CARYOLOGIA Vol. 58, no. 4: 293-299, 2005

Cytogenetic study on three species of the genus *Triatoma* (Heteroptera: Reduviidae) with emphasis on nucleolar organizer regions

Severi-Aguiar * Grasiela Dias de Campos and Maria Tercília Vilela de Azeredo-Oliveira

Departamento de Biologia, Instituto de Biociências, Letras e Ciências Exatas, IBILCE-UNESP, Rua Cristóvão Colombo n°2265, Caixa Postal 136, São José do Rio Preto, CEP 15054-000, São Paulo, Brasil. Fax +55 17 32212380.

Abstrat — The use of banding techniques allows the recognition of chromosomal pairs and karyotypical arrangements. However, its application in Heteroptera holocentric chromosomes is limited. Thus, little is known about their structure, specially their Nucleolar Organizer Regions (NORs). A comparative analysis of the nucleolar characteristics present during spermatogenesis in *Triatoma platensis, Triatoma protacta* and *Triatoma tibiamaculata* seems to indicate that in this group of insects nucleolar fragmentation occurs after prophase I. The study of chromosomal structure of these triatomines indicates that NORs are located at some telomeric and interstitial autosome regions and at sexual chromosomes (X/X_1X_2).

Key words: holocentric chromosomes, NOR, nucleolar fragmentation, silver impregnation, spermatogenesis, triatomines.

INTRODUCTION

Chagas' disease is a parasitosis of endemic nature that plays a major role in heart diseases in South America. Its vectors are the hematophagous heteroptera of the family Reduviidae, subfamily Triatominae. These insects are of great cytogenetic interest because they present an unusual form of meiosis, in which the segregation of sex chromosomes is postreductional, and chromosomes with diffuse kinetochores, known as holocentric (Tavares and Azeredo-Oliveira 1997; Tartarotti and Azeredo-Oliveira 1999a).

The silver-ion impregnation staining technique has been known for nearly half a century. It is used to mark nucleolar regions, namely the fibrillar centers (FC) and the Nucleolar Organizer Regions (NORs), in the chromosomes that carry the gene loci that code for ribosomal RNA, which are synthesized and processed, in the nucleolus, into pre-ribosomal subunits that will later form the ribosomes of the cytoplasm, essential for protein synthesis. In acidic conditions, the proteins stained by this technique reduce silver. They are found in the nucleolus at interphase and are specifically located in NORs during cell division (Goodpasture and Bloom 1975).

The purpose of the present work was to comparatively analyze the distribution pattern of the Nucleolar Organizer Regions and the nucleolar activity during spermatogenesis in three species of the genus *Triatoma* (*T. platensis, T. protacta* and *T. tibiamaculata*).

MATERIAL AND METHODS

Three species of the genus *Triatoma* (*T. protacta, T. platensis* and *T. tibiamaculata*,) all pertaining to the order Heteroptera, subfamily Triatominae and family Reduviidae (Lent and Wygodzinsky 1979; Manna 1995) were studied. The chromosomal number consisting of 20 autosomes and two sex chromosomes XY in *T. platensis* and three sex chromosomes X_1X_2Y in *T. protacta* and *T. tibiamaculata* (Panzera *et al.* 1998). An average number of 10 individuals per species were analyzed. The specimens (adult males) were provided

* Corresponding author:
e-mail: grazielaguiar@terra.com.br

by the Insectary of the Special Health Service (SESA), Araraquara (SP), organ of the Department of Epidemiology, Public Health School, São Paulo University,São Paulo (SP) Brazil. Testes were crushed, fixed in acetic acid and submitted to silver-ion impregnation (Howell and Black 1980, modified by Bicudo 1992). For documentation was used KODAK TMAX 100ASA film. Black-and-white photomicrographs were taken under an OLYMPUS BX40 microscope.

