Female-specific gene expression in *Schistosoma mansoni* is regulated by pairing

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SUMMARY

Gene expression studies in adult females of *Schistosoma mansoni* cultured *in vitro* revealed that the transcription of female-specifically expressed genes is influenced by pairing. In contrast, the activity of genes that are expressed in both genders was not affected by contact with the male. The transcription of genes was monitored in paired, separated and remated females. The transcript level of female-specifically expressed genes decreases within a few days following separation from males. Remating of uncoupled females with males leads to the reinitiation of transcription. These results provide strong evidence for the influence of the male on gene transcription in the female and contribute a molecular basis for the classical histological observation that the maturation of females is male dependent. The data also show that the culture system is suitable to monitor gene expression and, furthermore, they indicate *de novo* RNA synthesis *in vitro*.

Key words: *Schistosoma mansoni*, *in vitro* culture, female-specific gene expression, female maturation, *de novo* RNA synthesis.

INTRODUCTION

Pairing is an essential pre-requisite for the completion of female growth and reproductive morphogenesis in most schistosome species (Popiel, 1986*a*). From classical histological studies it is known that the direct contact of the male is required to achieve and maintain maturity of the female (Popiel, 1984). When mature female schistosomes are separated from their male partners, they stop egg laying and start to degenerate to an immature state. But, when these females are remated with males, they regenerate and produce viable eggs again (Clough, 1981). This shows that a permanent contact between the male and the female is indispensable for reproduction, and it has been suggested that a male stimulus is responsible for female maturation (Popiel, 1986*b*). In an *in vitro* culture system, it has been observed that DNA synthesis declined in separated females, and increased when these females were remated with males (Den Hollander & Erasmus, 1985).

The aim of this study was to investigate the influence of the male on the female at the gene expression level. We established an *in vitro* culture system which enabled us to perform pairing experiments and showed that this attempt is suitable to investigate gene activity. It is also demonstrated that the transcriptional activity of female-specifically expressed genes is directly regulated by pairing with the male.

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MATERIALS AND METHODS

Parasite stock

A Liberian isolate of *Schistosoma mansoni* (Greveldding, 1995) was maintained in *Biomphalaria glabrata* and in Syrian hamsters. Adult worms were obtained by perfusion at day 49 post-infection. Females and males were separated with a fine brush and checked under the microscope before culturing.

Parasite culture

After perfusion and collection, worms were immediately transferred into the *in vitro* culture medium. Aseptic techniques were maintained as stringently as possible using a clean bench. Adult males and females were kept in small Petri dishes using RPMI-1640 (Gibco) culture medium supplemented with 10% heat-inactivated calf serum (freshly added), 12 mM HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid), pH 7·4, and an antibiotic/antimycotic cell culture mixture (1·2%; Sigma, A-7292). Worms were washed 3 times with completely supplemented RPMI-1640 medium before they were kept at 37 °C and 5% CO_2 in an incubator for pairing experiments. A maximal number of 30 worms was kept per dish. The culture medium of the appropriate worm batches was changed every 2 days.

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RNA preparation and Northern blot analysis

At appropriate times, 30 females were collected from the *in vitro* culture for poly(A)$^+$-RNA isolation. The worms were homogenized (Omni 2000 homogenizer) for 3×10 sec on ice in 400 μl of lysis buffer (100 mM Tris(hydroxymethyl)aminomethane–HCl, pH 8·0, 500 mM LiCl (lithium chloride), 10 mM EDTA (ethylenediaminetetra-acetic acid) pH 8·0, 5 mM DTT (dithiothreitol). After homogenization, the volume was extended to 800 μl with lysis buffer and SDS (sodium dodecyl sulfate) was added to a final concentration of 1 %. After centrifugation to pellet the cellular debris, the supernatant was taken for mRNA isolation. Magnetic oligo(dT)$_{25}$ particles (Dynabeads, Dynal) were used for the extraction, following the instructions of the manufacturer with minor modifications (Schüßler *et al*. 1995*a*). The amount of extracted poly(A)$^+$-RNA varied from 150 to 500 ng.

