Acta Histochemica 114 (2012) 665–672

Contents lists available at SciVerse ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.de/acthis

Contribution of AT-, GC-, and methylated cytidine-rich DNA to chromatin composition in Malpighian tubule cell nuclei of *Panstrongylus megistus* (Hemiptera, Reduviidae)

Elenice M. Alvarenga[a], Mateus Mondin[b], Vera L.C.C. Rodrigues[c], Larissa M. Andrade[b], Benedicto de Campos Vidal[a], Maria Luiza S. Mello[a,*]

[a] *Structural and Physiological Biology for Anatomy, Cell Biology and Physiology, Institute of Biology, University of Campinas (UNICAMP), 13083-862 Campinas, SP, Brazil*
[b] *Department of Genetics, "Luiz de Queiroz" College of Agriculture-ESALQ, University of São Paulo (USP), 13418-900 Piracicaba, SP, Brazil*
[c] *SUCEN, 13840-000 Mogi-Guaçu, SP, Brazil*

ARTICLE INFO

Article history:
Received 16 August 2011
Received in revised form
25 November 2011
Accepted 28 November 2011

Keywords:
P. megistus
Heterochromatin
DNA
Epigenetics
Fluorescence microscopy
AgNOR

ABSTRACT

The Malpighian tubule cell nuclei of male *Panstrongylus megistus*, a vector of Chagas disease, contain one chromocenter, which is composed solely of the Y chromosome. Considering that different chromosomes contribute to the composition of chromocenters in different triatomini species, the aim of this study was to determine the contribution of AT-, GC-, and methylated cytidine-rich DNA in the chromocenter as well as in euchromatin of Malpighian tubule cell nuclei of *P. megistus* in comparison with published data for *Triatoma infestans*. Staining with 4′,6-diamidino-2-phenylindole/actinomycin D and chromomycin A_3/distamycin, immunodetection of 5-methylcytidine and AgNOR test were used. The results revealed AT-rich/GC-poor DNA in the male chromocenter, but equally distributed AT and GC DNA sequences in male and female euchromatin, like in *T. infestans*. Accumulation of argyrophilic proteins encircling the chromocenter did not always correlate with that of GC-rich DNA. Methylated DNA identified by immunodetection was found sparsely distributed in the euchromatin of both sexes and at some points around the chromocenter edge, but it could not be considered responsible for chromatin condensation in the chromocenter, like in *T. infestans*. However, unlike in *T. infestans*, no correlation between the chromocenter AT-rich DNA and nucleolus organizing region (NOR) DNA was found in *P. megistus*.

Introduction

P. megistus is a blood-sucking hemipteran reduviid that is considered to be the principal vector of Chagas disease in the Eastern, Southern and some Northeastern states of Brazil (Barbosa et al., 2001). It has 18 autosomes and multiple sex chromosomes (X_1X_2Y in males and $X_1X_1X_2X_2$ in females) (Schreiber and Pellegrino, 1950; Crossa et al., 2002). As in many other insect species, most organs of *P. megistus*, including the Malpighian tubules, grow by endopolyploidy, during post-embryonic development (Mello, 1975). In fully-nourished, unstressed specimens of *P. megistus*, the Malpighian tubules contain approximately 18,000 nuclei (Mello et al., 1986). In male specimens all these nuclei display a small heterochromatic body which constitutes a chromocenter (Fig. 1a and b) (Mello et al., 1986). Under stress conditions, some of the cells may undergo necrosis, apoptosis, nuclear fusion and/or heterochromatin unraveling, which affect the chromocenter organization (Garcia et al., 2000a,b). In single-chromocentered cells of *T. infestans*, another well-known vector of Chagas disease, the heterochromatic body contains contributions from several autosomes and the sex chromosomes (Schreiber et al., 1972). By contrast, the chromocenter of *P. megistus* is much smaller and is assumed to comprise only the Y chromosome, as this nuclear body has not been recognized or described in the somatic cells of female specimens (Fig. 1c) (Mello et al., 1986).

Knowledge of the composition of chromatin elements and epigenetic markers, as well as their spatial distribution, is particularly of interest in cellular models where differences in heterochromatin structures contribute to the definition of specific nuclear phenotypes. The spatial distribution of chromatin components is considered to play a part in the regulation of nuclear and cellular processes (Gilbert et al., 2005; Schneider and Grosschedl, 2007; Joffe et al., 2010; Rouquette et al., 2010; Stein et al., 2010; Cremer and Zakhartchenko, 2011). Chromatin organization has been demonstrated to involve a non-random distribution of chromatin elements in different mammalian species (Cremer and Cremer, 2011), but there is no information on this subject for reduviid hemipterans, except for the distribution of AT- and GC-rich

* Corresponding author.
E-mail address: mlsmello@unicamp.br (M.L.S. Mello).

0065-1281/$ – see front matter © 2011 Elsevier GmbH. All rights reserved.
doi:10.1016/j.acthis.2011.12.001