RESULTS

Triatoma protacta - The polyploid nuclei of the tubule wall nutritive cells showed one larger and several smaller nuclear corpuscles impregnated by silver ions (Figure 1a). The interphase nuclei of spermatogonial cells displayed peripheral or central nucleolar bodies (Figure 1b-c) and spermatogonial metaphase showed some slightly silver-ion

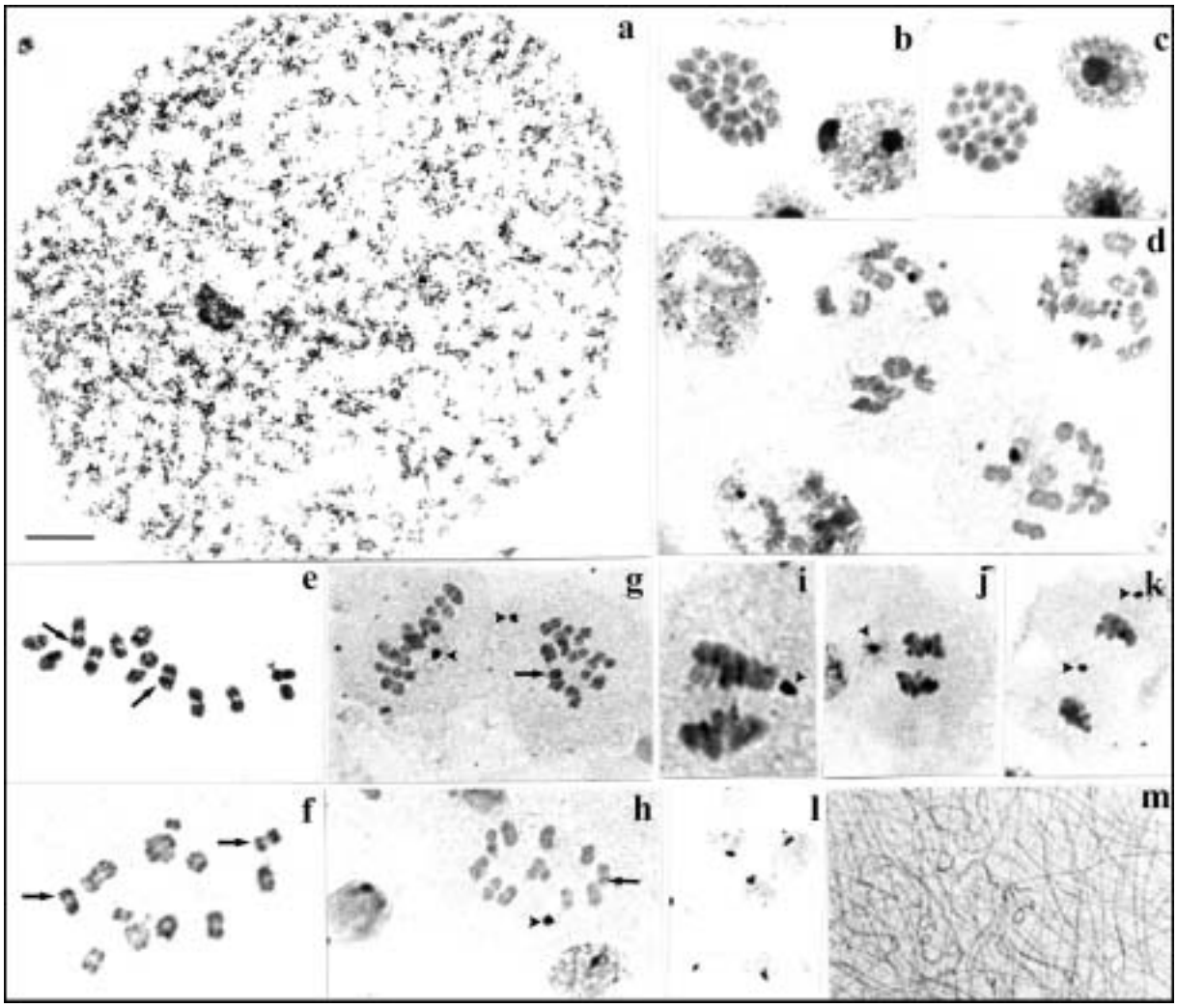

Fig. 1 — *Triatoma protacta* testicular tubules submitted to silver-ion impregnation. (a) Polyploid nucleus of tubule wall nutritive cells with a larger and several smallers silver-ion impregnated corpuscles. (b-c) Spermatogonial metaphase and nuclei: some silver-ion marked chromosomes. (d) Meiotic prophase I (confused stage): several nucleolar dots within the nucleus and a strongly impregnated nucleolar corpuscle. (e-f) First meiotic metaphase: some silver-ion marked bivalents (arrows). (g-h) Second meiotic metaphase: some chromosomes impregnated with silver (arrow) and one nucleolar fragment or corpuscle per cell (arrowheads). (i-j) Second meiotic anaphase: sexual chromosome with late migration and the presence of one nucleolar fragment or corpuscle by cell (arrowheads). (k) Second meiotic telophase: presence of one nucleolar fragment or corpuscle at each pole of the dividing cell (shown by arrowhead). (l) Spermatids at initial differentiation: a nucleolar corpuscle in every cell. (m) Spermatozoans. Bar= 18μm.

