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Partitioning of Large and Minichromosomes in Trypanosoma brucei
Klaus Ersfeld* and Keith Gull

The Trypanosoma brucei nuclear genome contains about 100 minichromosomes of between 50 to 150 kilobases and about 20 chromosomes of 0.2 to 6 megabase pairs. Minichromosomes contain nontranscribed copies of variant surface glycoprotein (VSG) genes and are thought to expand the VSG gene pool. Varying VSG expression allows the parasite to avoid elimination by the host immune system. The mechanism of inheritance of T. brucei chromosomes was investigated by in situ hybridization in combination with immunofluorescence. The minichromosome population segregated with precipitation, by association with the central intranuclear mitotic spindle. However, their positional relationship with the central intranuclear mitotic spindle in Trypanosoma cruzi has been examined by immunofluorescence and confocal microscopy. The intranuclear mitotic spindle is a novel mitotic spindle that is found in Trypanosoma cruzi and related flagellates. The intranuclear mitotic spindle is thought to play a role in chromosome segregation during cytokinesis. The intranuclear mitotic spindle is also found in other flagellates, such as Trypanosoma brucei and Leishmania donovani. These organisms are thought to have evolved from a common ancestor and share many similarities in their mitotic spindles. The intranuclear mitotic spindle in Trypanosoma brucei is a novel spindle that is unique to this organism and is not found in other eukaryotes. The intranuclear mitotic spindle is thought to play a role in chromosome segregation during cytokinesis and may be involved in the regulation of cell division. The intranuclear mitotic spindle is also found in other flagellates, such as Trypanosoma cruzi and Leishmania donovani. These organisms are thought to have evolved from a common ancestor and share many similarities in their mitotic spindles.
electron microscopic (EM) studies of dividing *T. brucei* nuclei indicate that the mitotic spindle contains insufficient microtubules to provide a conventional centromere-microtubule interaction for all of the more than 100 chromosomes (2, 10). Rather, the chromatin of dividing *T. brucei* cells seems to be closely associated with the nuclear envelope (10, 11). In a more recent study, in situ hybridization was used to demonstrate a peripheral localization of minichromosomes in the nucleus of interphase cells of trypanosomes (12).

Given genetic evidence of mitotic stability (13) yet uncertainty over the segregation mechanism, we addressed the question of where chromosomes of procyclic *T. brucei* are located during the cell cycle and how their segregation is achieved. To visualize spindle microtubules, we used a monoclonal antibody (KMX) specific for β-tubulin (14, 15). Fluorescence in situ hybridization (FISH) was used to visualize DNA segments of individual chromosomes (16, 17). To locate minichromosomes, we used a 177-base pair (bp) repeat sequence (MC177) as a probe (18). This sequence is specific for minichromosomes and has been used previously to study their distribution in interphase cells (12). To visualize a DNA segment on one of the larger chromosomes, we chose the 5S ribosomal DNA (R5S) because this gene exists as a linear array of hundreds of tandem repeats on one of the chromosomes larger than 4 megabase pairs (Mbp) (19, 20). The segregation pattern observed for the chromosome carrying the 5S genes is likely to be representative of the large chromosomes of *T. brucei* in general.

In support of this hypothesis, very similar results were obtained with genomic DNA clones covering about 150 kilobase pairs of the tubulin locus located in the center of a chromosome of about 1 Mbp (21). The small size of even the largest chromosomes of *T. brucei* and the size and position of the target DNA sequences used for FISH exclude trailing effects of those loci in relation to potential centromeres, which have not yet been identified.

Although chromosomes in trypanosomes do not visibly condense, probably as a result of their unusual histone composition (22), it is relatively easy to follow the progression of mitosis using nuclear elongation and segregation of the mitochondrial kinetoplast as markers (10, 23). Elongation of the nucleus indicates the onset of mitosis. The nucleolus does not disperse during mitosis but elongates and acquires a dumbbell shape before it splits into two entities preceding karyokinesis.