Northern blots were performed according to the method described by Sambrook, Fritsch & Maniatis (1989). The complete amount of extracted mRNA was separated by denaturing agarose-gel electrophoresis. Blotting was performed on Hybond NTM membranes (Amersham, UK), and mRNA was fixed by u.v. cross-linking. Hybridization experiments were carried out by *in vitro* transcription under stringent conditions (Sambrook *et al*. 1989) using ^{32}P-labelled T7 transcripts of cloned cDNAs (Kunz *et al*. 1987; Köster *et al*. 1988; Finken *et al*. 1994; Menrath, Michel & Kunz, 1995; Michel *et al*. 1995; Schüßler *et al*. 1995*b*) of the appropriate genes introduced in the Results section.

Densitometric quantification

The analysis of Northern blot autoradiographs was done by densitometric quantification. The relative values were obtained by integration using the bioScan software (Viper, Germany). Different exposures of each hybridization were scanned with a ScanJet 4c (Hewlett-Packard) and taken for the evaluation.

RESULTS

In vitro *maintenance*

Adult schistosomes from mixed infections were recovered by perfusion. To maintain worms *in vitro*, intact and well-developed individuals of the appropriate size were collected under a microscope. Different groups of worms were kept separately for a period of 1–2 weeks in this culture system. One group consisted of females that had been separated from males immediately after perfusion. Separated females of this group were split into 5 subgroups before they were kept in culture for different time-intervals as unisexual worms. Each subgroup was cultured *in vitro* for 1, 2, 3, 6 or 13 days, respectively. For each time-interval, about 30 individuals were collected.

Another group consisted of worm pairs that were collected after perfusion and transferred as couples into the *in vitro* culture for a 6-day period. Maintenance of pairing was checked on a half-day basis using the microscope.

For remating experiments, separated females were kept *in vitro* for a 6-day period without males. After this period, they were exposed to males again and, following pairing, couples were carefully separated from singles and immediately transferred into a separate dish. These remated worms were maintained for a further 7 days. Most females remated within 36 h.

Gene expression studies

In recent years, we have identified and characterized many genes with respect to their gender-dependent expression and tissue-specific localization (Kunz *et al*. 1987; Köster *et al*. 1988; Finken *et al*. 1994; Menrath *et al*. 1995; Michel *et al*. 1995; Schüßler *et al*. 1995*b*) to find those which are suitable candidates for the analysis of pairing-dependent gene expression in schistosomes. Three different examples for the regulation of female-specifically expressed genes are presented. The first example is the gene for the eggshell protein p14 (Bobek *et al*. 1986; Kunz *et al*. 1987; Bobek, Rekosh & LoVerde, 1988), a structural gene which is transcribed during cell differentiation in vitelline cells (Köster *et al*. 1988). The second example represents the iron storage protein gene FerI, which codes for yolk-ferritin and which is also expressed in vitelline cells (Schüßler *et al*. 1995*b*). Finally, the gene for the mucin-like protein A11 is shown which is transcribed in secretory epithelial cells of that part of the vitelloduct which is close to its entrance into the ootype (Menrath *et al*. 1995). For all these genes it was shown that they are gender-specifically transcribed in the female.

As examples for gender-independent expression, genes have been chosen that are expressed in several tissues in both genders, the protein disulfide isomerase (PDI) and cathepsin L (CatL). The PDI gene is equally expressed in males and females (Finken *et al*. 1994) and codes for an enzyme that is involved in protein secretion. CatL is a protease that occurs in several tissues as the epithelium around the vitelloduct, ovo-vitelloduct, ootype, uterus, but not the oviduct (Michel *et al*. 1995). In males, it appears in the subtegument of the gynaecophoric canal. The CatL gene is expressed 5 times more in females than in males, a quantitative difference in accordance with the higher number of female tissues showing cathepsin L activity.

