Published in final edited form as:

Int J Parasitol. 2011 January ; 41(1): 61–70. doi:10.1016/j.ijpara.2010.07.015.

Non-random organization of the *Biomphalaria glabrata* genome in interphase Bge cells and the spatial repositioning of activated genes in cells co-cultured with *Schistosoma mansoni*

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Abstract

Biomphalaria glabrata is a major intermediate host for the parasitic trematode *Schistosoma mansoni*, a causative agent of human schistosomiasis. To decipher the molecular basis of this host-parasite interaction, the Bge embryonic cell line provides a unique in vitro model system to assess whether interactions between the snail and parasite affect the cell and genome biology in either organism. The organisation of the *B. glabrata* genome in Bge cells was studied using image analysis through positioning territories of differently sized chromosomes within cell nuclei. The snail chromosome territories are similar in morphology as well as in non-random radial positioning as those found in other derived protostome and deuterostome organisms. Specific monitoring of four gene loci, *piwi*, *BgPrx*, *actin* and *ferritin*, revealed non-random radial positioning of the genome. This indicates that specific parts of the snail genome reside in reproducible nuclear addresses. To determine whether exposure to parasite is reflected in genome organization, the interphase spatial positioning of genes was assessed after co-culturing Bge cells with either normal or irradiation attenuated miracidia for 30 min to 24 h. The loci of *actin* and *ferritin*, genes that are up-regulated in the snail when subjected to infection, were visualized by fluorescence in situ hybridisation (FISH) and their radial nuclear positions i.e. their position in the interphase nucleus with respect to the nuclear edge/envelope, mapped. Interestingly, large scale gene repositioning correlated to temporal kinetics of gene expression levels in Bge cells co-cultured with normal miracidia while irradiated parasites failed to elicit similar gene expression or gene loci repositioning as demonstrated using the *ferritin* gene. This indicates that normal but not attenuated schistosomes provide stimuli that evoke host responses that are reflected in the host's nuclear architecture. We believe that this is not only the first time that gene-repositioning studies have been attempted in a mollusc but also demonstrates a parasite influencing the interphase genome organisation of its host.

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Keywords

Biomphalaria glabrata; *Schistosoma mansoni*; Bge; Chromosome territories; Interphase nuclei; Gene repositioning; Fluorescence in situ hybridization (FISH); Genome

1. Introduction

Schistosomiasis is a tropical disease that remains endemic in about 75 countries worldwide (Chitsulo et al., 2004; Gryseels et al., 2006). By using mass chemotherapy and molluscicides, efforts are being made to limit disease transmission in some of these countries, but persistent poverty, inadequate supply of clean water, poor sanitation and the expansion of irrigation projects and dam construction, all contribute in making schistosomiasis a disease that is difficult to control. In view of these challenges, alternative methods that will lead to the long-term control of schistosomiasis are being sought. These include the development of a protective vaccine and elucidating the molecular basis of the snail host-parasite relationship. It is hoped that a better understanding of this relationship will enable us to block transmission of the parasite during the intra-molluscan stage of its development (Han et al., 2009).

The host-parasite interaction between *Biomphalaria glabrata* and *Schistosoma mansoni* is initiated when a miracidium enters the snail via the surface epithelium and subsequently develops into a primary sporocyst. Naturally, the field of research into this relationship has focused on the genetic factors and potential markers that lead to parasite elimination in some snails and not in others (Knight and Raghavan, 2006). *Schistosoma mansoni*'s specificity for the *Biomphalaria* sp. points towards a co-evolutionary mechanism shaping the genetic makeup of both organisms (Van Valen, 1973; Webster et al., 2007). An elucidation of the genetic factors which influence resistance and susceptibility in the snail host has come from research investigating the transcriptional modulation of genes in the snail upon infection (Miller et al., 2001; Hertel et al., 2005; Lockyer et al., 2008). Investigations into *B. glabrata*'s relationship with *S. mansoni* have been aided by the development of an in vitro tissue culture model to support the intramolluscan sporocyst stage of *S. mansoni* (Yoshino and Laursen, 1995; Castillo and Yoshino, 2002) and in vitro development of cercaria (Basch and DiConza, 1977). Such a model system exists in the Bge cell line established in the 1970s from macerated embryonic tissue that spontaneously 'immortalized' (Hansen, 1976). The Bge cell line is able to maintain primary sporocysts and allow development of secondary sporocysts via co-culturing (Coustau et al., 1997; Laursen and Yoshino, 1999; Castillo et al., 2007) and supports the continuous in vitro propagation and differentiation of the intramolluscan stages of *S. mansoni* (Ivanchenko et al., 1999; Coustau and Yoshino, 2000; Kapp et al., 2003). Interestingly, bringing the cells together with parasite or parasite products gives rise to alterations in gene expression in Bge cells. Indeed, Humphries and Yoshino utilized the excretory and secretory (ES) products from *S. mansoni* to stimulate the p38 signalling pathway in Bge cells (Humphries and Yoshino, 2006). These cell cultures are also amenable to RNA interference (RNAi) using double stranded RNA as demonstrated by the knockdown of fibrinogen-related protein 2 (FREP 2) gene expression (Jiang et al., 2006). In terms of gene expression, it is not just the host-parasite relationship that has been investigated in Bge cells but also stress responses such as heat-shock (Laursen et al., 1997; Yoshino et al., 1998) and chemokinetic/tactic response to molecules such as cytokines (Steelman and Connors, 2009). Even though Bge cells show extensive aneuploidy in cell line isolates to the extent that the total complement of chromosomes greatly exceeds the original cell line's diploid number of 36 chromosomes (Odoemelam et al., 2009), they provide a manageable and responsive in vitro model system in which to study molluscan host-parasite interactions, stress responses and chemotaxis. Here we utilize the Bge cell in

vitro co-culture system to determine spatio-temporal affects on specific genes in the nuclei of Bge cells that have been co-cultured with *S. mansoni* miracidia.

