MOLECULAR APPROACH TO THE STUDY OF TREMATODE PARASITES : THE BLOOD FLUKE

Philip T. LoVerde

ABSTRACT

One important aspect of reproductive development in trematode parasites is the formation of a hardened eggshell which allows the zygote to develop into a miracidium in a hostile environment. The miracidium then can transfer the germline from the vertebrate host to snail intermediate host. Schistosome parasites, unlike other trematodes, have separate sexes and female reproductive development is known to depend on the presence of a male parasite. These facts make the blood flukes ideal material to study the mechanisms that underlie female reproductive development and eggshell formatian. We reasoned that the morphological and biochemical differences between the male and female must be reflected at the molecular level in the differential expression of sexually regulated genes. Radioactive single stranded cDNA was first transcribed from female RNA; and then sequences common to both male and female were removed by hybridization to an excess of male RNA. This probe was used to screen a cDNA library made from mRNA of adult worm paris. One hybridizing clone, pSMf 61-46, was shown to correspond to a 0.9 kilobase mRNA that is present only in mature female worms and is not detectable in female schistosomes from single-sex infections, in male worms or in eggs. Thus expression of the gene was female-specific. During normal bisexual infection this mRNA is first detected 28 days after infection (the time of worm pairing) and increases to a high level at 35 days postinfection, coinciding with egg production. Thus the temporal expression of the gene was dependent on paining with male worm. The nucleotide sequence of the gene shows an open reading frame that encodes a 16 kDA polypeptide that shows strong homology with eggshell proteins on insects. A second female-specific cDNA clone, F-4, represents a 1.6 kilobase mRNA whose expression is also correlated with worm pairing and subsequent egg production, encodes a different putative eggshell component of 44 kDA. The amino acid composition of the 16 kDA and 44 kDA polypeptiders show a strong correlation with the actual amino acid composition of the schistosome eggshell. Thus these two polypeptides appear to the major components of the schistosome eggshell. Analysis of the genomic arrangement of the eggshell genes show that p16 is represented by 5 gene copies and p48 is represented by 2-5 copies. The eggshell genes are expressed in the vitelline cell as recently demonstrated by in hybridization and immunocytochemical localization. The eggshell genes are being expressed in bacteria. The gene products will be used to study the biochemistry of eggshell formation.

INTRODUCTION

All trematode parasites have as their first intermediate host a mollusc. With few exceptions, the miracidium is the developmental stage that transfers the germline of the trematode parasite from the vertebrate host to the mollusc intermediate host. The development of the miracidium begins at fertilization with the zygote and subsequent formation of

* Department of Microbiology State University of New York Buffalo, New York 14214

the egg. Egg formation, as it is referred to in trematodes, is a complex process that results in the fertilized ovum (sometimes partly or fully embryonated) surrounded by a resistant polymer, the eggshell. The formation of the egg and the protective eggshell have been extensively reviewed.[1,2,3] The formation of the egg requires a large portion of the total energy budget (20-30%) of the trematodes.

The bulk of the material for the eggshel and for the yolk material is produced by vitelline cells. The structure and chemistry of the vitelline cells has been examined in a number of species.[2,3] The vitelline cells make up the vitellaria which is a characteristic and prominent structure in trematode parasites.

As reproductive development is a central issue for all trematode species, we began six years ago to study the molecular events that are important in reproduction, in particular, eggshell formation. We chose the blood flukes or the schistosomes as a model for this molecular approach because unlike other trematodes the sexes are separate, female reproductive development (formation of the vitellaria) is dependent on the presence of the male schistosome, and shistosomes can be cultured *in vitro*. Thus we could separate male and female physiological and biochemical processes and we could control the time at which these processes occur. Once the genes of interest were identified, we hoped to be able to study reproductive development in an *in vitro* system.

