A female-specific cDNA sequence of *Schistosoma mansoni* encoding an amidase that is expressed in the gastrodermis†

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SUMMARY

A female-specifically expressed cDNA clone was obtained by screening of a subtractive cDNA library enriched for RNA from *Schistosoma mansoni* females. The deduced protein shows significant homology to a class of enzymes functioning as amidases. Northern blots revealed a transcript of 4·0 kb which is absent in larval stages, but is expressed in adult female worms. By *in situ* hybridization, the expression site of the gene was exclusively localized in the gastrodermis of female schistosomes. This is the first report of a female-specifically transcribed sequence of *S. mansoni* that is not expressed in the reproductive organs.

Key words: *Schistosoma mansoni*, amidase, female-specific cDNA sequence, gastrodermis, gut epithelium.

INTRODUCTION

Adult schistosomes are closely paired with each other for their entire life, and the development and fertility of the female are totally dependent on a continuous pairing with the male (Clough, 1981). Unpaired females remain stunted and sexually immature (Armstrong, 1965). In an effort to shed more light on the molecular mechanism of these observations, several studies have been aimed at the isolation and characterization of female-specifically expressed genes. All such genes identified to date are expressed exclusively in the reproductive organs of mature, egg-laying females. The majority encode for egg-shell precursor proteins that have been localized in the vitellarium (Bobek *et al*. 1986; Johnson, Taylor & Cordingley, 1987; Kunz *et al*. 1987; Köster *et al*. 1988; Reis *et al*. 1989; Chen, Rekosh & Loverde, 1992). Beyond that, female-preferentially expressed yolk ferritin has been identified as a component of the vitelline cells (Schüßler *et al*. 1995), whereas a female-specific mucin-like protein is expressed in the reproductive duct (Menrath, Michel & Kunz, 1995). Little is known of female-specifically transcribed genes that are active in other tissues.

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† The sequence reported herein has been submitted to EMBL Data Library and assigned the accession number Y12802.

We utilized a subtractive cDNA library enriched for RNA of female worms (Menrath *et al*. 1995) to screen for further female-specifically expressed cDNAs. In this paper we report on the molecular characterization and localization of a cDNA sequence of *S. mansoni* which encodes a putative amidase that is selectively expressed in the gastrodermis of female worms.

MATERIALS AND METHODS

Parasites

A Liberian strain of *S. mansoni* was maintained in *Biomphalaria glabrata* and in Syrian hamsters or gerbils (Grevelding, 1995). Recovery of worms was done by perfusion according to Duvall & DeWitt (1967). Male and female worms were separated with a fine brush and stored in liquid nitrogen. Cercarial shedding of infected snails was induced by exposure to bright light. Miracidia were hatched from eggs isolated from the liver of infected hosts by incubation in water at 37 °C and exposure to bright light. To collect larval stages, miracidia and cercariae were incubated in tubes on ice for 10 min. The larvae were directly transferred from the bottom of the tubes into liquid nitrogen.

Screening of cDNA libraries

A subtractive cDNA library, enriched for female RNA, was used for screening as described by Menrath *et al*. (1995) and Michel *et al*. (1995). Screening of the *S. mansoni* female cDNA library was performed as described by Michel *et al*. (1995).

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1 (  1) MVSPvemsnsavdsvLKVWKEYFVLLVRHPV--RHILVACSLGlVSWKIItSIRTYQNQKlLKSKQMRVTDNMEK
2 (  1) MVLS-----------EVWTT---LSGVSGVclACSLLSAAVV-LRWT---GRQKARGAA-TRARQKQ-RASLET
3 (  1) M----------------IF--YLVLLVLGAI-----AFYVHFS-NNRKKL--IERLEIVA--QRRRDDLSKNVEQ
4 (  1) M--P-----------QSWEE---LAADKRA-----RLAKTIP-DEWK-------------VQTLPAE--DSVID
5 (  1) M--P-----------SASWED---LAADKRA-----RLEKSIP-DEWK-------------FKSVPIE--GSVID