The cell nucleus is a highly organized structure, with complex and dynamic architecture that controls the behaviour and function of the genome through regulating gene expression. Interphase chromosomes are not found in an unravelled state but as individual entities known as chromosome territories (Schardin et al., 1985; Cremer et al., 1993; Kurz et al., 1996; Zink et al., 1998; Croft et al. 1999; Foster and Bridger 2005; Meaburn and Misteli 2007; Meaburn et al., 2008; Cremer and Cremer, 2010; Mehta et al., 2010). The highly compartmentalized structure of the eukaryotic cell nucleus and the dynamic organization of chromosome territories and the gene loci within them, is believed to play an integral role in controlling gene expression (Kumaran et al., 2008). In a change in status to a cell that requires or induces altered gene expression, chromosome territories and/or individual gene loci within nuclei can be functionally and spatially repositioned i.e. during differentiation (Skalnikova et al., 2000; Kosak et al., 2002; Chambeyron and Bickmore, 2004; Kuroda et al., 2004; Foster et al., 2005; Ragoczy et al., 2006; Szczerbal et al., 2009; Solovei et al., 2009), in disease (Cremer et al., 2003; Zink et al., 2004; Meaburn et al., 2007, 2008; Li et al., 2009), and in cellular proliferation (Bridger et al., 2000; Branco et al., 2008; Mehta et al., 2010). This can either be due to whole chromosome territories being repositioned or due to activated gene loci looping away from the main body of the chromosome territory. A number of studies using mammalian models have correlated regional positions of gene loci within the nucleus with levels of gene expression (Volpi et al., 2000; Mahy et al., 2002; Williams et al., 2006; Finlan et al., 2008; Meaburn and Misteli, 2008; Takizawa et al., 2008a; Ballabio et al., 2009; Szczerbal et al., 2009), with activation of a gene being correlated with the movement of that gene towards the nuclear interior (Takizawa et al., 2008b; Denaid and Bickmore, 2009; Elcock and Bridger, 2010). During embryogenesis, a vital transcription factor Mash (AsII), is required for the production of neuronal precursor cells (Williams et al., 2006). In embryonic stem cells the *Mash 1* gene is transcriptionally repressed, but during neuronal differentiation it is preferentially repositioned from the nuclear periphery to the nuclear interior and transcription is up-regulated by more than 100 fold (Williams et al., 2006). Indeed, the impact of cellular differentiation upon specific gene loci repositioning and transcriptional activation has also been reported in porcine mesenchymal stem cells (Szczerbal et al., 2009). Seven genes involved in adipogenesis were found to adopt a more internal position upon the induction of adipogenesis and transcriptional up-regulation. In support of gene activation being connected to a movement of gene loci to the nuclear interior, one study by Takizawa et al. (2008a) reveals that copies of mono-allelically expressed genes localise in different nuclear compartments relative to each other, with the active allele more centrally located in nuclei.

Historically, the nuclear periphery has been associated with down-regulation of gene activity and gene silencing since there are many data that support repositioning of genes to the nuclear periphery with gene inactivation and transcriptional repression (Shaklai et al., 2007). When genome-wide screens of chromatin attached at the nuclear periphery were performed, the resultant fractions were generally gene-poor regions of the genome and heterochromatic in both *Drosophila* and human cells (Pickersgill et al., 2006; Guelen et al., 2008). However, as a caveat towards the dogma of the nuclear periphery being an exclusive region of gene suppression, there are active genes present at the nuclear edge and some active genes that are relocated to the nuclear periphery remain actively transcribed (Denaid and Bickmore, 2009). For example, the *INF- γ* gene in mice is located at the nuclear periphery depending on whether it switched on or off (Hewitt et al., 2004). In yeast and *Drosophila*, transcriptionally active genes have been found located around the nuclear pore complexes (Brown and Silver, 2007). This phenomenon is reminiscent of the gene-gating theory put forward by Blobel in the 1980s (Blobel, 1985) which states that the genome of a higher eukaryotic organism is

organized into a number of distinct three-dimensional (3-D) structures, each characteristic for a given differentiated state and the nuclear pore complexes are envisioned to serve as gene-gating structures. It is assumed that transcripts of a given gated gene would leave the nucleus by way of this complex.