In schistosomes sex is determined in the zygote by a chromosomal mechanism. The zygote which is enclosed in the egg develops into the miracidium. The miracidium then transfers the germline from the human to the snail. Inside the snail the parasite undergoes asexual reproduction resulting in an amplification of the genome, such that thousands of cercariae are produced. Thus a single miracidium will give rise to a clonal population of cercariae all the same sex. Male schistosomes, apparently, undergo normal morphological development whether isolated from single sex or bisex infection although behavioral, physiological and antigenic differences between males from single vs bisex infections have been reported.[4,5,6,7]

Female worms from single sex infections on the other hand, do show distinct differences from females obtained from bisex infections.[7,8,9,10] Females from a single sex infection are underdeveloped in that they physically are stunted and sexually exhibit an immature reproductive system. The ovary, ootype and uterus are developed in the immature female.[9,10] However the vitellaria whose cells produce the eggshell precursors and nutrients for the egg is not developed. Female worm physical development and reproductive maturation is dependent on the presence of a mature male worm.[4,8,9,10,11,12] Continuous stimulation (contact) by the male worm is necessary for the female to maintain reproductive maturation and to continue egglaying.[7,13,14]

To initiate a study of the molecular mechanisms that underly female reproductive development, we reasoned that the morphological and biochemical differences between the male and female must be reflected at the molecular level in the differential expression of sexually regulated genes. Therefore, we used subtractive hybridization to create a probe that was female-specific. Poly A+ RNA was isolated from mature female worms obatained from bisexual infections and reverse transcribed into single stranded cDNA in the presence of a ^{32}-P-dCTP. Poly A+ RNA was isolated from male worms from single-sex infections. The single-stranded radioactive cDNA which represents the genes expressed by the mature female worm was

hybridized to an excess of the male poly A+ RNA which represents the genes expressed by the male worm. The cDNA:RNA hybrids that formed represented expressed genes common to both the male and female worms. The population of single-stranded radioactive cDNAs that remained represented female-specific genes. These cDNAs were used as a hybridization probe to screen a cDNA library made from mRNA of adult worm pairs. One hybridizing clone, pSMf 61-46, that was identified has been extensively characterized[15,16], The mRNA corresponding to pSMf 61-46 is 0.9 kilo bases in size. The mRNA is present only in steady state RNA of mature female worms and is not detectable in female schistosomes from single sex infection, in male worms from single or bisex infections or in eggs. Thus expression of this gene was female specific. To determine at what time during development this female specific mRNA could be detected we took advantage of the fact that gene expression was not detected in male worms or immature female worms. We exposed hamsters to male and female cercariae at different times during development. Then we isolated the worms and performednorthern blots on total RNA using pSMf 61-46 as a hybridization probe. During normal bisexual infections this mRNA is first detected 28 days after infection (the time of worm pairing), increases to a high level at 35 days postinfection, coinciding with egg production and plateaus at 45 days postinfection when egg production is constant. Thus the time during development when the gene was expressed corresponded to pairing with male worms and subsequent egg production.

The nucleotide sequence of the cDNA shows two open reading frames in the coding strand. One of the open reading frames encodes a 16 kD polypeptide that shows strong homology with the eggshell (chorion) proteins of insects. The amino acid composition of the putative eggshell protein contains 44% glycine, 11% tyrosine, 6% lysine, 6% cysteine, 1% histidine and little or no arginine, methionine or tryptophan. There is a strong correlation between this protein and the actual amino acid composition of the eggshell.[17] A hydropathic plot shows a hydrophobic amino terminus suggestive of a leader sequence on a molecule that is destined to be secreted. The remainder of the protein can be separated into at least two additional domains separated by a proline containing hinge region. The first domain consists of many repeats of gly-gly-gly-tyr and gly-gly-gly-cys, with aspartic residues dispersed throughout giving the domain a sinusoidal appearance. The second domain consists of glycine repeats containing lysines which makes it very hydrophilic.[16] Antisera made against a peptide from the lysine rich domain recognizes an eggshell protein of approximately 14 kD on a western blot (unpublished data). Recently Koster *et al*.[18] demonstrated by genetic engineering that the reading frame coding for the glycine- and tyrosine-rich polypeptide produced an antibody that recognized droplets in vitelline cells. This same antibody recognized a 14 kDa molecule on a western blot of a mature female extract and did not recognize any molecules in a male worm extract. To summarize, the amino acid composition, the homology to insect chorion protein, the recognition of a 14 kD eggshell protein by anti-peptide antisera, the recognition of vitelline cells by antibody made againts a recombinant fusion protein, and the biological context of expression all support the notion that pSMf 61-46 encodes an eggshell protein.