          ..+...  .*........  ....*. . .+*..+.+...++...*  ++ ..++ .++... *+.+....+...
1 ( 74) MREALT--KLTQPSVPMTICSE-NLSYLCEQIKKKRMTPVDVLHAFQFRALQLQDNNNSGIAlFILEAEECAAN-
2 ( 57) MDKAVQrfRLQNPDLDSEALLTLPLLQLVQKLQSGELSPEAVFFTYLGKAWEVNKGTNCVTS-YLTD---CETQ-
3 ( 50) ARKAAD--KL--DTQRRDWIGSLDFEQLRDELQRGHVTCVEAIRAYFHKAILAHEKTNAVTC-FILDAE-RQAEE
4 ( 41) FPKKSG--ILSEAELKITEASAADLV---SKLAAGELTSVEVTLAFCKRAAIAQQLTNCAHE-FFPDAALAQARE
5 ( 42) LPEKSG--ILSPSEIKITNSSATELV---AQLANGTLKSVDVTLAFCKRAALAHQLVNCAHD-FFPELALAQARE

          *........ .....*+*+*+++*+ +.+**++..++++ . ......+++....++..*.+.*..+.+++..+.
1 (161) LMK-FPMNIDKDSE-LYGIPISIKEGIAIRGYDATMGIIKRCNQPIDEDCVLIKVLKSVGAIPFVTTVTTQLCRT
2 (145) LSQ------APRQGLLYGVPVSLKECFSYKGHDSTLGLSLNEGMPSESDCVVVQVLKLQGAVPFVHTNVPQSMLS
3 (134) LDE-QAKLPYYVKPPLFGVPLSLKECLKVKGYDTTRGFVQDAYHPATEDSIQVEHYKKLGLIPFCQTNVPQSLLS
4 (123) LDEYYAKHKRPVG-PLHGLPISLKDQLRVKGYETSMGYISWLNKYDEGDSVLTTMLRKAGAVFYVKTSVPQTLMV
5 (124) LDRYFETHKKPVG-PLHGLPISLKDQLRVKGTETCMAYISWLGKRDTSDSILTALLRKAGAVFLVKTSVPQTLMV

          .+..+.+++.+.+*......+**+++*++.+++. *+.+*+*++*+*+**+*..*. +.*++*...**. .....
1 (218) LDGFHVIYNDAENPFNKSRLPGGSSSGEAVLLAQCGSPVGIGTDIAGSIRIPCAFCNLAGLKPTSGRLSlLRIVS
2 (194) FDCSNPLFGQTMNPWKSSKSPGGSSGGEGALIGSGGSPLGLGTDIGGSIRFPSAFCGICGLKPTGNRLS-KSGLK
3 (191) YNCSNPLFGTTTNPYDSTRTCGGSSGGEGALIGAGGSLIGIGTDVGGSVRIPCHFTGTAGIKPSKMRFA-HRGGG
4 (181) CETVNNIIGRTVNPRNKNWSCGGSSGGEGAIVGIRGGVIGVGTDIGGSIRVPAAFNFLYGLRPSHGRLP-YAKMA
5 (182) CETVNNIIGRTSNPRNLNLSCGGSSGGEGAMIAMRGGAIGIGTDIGGSIRVPAAFNSLYGIRPSHDRLP-YGGMT

          ... +.  *.. .**+.+.++... .++.++ .. *..  ++...*++*++....  ........*.++** .++
1 (293) TAKKSVLYISPCLGPMARKVDDLPCVMRALLCPTMFDL--DPYVIPMSFDQVSYE---GK-NRSQLVIGYYsNFD
2 (268) GCVYGQTAVQLSLGPMARDVESLALCLKALLCEHLFTL--DPTVPPLPFREEVYR------SSRPLRVGYY-ETD
3 (265) ASVPGKPLIDANDGPMAKDVKTNVEFLRNVWGDIDFQSdrDPYCPPVHWNESVYS------SEKKLRVGYY--ID
4 (255) NSMEGQETVHSVVGPITHSVEDLRLFTKSVLGQEPWKY--DSKVIPMPWRQSESDIIASKIKNGGLNIGYY-NFD
5 (256) NSMEGQETIHSVVGPIAHSAQDVRLFLQSVLKEEPWKY--DSKVIPLPWREAEENAAQAKIAEKSLNFAFY-DFD