marked chromosomes (Figure 1b-c). At initial meiotic prophase I (confused stage), several nucleolar dots were observed within the nuclei as well as a strongly silver-ion impregnated nucleolar corpuscle (Figure 1d). At first meiotic metaphase, some bivalents were silver impregnated (Figure 1e-f). At second meiotic metaphase, these same characteristics were observed and scattered nucleolar fragments and corpuscles were observed within the cell (arrowheads) (Figure 1g-h). At meiotic anaphase the chromosomes progressively migrated to the cell poles, though one of them showed late migration. Still at this phase, nucleolar fragments or corpuscles were observed within the cell (arrowheads) (Figure 1i-j). At meiotic telophase was possible to verify the presence of silver impregnated fragments at each pole of the dividing cell (arrowheads) (Figure 1k). Spermatids at early differentiation presented a nucleolar corpuscle at the periphery of the cell (Figure 1l). Spermatozoans (m). Bar=18µm.

Triatoma platensis - The polyploid nuclei of the tubule wall nutritive cells showed several nucleolar blocks of different sizes (Figure 2a). Spermatogonial metaphase showed some silver-ion impregnated chromosomes (Figure 2b-d). At initial prophase I (confused stage) several nucleolar cor-

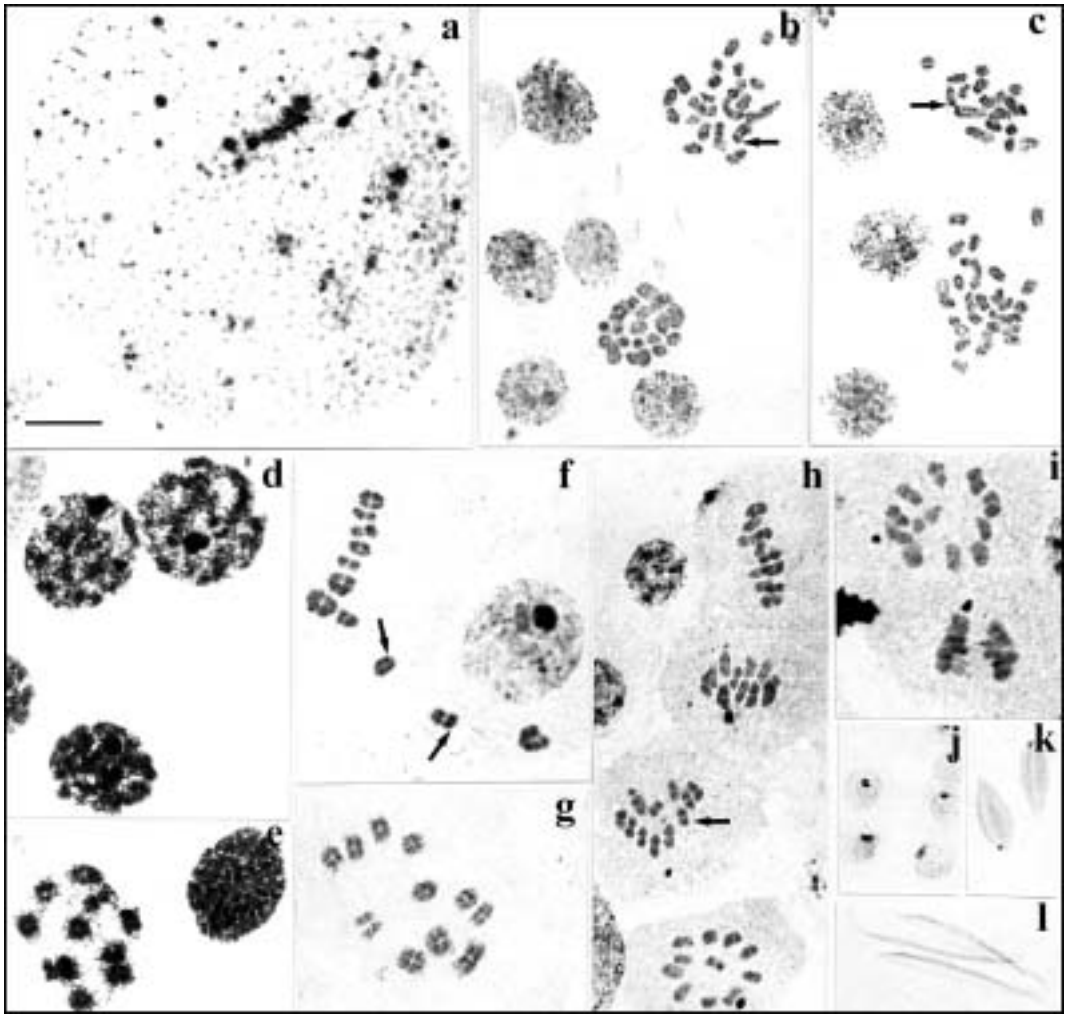

Fig. 2 — *Triatoma platensis* testicular tubules submitted to silver-ion impregnation. (a) Polyploid nucleus of tubule wall nutritive cells with several silver-ion impregnated corpuscles. (b-c). Interphase nuclei of tubule spermatogonial cells and spermatogonial metaphase with silver-ion