During interphase the minichromosomes were located in small clusters distributed asymmetrically around the periphery of the nucleus (Fig. 1, A and D). As the cells progressed toward M phase, a reorganization occurred. During the transition from G2 to M phase, the small minichromosome groups started to aggregate into larger and fewer clusters. Once the cell had clearly entered mitosis, indicated by the appearance of a small spindle, the minichromosomes congregated into one mass in the center of the nucleus (Fig. 1B). The mitotic spindle of trypanosomes consists of a central array of densely packed microtubules plus peripheral microtubules; the latter might represent pole-to-kinetochore microtubules because they terminate in electron-dense structures resembling the kinetochores described in higher eukaryotes (10, 11). After the establishment of the spindle, the minichromosomal DNA split into two equal-sized clusters that subsequently moved to the poles of the spindle (Fig. 1, C and D). As the central spindle elongated, the minichromosomes remained at their polar position (Fig. 1E). Late in mitosis the poles of the spindle were close to the nuclear envelope with the minichromosomes still attached. After the disassembly of the spindle, shortly before nuclear division, the minichromosomes congregated close to the nuclear envelope. This distribution pattern could still be observed in cells during and after cytokinesis; only later, during S and G1 phases, did the clusters disintegrate into smaller units (Fig. 1A, lower cell). This explained the nonrandom spatial

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**Fig. 1.** The distribution of minichromosomes during the cell division cycle. (A) In interphase, minichromosomes were asymmetrically distributed near the nuclear periphery (see also right cell in D). (B) After formation of a spindle, minichromosomes congregated in the nuclear center. (C to E) During progression of mitosis, the minichromosomes separated into two entities and relocated to the poles of the spindle. The minichromosomal signal is shown in red, the antibody to tubulin in green, and the total DNA in blue. The third and fourth frame of each row represent the merged signals and the phase-contrast image, respectively. The kinetoplasts as markers for cell cycle progression are labeled by arrows (first row only). Bar, 10 μm.
distribution of minichromosomes in G1 cells and, to a lesser extent, in S phase (12).

The segregation of large chromosomes, as deduced by the 5S ribosomal gene cluster localization, followed a different pattern (Figs. 2 and 3). Based on the analysis of many cells, the two dots representing the G1-phase diploid and, after S phase, the tetraploid chromosome complement did not exhibit a preferential localization within the nucleus (Fig. 2A). After DNA replication the RSS signals were still visible as two single dots because the sister chromosomes had not yet segregated (Fig. 2B). In early mitosis, when the minichromosomes were still congregated in the center of the nucleus, the RSS signals occupied a position near the nuclear periphery (Fig. 2B). Once the minichromosomes were positioned at the spindle poles, the RSS signals trailed behind and relatively closer to the midpoint of the spindle (Fig. 2C), suggesting different velocities of polar movement. As mitosis progressed, the RSS dots were frequently found at the outer periphery of the central spindle (Figs. 2, C and D, and 3, A and B), consistent with the position of kinetochore-like structures in spindles of dividing nuclei at these stages seen in EM images (11, 24). In late anaphase the RSS dots eventually moved closer to the poles but never overlapped with the minichromosomal locations (Figs. 2D and 3C).

To demonstrate the dependence of minichromosomal segregation on an intact mitotic spindle, we treated trypanosomes with the drug rhizoxin, which disassembles microtubules (25). A concentration of 5 to 20 nM rhizoxin affects the integrity of the spindle but leaves other microtubule-containing structures, such as the subpellicular cytoskeleton and the axoneme of the flagellum, largely unaffected. Treatment of cells for 4 hours with 5 nM rhizoxin resulted in the formation of aberrant spindle morphology in mitotic cells (Fig. 4A). Minichromosomes in these cells still associated with the malformed spindles but failed to segregate to the poles. Instead, they often formed rod-shaped structures along bundles of microtubules. After treatment with 10 nM rhizoxin for 4 hours a spindle was no longer visible, but in some cells small tubulin-containing structures could be detected at a position corresponding to the poles of the former spindle (Fig. 4B). Treatment with 20 nM rhizoxin prevented any reorganization of minichromosomes in cells that, according to the position of their kinetoplasts, should have entered mitosis (Fig. 4C).

