P19: a female and tissue specifically expressed gene in *Schistosoma mansoni*, regulated by pairing with the male

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SUMMARY

A female-specific sequence was isolated from a cDNA library of *Schistosoma mansoni* and further characterized¶. Expression of the corresponding gene (p19) depends on pairing with a male. *In situ* hybridization and immunohistology experiments revealed exclusive expression of the gene in the cells of the vitellarium, suggesting a function in egg formation. In addition, experimental evidence for cross-linking of the protein under oxidative conditions supports the assumption that the p19 gene may code for an egg-shell precursor protein.

Key words: *Schistosoma mansoni*, egg-shell, male-female interaction, female-specific gene expression, pairing-dependent gene expression.

INTRODUCTION

Egg formation in trematodes has been considered to be a major target for chemotherapeutic or immunological approaches to control schistosomiasis, not only because egg production is essential to maintain the life-cycle of the parasite, but also because in schistosomes, eggs are the causative agent for pathogenesis (Capron *et al*. 2002). The eggs of schistosomes are composed of cells produced by 2 different organs, the vitellarium and the ovary. About 40 vitelline cells and 1 oocyte are combined to a single ovum within the ootype. The vitelline cells synthesize most of the molecules used for egg-shell formation and for nutrition of the embryo (Kunz, 2001). The contribution of the oocyte itself for embryonic development appears to consist mainly in providing the genetic material, some stored mRNA and ribosomes.

Schistosomes are unusual in that the vitelline cell differentiation in females is dependent on pairing with the male worms. Gene expression in the vitellaria of females is dependent on signals from the male sexual partner (Grevelding, Sommer & Kunz, 1997). If the female is separated from the male, gene expression is stopped and egg production ceases. If the female remates with a male, transcription in the vitellaria starts again, and egg production continues. Male-regulated gene expression in schistosomes has been shown for egg-shell proteins, ferritin and mucin (Grevelding *et al*. 1997). Here, we characterize another gene (p19) that is specifically expressed in the vitellaria of females, and that is regulated by the male.

MATERIALS AND METHODS

Parasites and nucleic acid extraction

Adult worms of the Liberia strain of *Schistosoma mansoni* were reared in Syrian hamsters and obtained by perfusion of the hepatic portal system (Duvall & DeWitt, 1967). Male and female worms were separated with fine brushes and stored in liquid nitrogen. DNA from female and male worms was obtained by a proteinase K-based extraction method (Grevelding, 1995). Total RNA was isolated using commercially available kits following the instructions of the manufacturer (RNeasy Kit, Qiagen). Poly(A)$^+$ RNA was isolated with 200 ml Dynabeads Oligo $(dT)_{25}$ solution according to the manufacturer's protocol (Dynal).

In vitro *culture*

Freshly perfused worm pairs were separated, washed 3 times in culture medium and incubated at 37 °C with 5% CO_2 in Petri dishes (Grevelding *et al*. 1997). After increasing numbers of days, samples of 10 females were used for RNA extraction.

Synthesis of cDNA libraries and differential hybridization

Samples of 5 μg of poly(A)$^+$ RNA from male and female worms were used for cDNA synthesis following the instructions for the ZAP synthesis kit

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¶ Sequences reported here are available from DDBJ/EMBL/GenBank under the Accession number Z27402.