          +  ..+.+.+ .*.+......*+..**.+.+*.   *.+..+..+ *  ... .+*+..+...+...+ .+*.+.
1 (363) DPNLIQVLDVnQRVVEKAAKALESAGHRLVKFTvphPYK-AFILG-L--HALFADGGQELRSHL-QGEPLSPHIK
2 (336) N-YTMPSPAM-RRALIETKQRLEAAGHTLIPFL---PNNIPYALEvLSAGGLFSDGGRSFLQNF-KGDFVDPCLG
3 (335) DGWFTPTPAL-QRAVLESKKHLEAAGHTVIPFY---PPRLPSVMQ-LYFRAVCLDGGQYVLNKL-LKDIIEPTIR
4 (328) G-NVLPHPPI-LRGVETTVAALAKAGHTVTPWT---PYKHDFGHD-LISHIYAADGSADVMRDISASG--EPAIP
5 (329) G-VVRPHPPI-TRGVEIVRSTLEKDGHTVAPWT---PYKHAFAVD-LANKIYAADGSTDVYKHINASG--EPAIP

           ...+.....  ..+.... ..*  .+. .. ...+... +... +....*.... ..... ... ........
1 (436) LINTITVIPDILKPIVEFISKHFIGKPVCPLGAFQKLASGQ-AAVNLISEIKAYRYDLQKLGMKQALDALICPVF
2 (404) DLILILRLPSWFKRLLSLLLKPLF-PRLAAFLNSMRPRSAE-KLWKLQHEIEMYRQSVIAQWKAMNLDVLLTPML
3 (403) FQVTLWMVPVWIQRILSY-PVSLVFPRMGMLMQSLTRDTFE--LREAYADIEAYREEFVGLMMKDNLDVILCPAS
4 (397) NIKDLLNPNIKAVNMNELWDTHLQ-KWNYQMEYLEKWREAEEKAGKELDAIIAPITPTAAVRHDQFRYY------
5 (398) NIKDLMNPNLPKADLNEVWDAQLQ-KWRYQCEYLDKWREWEERTGKELDAIIAPVAATAAVRHNQFRYY------

           .... ..... .. ....*+ +*+.*+*...++*++.....+.    ..... *.. ..+.. ..........
1 (507) PYPAPPKDAPQIyISPSIIYTFLYNILDYPAGAVPAGFVTEDDVrnslIKSESLYSS--GDFYMSKVYKLLD--G
2 (487) G-PALDLNTPGR-ATGAISYTVLYNCLDFPAGVVPVTTVTAEDD-----AQMELYKGyFGDIWDIILKKAMK--N
3 (485) IMPAPQHDIPSK-VVSGVSYTCLYNLLDFGAGVVPVTAVSKSDE----EKLINEYPE--TDKWYQITKKA--TLG
4 (468) ------------------GYASVINLLDFTSVVVPVTFA-DKNI----DKKNESFKA-VSEL-DALVQEEYDPEA
5 (469) ------------------GYATVFNVLDYTSVVVPVTYA-DKAV----DHRLADYQP-VSDM-DKAVYAEYDPEV

           .+.*+.++.++.+..++ .*.....++  ..*.+. . ..
1 (577) GENLPLSIQVVGKPYHEETVLRVMREIEscFSLQTESVISEeyfylflsvfplylilcrlcfstsilfmflklaf
2 (542) SVGLPVAVQCVALPWQEELCLRFMREVE---QLMTPQKQPS
3 (539) AVGMPIGVQVAAPPYREEAVLRTMREIE--IAVTGK-----
4 (512) YHGAPVAVQVIGRRLSEERTLAIAEEVG---KLLGNVVTP-
5 (513) YHGAPVAVQIIGRRLSEERTLAIAEYVG---KLLGH-----

1 (652) vlspsvlrsmvnvlyssfllitdiyffwsfsfrscdncvc
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Fig. 1. Multiple alignment of the predicted 25-5 polypeptide (1) with amidase amino acid sequences from *Rattus norvegicus* (2), *Caenorhabditis elegans* (3), *Emericella nidulans* (4), and *Aspergillus oryzae* (5). The alignment program of the GeneBee service was used (Belozersky Institute of Physico-Chemical Biology, Moscow State University; http://www.genebee.msu.su/services/malign_full.html). Point (.), weak similarity; cross (+), similarity or identity of amino acids with low score; star (*), identity or similarity of amino acids with high score.