A second female-specific cDNA clone (F4) that represents 640 bp of the 3' end of a

corresponding mRNA estimated to be 1.5 kb in length does not cross hybridize with pSMf 61-46.[19] The deduced amino acid sequence also showed considerable homology with insect chorion genes and strongly suggested that it encoded a different eggshell component. This peptide has much less glycine but contains 24% tyrosine, 17% lysine, 17% aspartic acid and 15% histidine. Together this polypeptide and the one encoded by pSMf 61-46 appear to be the major components of the schistosome eggshell.[3,17] Interestingly, in *Fasciola hepatica* there seems to be three major eggshell components.[20]

S. *mansoni* female worms produce approximately 300 eggs per day. In order to maintain the level of reproductive activity, it has been estimated that the female worm converts nearly her own body weight into eggs every day.[21] The high demands of egg production dictate an active metabolic role for the vitelline gland. In order to maintain an output 300 eggs per day, each female worm is calculated to produce 11,000 mature vitelline cells per day.[7,22] This is consistent with estimates that the mRNA for the the glycine-rich eggshell protein makes up 5-10% of the total poly A+ RNA of the mature female worm.[15,23] That the mRNA is expressed and translated in vitelline cells and the gene product is associated with the vitelline droplets has been recently demonstrated by in situ hybridization and immunocytochemical localization.[18] As the male stimulus controls female vitelline development, it is not surprising to find this form of tissue-spesific expression.

Analysis of the genomic arrangement of the eggshell gene family represented by pSMf 61-46 shows a simple hybridization pattern with Eco R1 giving two fragments; 3.0 and 0.9 kbp. The genes do not amplify or rearrange during schistosome development[15,16].

Reconstruction experiments and densitometric scanning of genomic blots indicate five copies of the eggshell protein gene per haploid genome. Detailed restriction enzyme maps and positioning of gene sequences within six independent recombinant genomic clones allowed the generation of a linkage map of five overlapping clones spanning 35 kbp of S. *mansoni* genome with two eggshell genes closely linked, separated by 7.5 kbp of intergenic DNA. The two genes of the cluster are organized in direct orientation, that is they are transribed from the same strand. The sixth clone represents a third copy of the eggshell gene.[24]

The 5' end of the eggshell protein mRNA was defined by primer extension and the cap-site sequence by direct RNA sequencing. The ATCAT cap-site sequence is homologous to a silkmoth chorion PuTCATT cap-site sequence.[25] DNA sequence analysis showed that there are no introns in these genes and thus the mRNA is not spliced. DNA sequences of three genes are very homologous to each other and to cDNA pSMf 61-46, differing only in three or four nucleotides which do not alter the open reading frame. A multiple TATA box is located at positions -23 to -31 and CAAT sequence at -50 upstream of the eggshell protein transcription unit. S. *mansoni* eggshell genes like chorion genes of Drosophila and silkmoth are developmentally regulated showing sex-, tissue- and temporal-specific expression. Sequence comparison of further upstream sequences to those of silkmoth and Drosophila revealed several short elements that are shared. One such element, TCACGT, has beem shown to be essential for sex, tissue and temporal gene expression in Drosophila and silkmoth.[26,27] This element with a reversed polarity, is found in all S. *mansoni* eggshell protein genes sequenced to date.[24,28] A very similar ele-

ment of sequence, TCACGCT, is located at -157 from the cap-site. Two other elements, GTAGAAT at position -215 and AGTGTAT-TC at position -298, are homologous to those found in Drosophila or silkmoth chorion genes. These cis-acting homologous elements are thought to bind trans-acting factors that regulate chorion gene expression in Drosophila and silkmoths.[26,27]