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1 ACTACTTACAACTAGACATTATAGTAGAGTAGAGATTATTTCTAAAAAAAA
52 CAAAACA**ATG**AAAAGTGTTATCTCATTGTATTTATTAGTAATTATATCATG
M K S V I S L Y L L V I I S C
103 TACATTTGGCGGTGAATCTTATATACCATATACACCATATTCAAATGACAA
T F G G E S Y I P Y T P Y S N D N
154 TTATGGTACATATACTGATAATGGACAATATAACAATAATAATGATTATTA
Y G T Y T D N G Q Y N N N N D Y Y
205 TGGTGGAGATAAAAATCATGGCAATGATAATCATGGTAAAATACATACACA
G G D K N H G N D N H G K I H T Q
256 AGGCAAATTTATAGCATATGGTAATGCAGATAAAGGATTAAAATATCATCA
G K F I A Y G N A D K G L K Y H Q
307 AACTACATATTTCATAAAGGTAGTAAATATCAAAAGAAATTTGCTAATTAT
T T Y F I K V V N I K R N L L I M
358 GATACAAAAGGTAAAGTTCATTCATATGGTAACAAATGTAAAAGCTAAATT
I Q K V K F I H M V T N V K A K F
409 TAATATTGAAAGTAAATTATATTCTAAAGGAAAATATAATGGTGGTAAATT
N I E S K L Y S K G K Y N G G K L
460 GATCAACTTGCTGGTACTGGAAGTGGAACAATATAGTGAAGGACAAATACC
I N L L V L E V E Q Y S E G Q I P
511 AATTGAATATGCTGCATATGGAAGTGGTTCAACTAGTAATGCTCCAACAAA
I E Y A A Y G S G S T S N A P T N
562 TTATGATACTACTAATAATAATTATTATGATCAAAACAATGGAGAATCATC
Y D T T N N N Y Y D Q N N G E S S
613 AAATGATAATGGATATTCTGCTGACACTTATCAACCGCAATAT**TAA**AAAAA
N D N G Y S A D T Y Q P Q Y
664 TAAGAAAAAATTCAGTTTGACTAATAATAAGTAAGATATTATCAGAAAAAGT
715 CTAATACTAGTTTGTAAAGTGAACACATAATAATTAAAATAAATTGTTTAA
766 AG

Fig. 1. cDNA sequence of 3H11 and its open reading frame. The putative start and stop codon as well as the signal sequence are shown in bold.

(Stratagene). A subtractive female-specific cDNA library was constructed as described (Menrath, Michel & Kunz, 1995). In total, 600 colonies of this library were replica plated onto duplicate filters. Colony hybridization with radio-isotope labelled cDNA of male and female RNA was done according to Sambrook, Fritsch & Maniatis (1989).

Labelling and blotting of nucleic acids

Radioactive *in vitro* transcripts were obtained with T7 RNA polymerase using (alpha-^{32}P)dUTP. digoxigenin-labelled *in vitro* transcripts were synthesized following the manufacturer's protocol (Roche). Northern blotting was carried out as described earlier (Knobloch *et al*. 2002).

Sequence analysis

Nucleotide sequencing was performed with an ABI 373 DNA sequencer from Applied Biosystems, using the dye terminator cycle sequencing kit with AmpliTaq DNA Polymerase FS (Perkin Elmer). Amino acid analyses were done using the programs FastA, Blast and WinPep.

Expression of fusion protein, purification of antibodies and immunoblotting

The complete insert of clone 3H11 was recloned into the *Bam*HI/*Sal*I sites of the pUR 288 vector. Expression of recombinant beta-galactosidase was carried out as previously described (Köster *et al*. 1988). Correct cloning was confirmed by Western blotting. Antibodies were prepared from rabbit antisera against female schistosomes essentially according to Whitfield *et al*. (1988), but immunopurification was carried out on a bacterial lawn, not on single colonies. Preparation of worm extracts and immunoblotting were done as described (Wippersteg *et al*. 2002).

In situ *hybridization and immunohistology*

Adult parasites were fixed in Bouin's solution (picric acid/acetic acid/formaldehyde 15 : 1 : 5) and embedded in Paraplast. Sections were treated as described (Köster *et al*. 1988). Dig-transcripts were hybridized and detected as described (Finken *et al*. 1994). Anti-p19 antibodies (1 : 100) were detected with alkaline phosphatase-conjugated goat anti-rabbit IgG (Dianova) with naphthol-AS-MX-phosphate and Fast Red TR (Sigma) as substrate.