Very little is yet known about how molluscs organize their genome in interphase nuclei. However, since organisms such as *Hydra* (Alexandrova et al., 2003) and *Caenorhabditis elegans* (personal communication Dr. Kentaro Nabeshima, University of Michigan, USA) contain individual chromosome territories, we predicted that molluscs would too, as they are more organized and rank higher in the evolutionary scale. Indeed, in one of the very few studies that has assessed interphase genome organisation in molluscs, the red abalone mollusc displays telomeric repeats throughout interphase nuclei that strongly suggests a non-Rabl distribution of chromosomes (Gallardo-Escarate et al., 2005). If it were a Rabl distribution the telomeric repeats will be present only at one side of the interphase nuclei and the centromeres at the other. In the non-Rabl model, both centromeres and telomeres are dispersed throughout the interphase nucleus, except in the area occupied by the nucleolus. Such a distribution was also corroborated by studies in the king scallop where repetitive DNA was found localized in discrete regions, also throughout interphase nuclei (Biscotti et al., 2007). However, we cannot speculate on whether these territories in these molluscs might be organized in a non-random or random fashion, and if non-random, whether it is a radial organization as seen in more complex organisms such as chordates and correlated with gene density or chromosome size, as in mouse and human nuclei (Boyle et al., 2001; Bolzer et al., 2005; Foster and Bridger, 2005; Mayer et al., 2005; Mehta et al., 2007, 2010; Meaburn et al., 2008).

However, using Bge cells, we have determined for *B. glabrata* that the interphase chromosomes are non-randomly organized as individual territories in a radial distribution, as were the specific gene loci for *actin*, *ferritin*, *piwi* and *peroxiredoxin*. We further determined that there is specific temporal repositioning or relocation of gene loci within interphase nuclei after in vitro schistosom exposure. We found significant gene loci repositioning within nuclei tightly correlated with gene expression, with one gene (*ferritin*) moving to the nuclear interior and one gene (*actin*) moving to the nuclear periphery concurrently with up-regulated gene expression. These dynamics for *ferritin* gene loci were not elicited when radiation attenuated parasites were similarly co-cultured with Bge cells.

2. Materials and methods

2.1. Culturing of Bge cells

Bge cells used in this study were derived from Hansen's original Bge cell line (Hansen, 1976), and were grown in the absence of carbon dioxide, at 26°C in sterile medium that comprised 220 ml/L (v/v) Schneider's *Drosophila* medium (Invitrogen), 1.3 g/L galactose (Invitrogen), 4.5 g/L lactalbumin hydrolysate (Invitrogen), 8.1 mg/L phenol red and 50 µg/ml gentamicin (Invitrogen). The Bge medium was made complete by adding 10% (v/v) heat inactivated FBS (Hyclone).

For the parasite co-culture experiments, 3–4 day old Bge cells (1.2×10^6 cells/flask) in T25 flasks were exposed to 50–100 *S. mansoni* miracidia (either normal or irradiated) for 0, 0.5, 2, 5 and 24 h. Irradiation of miracidia (20 Krad) (which did not alter the ability of penetration and was similar to that observed in normal miracidia) was performed as described by Ittiprasert et al. (2009). *Schistosoma mansoni* miracidia were isolated under sterile conditions in the laminar hood to prevent contamination of tissue culture cells. All reagents used in the process of isolating miracidia contained gentamicin (50 µg/ml), penicillin (50–100 µg/ml) and streptomycin (50–100 µg/ml). At the end of the incubation

period, parasites were removed by pipetting without disturbing the Bge cells. The cells were washed while attached to the flask by rinsing with Bge culture medium without FBS and removing the wash fluid with a pipette. The entire flask was examined thoroughly for any residual parasite material after each wash and washed until free of parasite. The cells were then detached from the flask using a cell scraper and centrifuged at 90 g for 10 min at 15°C. The pellet was re-suspended in 5 ml of hypotonic potassium chloride solution (0.05 M) for 15 min at room temperature and centrifuged at 90g for 10 min at 15°C. The cells were subsequently fixed with ice-cold methanol and acetic acid (3:1 v/v) for 1 h on ice. The fixed cells were washed twice with ice-cold methanol and acetic acid (3:1 v/v) and stored at 4°C till further use. The cells were then dropped onto damp glass microscope slides (Odoemelum et al., 2009) to prepare both interphase nuclei and/or metaphase spreads samples for Fluorescence in situ hybridization (FISH).

2.2. Incorporation and visualization of Bromodeoxyuridine

Bge cells were grown on sterile 13 mm diameter circular glass coverslips and seeded at a density of 2.5×10^5 . The cells were grown in complete Bge medium overnight before the addition of 0.1% 5-bromo-2-deoxyuridine (BrdU) and 5-fluoro-2'-deoxyuridine (FrdU) (Sigma-Aldrich) for 48 h after which the medium containing the BrdU and FrdU was removed and replaced with complete Bge medium. The Bge cells were subsequently allowed to grow for 10 days in complete Bge medium (devoid of BrdU and FrdU) before fixation.

The Bge medium was removed from the dish and the cells washed thrice with 1 X PBS, (10 mM). The cells were then fixed with 10 ml of ice cold 1:1 methanol:acetone (v/v) for 4 min, after which the fixative was removed and the coverslips washed thrice in PBS. The coverslips were kept in ice cold 1 X PBS before the subsequent indirect immunofluorescence.

Immunological detection of BrdU incorporation requires the pre-treatment of Bge cells with acid. The coverslips were washed with 10 ml of 2 N HCl for 30 min, after which the acid was removed and slides washed 10 times in PBS to eliminate any residual acid. Mouse anti-BrdU antibody (Beckton and Dickenson) was diluted 1:100 in 1% newborn calf serum (NCS) in PBS (v/v). The coverslips were incubated with the anti-BrdU for 1 h at room temperature. The cells were then washed thrice in PBS before the addition of the secondary antibody, donkey anti-mouse FITC (Jackson laboratories) 1:80 dilution 1% NCS/ PBS for 1 h at room temperature. The cells were mounted in Vectorshield anti-fade mountant containing DAPI (Vectorlabs).