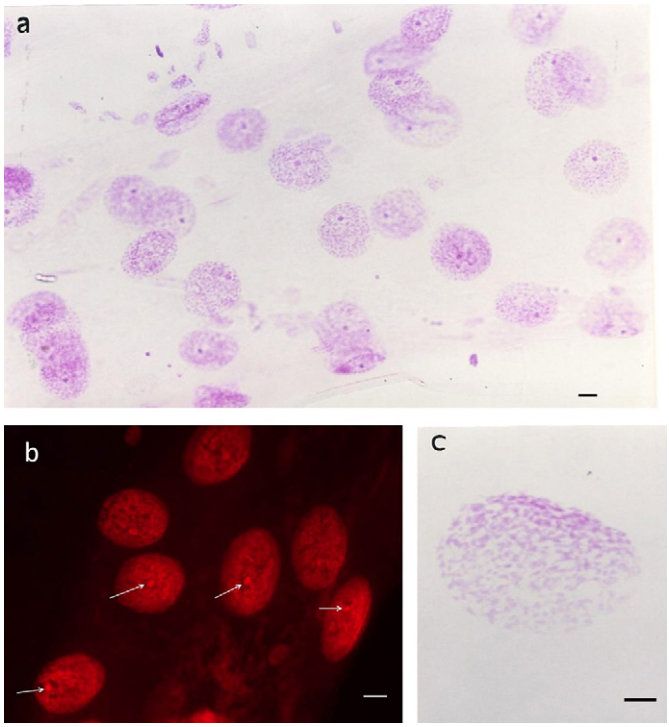

Fig. 1. *P. megistus* whole-mounted Malpighian tubule preparations subjected to ordinary (a and c) and fluorescent (b) Feulgen reactions. A single chromocenter is present in male (a and b, arrows) but not in female cell nuclei (c). Scale bars = 10 μm.

DNA as well as methylated DNA in chromatin compartments of *T. infestans* (Alvarenga et al., 2011).

Although the sequencing of whole genomes of hemipteran reduviids is not yet available, there are data that indicate an AT-biased DNA in *T. infestans* (Marcilla et al., 2001; Mas-Coma and Bargues, 2009; Segura et al., 2009; Alvarenga et al., 2011). This observation is especially valid for the heterochromatin in single- or multichromocentered nuclei, although topochemical and immunochemical tests have identified some GC-rich DNA at the edges of the chromocenters that contact the nucleolus and in the euchromatin of the Malpighian tubule cell nuclei of this species (Alvarenga et al., 2011).

Despite the fact of a relatively close phylogenetic relationship between *P. megistus* and *T. infestans* (Stothard et al., 1998; Garcia et al., 2001; Marcilla et al., 2001; Crossa et al., 2002; Maranhão, 2008), they differ drastically in their chromosome contributions to chromocenter composition. These models are therefore important for the investigation of the nature of the chromocenter and the roles of enrichment for specific DNA nucleotides and DNA methylation in chromatin territories, a step that necessarily precedes any studies on protein epigenetic markers in these models.

In this study, we have investigated the distribution of AT- and GC-rich DNA in various chromatin regions of the Malpighian tubule polyploid cell nuclei of *P. megistus* specimens with or without a chromocenter (males and females, respectively) and have compared these results with previously described data for *T. infestans*.

Materials and methods

Insects and cell preparations

Fifth instar male and female nymphs of a domestic population of *P. megistus* (Burmeister) (Hemiptera, Reduviidae) reared at 28 °C and 80% relative humidity, under a short light regime, and fed hen's blood once a week in the laboratory at SUCEN (Mogi-Guaçu, SP) were used. The domiciliary specimens of *P. megistus* reared at SUCEN originated from natural populations in the lower slopes of the Serra da Mantiqueira in Eastern São Paulo state (Caconde, Divinolândia, São Sebastião da Grama, São João da Boa Vista) in Brazil (approximately between the latitudes 21°31′S and 21°58′S and close to the longitude 46°40′W). The number of specimens used varied depending on the experimental assay.

Gently squashed whole mounts of Malpighian tubules were fixed in absolute ethanol–glacial acetic acid (3:1, v/v) for 1 min, rinsed in 70% ethanol for up to 5 min and air-dried.

DAPI/CMA_3 differential stainings

The fixed preparations were kept in the freezer for two weeks before staining. Some of the squashes ($n = 10$ for each sex) were treated with 2 μg/ml DAPI (Sigma–Aldrich, Chemical Co., St. Louis, MO, USA) for 30 min. Of these samples, some were treated with 0.2 mg/mL actinomycin D (AMD) (Sigma–Aldrich) for 15 min in the dark, while others were left untreated. Another set of preparations ($n = 5$ for each sex) was treated with 0.5 mg/mL CMA_3 (Sigma–Aldrich) for 3 h in the dark. In still other preparations ($n = 5$ for each sex) CMA_3 staining was followed by treatment with 0.2 mg/mL distamycin (Sigma–Aldrich) for 10 min in the dark (Schweizer, 1976; Friebe et al., 1996). After staining, all preparations were rinsed in distilled water, air dried in the dark, mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA), and examined 15 days later under a Zeiss Axiophot 2 microscope (Oberkochen, Germany) equipped for epifluorescence and using a Zeiss AxioCam HRc color video camera. At least 200 cells from each specimen were examined.

Similarly treated Malpighian tubules of *T. infestans* were used for comparison.

Immunoassay for 5-methylcytidine

Malpighian tubule preparations from five specimens of each sex were heated at 60 °C for 30 min, postfixed in 4% paraformaldehyde (pH 7.0) for 5 min, dehydrated in 70%, 96% and 100% ethanol, and air dried. The squashes were then treated with 70% formamide in 2× SSC solution for 3 min at 75 °C followed by cold absolute ethanol. Next, the preparations were treated with 1% bovine serum albumin (BSA) in 1× phosphate-buffered saline (PBS) for 1 h at 37 °C to prevent non-specific protein reactions. The preparations were then incubated overnight with sheep anti-5-methyl-cytidine (Abcam, Cambridge, MA, USA) diluted 1:200 in 1× PBS with 1% BSA, followed by a 90 min treatment with rabbit anti-sheep-FITC diluted 1:100 in 1× PBS with 1% BSA (Zhang et al., 2008). Early prophase nuclei of the maize root meristem, in which 5-methylcytosine is distributed homogeneously (Andrade et al., 2009), were used as a positive control. The preparations were mounted in DAPI (200 μg/mL)-containing Vectashield® (Vector Labs, Burlingame, CA, USA) and examined under a Zeiss Axiophot 2 microscope equipped for fluorescence.