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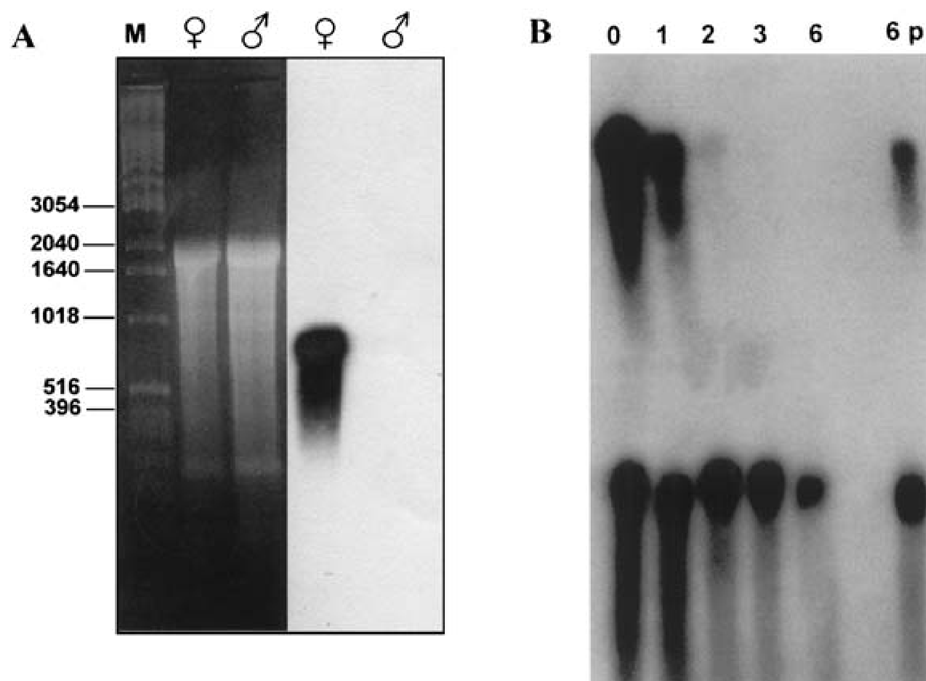

Fig. 2. (A) Northern blot analysis of 3H11. Total RNA from adult female and male schistosomes was separated on a denatured ethidium bromide agarose gel (left), blotted and hybridized with antisense transcripts of clone 3H11 (right). Molecular weight marker in nucleotides (M) is 1 kb DNA ladder (BRL). (B) Expression of p19 in females cultivated *in vitro* that were separated from males for 1, 2, 3, and 6 days, in comparison with freshly perfused females (0) and those that remained paired for 6 days (6p). Poly $(A)^{+}$ RNA was probed with antisense transcripts of 3H11 (top) and, as a control, with transcripts of the schistosome protein disulfide isomerase (bottom).

RESULTS

Isolation and characterization of clone 3H11

In order to isolate clones coding for female-specific sequences, a subset of 600 colonies of a subtractive cDNA library was screened with male and female cDNA. Eight clones not reactive with male cDNA were isolated, and the plasmid DNA reprobed with each male or female cDNA. Only 1 of the clones was found to hybridize exclusively with female cDNA. This clone, containing a 839 bp insert, was named 3H11 (accession number Z27402).

There was 1 long open reading frame starting with a methionine at position 59 of the cDNA insert. The deduced translation product of 199 amino acids (Fig. 1) had a calculated molecular mass of 22 kDa. Since the molecular mass on Western blots was found to be 19 kDa (see below), the protein was called p19. Frequent amino acids were Asn (13·3%), Tyr (11·6%) and Gly (9·6%). The base composition of the coding sequence was determined to be 73% A/T. At the 3′ end of the sequence, a poly(A) tail and a polyadenylation signal with the canonical consensus sequence of AATAAA were found.

Expression and regulation of p19 mRNA

We analysed the hybridization of *in vitro* generated antisense transcripts of 3H11 with total RNA prepared from male as well as female parasites (Fig. 2A). On Northern blots, a hybridization signal at the position of 850 nucleotides was detected only in the lane containing RNA from female worms demonstrating that the p19 gene is expressed exclusively in female schistosomes.

We studied the expression of the p19 gene in female worms that were cultured *in vitro*. Females separated from males were compared with paired females. RNA from worms of increasing separation times was probed with transcripts of 3H11 and, as an internal control, with transcripts of the schistosome protein disulfide isomerase (PDI), an ER-luminal enzyme responsible for protein folding (Finken *et al.* 1994). The signal on the Northern blot revealed that the expression of p19 decreased tremendously within the first 2 days after separation and totally disappeared after 3 days, whereas in paired females after 6 days of *in vitro* culture a signal was still detectable (Fig. 2B). In contrast, PDI mRNA in separated females showed no decrease in expression during culture. This demonstrates that, in comparison with PDI, the expression of p19 in females is dependent on pairing with a male.