In the silkmoth choriogenesis (eggshell formation) occurs over a 55 hour period, involves approximately 200 genes in three gene families that are clustered as tandem duplications in a giant locus, exceeding a million base pairs. The very high rates of chorion gene expression required for eggshell synthesis are achieved by the redundancy of the chorion locus.[29,30] In Drosophila choriogenesis occurs over a 5-6 hour period, involves approximately 20 genes expressed in about 1,000 follicle cells that surround each maturing oocyte. The chorion genes are located in two clusters of about 10 kilobasepairs. The high rates of chorion gene expression in Drosophila are achieved by specific amplification of chorion genes and their flanking sequences during development.[29,31] In schistosomes eggshell formation is continuous (but male-dependent), involves at least two different genes that are each present in a few (five) copies on chromosome 3 (unpublished data). The eggshell genes are expressed in vitelline cells of which 11,000 are produced and released by the vitellaria every day. Thus the high rate of gene expression in schistosomes seems to be due to the large number of vitelline cells produced each day rather than by gene amplification or a large gene family. The eggshell genes of schistosomes represent one example of a molecular approach to the study of trematode reproductive development.

REFERENCES

1. Smyth,J.D. and J.A. Clegg (1959). Egg-shell formation in trematodes and cestodes. *Experimental Parasitology* 8: 286-323.
2. Smyth, J.D. and D.W. Halton (1983). The physiology of trematodes. *2nd Edition, Combridge University Press, New York*, 446 pgs.
3. Cordingley,J.S. (1987). Trematode Eggshells : Novel Protein Biopolymers. *Parasitology Today* 3: 341-344.
4. Armstrong,J.C.(1965). Mating behavior and development of schistosomes in the mouse. *Journal of Parasitology* 51: 605-615.
5. Aronstein, W.S., and M.Strand (1984). Gender-specific and pair dependent glycoprotein antigens of *Schistosoma mansoni. Journal of Parasitology* 70: 545-557.
6. Cornford, E.M., and A.M.Fitzpatrick (1985). The mechanism and rate glucose transfer from male to female schistosomes. *Molecular and Biochemical Parasitology* 17: 131-141.
7. Popiel,I.(1986). The reproductive biology of schistosomes. *Parasitology Today* 2: 10-15.
8. Moore, D.V., T.K., Yolles, and H.E. Meleney (1954). The relationship of male worm to the sexual development of female *Schistosome mansoni. Journal of Parasitology* 40: 166-185.
9. Erasmus,D.A. (1973). A comparative study the reproductive system of mature, immature and 'unisexual' female *Schistosome mansoni. Parasitology* 67: 165-183.
10. Shaw, J.R.(1977). *Schistosoma mansoni* : Pairing *in vitro* and development of females from single sex infections. *Experimental Parasitology* 41: 54-65.
11. Erasmus,D.A., and J.R.,Shaw (1977). Egg production in *Schistosoma mansoni. Transactions of the Royal Society of Tropical Medicine and Hygiene* 71: 289.
12. Basch, P.F., and W.D. Rhine (1983). *Schistosoma mansoni* : Reproductive potential of male and female worms cultured *in vitro. Journal of Parasitology* 69: 567-569.

13. Clough,E.R. (1981). Morphology of reproductive organs and oogenesis in bisexual and unisexual transplants of mature *Schistosoma mansoni* females. *Journal of Parasitology* **67**:535-539.

14. Popiel, I.(1986). The reproductive development of female *Schistosoma mansoni* (Digenea: Schistosomatidae) following bisexual pairing of worms and worm segments. *Journal of Experimental Parasitology* **232**: 141-150.

15. Bobek,L.A., D.M. Rekosh, H., van Keulen., and P.T. LoVerde (1986). Characterization of female-specific cDNA derived from a developmentally regulated mRNA in the human blood fluke *Schistosoma mansoni*. *Proceedings of the National Academy of Sciences USA* **83**: 5544-5549.

16. Bobek L.A., P.T. LoVerde., H., van Keulen., and D.M. Rekosh (1987). A developmentally regulated genen in *Schistosoma mansoni* that may encode an eggshell protein. *UCLA Symposium on Molecular and Cellular Biology, New Series* **42**; 133-143.