AgNOR test

Ethanol–acetic acid-fixed Malpighian tubules from eight male specimens and 10 female specimens were subjected to AgNOR staining as described by Ploton et al. (1986), Derenzini and Ploton (1991) and Mello et al. (2008). Briefly, the tubules were treated with a solution containing 2 volumes of 50% aqueous silver nitrate (Merck, Darmstadt, Germany) and 1 volume of 2% gelatin in 1% aqueous formic acid (v/v) for 20 min at 37 °C, rinsed in deionized water, air dried, cleared in xylene and mounted in Canada balsam. In order to provide a better cell and nucleus permeabilization, silver impregnation was preceded by treatment with a 1% Triton X-100 solution in the presence of 4 M glycerol for 15 min at 37 °C

(Vidal et al., 1994). Imprints of mouse hepatocytes also fixed in ethanol–acetic acid were used as a positive control of the reaction (Vidal et al., 1994).

The analysis was performed in a Zeiss Axiophot 2 microscope and images were captured using a Zeiss AxioCam HRc color video camera.

Results

DAPI/CMA_3 fluorochrome differential stainings

A positive affinity for DAPI or DAPI/AMD was found in the euchromatin and the chromocenter of the Malpighian tubule epithelial cells of *P. megistus* (Fig. 2b). However, in all cell nuclei examined, the affinity for DAPI, indicating the presence of AT-rich DNA, was much more intense in the chromocenter (Fig. 2b), resembling findings in *T. infestans* control cells (Fig. 2a). The image of the chromocenter visualized by DAPI staining in *P. megistus* was consistent with images obtained using the Feulgen reaction (Fig. 1a and b).

A CMA_3-positive response even after DA treatment, indicative of GC-rich DNA, was evident in all cell nuclei examined, seen as either fine or coarse granules (Fig. 2d–h). In male cell nuclei, this response was observed only in the euchromatin (Fig. 2d–f). Images of the CMA_3-positive response showing a more intense ring of signal encircling the chromocenter (Fig. 2e and f), similar to that found in most Malpighian tubule cells in *T. infestans* (Fig. 2c) were observed in ≤5% of the male *P. megistus* cell nuclei examined (n = ~200 per specimen). However, there is no certainty that this proportion is maintained along the whole organ. Examination of a larger number of nuclei than the number used in this study is difficult owing to the rapid fading of the CMA_3 fluorescence.