2.3. Quantitative reverse transcription-PCR

Quantitative reverse transcription-PCR (qRT-PCR) was performed using Applied Biosystems 7300 Real Time PCR System (Applied Biosystem, Foster City, CA, USA). Reactions were performed in a one step format with total Bge cell RNA (80 ng). Synthesis of first strand cDNA and amplification by PCR were performed sequentially in a single tube using Full velocity SYBR Green QRT-PCR Master mix according to the manufacturer's instructions (Stratagene). Reactions (25 µl final volume) contained the following; 200 nM each of specific primers for *B. glabrata* *ferritin*, (F: 5'-GGAGGAGAGAGAACATGC- 3'; R: 5'-CACCAATCTGCTTGATGGAC-3'; GenBank Acc. No. [AW739595](#)), and *actin* (F: 5'-GTCTCCACACTGTACCTATC-3'; R: 5'-CGGTCTGCATCTCGTTTC-3'; GenBank Acc. No. [CO501282](#)). A parallel reaction was performed with the constitutively expressed *myoglobin* gene (50 nM of *B. glabrata*-specific myoglobin primers F: 5'-GATGTTGCGCAATGTTCCC-3'; R: 5'-AGCGATCAAGTTTCCCCAG-3'; GenBank Acc. No. [U89283](#)) was used to assess the comparability of samples and confirm that template

cDNA was used in equivalent amounts for each amplification reaction (Raghavan et al., 2003). All reactions contained 300 nM of reference dye; 1X Full Velocity SYBR Green QRT-PCR master mix containing RT-PCR buffer, SYBR green I dye, MgCl₂ and nucleotides. The amplification protocol included an initial incubation at 48°C for 45 min for cDNA synthesis, a 95°C initial denaturation for 10 s, and annealing/amplification at 58°C for 1 min. Detection of the fluorescent product was carried out at the end of the amplification period. All amplifications were run in triplicate and the fluorescence threshold value (Ct) was determined using the 7300 System v1.3.1 SDS software (Applied Biosystems). Comparison of the expression of the *ferritin* and *actin* genes between pre- and post-exposure of Bge cells was determined using delta-delta ($\Delta\Delta$) Ct (Livak and Schmittgen, 2001). Results were transformed into 'fold increase' according to the following formula:

$$\text{Fold change} = 2^{-\Delta\Delta Ct}$$

$$= 2^{-[(Ct_{\text{gene, exposed}} - Ct_{\text{myoglobin, exposed}}) - (Ct_{\text{gene, unexposed}} - Ct_{\text{myoglobin, unexposed}})]}$$

These experiments were repeated four times with different batches of Bge cell exposures to *S. mansoni*. In order to determine the significance of differences ($P < 0.05$) in gene expression for the different time points, the P -value was calculated by comparing delta Ct values using the Student's t -test between the Bge cells with and without parasite co-culture as described previously (Myers, 2008; Ittiprasert, 2009).

2.4. FISH

Probes were derived from clones of *B. glabrata* bacterial artificial chromosome (BAC) libraries (BB02 and BS-90 stocks), previously determined to contain *actin*, *ferritin*, *peroxiredoxin* and *piwi* encoding sequences (Adema et al., 2006; Knight et al., 2009). The BAC clones used as probes were identified using cDNA probes of specific genes and validated by PCR using gene-specific primers but were not fully sequenced. The BAC DNA was extracted using a Qiagen midi kit (Qiagen) as described previously (Raghavan et al., 2007) and labelled with Biotin-14 - dATP via nick translation using the BioNickTM kit (Invitrogen). Five hundred ng of the labelled gene probe and 3 µg of herring sperm DNA as carrier were combined with 40 µg of sonicated Bge genomic DNA. These constituents were co-precipitated with ethanol at -80°C for 30 min. After washing twice with 70% ethanol, the DNA was subsequently dissolved in 12 µl of hybridization buffer at room temperature for 24 h. The probes were denatured at 75°C for 5 min and then incubated at 37°C between 30 and 120 min prior to use.

The slides of Bge metaphase spreads were aged for 2 days at room temperature and then dehydrated through a series of ethanol solutions of 70%, 90% and 100% (5 min each). The cells were denatured in a solution of 70% formamide/2 X SSC at 70°C for 1.5 min. Immediately after denaturation the slides were immersed in ice cold 70% ethanol for 5 min before an additional 90% and 100% ethanol cycle. The slides were allowed to dry on a hot block (37°C) before the addition of the denatured probe.

Eight microliters of the biotin-labelled probe were placed onto the slide, covered with a 24 × 40 mm coverslip and sealed with rubber cement. The denatured slides and probes were hybridized overnight (12–16 h) in a humidified chamber at 37°C. Following hybridization, the rubber cement and coverslips were removed and the slide was washed three times for 5 min in a neutral buffered solution of 50% formamide and 2 X SSC at 45°C. A second wash followed. The slides were transferred to Coplin jars containing pre-warmed 0.1 X SSC at 60°C, which were transferred to a 45°C water bath. The slides were washed three times for 5 min. Subsequently, the slides were placed in a solution of 4 X SSC at room temperature for 10 min after which 100 µl of blocking solution was added to each slide (4% BSA, in 4 X

SSC). A 22 × 50 mm coverslip was placed on the slide for 10 min at room temperature. The coverslips were then removed and 100 µl of streptavidin conjugated to cyanine 3 in 1% BSA/4 X SSC (1:200 dilution) were added to each slide and a coverslip applied. The slides were incubated at 37°C for 30 min in the dark. After this incubation, the slides were washed three times for 5 min in 4 X SSC with 0.1% Tween 20 (v/v) at 42°C in the dark. The slides were rinsed in deionized distilled water followed by counterstaining with DAPI in Vectorshield anti-fade mountant (Vectorlabs).