To investigate the influence of pairing on the expression of female-specifically versus gender-

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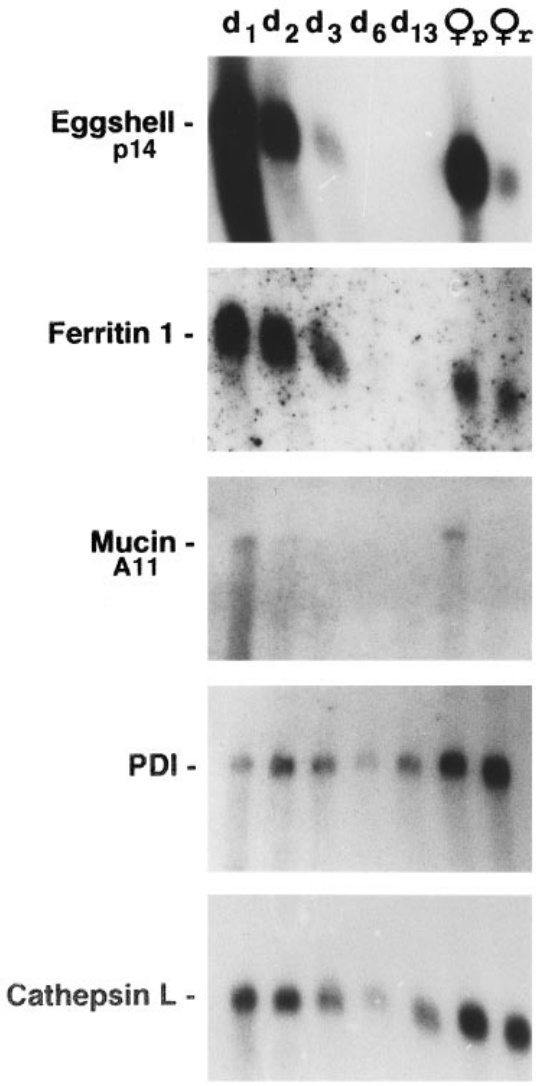

Fig. 1. Northern blot analysis of poly(A)$^+$-RNA from *Schistosoma mansoni* females kept *in vitro* hybridized with ^{32}P-labelled transcripts of cloned cDNAs of the following genes: the egg-shell protein p14, the iron storage protein FerI, the mucin-like protein A11, the protein disulfide isomerase (PDI) and the protease cathepsin L (CatL). Lanes d1–d13 contain RNA from mature females that originated from couples, before they were kept isolated in culture for 1, 2, 3, 6 or 13 days as unisexual worms. ♀$_p$, females that were kept paired with males for 6 days in culture; ♀$_r$, females that were separated from males for 6 days and afterwards remated with males for a further 7 days. All hybridizations were performed on one Northern blot filter by stripping and re-probing.

independently-expressed genes, poly(A)$^+$-RNA was extracted from the different groups for Northern blot analysis (Fig. 1). A direct comparison of the different hybridization experiments was achieved by continuous rehybridization of the same Northern filter using the following gene probes. The apparently different slanting in the migration pattern of some of the panels is due to a smiling effect of the gel.

With p14 as a probe, transcripts were detected in females that were kept as paired worms for 6 days in culture (Fig. 1, eggshell). In contrast to paired worms, the level of p14 mRNA sharply decreased in uncoupled females within the first 3 days of separation from the male and completely disappeared after 6 days of separation. However, if females were separated from males for 6 days and remated with males for a further 7 days, a distinct signal reappeared.

A similar pairing-dependent transcript profile is observed when ferritin is used as a probe. In females that remained paired for 6 days, transcripts were still present (Fig. 1, ferritin). FerI transcripts decreased, however, in uncoupled females during the first 3 days of separation from the male and disappeared on days 6 and 13. In remated females, FerI transcripts were observed again, similar to the expression profile of the p14 gene.

The regulation of A11 gene expression was again similar. Figure 1 (mucin) reveals transcripts in paired females and a reduction during the first 3 days of separation. A remating signal was not observed. Since A11 was expressed at a distinctly lower level as p14 and FerI, the signals were not as strong. This probably explains why transcripts cannot be observed in remated females.