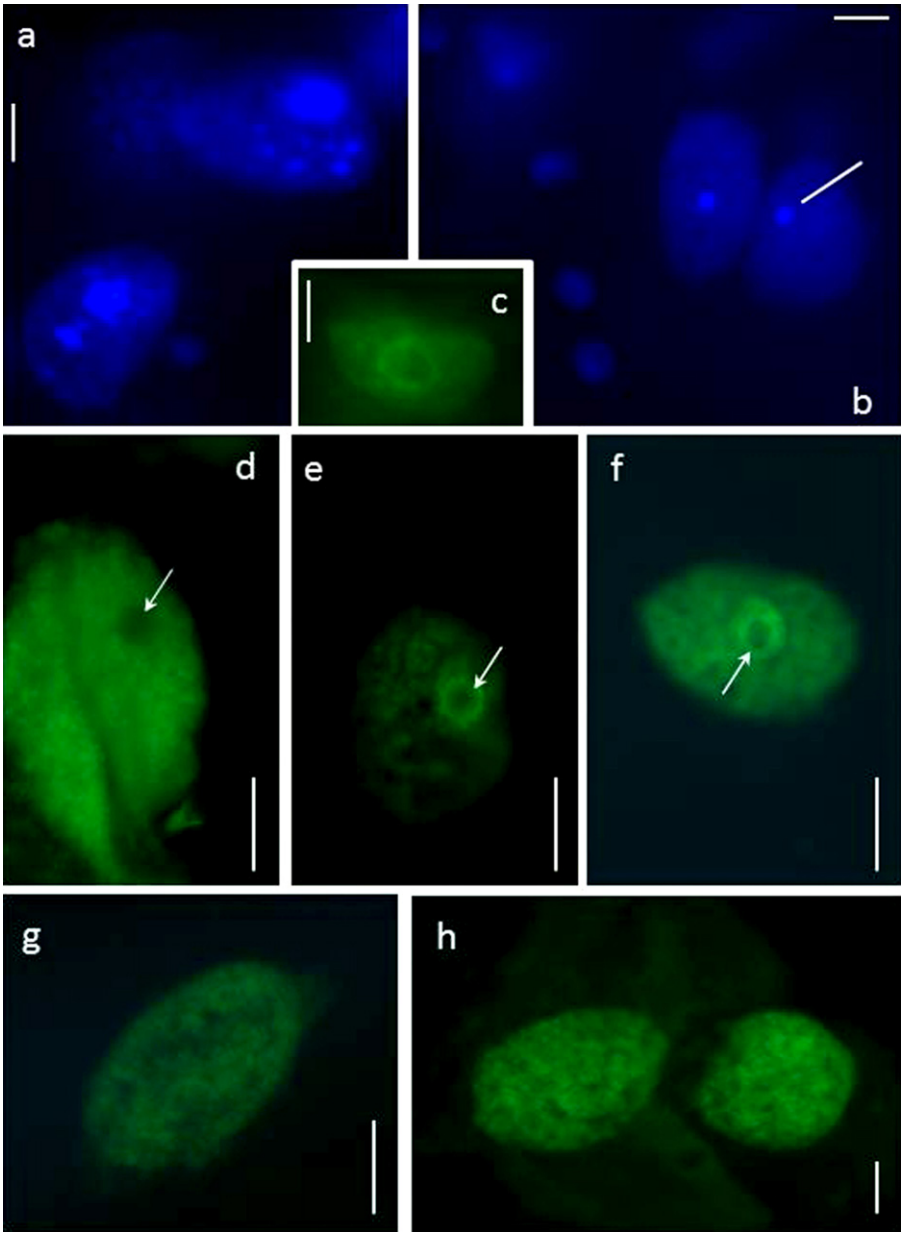

Fig. 2. Malpighian tubule cell nuclei of *P. megistus* nymphs after staining with DAPI/AMD (b – male) and CMA_3/DA (d–f, males; g and h, females). Images of Malpighian tubule cells from *T. infestans* (a, DAPI/AMD; c, CMA_3/DA) are shown for comparison. The arrows in (b) and (d) indicate the chromocenter region. The arrows in (e) and (f) indicate a ring of more intense response to CMA_3/DA surrounding the chromocenter. Scale bars = 10 μm.

5-Methylcytidine immunofluorescence assay

Bright green fluorescent 5-methylcytidine signals were found as small dots distributed throughout the euchromatin of the Malpighian tubule cells of male and female *P. megistus* nymphs (Fig. 3A–F). In males, the signals appeared to be positioned on part of the chromocenter's edge (Fig. 3A–C). No direct correlation could be made between 5-methylcytidine signals and the chromocenter. Preparations in which the primary antibody was omitted did not revealed green signals on chromatin (Fig. 3G–I). In the maize somatic cell nuclei used as a positive control, intensely bright 5-methylcytidine signals were shown to be homogeneously distributed and the negative image of the nucleolus was evident (Fig. 3J–L).

Certain small irregularly shaped and sparsely distributed nuclear areas, especially in female specimens, were observed to respond negatively to both the 5-methylcytidine immunoassay and the DAPI staining (Fig. 3E and F). Similar findings have been observed in CMA_3/DA-stained female cell nuclei (Fig. 2g).

AgNOR assay

As a positive control of the reaction, differently sized mouse hepatocyte nuclei showed well known characteristic positive responses in their NORs (Fig. 4a). In male *P. megistus* specimens, a positive response to the AgNOR test was detected at the periphery of the chromocenter and as coarse aggregates of granules extending from the chromocenter region to the periphery of the nuclei (Fig. 4b–d). In female specimens, the AgNOR positive response also generally occurred agglomerated on coarse structures (Fig. 4e). In males and females, some nuclei were found which did not respond to the AgNOR assay (Fig. 4d) or appeared less intensely impregnated by the silver (Fig. 4f and g). The responses detected were observed in variable numbers of the analyzed samples (Table 1), which does not