2.5. Image analysis

Digital images were captured using an epifluorescence microscope (100X oil immersion objective, Zeiss, Axioplan 2), equipped with a charged coupled device (CCD) camera (RS Photometrics Sensys camera model KAF1401E G2) and the program Smart capture 3.00 (Digital Scientific). Fifty to 60 images of BrdU labelled chromosome territories and the hybridized *B. glabrata* genes in interphase nuclei of the Bge cell line were analysed using an erosion analysis script developed by Dr. Paul Perry in IPLab software (Croft et al., 1999) a kind gift from Prof. Wendy Bickmore (Medical Research Council Human Genetics Unit,, Edinburgh). The interphase positioning of both the BrdU labelled chromosome territories and the mapped *B. glabrata* genes was analysed by partitioning DAPI images of the nuclei into five concentric shells of equal area from the nuclear periphery to the nuclear centre with background removal by subtracting the mean pixel intensity of the nuclei. The data were normalized for each of the five shells by dividing the chromosome/gene signal intensity with the DAPI intensity measurement. Statistical analyses were performed by using unpaired, unequal variances, two-tailed Student's *t* test and one-way ANOVA.

3. Results

3.1. Presence of non-random, radially positioned chromosome territories in Bge cells

Presently there are no *B. glabrata*, other snail or molluscan chromosome painting probes that could be employed to elucidate chromosome positioning in the Bge cells by straight-forward FISH or ZOO-FISH. However, there is a method that can be used to analyse individual chromosomes, involving the incorporation of exogenous labelled nucleotides into cells (Visser et al., 1998; Zink et al., 1999). By incorporating BrdU we were able to use indirect immunofluorescence to delineate the labelled chromosomes (Fig. 1A). The interphase chromosomes within the Bge nuclei were indeed found in chromosome territories of a similar morphology and structure to those of mammalian cells. The *B. glabrata* chromosome territories contain invaginations at their edges that likely allow access to the core regions of the territories. Indeed from the representative image shown in Fig. 1A the two large chromosome territories (which will most likely not be homologs due to non-random segregation at mitosis) are located adjacent to the nuclear periphery (large arrow) and a chromatin-poor region of the nucleus, most probably the nucleolus (small arrow). This implies that the chromosomes in Bge cells/*B. glabrata* have interactions with the nuclear envelope and nucleolus as they do in other species.

Since more complex organisms such as vertebrates organise their genome in interphase nuclei in a radial non-random manner, i.e. being positioned within the nuclear space with respect to the nuclear edge/envelope; we sought to determine whether this type of organisation was conserved in the cells derived from a gastropod mollusc such as *B. glabrata*. The methodology used does not permit us to determine which chromosome territories correspond to particular chromosomes in the Bge karyotype (Odoemelam et al., 2009). However, size selection is possible and chromosomes were subdivided into two clear categories, large and small. The medium sized chromosomes were difficult to categorize since they may represent larger chromosomes that are more condensed than others. The cells

were fixed so that when they were dropped onto microscope slides they were flattened. This allowed us to use a well-tested chromosome positioning erosion analysis script that enables the position of chromosome location in 50–100 images (Croft et al., 1999; Bridger et al., 2000; Meaburn et al., 2007; Szczerbal et al., 2009; Mehta et al., 2010). The script generates outlines of cell nuclei that contain DAPI-stained DNA and creates five shells of equal area in which the signal intensity of the chromosome fluorescence and the DAPI fluorescence is measured (Fig. 1B). From this analysis, a composite nucleus made up of images of a cell nucleus, representative chromosome territories (green) and gene signals (red) can be deduced. Because the analysis is performed in 2-D and there is more DNA within the interior shells due to the nuclear flattening the chromosome fluorescence signal intensity, data are normalized by division with the DAPI fluorescence signal intensity for each of the five shells. When the data were plotted as histograms it became apparent that Bge chromosome territories were organized in a non-random way with larger chromosomes towards an intermediate position and the smaller chromosomes towards the interior, implying a size correlated distribution. Attempts at chromosome position correlation with gene density, as is seen in humans (Boyle et al., 2001), amphibians and reptiles (Federico et al., 2006), will have to await completion of the *B. glabrata* genome sequencing project (Raghavan and Knight, 2006).