We propose the following model for the mechanism of chromosome segregation in T. brucei. Minichromosomes congregate in the center of the nucleus at the onset of mitosis. This aggregation may, or may not, Fig. 2. Minichromosomes (red) and large chromosomes (yellow) exhibit different positional dynamics during mitosis. (A) During interphase the RSS signal was located in a central position within the nucleus, whereas minichromosomes were close to the nuclear envelope. (B) On the onset of mitosis minichromosomes congregated in the center of the nucleus, whereas the 5S signals were near the periphery of the nucleus. (C and D) As mitosis progressed, minichromosomes became localized at the spindle poles and the 5S dots were closer to the center of the spindle. The approximate position of the spindle corresponds to the black exclusion zone between the DNA (blue) and by the dark structure inside the nucleus in the corresponding phase-contrast images, which is caused by the spindle and the persistent nucleolus. Bar, 10 μm.

Fig. 3. The localization of a large chromosome during mitosis. (A and B) In early stages of anaphase the 5S ribosomal gene signal (yellow) was located outside the central spindle (green). (C) In late anaphase, shortly before karyokinesis, the dots were much closer to the spindle poles. Phase-contrast images are shown on the far right. Bar, 10 μm.

Fig. 4. The effect of the anti-microtubule drug rhizoxin on the segregation of minichromosomes (color designation as in Fig. 1). (A) Rhizoxin (5 nM) still allowed a small spindle to form but prevented the polar organization of minichromosomes; they remained distributed alongside the entire spindle. (B) At 10 nM the drug prevented spindle formation but left two small structures. Minichromosomes colocalized with these structures, which were interpreted as spindle pole remnants. (C) Rhizoxin (20 nM) inhibited spindle formation completely and prevented minichromosomes from reorganizing in the nuclear center at the onset of mitosis. The interkinetoplast distance (the two DAPI-stained dots) indicated that the cell should have entered mitosis by this time. Bar, 10 μm.
be preceded by a condensation of the chromosomes. After association with the emerging central spindle, they separate into two clusters that move to opposite spindle poles. They remain at the spindle poles during spindle elongation until they are in close proximity to the nuclear envelope. Their asymmetrical distribution within the nucleus is maintained after spindle disassembly until late S phase when they are distributed almost randomly near the nuclear envelope. As the ploidy is uncertain and individual minichromosomes cannot be visualized owing to the lack of large enough specific target DNA sequences, it is not clear whether there is faithful segregation of each minichromosome. However, detailed microscopic analysis of many cells (>100) showed that the minichromosomal clusters segregated on the spindle and inherited by each daughter cell were of equivalent size, indicating a precise segregation mechanism.

The existence of a highly coordinated segregation mechanism for minichromosomes suggests that they play an important role in the biology of this parasite. In addition, owing to their small size, minichromosomes may serve as an excellent model for the study of mitotic segregation, particularly with respect to the evolution of DNA partition mechanisms. The diploid large chromosomes, as exemplified by the chromosome harboring the 53 ribosomal gene, are likely segregated by peripheral pole-to-kinetochore microtubules. There is, however, an intriguing discrepancy between the number of large chromosomes, estimated to be at least 20 for the diploid set (4, 21), and the number of kinetochore-like structures, estimated to be approximately 10 (11, 24).

REFERENCES AND NOTES


Continuous in Vitro Evolution of Catalytic Function

Martin C. Wright and Gerald F. Joyce*

A population of RNA molecules that catalyze the template-directed ligation of RNA substrates was made to evolve in a continuous manner in the test tube. A simple serial transfer procedure was used to achieve approximately 300 successive rounds of catalysis and selective amplification in 52 hours. During this time, the population size was maintained against an overall dilution of 3 × 10^9. Both the catalytic rate and amplification rate of the RNAs improved substantially as a consequence of mutations that accumulated during the evolution process. Continuous in vitro evolution makes it possible to maintain laboratory “cultures” of catalytic molecules that can be perpetuated indefinitely.

The principle of Darwinian evolution is applicable in vitro when a population of informational macromolecules is subjected to repeated rounds of selective amplification and mutation. An earlier extracellular Darwinian evolution experiment was done with variants of Qβ bacteriophage genomic RNA that were amplified on the basis of their ability to serve as a substrate for the Qβ replicase protein (1). Evolution was made to occur in a continuous manner by serial transfer of the RNAs to successive reaction vessels. In recent years, in vitro evolution procedures have been generalized to encompass almost any nucleic acid molecule, including those that have catalytic function (2). Unlike the Qβ evolution experiments, however, the evolution of catalytic function has been carried out in a stepwise rather than continuous fashion. Stepwise evolution requires intervention by...