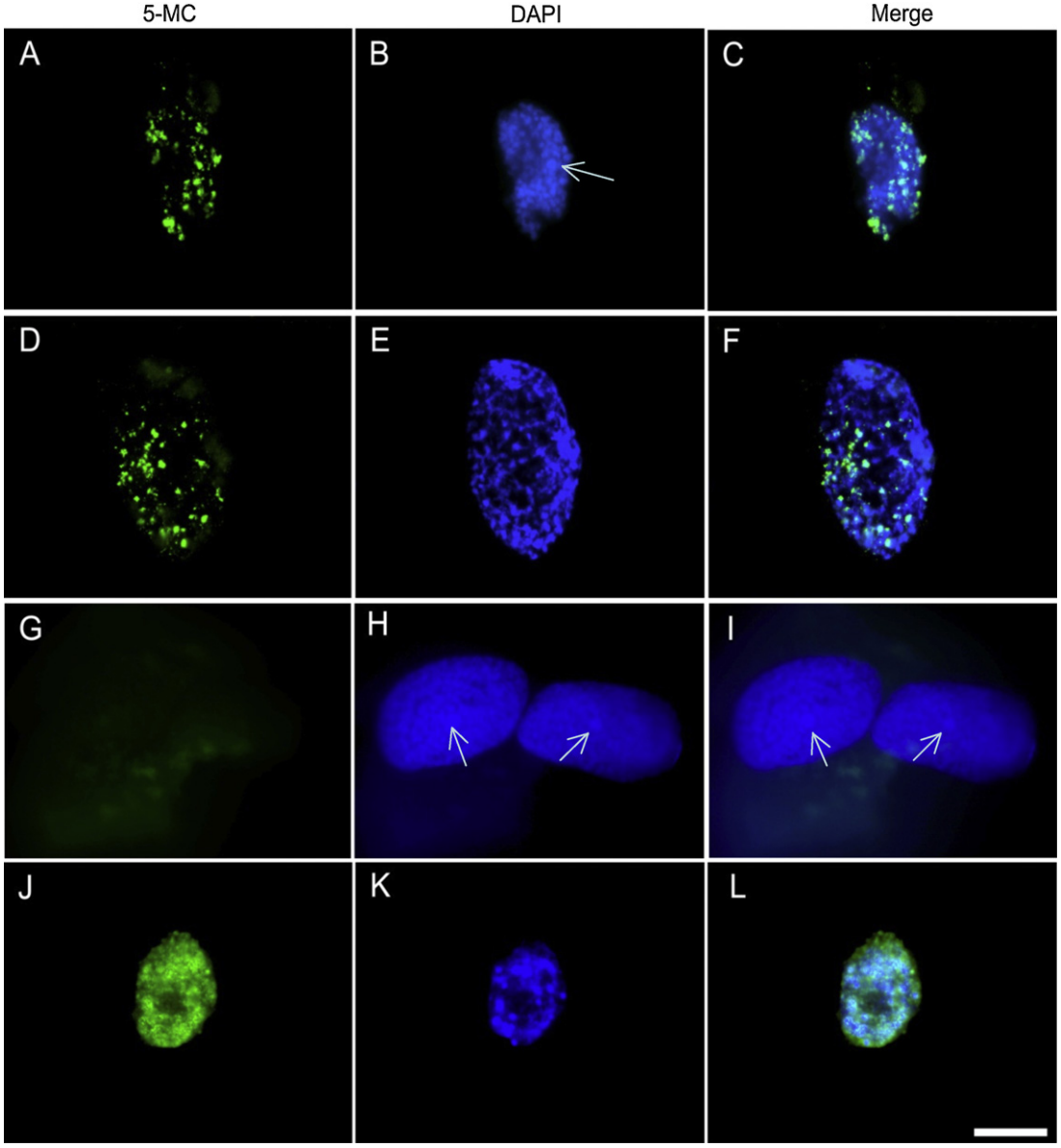

Fig. 3. 5-methylcytidine signals (A, C, D and F) in cell nuclei of *P. megistus* counterstained with DAPI (B, C, E and F). A–C are from a male nymph, whereas D–F are from a female nymph. The arrow in B indicates the chromocenter area. Images of a male *P. megistus* nymph in which the primary antibody was omitted are shown as a negative control (G–I). Images of an early prophase nucleus of a maize root meristem cell treated for 5-methylcytidine signals are included as a positive material control (J–L). Scale bars = 10 μm.

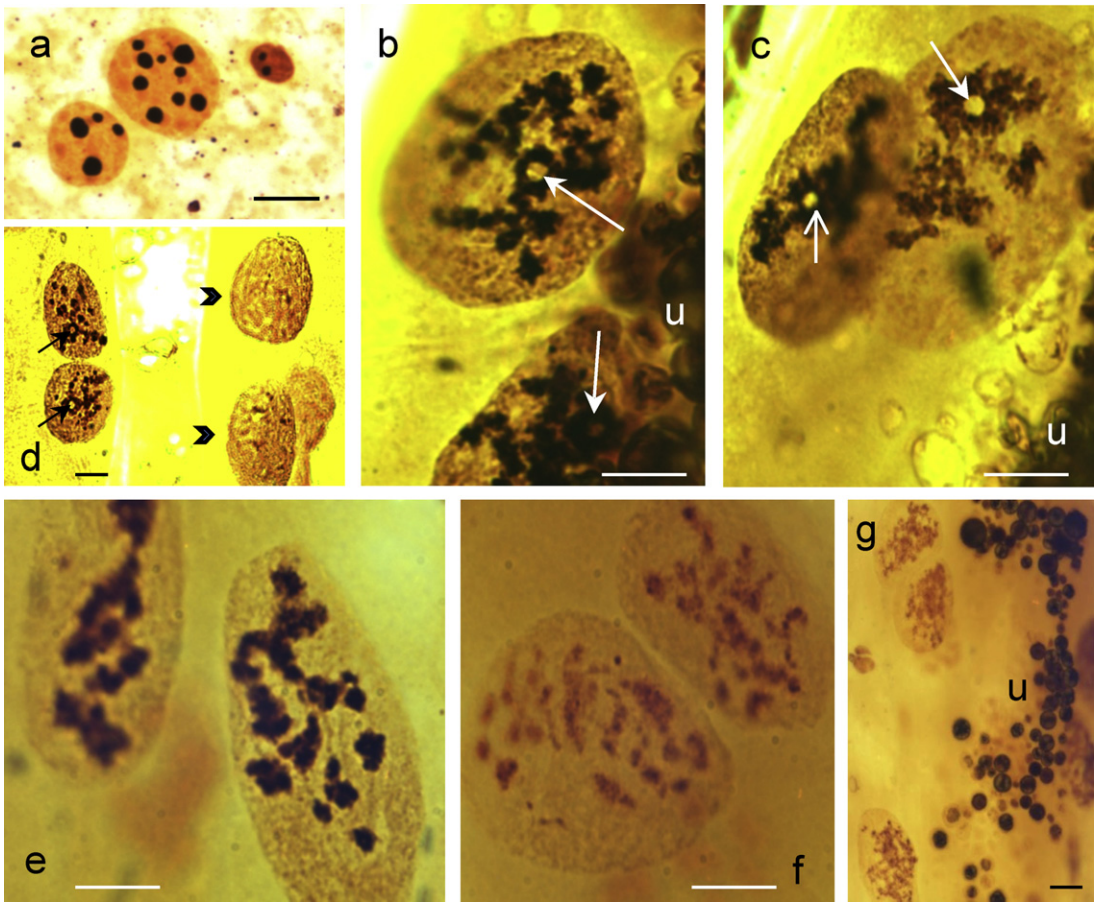

Fig. 4. AgNOR-positive response in the form of black granules spread in the cell nuclei, including the periphery of the chromocenter (b–d, arrows) in male *P. megistus* Malpighian tubule cell nuclei. Some nuclei do not show a positive response to the test (d, arrowheads). The AgNOR-positive response in female cell nuclei appears as coarse (e) or thin (f and g) granules forming filamentous agglomerates. Images of similarly treated imprints of mouse liver cells are shown as a positive control of the reaction. (a) u, urate spherulites. Scale bars = 10 μm.

justify accepting their mean value as representative of the whole organ's response to the AgNOR assay. In addition, examination of the total number of nuclei present in the Malpighian tubules of *P. megistus* (approximately 18,000) by the AgNOR assay could not be performed as the discrimination of individualized nuclei in such a material is severely jeopardized by the elevated amount of black precipitates on the abundant urate spherulites (Mello and Vidal, 1985) that this tissue contains (Fig. 4b, c and g).

