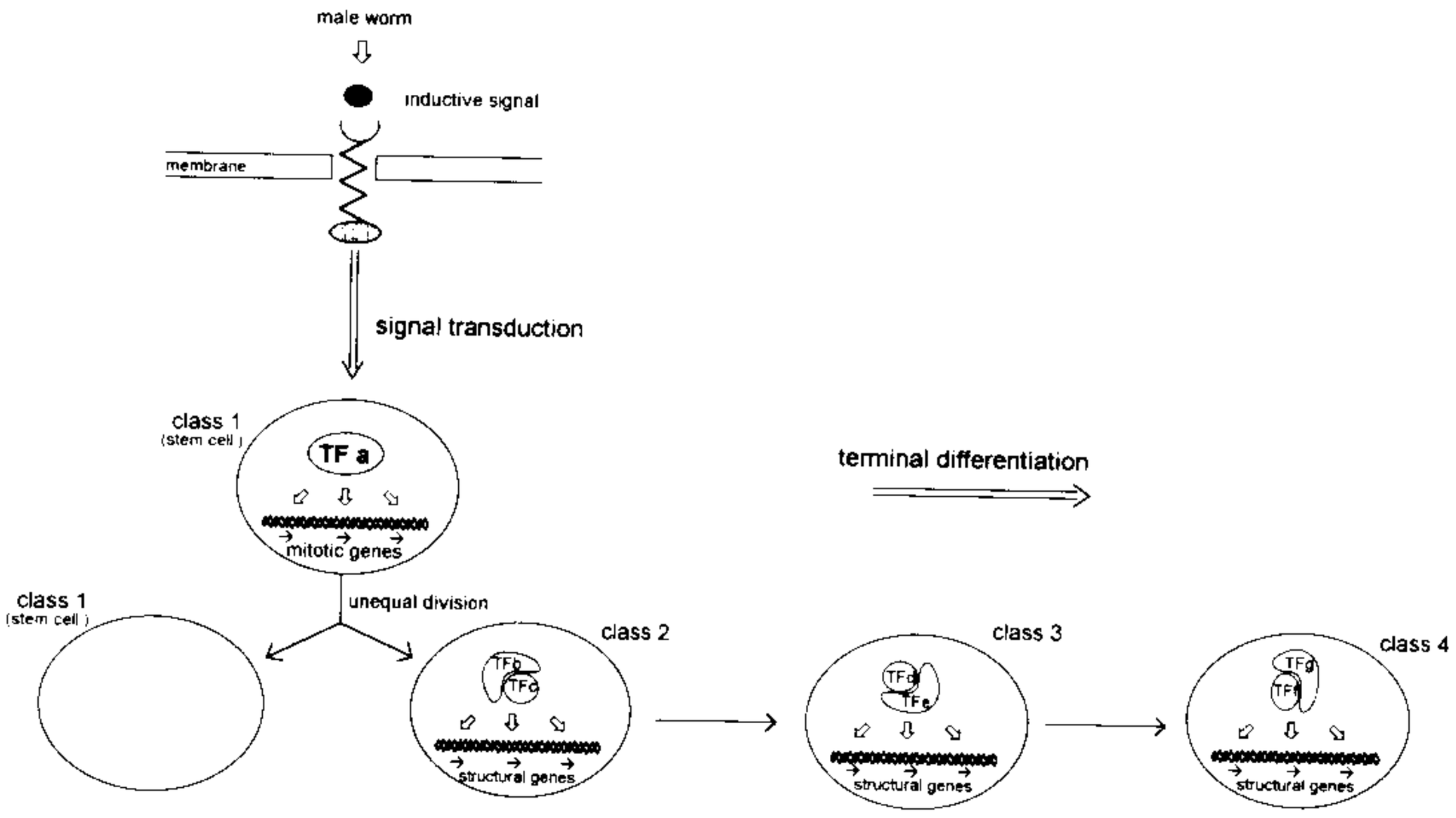

Fig. 4: hypothetical scheme of signal transduction in the female following the stimulation by the male inductive signal. The direct target genes for the signal appear not to be the structural genes in the differentiated vitelline cells of classes 2 to 4. Instead, the signal appears to activate a growth factor that acts as a mitogen stimulating the stem cells of the vitellaria to undergo mitoses and subsequent unequal divisions. Vitelline cells of classes 2 to 4 are believed to be equipped with a set of transcription factors (TFa - TFg) for the coordinated program of expression of the structural genes.

Very important for our concept is the finding that an artificially generated increase in inositol phosphate was significantly higher in females compared to males (Wiest et al. 1992c). The well-known drug against schistosomiasis, praziquantel, appears to interact with inositol phosphate production (Wiest et al. 1992b). Progressive knowledge on signal transduction molecules which transfer the male inductive signal to the female target genes shall open the possibility to study the influence of blockers of signal transduction and, thus, elucidate their function.

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