17. Byram, J.E., and A.W. Senft (1979). Structure of the schistosome eggshell: Amino acid analysis and incorporation of labelled amino acids. *American Journal of Tropical Medicine and Hygiene* **28**: 539-547.

18. Koster, B., H. Dargatz., J. Schroder. J., Hirzmann., C.Haarmann., P.Symmnons and W.Kunz (1988). Identification of the products of a putative eggshell precursor gene in the vitellarium of *Schistosoma mansoni*. *Molecular and Biochemical Parasitology*, in press.

19. Johnson,K.S., D.W. Taylor., and J.S.Cordingley (1987). Possible eggshell protein gene from *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* **22**: 89-100.

20. Waite, J.H. and A.C. Rice-Ficht (1987). Presclerotized eggshell protein from the liver fluke *Fasciola hepatica*. *Biochemistry* **26**: 7819-7825.

21. Becker, W.(1977). Zur Stoffwechselphysiologie der Miracidien von Schistosoma mansoni wahrend ihrer Aktivierung innerhalb der Eischale. *Zeitchrift fur Parasitenkunde* **52**: 69-79.

22. Erasmus, D.A., and J.R. Shaw (1977). *Schistosoma mansoni* and *S.Haematobium*: calcium metabolism of the vitelline cell. *Experimental Parasitology* **47**: 91-106.

23. Simpson,A.J.G., and M.Knight (1985). cloning of a major developmentally regulated gene expressed in mature females of *Schistosoma mansoni*. *Molecular and Biochemical Parasitology*, **18**: 25-35.

24. Bobek,L.A., D.M. Rekosh and P.T. LoVerde (1988). Small gene family encoding an eggshell (chorion) protein of the human parasite *Schistosoma mansoni*. *Molecular and Cellular Biology* **8**: 3008-3016.

25. Mitsialis,S.A., and F.C. Kafatos (1985). Regulatory elements controlling chorion gene expression are conserved between flies and moths. *Nature (London)* **317**: 453-456.

26. Mitsialis,S.A., N. Spoerrel., M. Leuiten and F.C.Kafatos (1987). A short 5' flanking DNA region is sufficient for developmentally correct expression of moth chorion genes in Drosophila. *Proceeding of the National Academy of Sciences USA* **84**: 7987-7991.

27. Mariani,B.D., J.R. Lingappa and F.C.Kafatos (1988). Temporal regulation in development: Negative ad positive cis regulators dictate the precise timing of expression of a *Drosophila* chorion gene. *Proceedings of the National Academy of Sciences USA* **85**: 3029-3033.

28. Kunz,W., K.,Opatz. M. Finken and P.Symmons (1987). Sequences of two genomic fragments containing identical coding region for putative eggshell precursor protein of *Schistosoma mansoni*. *Nucleic Acids Research* **15**: 5894.

29. Kafatos,F.C. (1983). Structure, evolution, and developmental expression of the chorion multigene families in silkmoths and Drosophila. *In* "Gene structure and regulation in development". (S. Subtelny and F.C. Kafatos,Eds.), pp. 33-61. A.R. Liss, New York.

30. Goldsmith, M.R., and F.C. Kafatos (1984). Developmentally regulated genes in silkmoths. *Annual Reviews of Genetics* **18**: 443-487.

31. Mahowald,A.P., and M.P.Kambysellis (1980). Oogenesis. *In* "The genetics and biology of Drosophila" (M.Ashbuner and T.R.F. Wright Eds.), pp. 141-224. Academic Press, New York.

QUESTIONS AND ANSWERS :

1. Question: 1. What was the reason that the immature one failed to hybridize.
   2. I did not quite get whether you mentioned also, the function of the cysteine-domain compaired to/relative to the lysine domain of the gene.

   Answer : 1. The gene does not appear to be expressed in immature female worms. The expression of the gene is related to mating with the male parasite.
   2. The cysteine domain contains sequences that give the protein a Beta sheet structure and contains a number of amino acids that may participate in crosslinking. This crosslinking gives the eggshell its rigidity.