Table 1
Negative or weak response to the AgNOR assay in Malpighian tubule cell nuclei of *P. megistus*.

Sex	No. of specimens	No. of nuclei counted	Negative or weak response (%)
Male	8	188	6.3
		205	3.4
		180	7.2
		181	4.9
		234	4.1
		178	2.8
		188	2.6
		363	3.3
			X = 4.3, SD = 1.7
Female	10	359	10.5
		243	6.5
		302	1.3
		120	11.6
		286	2.8
		886	1.3
		284	1.4
		277	0.7
		310	1.9
		249	1.6
			X = 4.0, SD = 4.1

X, arithmetic mean; SD, standard deviation.

Discussion

Our results indicate that as a rule, AT-rich and GC-poor DNA occurs in the chromocenter of male *P. megistus* nymphs, as it does in *T. infestans* chromocenters (Alvarenga et al., 2011), although the chromocenter size and the identities of the chromosomes which participate in the chromocenter differs between these species (Schreiber et al., 1972; Mello, 1971; Mello et al., 1986). In comparison to *T. infestans*, where three or four autosomal chromosome pairs and the X and Y chromosomes compose the chromocenters, which are thus detectable in specimens of both sexes (Schreiber et al., 1972; Morielle-Souza and Azeredo-Oliveira, 2007), the small chromocenter of *P. megistus* is detectable only in males, a fact that suggests that it is composed of the Y chromosome (Mello et al., 1986). Occurrence of heterochromatin in the Y chromosome of all species of Triatominae has been recently emphasized (Panzera et al., 2010).

The similarly intense DAPI/AMD affinity for DNA in the chromocenters of *P. megistus* and *T. infestans* is probably a consequence of their A+T-biased DNA composition, phylogenetic proximity (Stothard et al., 1998; Garcia et al., 2001; Marcilla et al., 2001; Maranhão, 2008), and chromatin condensation.

Although GC-rich DNA responsive to CMA_3/DA may contribute to the coarsely filamentous structures evident in the euchromatin of *P. megistus* and even encircle the chromocenter, recognizably methylated DNA is scarce and sparsely distributed in the euchromatin of interphase cells of this species. Sometimes, fluorescent signals associated with methylated DNA appear close to the chromocenter, giving the impression that they occurred partly inside this heterochromatic body. However, the fluorescent signals that identify 5-methylcytidine are not expected to occur inside the chromocenter of *P. megistus* because positive affinity for CMA_3/DA indicating the presence of GC-rich DNA has never been found inside this body, but only around it. In conclusion, as has previously been found for *T. infestans* (Alvarenga et al., 2011), cytosine methylation does not appear to be involved in the maintenance of chromatin condensation in the chromocenter of *P. megistus*. To establish the role of epigenetic markers in chromatin condensation in *P. megistus*, it was necessary to begin by analyzing DNA modification, a goal that was preliminarily reached in the present investigation. Further studies involving chromocenter removal by laser surgery from the highly polyploid cell nuclei of the Malpighian tubules of male *P. megistus*, followed by DNA analysis by PCR and restriction enzyme assays, should certainly reveal whether immunocytochemically undetectable cytosine methylation occurs in the DNA sequences of the chromocenter in these nuclei. However, current studies in our laboratory already suggest that histone modifications are linked to chromatin condensation in the chromocenter of *P. megistus* (manuscript in preparation).

Most of the nuclear areas that responded negatively to both DAPI and CMA_3 staining in *P. megistus* seem to be nucleolar zones because in male specimens of *P. megistus*, nucleolar structures have been identified by electron microscopy close to the chromocenter and extending from it to peripheral nuclear areas (Mello, 1987). In addition, part of the small round areas presenting DAPI- and/or CMA_3-negative results in *P. megistus*, and also in *T. infestans* (Alvarenga et al., 2011; Fig. 5D–F), may represent sites of intranuclear vesicular inclusions, which occur close to nucleolar elements in association with a very common RNA virus infection that has been reported for triatominae fed hen's blood in several laboratory facilities (Dolder and Mello, 1978a,b). Chromatin regions containing histone H3 gene clusters have also been reported to lack DAPI staining in mussel cells, a finding that was assumed to be due to a high degree of chromatin local decondensation (Pérez-García et al., 2010).

With regard to the results obtained with the AgNOR test in *P. megistus* interphase cell nuclei, it is worth mention that an intense AgNOR-positive response has often been related to GC-rich DNA in the NORs of several other organisms (Mandrioli et al., 1999; Kuznetsova et al., 2003; Golub et al., 2004; Das and Khuda-Bukhsh, 2007). However, in *T. infestans*, 45S rDNA has been associated only with an AT-rich DNA region of the X chromosome (Bardella et al., 2010), which makes up part of the chromocenters in interphase cells (Schreiber et al., 1972).

Although similar to the chromocenters of *T. infestans* in terms of their composition of AT-rich DNA and the surrounding GC-rich DNA, in the case of *P. megistus*, the chromocenter cannot simply be considered as containing the NOR for several reasons. First, in the highly polyploid epithelial cells of *P. megistus,* the chromocenter is assumed to contain several copies of the Y chromosome only (Mello et al., 1986). Second, FISH signals for a 28S- 12 kb rDNA probe of *Drosophila melanogaster* have been reported to be displayed by several chromosome regions in *P. megistus*, and NOR sites have been identified by silver staining in autosomes and in one sex chromosome which was not identified as Y, X_1 or X_2 (Morielle-Souza and Azeredo-Oliveira, 2007). Indeed, female interphase cells of *P. megistus* do not display a chromocenter, but contain a large nucleolus (data not shown) similar to that in male cells, and generally an intense AgNOR response spread along the cell nuclei. Images of the accumulation of argyrophilic proteins sometimes resemble images of the distribution of GC-rich DNA responsive to CMA_3/DA staining. In conclusion, although the establishment of FISH signals for rDNA in clearly identified chromosomes and in chromatin of interphase somatic cells of *P. megistus* may still be required to correlate NORs with the spatial distribution of DNA of a specific composition in individual interphase cell nuclei, present findings are indicative that chromocenters may vary in their relationship with NORs in the Triatominae subfamily. These differences may result from distinctive evolutionary trends in which heterochromatin is the main source of karyological differentiation, as proposed by Panzera et al. (2010).