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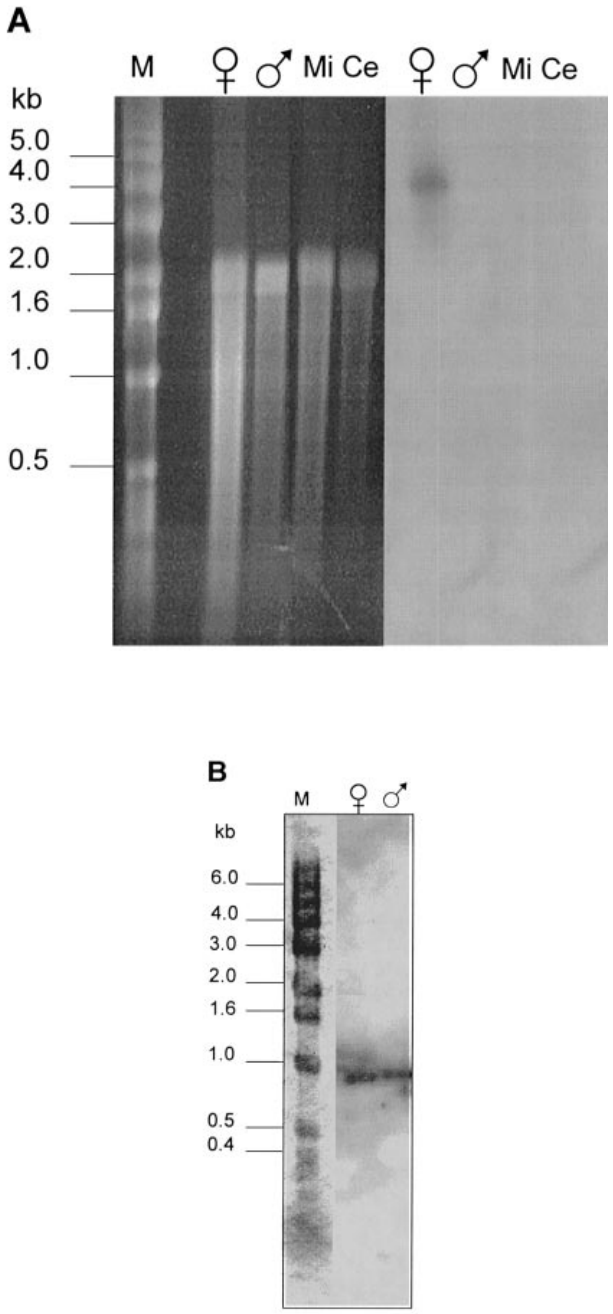

Fig. 2. Northern blot (A) and Southern blot analysis (B) of clone 25-5. (A) Approximately 20 μg total RNA of adult female and male schistosomes, miracidia (Mi), or cercariae (Ce) were separated on a 1·3% formaldehyde agarose gel. (B) 3·5 μg of genomic DNA from female and male worms, digested with *Eco*R I, were used for electrophoresis in 1% agarose. Both blots were hybridized with a ^{32}P-labelled 250 bp *Eco*R I/*Hin*d III fragment from the 5′-region of the 25-5 cDNA. The 1 kb-ladder was used as molecular weight marker (M).

DNA sequencing

Sequencing was done with double-stranded plasmid DNA templates isolated with the Qiagen BioRobot 9600 system (QIAGEN, Hilden). The nucleic acid composition of cDNA clones was determined by sequencing both strands with standard primers and primers specific for the insert sequence ('primer walking'). Reaction products were analysed with an ABI 373 DNA sequencer from Applied Biosystems, using the dye terminator cycle sequencing kit with AmpliTaq DNA Polymerase FS (Perkin Elmer).