The gender-independently-expressed genes PDI and CatL were not influenced by pairing. As shown in Fig. 1 (PDI and cathepsin L), a continuous transcript level was observed in females during the whole period of separation from the male, in females that remained paired for 6 days, and in remated females.

mRNA quantification

To measure the relative intensities of the hybridization signals resulting from the Northern blot (Fig. 1), a densitometric quantification analysis was performed on autoradiographs exposed for different times according to the activity of the different probes used (result not shown). The analysis of the gender-independently-expressed genes PDI and CatL shows a continuous transcript level, but variations in the strengths of the signals which are due to the varying amounts of RNA obtained from the different worm batches. To normalize the differences in the amounts of mRNA, the CatL hybridization result was taken as a basis (Fig. 1, cathepsin). The intensities of the CatL signals were related to the day 1 value which was defined as 1. To determine the values for genes that depend on pairing, their densitometric values were corrected by factors regarding the differences in the mRNA amounts loaded on each lane. PDI was not considered as a reference for normalization, since the low amount of transcripts of day 1 could not be explained (Fig. 1, PDI).

In females that were kept paired with males for a

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6-day period, transcripts of the female-specifically expressed genes were detected, but at a lower level compared to the first day in culture, shortly after perfusion. The level of p14 transcripts was reduced to 42 %, A11 to 51 % and FerI to 18 %. The low level of FerI will be discussed later. This shows that in spite of the continuous presence of the male, the state of maturity of the female is not maintained at full level under *in vitro* conditions.

In contrast to paired females, the p14 signal decreased much more distinctly in females that were separated from males. It was diminished to 14·5 % after 3 days, and it completely disappeared after 6 days of separation. In females, however, that had been separated from males for 6 days and remated for 7 days, transcripts were observed again and represented 7 % of the original day 1 value.

For FerI, the expression profile was similar. After 3 days of separation the amount of transcript was reduced, and only 48 % of the transcripts were present. At days 6 and 13, signals were no longer detected. In remated females, transcripts exhibited a level of 8 % again, compared to day 1, a value which is similar to that of p14.

A reduction following separation from the male was also observed for the amount of A11 transcripts. The level was reduced to 30 % in females during the first 3 days of separation and disappeared on day 6, but no signal could be quantified after remating.

DISCUSSION

In 1973, Erasmus provided histological evidence that the vitelline gland development in female *S. mansoni* is directly influenced by the male (Erasmus, 1973). He showed that the presence of a male leads to mitogenic activity of undifferentiated stem cells of the vitellarium which undergo an irrevocable, terminal differentiation programme. Following separation from males, only these stem cells stop proliferation, whereas cells that already have entered the differentiation process will complete their developmental programme. These results indicated that vitelline cell differentiation does not stop immediately after separation, but decays continuously. In 1981, Clough showed by *in vivo* transplantation experiments that the vitellaria of unmated females degenerated within 6 days following separation from the male (Clough, 1981). Upon remating with males, these females regenerated and produced viable eggs.

Early molecular studies have shown that the expression of egg-shell genes of *S. mansoni* occurs in a stage-, tissue-, and time-specific manner (Bobek *et al.* 1986; Köster *et al.* 1988; Chen, Rekosh & LoVerde, 1992). It was hypothesized that the appearance of egg-shell gene transcripts may somehow be influenced by the presence of the male (Bobek *et al.* 1986) since egg-shell gene transcripts appeared in female worms obtained from bisexual infections, but not in female worms from unisexual infections.

The results presented in this study provide the first molecular evidence that female schistosomes need a constant stimulus from the male to maintain female-specific gene expression. An *in vitro* culture system was established to perform separation and pairing experiments. For separation experiments, females were disconnected from intact couples and kept isolated from males for different time-intervals in culture. For pairing experiments, paired females were kept in culture, or separated females were remated with males. Using Northern blots, the transcription of female-specifically-transcribed genes and gender-independently transcribed genes was monitored in these females. The analysis of the gender-independently transcribed genes for the protein disulfide isomerase (PDI) and cathepsin L (CatL) showed that the expression profile of these genes remained relatively constant in separated, paired or remated females. This result proves that these two genes are not influenced by pairing. Furthermore, it demonstrates maintenance of good levels of RNA synthesis *in vitro*.