marked chromosomes (arrows). (d-e) Meiotic prophase I (confused stage): several nucleolar dots within the cell and a strongly impregnated nucleolar corpuscle (f-g) First meiotic metaphase: some silver-ion impregnated bivalents (arrows). (h-i) Second meiotic metaphases: silver-ion impregnated autosomal bivalents (arrows) and one nucleolar fragment or corpuscle per cell. (i) Second meiotic anaphase: presence of one nucleolar fragment or corpuscle per cell. (j-k) Spermatids at initial differentiation with one nucleolar corpuscle in every cell. (l) Spermatids at a more advanced differentiation stage: absent silver-ion impregnation. Bar= 18µm.

puscles were seen within the nucleus as well as a nucleolar region more strongly impregnated with silver-ions (Figure 2d). At first meiotic metaphase some autosomes and sexual chromosome were marked with silver-ions (Figure 2f-g). At second meiotic metaphase, the chromosomes were interconnected and positioned in the center of the autosomal ring. Some chromosomes were silver-impregnated (Figure 2h-i). At meiotic anaphase, the chromosomes progressively migrated to the cell poles and nucleolar fragments or corpuscles were observed within the cell (Figure 2i). At initial spermiogenesis, spermatids showed peripheral nucleolar corpuscles (Figure 2j-k). Bar=18µm.

Triatoma tibiamaculata - The polyploid nuclei of the tubule wall nutritive cells showed a nucleolar corpuscle impregnated with silver-ions (Figure 3a). In the interphase nuclei of the spermatogonial cells, peripheral or central nucleolar bodies were seen (Figure 3b) and spermatogonial metaphase