Isolation of nucleic acids and blotting procedures

For the preparation of total RNA from the larval stages, 20000 cercariae, 100000 miracidia and about 1000 adult worms were used. The RNA was isolated by a guanidinium isocyanate method with modifications as described elsewhere (Schüßler *et al*. 1995). DNA was extracted by a proteinase K based method (Grevelding, 1995). Northern and Southern blots were performed on nylon membranes (Hybrid N, Amersham) using standard protocols. Hybridization procedures were carried out as described by Schüßler *et al*. (1995).

In situ *hybridization*

Adult worm pairs were fixed in Bouin's solution (picric acid/acetic acid/formaldehyde 15:1:5) and embedded in Paraplast. Sections for *in situ* hybridization were treated as described elsewhere (Köster *et al*. 1988). *In vitro* transcripts were labelled with digoxigenin according to the protocol of the manufacturer (Boehringer-Mannheim). The *in situ* hybridization was carried out following the procedure of Finken *et al*. (1994). Detection of hybrids was achieved with alkaline phosphatase-conjugated anti-digoxigenin antibodies, NBT and BCIP (West, Schröder & Kunz, 1990).

RESULTS

To isolate cDNA sequences that are female-specifically expressed, we analysed all 2547 primary clones from a subtractive library on triplicate filters (see Materials and Methods section). The first filter was hybridized to radioactive-labelled cDNA prepared from male worms, the second one to cDNA from females, and the third to DNA prepared from known clones which have previously been shown to be female specific (see Introduction section). In total, 56 clones were found to react neither with the male probe nor with the known female-specific sequences. A strong-reacting clone, termed 25-5, was selected for further investigation.

Sequencing of the clone 25-5 revealed a total length of 2191 bp. The insert contained an open reading frame that codes for a protein of 691 amino acids and ends with a stop codon at position 2087. The 3′-untranslated region of 67 bp includes the polyadenylation signal and terminates with a poly(A) tail of 39 nucleotides.

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Comparison of the deduced amino acid sequence of the clone 25-5 to protein data banks using the alignment algorithm BlastP, FastA, and Blitz revealed a significant similarity to amidases from different organisms. Highest scores were obtained with amidases of *Rattus norvegicus*, *Caenorhabditis elegans*, *Emericella nidulans* and *Aspergillus oryzae*. Fig. 1 shows a multiple alignment of these amino acid sequences and the predicted 25-5 polypeptide. Over a wide range, the sequences match stringently. However, the amidase of *S. mansoni* differs significantly at both ends. At the N-terminus, the alignment program inserts a gap of 11 amino acids, and at the C-terminus, the *S. mansoni* amidase possesses additional 74 amino acids. Furthermore, the alignment suggests that the methionine at position 1 is the potential start methionine. To document the percentages of identity and similarity of the schistosome sequence with the other 4 amidases, a pairwise alignment using the GENESTREAM SSEARCH program has been carried out (CRBM, Montpellier, France; http://genome.eerie.fr/bin/align-guess.cgi). The following identities/similarities have been found: 30·5 %/60·5 % with *R. norvegicus*, 29·8 %/60·2 % with *C. elegans*, 26·6 %/52·9 % with *E. nidulans* and 24·5 %/51·3 % with *A. oryzae*. The 74 amino acid C-terminus of the *S. mansoni* amidase has not been considered in this alignment.

Northern blot analysis was performed with total RNA from adult male and female schistosomes and from miracidia and cercariae. A transcript of about 4 kb was detected with RNA from adult females, but not in adult males or larvae (Fig. 2A). A repetition of this Northern blot experiment with poly(A^+) RNA from 80 adult females and 80 males confirmed the female-specific expression of the putative amidase of *S. mansoni* (not shown). A comparison of the length of the detected transcript with the cDNA clone 25-5 indicated that the 5′ untranslated region of the corresponding mRNA is probably about 1·5 kb.