In contrast to the gender-independently expressed genes, the transcription of female-specifically expressed genes significantly decreases in isolated females within 3 days of separation from the male and totally disappears after 6 days. Transcription of the egg-shell gene p14 and the mucin gene A11 decreases exponentially and, in the case of the ferritin gene FerI, the decay is linear. In paired females, however, transcription of these genes continues during a 6-day interval which documents the maintenance of RNA synthesis *in vitro*, although at a lower rate. Roughly 40–50 % of the transcript level of day 1 is maintained for p14 and A11 as determined by densitometric quantification. The expression of FerI is maintained at a proportionally lower level of 18 %, which may be explained by a reduced need for ferritin in an iron-free culture medium without haemoglobin. The observed decrease of female-specifically expressed genes in paired females is not surprising, since it is known that the number of eggs produced in culture declined to nearly 20 % after a period of 6 days (Mercer & Chappel, 1985), documenting that the presence of males is not sufficient to fully maintain the reproductive activity *in vitro* (Basch & Rhine, 1983).

In females that have been separated from males for 6 days and remated with males for another 7 days, however, distinct signals for p14 and FerI are detected again. This result strongly indicates that males are able to reinitiate the transcription of genes that were repressed during a preceding separation period since after 6 and 13 days p14 expression has completely stopped in separated females. Additionally, it demonstrates that males have the capacity to

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exert an obvious stimulation under culture conditions. Furthermore, this result indicates *de novo* RNA synthesis *in vitro*, since the reinitiation of transcription within 7 days following remating compared to the disappearance after 6 days of separation cannot be explained by changes in the steady-state levels of transcripts. This again shows that the system is suitable to monitor gene expression.

These molecular data perfectly correlate with the classical morphological observations mentioned above showing that the degeneration of the vitellaria occurs gradually over a period of 6 days following separation from the male. In agreement with these data, structural genes expressed during the differentiation process of the vitelline cells, such as egg-shell genes, are not immediately blocked after separation of the two genders, but transcription is gradually reduced during the first 6 days.

There is a remarkable difference between the A11 and the CatL gene. Both genes are expressed in the genital tract of the female, A11 in that part of the vitelloduct which is close to its entrance into the ootype (Menrath *et al*. 1995) and CatL in the subtegument of the uterus (Michel *et al*. 1995). Interestingly, transcription of A11 is pairing dependent, whereas CatL is regulated independently from the male. This perfectly agrees with the observation that in immature females the uterus is clearly visible in contrast to the vitelline duct which is not developed (Erasmus, 1973).

In the past, many experiments have been performed to elucidate the nature of the male–female interaction. Many authors favour the hypothesis that a male stimulus is involved, but it remains unclear whether this stimulus is of physical or chemical nature (Den Hollander & Erasmus, 1985; Popiel, 1986*b*). The data presented in this study confirm the classical observations and strongly support the idea that the expression of female-specific genes is the final result of the male signal. Since the stimulation has an effect on proliferating tissue, we believe that the presumptive signal activates a mitogen which stimulates the stem cells of the vitellaria to undergo mitosis and subsequent unequal cell division (Kunz *et al*. 1995). This is supported by the observation that the DNA synthesis rate was increased when immature females were paired with males. In turn, the rate declined when females were separated from males (Den Hollander & Erasmus, 1985).

The differentiation of stem cells belongs to the kind of developmentally regulated processes that are governed by signal transduction events which can act gender, time or tissue specifically (Pawson, 1991; Hanley & Feramisco, 1992). First indications for the existence of signalling molecules that participate in this male–female interaction of schistosomes are provided in the accompanying paper by Schüßler, Grevelding & Kunz (1997).

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