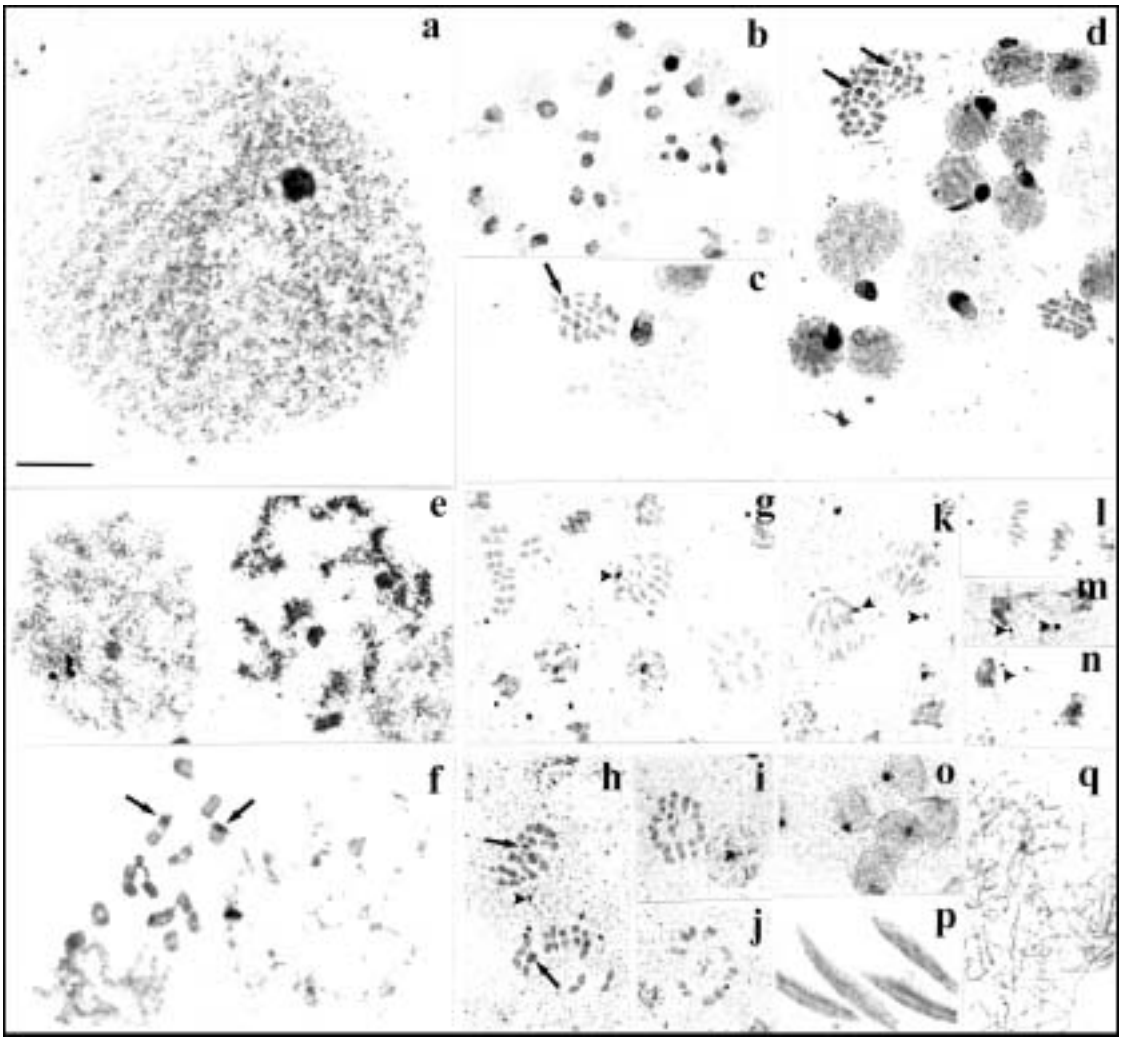

Fig. 3 — *Triatoma tibiamaculata* testicular tubules submitted to silver-ion impregnation. (a) Polyploid nucleus of the tubule wall nutritive cells with a larger and several smaller silver-ion impregnated corpuscles. (b) Interphase nuclei of tubule spermatogonial cells: peripheral or central nucleolar regions. (c-d) Spermatogonial metaphase with silver-ion marked chromosomes (arrows). (e) Nuclei at meiotic prophase I (confused stage): several nucleolar dots within the nucleus and a strongly impregnated nucleolar corpuscle. (f) Metaphase at first meiotic division: some bivalents marked with silver (arrows). (g-j) Metaphase at second meiotic division: some autosomal bivalents impregnated with silver (arrows) and one nucleolar fragment or corpuscle per cell (arrowheads). (k-m) Second meiotic anaphase: sexual chromosome with late migration and the presence of one nucleolar fragment or corpuscle per cell (arrowheads). (n) Second meiotic telophase: presence of one nucleolar fragment or corpuscle at each pole of the dividing cell (shown by arrowhead). (o) Spermatids at initial differentiation; one nucleolar corpuscle present in every cell. (p) Spermatids at a more advanced differentiation stage: absent silver-ion impregnation. (q) Spermatozoans. Bar= 18µm.

showed some silver-ion marked chromosomes (arrows) (Figure 3c-d). At initial meiotic prophase I (confused stage) several nucleolar dots were seen within the nuclei as well as a strongly silver-ion impregnated nucleolar corpuscle (Figure 3e). At first meiotic metaphase some of the bivalents were silver-impregnated and sex chromosomes were individualized. At this phase, scattered nucleolar fragments and corpuscles were observed within the cell (Figure 3f). At second meiotic metaphase, these same characteristics were observed (Figure 3g-j). At meiotic anaphase, the chromosomes progressively migrated to the cell poles and nucleolar fragments or corpuscles were observed within the cell (Figure 3k-m). At meiotic telophase was possible to verify the presence of silver-ion impregnated fragments at each pole of the dividing cell (Figure 3n). Spermatids at initial differentiation showed at the cell periphery a nucleolar corpuscle (Figure 3o) that disappeared as differentiation progressed (Figure 3p-q). Bar=18μm.