By Southern blot hybridization using a 250 bp *Eco*R I/*Hind*III fragment from the 5′-region of the 25-5 cDNA, a band of about 0·9 kb was detected with *Eco*R I-restricted DNA (Fig. 2B). Since 25-5 possesses an *Eco*R I site at position 1354, the 0·9 kb band can only be explained by the existence of an additional *Eco*R I site within the amidase gene which must be located in an intron. From this result we conclude that the amidase gene is a single copy gene in *S. mansoni*. Southern blot further shows that the amidase gene is present in male worms although it is not transcribed in males. This excludes a W chromosomal localization.

In order to elucidate the role of the putative amidase in female worms, we localized the sites of gene expression by *in situ* hybridization. For this purpose, we used the full-length clone 25-5 to generate digoxigenated anti-sense transcripts. Sense transcripts of the same clone served as a negative control. In 2 independent experiments, using the anti-sense probe, only the gastrodermis (or gut epithelium) of females was found to be labelled (Fig. 3). No staining was detected in male worms.

DISCUSSION

In this paper we report on a gene in *S. mansoni* (clone 25-5) which is developmentally regulated and transcribed only in adult females. Remarkably, this gene is not expressed in cells of the reproductive organs, but in the gastrodermis. The deduced protein shows significant homology to a class of enzymes functioning as amidases.

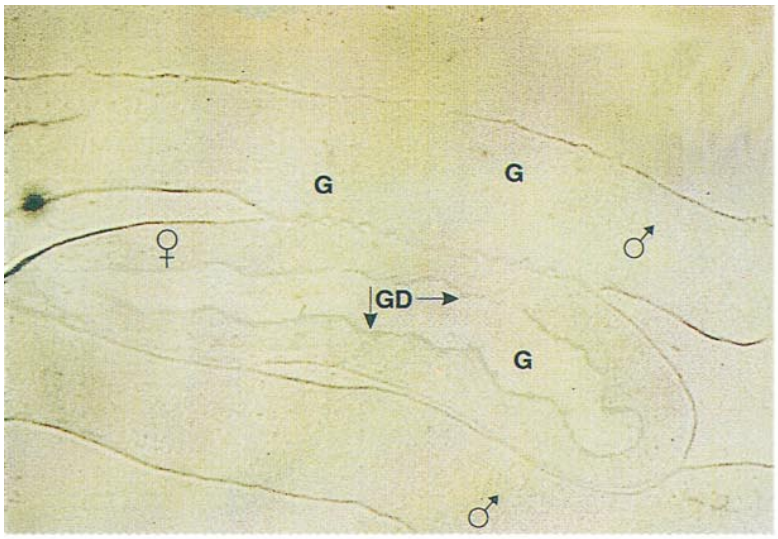
Amidases belong to the group of hydrolases and catalyse the reaction of an amide with water into a carboxy-acid and an amino-derivative (McGilvery, 1970). Typical members of the class of amidases are asparaginases, glutaminases, arginases or ureases. In contrast to most exo- and endo-peptidases, the substrates of amidases are restricted to several and specific amide compounds (Jakubke & Jeschkeit, 1982).

In eukaryotes, amidases play an important role in the digestion of protein during the catabolic pathway (Soberon & Gonzalez, 1987). Furthermore, several amidases stabilize proteins by an N-terminal amidation reaction (Baker & Varshavsky, 1995; Grigoryev *et al*. 1996). For example, an amidation of asparagine or glutamine at the N-terminus increases significantly the half-life of normally unstable proteins. Finally, amidases may represent an important component of the immuno-defence strategy due to their ability to split amide compounds specific for bacteria (Vanderwinkel *et al*. 1990, 1995).