The negative or weak positive response to the AgNOR assay that was observed in some of the *P. megistus* nuclei examined in this study may be caused by a decrease in transcriptional activity preceding occasional necrosis, a phenomenon that has been observed under the influence of occasional stressing effects unintentionally induced in insects reared in the laboratory (Garcia et al., 2000b).

Acknowledgments

This investigation was sponsored by the Brazilian National Council for Research and Development (CNPq, grant no. 47303/2009-7) and the São Paulo State Research Foundation (FAPESP, grant no. 2010/50015-6). EMA and MLSM received fellowships from CNPq (grants no. 132341/2010-7 and 301943/2009-5, respectively). One of the authors (MLSM) is indebted to Dr. Luciana Bolsoni Morandini (Unicamp, Brazil) for valuable discussions on AT- and GC-rich DNA in chromosome banding.

References

Alvarenga EM, Mondin M, Martins JA, Rodrigues VLCC, Vidal BC, Rincones J, et al. Spatial distribution of AT- and GC-rich DNA within interphase cell nuclei of *Triatoma infestans* Klug. Micron 2011;42:568–78.

Andrade LM, Fernandes R, Mondin M. Immunodetection of methylcytosine in maize chromatin by a denaturating protocol. Maize Genet Coop Newslett 2009;83:39–40.

Barbosa SE, Soares RPP, Pires HHR, Diotaiuli L. Experimental evidence for a demographic cline in *Panstrongylus megistus* populations. Mem Inst Oswaldo Cruz 2001;96:773–5.

Bardella VB, Gaeta ML, Vanzela AL, Azeredo-Oliveira MTV. Chromosomal location of heterochromatin and 45S rDNA sites in four South American triatomines (Heteroptera: Reduviidae). Compa Cytogenet 2010;4:141–9.

Cremer T, Cremer M. Chromosome territories. In: Misteli T, Spector DL, editors. The nucleus. Cold Spring Harbor: Cold Spring Harbor Lab. Press; 2011. p. 93–114.

Cremer T, Zakhartchenko V. Nuclear architecture in developmental biology and cell specialisation. Reprod Fertil Dev 2011;23:94–106.

Crossa RP, Hernández M, Caraccio MN, Rose V, Valente SAS, Valente VC, et al. Chromosomal evolution trends of the genus *Panstrongylus* (Hemiptera, Reduviidae), vectors of Chagas disease. Infect Genet Evol 2002;2:47–56.

Das JK, Khuda-Bukhsh AR. GC-rich heterochromatin in silver stained nucleolar organizer regions (NORs) fluoresces with Chromomycin A (3) (CMA(3)) staining in three species of teleostean fishes (Pisces). Indian J Exp Biol 2007;45:413–8.

Derenzini M, Ploton D. Interphase nuclear regions in cancer cells. Int Rev Exp Pathol 1991;32:149–92.

Dolder H, Mello MLS. Vesicular inclusions in the nuclei of epithelial cells of Malpighian tubes of the hemipteran, *Panstrongylus megistus*. Experientia (Basel) 1978a;34:794–5.

Dolder H, Mello MLS. Virus-like particles in the Malpighian tubes of blood-sucking hemipterans. Cell Mol Biol 1978b;23: 299–310.

Friebe B, Endo TR, Gill BS. Chromosome-banding methods. In: Misteli K, Shigeki N, editors. Plant chromosomes: laboratory methods. Boca Raton: CRC Press; 1996. p. 123–54.

Garcia BA, Moriyama EN, Powell JR. Mitochondrial DNA sequences of Triatomines (Hemiptera: Reduviidae): phylogenetic relationships. J Med Entomol 2001;38:675–83.

Garcia SL, Mello MLS, Garcia NL, Rodrigues VLCC. Changes in nuclear phenotypes following cold shock in *Panstrongylus megistus* (Burmeister). Mem Inst Oswaldo Cruz 2000a;95:893–8.

Garcia SL, Mello MLS, Rodrigues VLCC, Garcia NL. Nuclear phenotype changes after heat shock in *Panstrongylus megistus* (Burmeister). Mem Inst Oswaldo Cruz 2000b;95:271–7.

Gilbert N, Gilchrist S, Bickmore WA. Chromatin organization in the mammalian nucleus. Int Rev Cytol 2005;242:283–336.

Golub NV, Nokkala S, Kuznetsova VG. Holocentric chromosomes of psocids (Insecta, Psocoptera) analysed by C-banding, silver impregnation and sequence specific fluorochromes CMA(3) and DAPI. Folia Biol (Krakow) 2004;52:143–9.

Joffe B, Leonhardt H, Solovei I. Differentiation and large scale spatial organization of the genome. Curr Opin Genet Dev 2010;20:562–9.

Kuznetsova VG, Maryanska-Nadachowska A, Nokkala S. A new approach to the Auchenorrhyncha (Hemiptera, Insecta) cytogenetics: chromosomes of the meadow spittle bug *Philaenus spumarius* (L.) examined using various chromosome banding techniques. Folia Biol (Krakow) 2003;51:33–40.