3.2. *Biomphalaria glabrata* gene loci are positioned non-randomly in Bge cell nuclei

Even though chromosomal painting probes do not yet exist for *B. glabrata*, specific gene sequences have been cloned and identified to BACs. Such BACs can be labelled by nick translation for use as probes in FISH. Using this approach we have delineated four gene loci in interphase Bge cell nuclei. They are *piwi* and *BgPrx* (Knight et al., 2009), identified in the resistant BS-90 snails, both of which have been mapped to two homologous chromosomes in the Bge karyotype (Odoemelam et al., 2009). The two other genes *actin* and *ferritin* mapped to BACs from BB02 snails (Adema et al., 2006) were studied and found to be up-regulated after infection with *S. mansoni*. Fig. 2 displays the hybridization by FISH of the four gene loci onto mitotic chromosomes derived from Bge cells. Although these cells are aneuploid, they contain only two gene loci signals on two homologous chromosomes for all four of these genes, indicating that for these genes and the chromosomes they are housed on there are no extra copies. The *actin* gene is localized on a single pair of large acrocentric chromosomes (Fig. 2A) and *ferritin* is localized onto a pair of large metacentric chromosomes (Fig. 2B). The chromosomal mapping of *piwi* and *BgPrx* genes has been shown previously (Odoemelam et al., 2009) (Fig. 2C and D, respectively) and is included here for completeness. All four gene loci were then also revealed in interphase Bge cell nuclei, displaying two signals (Fig. 3A–D) and 50+ images subjected to the radial positioning as the chromosomes were, with the erosion analysis script in Fig. 2B. All four gene loci displayed non-random radial positioning in interphase nuclei of Bge cells, with *actin* (Fig. 3E), *piwi* (Fig. 3G) and *BgPrx* (Fig. 3H) all being located towards the nuclear interior and *ferritin* being located very much at the nuclear periphery (Fig. 3F). The distribution of *actin* appeared slightly more random than the others but the majority of signal was found in shell 4, revealing a more interior distribution.

3.3. Gene positioning is altered when Bge cells are co-cultured with *S. mansoni* miracidia and is correlated with gene expression

It is known that infection of *B. glabrata* with *S. mansoni* miracidia modulates host gene expression and certain genes are up-regulated, such as *actin*, *ferritin* and heat shock protein (*hsp*)70 (Ittiprasert et al., 2009). We took advantage of the Bge cell-miracidia co-culture model system of infection (Ivenchenko et al., 1999; Coustau et al., 2003) to analyze how these genes behave after the addition of parasite to the cell culture media. As genes and chromosomes can move within a matter of minutes to hours within the nuclear environment

upon activation (Volpi et al., 2000; Hu et al., 2009; Mehta et al., 2010) we took time points at 0, 30 min., 2 h, 5 h and 24 h. Fig. 4 displays graphically the gene positioning of *actin* at the different time points. Interestingly, the *actin* gene loci move from the nuclear interior towards the nuclear periphery at 30 min. The bimodal distribution at this time may indicate that one allele has moved or that only a proportion of the cells have responded by transcribing *actin* (Fig. 4B). In the 2 h sample the gene loci are almost always found towards the nuclear periphery (Fig. 4C). At 5 h of co-culture, the *actin* gene loci are repositioned back in the nuclear interior (Fig. 4D). However, at 24 h the genes are positioned back to the nuclear periphery (Fig. 4E). To correlate the alteration in the spatial positioning with gene expression, we performed qRT-PCR using RNA isolated from cells co-cultured with miracidia for the same time points (Fig. 4F). There is a direct correlation between the up-regulation of gene expression as shown by qRT-PCR and repositioning of *actin* loci to the nuclear periphery for the 2 h and 24 h samples. There is no correlation with up-regulation for the 30 min bimodal redistribution of interphase gene position; however, there was a three-fold up-regulation of *actin* gene expression at 15 min as shown by qRT-PCR (data not shown) suggesting that the *actin* loci may well be repositioning back to the default interior position after moving to the nuclear periphery.

3.4. Irradiated attenuated miracidia do not elicit a gene repositioning response in Bge cells

In order to determine whether the gene repositioning is purely a reaction to the presence of miracidia rather than a response to an active infection mechanism elicited by the parasite, live irradiated miracidia were co-cultured with the Bge cells. The nuclei were collected from cells that had been treated with attenuated miracidia at 0 min, 30 min, 2 h, 5 h and 24 h. The nuclei were subjected to FISH with labelled BACs containing *ferritin*. Fig. 5 displays the data for the positioning of the *ferritin* gene in timed samples prepared for FISH. The *ferritin* gene loci are located at the nuclear periphery at 0 h (Fig. 4A). Co-culture with normal miracidia dramatically altered the interphase gene positioning at 5 h with the gene loci located exclusively in the third and fourth shells of the erosion analysis i.e. towards the interior of the nucleus (Fig. 5D). By 24 h the gene loci have returned to a more peripheral location. This dramatic and very specific relocation from the periphery to the interior is once again correlated with levels of gene expression as determined by qRT-PCR. Indeed, a large increase in gene expression is seen at 5 h (Fig. 5F). However, when irradiated miracidia are used in the co-culture system there is no evidence for the relocation of the *ferritin* gene loci and no induction of *ferritin* expression was detected by qRT-PCR. However both the normal and attenuated miracidia appear to be morphologically similar after 24 h with the shedding of ciliary plates and slower motility. However in an in vitro system such as this, 24 h is too short a time to follow miracidial development and transformation criteria in depth.