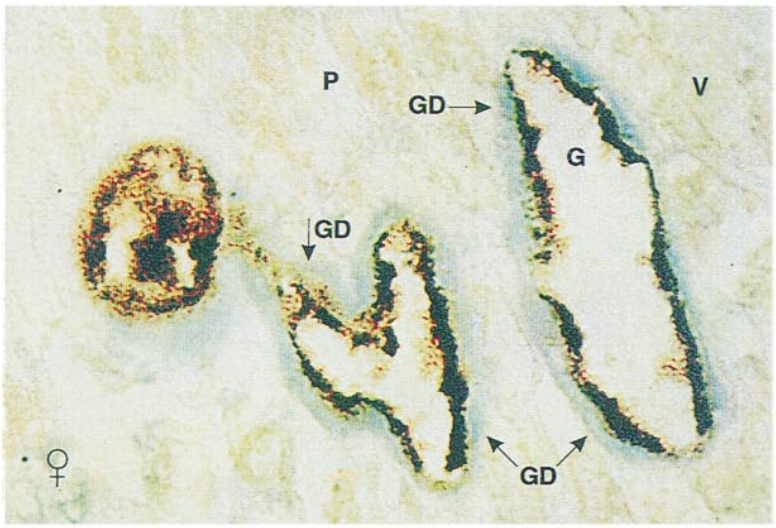
The amidase 25-5 of *S. mansoni* shows significant homology to the sequences of distantly related organisms such as yeast, nematodes or vertebrates. It aligns with amidases of different or unknown functions, making it impossible to specify its precise biochemical role. However, its localization exclusively in the gastrodermis suggests a role in digestion, as known for other eukaryotes.

The gastrodermis of schistosomes exhibits a syncytial structure and surrounds the gut as an epithelium (Morris, 1968; Spence & Silk, 1970; Bogitsh, 1991). It has been shown that the gastrodermis of schistosomes is involved in both absorption and secretion (Bogitsh & Shannon, 1971). The absorption of nutrients such as carbohydrates, lipids, or proteins is achieved by pinocytosis or specific carriers (Erasmus, 1972).

The amidase 25-5 of *S. mansoni* is regulated in a developmental and gender-specific manner. Transcripts are synthesized only in adult female worms. Female schistosomes are characterized by extended



A



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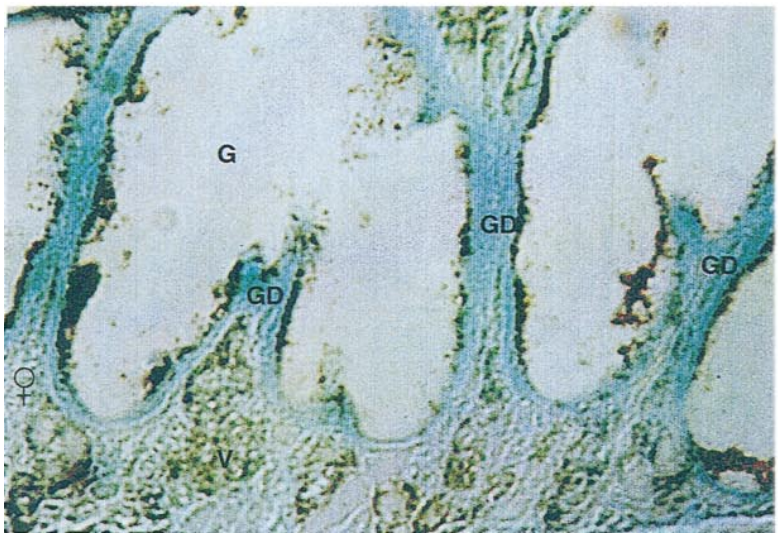

C

Fig. 3. Localization of 25-5 mRNA in the gastrodermis of female schistosomes by *in situ* hybridization. (A) Overview showing a section of a worm pair. Only the gastrodermis of the female reacted positively. In the section of the male, the gastrodermis is present but hardly visible since it does not hybridize with the probe. (B) Higher magnification of the gastrodermis and the surrounding tissue of a female worm. (C) The same obtained by an independent experiment. G, gut; GD, gastrodermis; P, parenchyma; V, vitellarium.

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metabolic activities to maintain the cell proliferation and differentiation processes in the reproductive organs. They feed much more on erythrocytes than males (Lawrence, 1973) and use the digested haemoglobin as a protein supply (Morris, 1968). The amidase that is selectively expressed in the gastrodermis of the female may be involved in these processes.

Future experiments will elucidate its precise role and investigate whether the transcription of 25-5 is regulated by pairing with the male, as has been demonstrated for other female-specifically expressed genes (Grevelding, Sommer & Kunz, 1997).

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