DISCUSSION

In some chromosomes of *Triatoma protacta*, *Triatoma platensis* and *Triatoma tibiamaculata* nucleolar organizer activity specially occurs at the extremities and the nucleolus is fragmented after meiotic prophase I, when the presence of nucleolar fragments or corpuscles are observed within the division cell.

MEDINA *et al.* (1986) suggested that NOR silver staining was associated with decondensation of the chromatin that carries rDNA rather than transcriptional activity. Additional support for this idea was provided using transcription inhibitors that do not weaken silver staining (HERNÁNDEZ-VERDÚN *et al.* 1984). It does not seem that a single protein or protein group is responsible for silver staining. In fact, neither the presence of NOR nor transcriptional activity are characteristics common to all silver impregnated structures (MEDINA *et al.* 1986).

The genes that code for ribosomal RNA transcription form the nucleolus (FISCHER *et al.* 1991). Within the nucleolus, the production of pre-ribosomal components takes place including the transcription of rRNA genes, primary transcript processing of 45S rRNA into mature 18S; 5,8S and 28S rRNA, protein addition to pre-ribosome synthesis and incorporation of 5S-rRNA, that transcribed outside the nucleolus (SCHEER and WEISENBERGER 1994; HERNÁNDEZ-VERDÚN 1991).

The main nucleolar ultrastructural components are the fibrillar centers (FC), dense fibrillar components (DFC) and granular components (GC) (WACHTLER and STAHL 1993; RASKA 1995). FC is the component most frequently stained with silver.

Even though nucleolar function has already been elucidated, the functional significance of the different nucleolar components remains unclear. The localization of early ribosome biogenesis takes place, specially rRNA transcription, is still a controversial issue (WACHTLER and STAHL 1993).

HERNANÁNDEZ-VERDÚN (1991) found that chromosomes occupy a specific territory within the nucleus, called chromosomal domain, has been recently demonstrated by chromosome painting using specific probes that correspond to a single chromosome and *in situ* hybridization to localize specific chromosomes during interphase. Therefore, it is not surprising that ribosomal genes are orderly distributed throughout the nuclear structure. Nucleolar Organizer Regions are strongly involved in nuclear polarity. Besides being a traditional cytological mark, polarity is also evident between different NORs and, between NORs and the nuclear envelope. In the metaphase plate, the NORs of the adjacent chromosomes are close to each other, better than it would be expected from a random distribution. This observation indicates that NORs of the adjacent chromosomes are in a polarized arrangement in the interphase nucleus.

The chromosomes that carry NORs may move from different parts of the nucleus to aggregate and form a nucleolus and may be dispersed in one or two cell cycles. This means that they probably move free within the nucleus without affecting the order of the other chromosomes. This positioning seems to be associated with the presence of a specific skeletal structure where the nucleolus connects to the envelope (HERNÁNDEZ-VERDÚN 1991).

The specific compartmentalization of the nucleolar process in the nucleus demonstrates the existence of some kind of cellular mechanism that generates this nuclear and nucleolar organization. The localization of nucleolar proteins in specific regions of the nucleolus indicates that these proteins may show signs that determine their final nucleolar fate (HERNÁNDEZ-VERDÚN 1991).

WACHTLER and STAHL (1993), has demonstrated by electron microscopy that, during mitosis, the nucleolus usually disappears at final prophase and reappears at telophase. During this process, rRNA synthesis ceases. However, this

disappearance is not complete as silver-stained material can be observed in chromosomal NORs during mitosis. At telophase, the dense fibrillar component reappears and is later followed by granular material corroborating that the granular component corresponds to a later step in the ribosome biogenesis than the fibrillar component.

It is probable that the relatively quick reappearance of the nucleulus after karyokinesis is not only due to the "de novo" synthesis of the nucleolar material, but also to the reconstruction of the nucleolus from the nucleolar material that is dispersed in the nucleus and cytoplasm at prophase. Some observations found in the literature support this idea. The increase observed in the silver stained material after mitosis is not weakened by protein synthesis inhibition, indicating that nucleolar reconstruction does not depend solely on material that is once more synthesized. The disintegration and subsequent reintegration of nucleolar material during mitosis has been corroborated by *in situ* hybridization combined with the use of antibodies against nucleolar proteins. These studies indicated that nucleolar proteins are again specifically localized around the chromosomes during mitosis (Wachtler and Stahl 1993; Mello 1995). The phenomenon called nucleolar persistence, that is, the presence of nucleolus during mitosis, is observed in dynoflagellates and has been described in the malign cells of mammals. In embryo cells with carcinoma, the nucleulus is present and transcriptionally active throughout mitosis, showing mitosis itself is not an insuperable barrier for rRNA transcription (Wachtler and Stahl 1993).