Mandrioli M, Bizarro D, Manicardi GC, Gionghi D, Bassoli L, Bianchi U. Cytogenetic and molecular characterization of a highly repeated DNA sequence in the peach potato aphid *Myzus persicae*. Chromosoma 1999;21:436–42.

Maranhão ACB. Proteoma e transcriptoma comparativo das glândulas salivares de barbeiros das espécies de *Rhodnius brethesi*, *Rhodnius robustus* e *Panstrongylus megistus*. PhD thesis, Brasilia: University of Brasília, 2008.

Marcilla A, Bargues MD, Ramsey JM, Magallon-Gastelum E, Salazar-Schettino PM, Abad-Franch F, et al. The ITS-2 of the nuclear rDNA as a molecular marker for populations, species, and phylogenetic relationships in Triatominae (Hemiptera: Reduviidae), vectors of Chagas disease. Mol Phylogenet Evol 2001;18: 136–42.

Mas-Coma S, Bargues MD. Populations, hybrids and the systematic concepts of species and subspecies in Chagas disease triatomine vectors inferred from nuclear ribosomal and mitochondrial DNA. Acta Tropica 2009;110:112–36.

Mello MLS. Nuclear behavior in the Malpighian tubes of *Triatoma infestans*. Cytologia (Tokyo) 1971;36:42–9.

Mello MLS. Feulgen-DNA values and ploidy degrees in the Malpighian tubes of some triatomids. Rev Brasil Pesq Méd Biol 1975;8:101–7.

Mello MLS. Nucléolo: estrutura, ultraestrutura e composição. In: Vidal BC, Mello MLS, editors. Biologia celular. Rio de Janeiro: Livraria Atheneu; 1987. p. 183–95.

Mello MLS, Vidal BC. Microspectrofluorimetry of the naturally fluorescent substances of the Malpighian tubules of *Triatoma infestans* and *Panstrongylus megistus*. Acta Histochem Cytochem 1985;18:365–73.

Mello MLS, Randi MA, Giorgio S, Ferraz-Filho AN, Rodrigues VLCC, Rocha-e-Silva EO, Cordeiro JA. Number of chromosomes, Feulgen-DNA content and nuclear phenotypes in domestic and wild specimens of *Panstrongylus megistus*. Ann Trop Med Parasitol 1986;80:641–8.

Mello MLS, Vidal BC, Russo J, Planding W, Schenck U. Image analysis of the AgNOR response in *ras*-transformed human breast epithelial cells. Acta Histochem 2008;110:210–6.

Morielle-Souza A, Azeredo-Oliveira MTV. Differential characterization of holocentric chromosomes in triatomines (Heteroptera, Triatominae) using different staining techniques and fluorescent in situ hybridization. Genet Mol Res 2007;6: 713–20.

Panzera F, Pérez R, Panzera Y, Ferrandis I, Ferreiro MJ, Calleros L. Cytogenetics and genome evolution in the subfamily Triatominae (Hemíptera, Reduviidae). Cytogenet Genome Res 2010;128:77–87.

Pérez-García C, Cambeiro JM, Morán P, Pasantes JJ. Chromosomal mapping of rDNAs, core histone genes and telomeric sequences in *Perumytilus purpuratus* (Bivalvia: Mytilidae). J Exp Mar Biol Ecol 2010;395:199–205.

Ploton D, Menager M, Jeannesson P, Himber G, Pigeon F, Adnet JJ. Improvement in the staining and in the visualization of the argyrophilic proteins of the nucleolar organizer region at the optical level. Histochem J 1986;18:5–14.

Rouquette J, Cremer C, Cremer T, Fakan S. Functional nuclear architecture studied by microscopy: present and future. Int Rev Cell Mol Biol 2010;282:1–90.

Schneider R, Grosschedl R. Dynamics and interplay of nuclear architecture, genome organization, and gene expression. Genes Dev 2007;21:3027–43.

Schreiber G, Pellegrino J. Eteropicnosi di autosomi come possible meccanismo di speciazione (Ricerche citologiche su alcuni Emitteri neotropici). Sci Genet 1950;3:215–26.

Schreiber G, Bogliolo AR, Pinto AC. Cytogenetics of Triatominae: caryotype, DNA content, nuclear size and heteropyknosis of autosomes. Braz J Biol 1972;32:255–63.

Schweizer D. Reverse fluorescent chromosome banding with chromomycin and DAPI. Chromosoma 1976;58:307–24.

Segura EL, Torres AG, Fusco O, Garcia BA. Mitochondrial 16S DNA variation in populations of *Triatoma infestans* from Argentina. Med Vet Entomol 2009;23:34–40.

Stein GS, van Wijnen AJ, Imbalzano AN, Montecino M, Zaidi SK, Lian JB, et al. Architectural genetic and epigenetic control of regulatory networks: compartmentalizing machinery for transcription and chromatin remodeling in nuclear microenvironments. Crit Rev Eukaryot Gene Expr 2010;20:149–55.

Stothard JR, Yamamoto Y, Cherchi A, Garcia AL, Valente SAS, Schofield CJ, et al. A preliminary survey of mitochondrial sequence variation in Triatominae (Hemiptera: Reduviidae) using polymerase chain reaction-based single strand conformational polymorphism (SSCP) analysis and direct sequencing. Bull Entomol Res 1998;88:553–60.

Vidal BC, Planding W, Mello MLS, Schenck U. Quantitative evaluation of AgNOR in liver cells by high-resolution image cytometry. Anal Cell Pathol 1994;7:27–41.

Zhang WL, Wang X, Yu Q, Ming R, Jiang J. DNA methylation and heterochromatinization in the male-specific region of the primitive Y chromosome of papaya. Genome Res 2008;18:1938–43.