4. Discussion

To date, there have been no published studies on how the genome is organized in interphase nuclei of molluscan organisms – on whether individual chromosome territories are present or how specific gene loci behave. Using the in vitro co-culture assay employing Bge cells and schistosome miracidia we have revealed major gene repositioning events in the interphase nuclei of the snail Bge cells. The dynamics of repositioning of loci for both *actin* and *ferritin* genes are tightly correlated with up-regulation of gene expression as determined by qRT-PCR. However, only the peripherally located *ferritin* gene loci moved to the interior during co-culture with parasite at the same time point when gene expression was up-regulated. This is consistent with the behaviour of actively expressing genes to move to the nuclear interior or becoming more internally located as was expected from other studies (Takizawa et al., 2008a; Szczerbal et al., 2009). However the *actin* gene, which is internally located within interphase nuclei, upon activation became more peripheral when it migrated

from the interior towards the nuclear edge. While repositioning towards the nuclear periphery with gene expression has not been seen in higher organisms such as vertebrates it may be a novel snail-specific response similar to that seen in simpler organisms. Active genes become associated with and/or are located at the nuclear periphery in yeast (Brickner and Walter, 2004; Casolari et al., 2005; Taddei et al., 2006; Brown and Silver 2007) and *Drosophila* (Mendjan et al., 2006; Pickersgill et al., 2006). Most interestingly, transcriptionally activated *var* genes in the malaria parasite *Plasmodium falciparum* (Apicomplexa), are located at the nuclear periphery (Duraisingh et al., 2005). In contrast, in mammalian cells the tethering of parts of the genome to the nuclear membrane or lamina either results in down-regulation of gene expression or does not alter the level of expression (Finlan et al., 2008; Kumaran and Spector, 2008; Reddy et al., 2008; reviewed in Denaiud and Bickmore, 2009). Our observations on gene positioning indicate that gene expression in *B. glabrata* may occur in both the nuclear interior and the nuclear periphery. Therefore this lophotrochozoan (the intact snail) could be a model system to study involvement of different nuclear compartments in genomic regulation at the phylogenetic interface between yeast/*Drosophila* and higher organisms.

Instead of the movement of individual genes towards different nuclear compartments, the repositioning of genes may also be explained by a global reorganisation of the whole genome upon exposure to the parasite (Strasak et al., 2009). This however is unlikely, since the repositioning of the genes is non-random and even though the *hsp70* gene of *B. glabrata* is up-regulated following exposure to the parasite, the gene loci do not change their nuclear localisation at any of the time points (data not shown). Thus, we believe that the repositioning is a specific response elicited for specific genes. However, it will be important not only to look at spatial repositioning but also at chromatin modifications for the activated genes and alterations in association with regions of specific types of chromatin.

We believe this study is also the first to reveal the occurrence of chromosome territories in cell nuclei of molluscs, with characteristics and morphology that seem similar to chromosome territories found in higher eukaryotes (Meaburn and Misteli, 2007). We also provide some evidence that these chromosome territories are non-randomly positioned in cell nuclei with a bias towards a size-correlated distribution with small chromosomes towards the nuclear interior and larger ones more towards the nuclear periphery. Even by using a cell line, we have clearly shown that the snail chromosome territories are similar in morphology as well as in non-random radial positioning as those found in other derived protostome and deuterostome organisms (Haszprunar, 2000; Lindberg et al., 2004). It is too early to make predictions about how the snail genome is organized in interphase nuclei, but the progression of the *B. glabrata* genome sequencing project, as well as studies with the intact snail itself, not the cell line, may enable us to correlate gene density with gene positioning and provide us with much needed chromosome painting probes for this organism.

We believe this is the first example of spatial reorganisation of a genome after exposure to a parasite, although viral infection has been shown to elicit a response whereby the nucleus is remodelled (Li et al., 2009). Such a response could not be observed using attenuated parasites, perhaps indicating the absence of a signalling molecule(s). Since the snail and the schistosome have co-evolved and would benefit from being able to influence each others' genomes for their own benefit, it seems possible that this spatial repositioning is initiated by the parasite rather than being only a response by the host. Thus, it would be interesting to identify the factor(s) in the miracidial ES product that may be responsible for this rapid communication either directly or indirectly with the snail host nucleus. By examining ES products from normal and attenuated miracidia, and by subtractive cDNA cloning strategies, we may be in a position to isolate this parasite factor. The identification of this factor will be

an important first step in addressing the process by which schistosomes manipulate their hosts' genome for their developmental biology.

Acknowledgments

We would like to thank Dr. Christopher Bayne (Oregon State University, USA) for the Bge cell line (isolate 2) that was used in this study. We also thank Drs Paul Perry and Prof Wendy Bickmore, (Medical Research Council, Human Genetics Unit, Edinburgh, UK), for their bespoke erosion analysis script, and Dr Fred Lewis (Biomedical Research Institute, USA) for his helpful suggestions and comments with the writing of this manuscript. CMA acknowledges support from National Institutes of Health (NIH), USA AI52363 and 1P20RR18754. This work was funded by a grant from NIH AI63480.

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Figure 1.