Cytological localization and Western blotting

In situ hybridizations were carried out with antisense transcripts of clone 3H11. Only the cells of the

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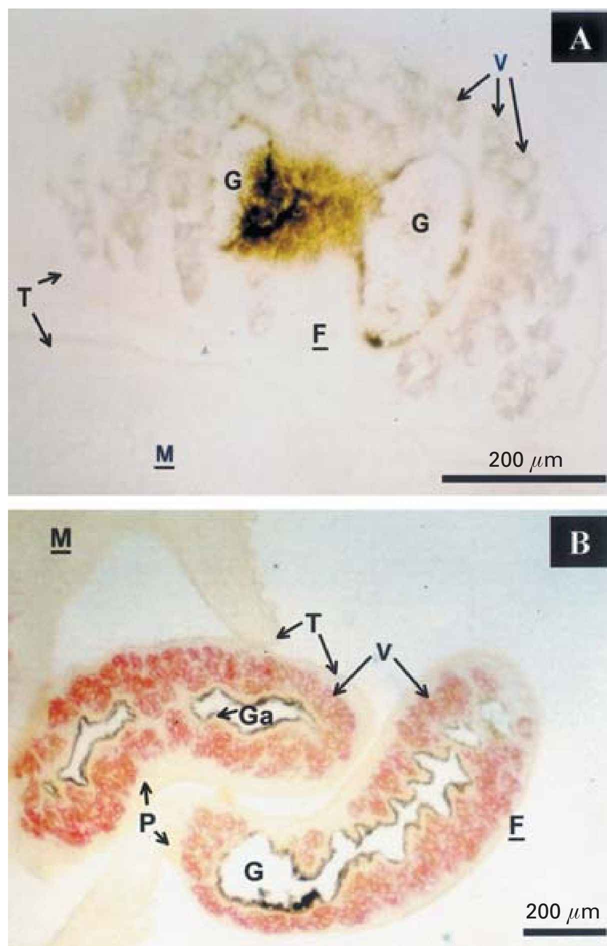

Fig. 3. (A) Localization of p19 mRNA in a cross-section of a schistosome female (F) showing greyish blue staining of the cytoplasm of the vitelline cells (V) with BCIP/NBT. The surrounding parenchyma and the tegument (T) are not labelled. The dark staining material in the gut (G) is digested haemoglobin. At the bottom left of the figure, part of a male (M) is shown. (B) Immunohistological localization of the p19 protein, stained with naphthol-AS-MX-phosphate and Fast Red TR in a tissue section documenting selective labelling of the cytoplasm of the vitelline cells (V) in the female. The surrounding parenchyma (P), the gastrodermis (Ga) and the tegument (T) are not labelled. At the upper left side of the figure, part of a male (M) is shown.

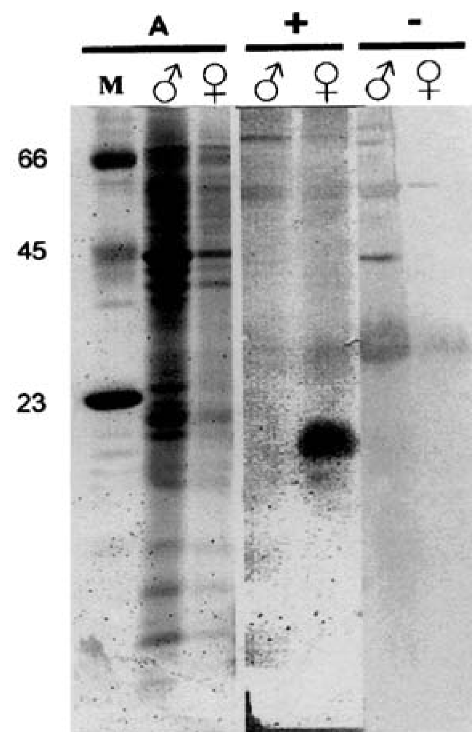

Fig. 4. Immunoblot of male and female proteins, extracted with (+) and without antioxidant (−), probed with the anti-p19 antibody showing a single band of 19 kDa only in females. Left: amidoblack-stained total protein extracts to document that the female lane is not overloaded (A). High molecular weight marker in kDa (M) (Sigma).

vitellaria were labelled (Fig. 3A). The label was clearly restricted to the cytoplasm of the cells. No hybridization signal was visible in the male nor in other tissues of the female, clearly documenting that the vitellaria are the only site of p19 expression. Sense transcripts as a negative control did not show any reaction (data not shown).