During mitosis, proteins that play a key role in the transcription of rDNA genes, such as RNA-polymerase I and DNA topoysomerase, remain associated with chromosomal NOR, that also anchors rRNA genes. The mechanism that modulates the activity of the rRNA genes during mitosis may provide an interesting case for the analysis of the control of gene activity and structure. The post-mitotic nucleolar reformation depends on the interaction of two separate entities, NOR and pre-nucleolar bodies (PNBs). PNBs appear at telophase and are completely dispersed at the beginning of the reformation of the son nuclei when they progress to phase G1 (Scheer and Weisenberger 1994).

The analysis of meiosis in the triatomines under study indicates that our results are in agreement with nucleolar material persistence during mitotic cell cycle. In all species studied, it was possible to observe nucleular fragments or corpuscles at the more advanced stages of division such as anaphase and telophase. These fragments or corpuscles may be nucleular remains. Thus, the nucleulus may not completely disappear during the spermatogenesis of triatomines, but may persist in the form of small pre-nucleolar corpuscles that reunite to form the next nucleolar cycle, that, in the case of meiosis, will only be completed if the fertilization and formation of a zygote occurs.

The persistence of bodies such as nucleoli during mitosis supports the idea that nucleolar material does not completely break at metaphase and anaphase though its functional significance is still not very clear. It may be hypothesized that these bodies carry primary or new nucleolar material or even supply nucleolar RNA to the daughter cells while the new nucleolus is being organized (Mello 1995).

Studies on different species show that Ag-NOR staining sites on chromosomes correspond to ribosome genes sites identified by *in situ* hybridization. However, among humans, for example, all 10 rDNA sites, i.e. the acrocentric chromosomes 13, 14, 15, 21 and 22 that form a characteristic secondary constriction in each one of these chromosomes at metaphase (Fakan and Hernández-Verdun 1986), are very rarely stained with silver. This is likely to be due to the fact that some sites contain less rDNA genes, below the limit of silver impregnation sensitivity (Sumner 1990). In such studies, NORs were observed at the borders of some chromosomes.

In the present work, arginophylic markings were observed in the initial spermatids of the three *Triatoma* species. The analysis of spermatogenesis in others triatomines species, with this same technique, showed similar results (Tavares and Azeredo-Oliveira 1997; Tartarotti and Azeredo-Oliveira 1999b; Morielle and Azeredo-Oliveira 2004). This silver-ion impregnation disappeared as spermatids underwent elongation as reported in the literature about a series of vertebrates and some cephalochordates. Thus, our study corroborates the hypothesis of RNA gene post-meiotic reactivation, allowing this phenomenon to be also applied to invertebrates, and reinforcing the initial idea that RNA synthesis at gametogenesis is a possibly universal pattern among organisms with sexual reproduction.

It is probable too that many messenger RNAs that code for spermatozoa proteins are synthesized before cell division in the spermatocytes and stored in a "long-lived" form as "chromatoid bodies", for translation during late spermiogenesis where the genome is not active. Subsequently, this

body moves to the area where the flagellum connects with the spermatid nucleus and forms a ring-shaped structure. Maybe, the most interesting function proposed regarding the "chromatoid body" is the participation of RNA metabolism in spermatogenic cells. Even though very little understood, there is evidence that considerable amounts of RNA synthesized in the spermatocytes of mammals at pachytene are preserved during spermatid development until final spermiogenesis (PARVINEN *et al*. 1986). Therefore, we suggest that this same mechanism may also explain the presence of silver-ion impregnated corpuscles in initial spermatids of these *Triatoma* species.

Acknowledgements — The authors are thankful to Dr. José M. Soares Barata (director), João Luis Molina Gil and João Maurício R. da Silva Filho, technicians of the Insectary of the Special Health Service (SESA) (Araraquara, SP), organ of the Department of Epidemiology- São Paulo Public Health School (São Paulo, SP) for supplying the specimens studies. Special thanks to FAPESP (98/12876-6) and CAPES for their financial support.

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Received VI.01.2005; accepted IX.09.2005