The presence and non-random distribution of chromosome territories in *Biomphalaria glabrata* Bge cells. A) Two separate chromosome territories (green) in a Bge cell nucleus (blue), revealed by using the incorporation of 5-Bromo-2-deoxy Uridine (BrdU) into newly synthesised DNA during the cell's S-phase after a 48 h pulse, followed by 10 days in BrdU-free whole medium. The DNA within the cell nucleus is stained with the DNA, DAPI and the incorporated BrdU is revealed using a primary antibody recognising BrdU and a secondary antibody conjugated to FITC. One territory (thick white arrow) is located at the nuclear periphery and one (thin white arrow) at a chromatin-free region, the nucleolus. B) A composite cartoon of the nucleus where a computer script has outlined the DAPI signal and five shells of equal area created by erosion analysis (see Croft et al., 1999; Szczerbal et al., 2009). Two gene signals are in the red and two in the green chromosome territories that are

pseudo-coloured using a computer generated program. The script measures the intensity of fluorescent signal from either the genes or the DAPI and records those. In order to normalise the data, because the analysis is in two dimensions (2-D) for a 3-D object, the percentage of chromosome/gene signal in each shell is divided by the DAPI signal for the corresponding shell. These data for large and small chromosomes are plotted as a bar chart in C, displaying large chromosomes as having a non-random intermediate distribution within Bge cells, while the smaller chromosomes were found in the nuclear interior. Error bars = S.E.M. Scale bar = 5 μ m. Statistically significant differences, as assessed by Student's *t* test, between normalised chromosome signal in each shell of small chromosome compared with large chromosome are indicated by an asterisk ($P < 0.05$).

Figure 2.

Gene alleles studied are present as two copies on two homologous metaphase chromosomes in *Biomphalaria glabrata* Bge cells. Actively growing Bge cells were treated with colcemid to increase the number of cells in metaphase (see Odoemelam et al., 2009). Metaphase chromosomes were subjected to fluorescence in situ hybridisation (FISH) with labelled bacterial artificial chromosome (BAC) probes containing specific *B. glabrata* genes (red). All four genes *actin* (A), *ferritin* (B), *piwi* (C) and *BgPrx* (D) were delineated as two loci (indicated by white arrows) on sister chromatids of a pair of homologous chromosomes stained with DAPI. The inset in each panel shows a grayscale inverted DAPI image of similar chromosomes from another metaphase-spread preparation. Scale bar = 5µm.

Figure 3.

Genes are non-randomly positioned in the interphase nuclei of *Biomphalaria glabrata* Bge cells. Fluorescence in situ hybridisation (FISH) was used to delineate the gene signals in interphase nuclei that were visualised in metaphase (Fig. 2), prepared in the same way as the metaphase chromosomes but without the colcemid treatment. The gene signals have been pseudo-coloured green and there are only two distinct signals within each representative nucleus - *actin* (A), *ferritin* (B), *piwi* (C) and *BgPrx* (D). Fifty images of nuclei for each gene (E–H) were captured and the gene position assessed using the erosion script analysis (see Fig. 1). All genes are non-randomly located with *actin* (E), *piwi* (G) and *BgPrx* (H) being located towards the nuclear interior and *ferritin* (F) towards the nuclear periphery. Scale bar = 5 μ m. Statistically significant differences, as assessed by one-way ANOVA, between the normalised gene signal in each shell are indicated by the asterisk ($P < 0.05$).

Actin

Figure 4.

The *actin* gene is repositioned within interphase nuclei after co-culture with parasite and this is correlated with changes in gene expression. *Biomphalaria glabrata* Bge cells were co-cultured with *Schistosoma mansoni* miracidia in tissue culture flasks, washed, fixed, subjected to fluorescence in situ hybridisation (FISH) and 50 images collected for each time point pre- (0 h) and post-exposure to parasite i.e. 0.5 h, 2 h, 5 h and 24 h. The images were analysed by the erosion script (IPLab software; Croft et al., 1999). The *actin* gene had altered its location at the nuclear interior (A) to a bimodal distribution at 30 min (B) and then was located towards the nuclear periphery at 2 h (C) and 24 h (E), with relocation to the nuclear interior at 5 h (D). Reverse transcription (RT)-PCR (F) was performed on three samples of RNA extracted at the same time points and there was an increase in *actin* expression at 2 h and 24 h p.i., correlating with the gene repositioning. Numbers 1–5 on the X-axis refer to the shell numbers denoting nuclear periphery > nuclear interior (see Fig. 1). Statistically significant differences, as assessed by one-way ANOVA, between the normalised gene signal in each shell are indicated by an asterisk ($P < 0.05$).

Ferritin

Figure 5.

The *ferritin* gene is repositioned within interphase nuclei after co-culture with normal parasite but not with attenuated parasite that elicits no gene expression response. *Biomphalaria glabrata* Bge cells were co-cultured with normal *Schistosoma mansoni* miracidia or with miracidia that had been attenuated by irradiation in tissue culture flasks washed, fixed, subjected to fluorescence in situ hybridisation (FISH) and 50 images collected for each time point pre- (0 h) and post-exposure to parasite i.e. 0.5 h, 2 h, 5 h and 24 h. The images were analysed by the erosion script. When incubated with normal parasite the *ferritin* gene had altered its location at the nuclear periphery (A) to the nuclear interior at 5 h (D) with a slight change in position at 2 h (C). Reverse transcription (RT)-PCR was performed on three samples of RNA extracted at the same time points and there was a large fold increase of *ferritin* expression at 5 h as revealed by dark grey triangles in a line graph (F), correlating with the gene repositioning. By 24 h the gene loci had relocated back to towards the nuclear periphery (E). When irradiated parasite was used, no gene repositioning for the *ferritin* gene was observed (G–K) at any time point and no up-regulation of gene expression was measured by RT-PCR as displayed by light grey squares on the line graph (F). Numbers 1–5 on the X-axis refer to the shell numbers denoting nuclear periphery > nuclear interior (see Fig. 1). Statistically significant differences, as assessed by one-way ANOVA, between the normalised gene signal in each shell are indicated by the asterisk ($P < 0.05$).