To obtain specific anti-p19 antibodies, antisera against protein homogenates of female schistosomes were affinity purified on the 3H11-beta-galactosidase fusion protein and, for further purification, post-adsorbed on beta-galactosidase. The size of the p19 protein predicted from the 3H11 sequence was expected to be 22·5 kDa. On Western blots of male and female proteins, the antibodies detected no proteins in this size range (Fig. 4). Since egg-shell precursor proteins are known to cross-link under oxidative conditions, we added the antioxidant n-propyl gallate (10 mM) during protein extraction (Köster *et al.* 1988). Under these conditions, the antibodies detected a prominent band of 19 kDa that was present exclusively in females. Some minor bands in the upper range were detected by the antibody in both the male and the female lane, and thus resulted from unspecific cross-reaction. Pre-immune sera, as a negative control, did not show any reaction (data not shown). This result confirmed that the p19 protein predicted from the cDNA is expressed in schistosomes. Computer-assisted analysis indicated that the amino terminus had a hydrophobic region of 18 amino acids that could function as a signal sequence for ER entry. Reduced by this signal sequence of 2 kDa, the calculated value falls into the size range seen on the Western blot.

The antibodies were used to localize the p19 protein in tissue sections. The label was clearly restricted to the cytoplasm of the vitelline cells only. No signal was visible in the nuclei (Fig. 3B). No other cells were labelled nor was there any reaction in the male. Again, pre-immune sera as a negative control did not show any reaction (data not shown).

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DISCUSSION

Several different genes are known in *S. mansoni* that are exclusively or preferentially expressed in the vitelline cells of mature females. Some with high probability code for egg-shell precursor proteins (Chen, Rekosh & LoVerde, 1992). Others code for the yolk protein ferritin (Schüssler *et al*. 1995), for phospholipid hydroperoxide glutathione peroxidase (Roche *et al*. 1996), or are of unknown function (fs800) (Reis *et al*. 1989). For the egg-shell precursor genes and for the ferritin gene it has been shown that their expression in the female depends on pairing with a male (Grevelding *et al*. 1997), whereas for the 2 other genes the situation has not yet been investigated. In this paper, we introduce a new gene (p19) whose activity is restricted exclusively to the vitellarium, and, in addition, it is expressed only in females that are paired with a male.

Since during egg formation in the ootype, the content of the vitelline cells completely merges with the zygote's cytoplasm to form the early embryo, one can assume that most gene products that are synthesized in the vitelline cells, have a function in egg or embryonic differentiation or nutrition.

On the basis of sequence alignments, no convincing information about the function could be obtained. Comparison of the deduced protein sequence with protein data banks showed weak similarity to the egg-shell precursor protein B1 of *Fasciola hepatica* (Zurita *et al*. 1987). Permutation analysis (Altschul & Erickson, 1985), however, revealed that there is no similarity in amino acid sequence, but instead a similarity in amino acid composition. This results from the high glycine and tyrosine content that the p19 protein and the B1 protein have in common. Therefore, the conclusion of a common evolutionary origin must not necessarily be justified.

A strong bias towards high glycine/tyrosine content and the frequent occurrence of the Tyr-Gly sequence motif are general characteristics of trematode egg-shell precursor proteins (Rice-Ficht *et al*. 1992; Bobek, Rekosh & LoVerde, 1991). This suggests that the p19 gene may code for an egg-shell precursor. This assumption is supported by the observation of cross-linking of the monomeric protein under oxidative conditions. Several of the other female-specific genes expressed in vitelline cells, as p14, p48 (Chen *et al*. 1992), and fs800 (Reis *et al*. 1989), all show multiple ORFs; this, however, is not the case in the p19 sequence.

The assumption that the p19 is an egg-shell precursor protein needs further substantiation. The most obvious and indispensable set of experiments here would be to analyse monomeric egg proteins isolated from schistosome egg-shells to compare the native protein sequence with the nucleotide sequence of the clone.

Note added after completion

As this work was nearing completion, a sequence of *Schistosoma japonicum* has been submitted to DDBJ/EMBL/GenBank under the Accession number AAP05897.1 that has 55·9% homology to p19 along its entire amino acid sequence (AliBee-Multiple Alignment, http://www.genebee.msu.su).

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