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REPLICATION OF THE KINETOPLAST DNA OF *LEISHMANIA TARENTOLAE* AND *CRITHIDIA FASCICULATA*

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Summary

1. Replicating kinetoplast DNA networks from both *Crithidia fasciculata* and *Leishmania tarentolae* have an equilibrium density in ethidium bromide—CsCl which is less than that of covalently closed non-replicating networks. After a few hours of chase, these networks assume the covalently closed position in the gradient.

2. Pulse-labeled minicircles isolated from sonicated networks band in the upper position in ethidium bromide—CsCl equilibrium gradients. Both "free" and network minicircles incorporate [^{3}H]thymidine at the same rate in a pulse. Labeled single stranded fragments of less than unit minicircle length are released from pulse-labeled minicircles in alkali.

3. Intact networks of *Leishmania* and *Crithidia* can be isolated in the process of replication: replication involves a doubling of the surface area of the networks as visualized by spreading the kinetoplast DNA on glass slides, staining and examining in the light microscope.

4. DNA replication within the kinetoplast DNA networks of both *Leishmania* and *Crithidia* in all parts of the S phase is restricted to the periphery of the structures. In *C. fasciculata* the pulse-labeled DNA remains in position as the network enlarges by peripheral growth, and then becomes redistributed throughout the network sheet by an unknown mechanism after one cell generation.

5. In the case of *L. tarentolae* it was demonstrated by density transfer experiments that all network minicircles replicate by an apparent semi-conservative pattern in one cell generation. This implies that there is some type of

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Abbreviation: SSC, 0.15 M NaCl—0.015 M sodium citrate, pH 7.0.

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lateral mobility of molecules within the network, by which minicircles move past the peripheral locus of replication.

Introduction

Very little is known about the replication of the kinetoplast DNA of the hemoflagellates. The very complexity of the kinetoplast DNA structure — a large "network" of approx. 10^4 intercatenated minicircles and other molecules — makes the problem formidable [1,2]. It has been suggested from qualitative electron microscope autoradiography studies of [^{3}H]thymidine incorporation in *Trypanosoma lewisi* [5] and *Crithidia fasciculata* [6] that DNA synthesis is limited to the end portions of the kinetoplast DNA in situ structure in thin sections of intact cells.

This report presents light microscope autoradiographic evidence for a peripheral replication pattern in kinetoplast DNA networks from two species of hemoflagellates (*Leishmania tarentolae* and *Crithidia fasciculata*). The replication of kinetoplast DNA networks was also investigated in terms of the component molecules by means of density labeling and pulse-chase studies.

Methods

Cell culture

L. tarentolae (clonal strain Lt-Cl) and *C. fasciculata* (clonal strain Cf-Cl) cells were grown as described previously [4] in Difco brain heart infusion medium (BHI) or in defined media. The defined medium for *L. tarentolae* (Medium CA) was modified Medium C of Trager [10] containing 20 μg/ml adenine in place of the purine-pyrimidine mixture. The defined medium for *C. fasciculata* was that of Kidder and Dutta [11].

Ethidium bromide—CsCl equilibrium gradients

These were carried out, fractionated and counted as described elsewhere [12].

Band velocity sedimentation in sucrose gradients

This was performed as described previously both for neutral and alkaline separations [8].

Light microscopy of kinetoplast DNA networks

Purified networks in 0.15 M NaCl—0.015 M sodium citrate (SSC) were mixed with 1% bovine serum albumin in SSC and smeared onto gelatin-subbed glass slides, air-dried, fixed in methanol for 5 min and stained in Giemsa for 1 h.

For autoradiography the unstained smears were coated with 1:1 diluted Ilford L-4 emulsion. Slides were developed for 4 min in D-19 developer, fixed and stained with Giemsa for 1 h. Micrographs were taken using Pan X film and a Zeiss Universal microscope with 100 × Neofluor phase contrast objective.

Analytical CsCl centrifugation

This was carried out as described previously [2].

Isolation of kinetoplast DNA networks

This was carried out by the hot sarkosyl-pronase lysis and differential centrifugation technique described elsewhere [12].

Isolation of labeled reference DNAs

^{14}C-labeled nuclear DNA was isolated from total cell lysate after removal of the kinetoplast DNA networks by centrifugation. The isolation procedure has been described previously [2]. The cells had been grown in Medium CA with 0.5 μCi/ml [^{14}C] thymidine (36 Ci/M) for 4 days.

Partial synchronization of C. fasciculata cells

Cells were treated with 200 μg/ml hydroxyurea in BHI medium for 6 h at 27°C and were then washed and resuspended in BHI without hydroxyurea. Samples were taken at different times and pulsed with 50 μCi/ml [^{3}H]-thymidine in Medium CA to measure the rate of DNA synthesis per 10^6 cells. Smears were also prepared and stained with Giemsa for light microscopy, and the percentage of dividing cells was scored.

Results

Ethidium bromide—CsCl analysis of replicating networks

It has been demonstrated that essentially all of the kinetoplast DNA networks from stationary phase *L. tarentolae* and *C. fasciculata* cells can be recovered in a covalently closed configuration (ref. 12, and Simpson, A.M. and Simpson, L., unpublished results). However, a significant fraction of kinetoplast DNA networks from log phase cells possess an equilibrium density in ethidium bromide—CsCl which is less than that characteristic of covalently closed molecules. This phenomenon was visualized by centrifuging purified *L. tarentolae* network DNA to equilibrium in ethidium bromide—CsCl, as shown in Fig. 1. The network preparation from early log phase cells in Fig. 1a showed an intermediate and upper region in the gradient in addition to the major lower band, whereas the network preparation from stationary phase cells in Fig. 1b showed only a lower band.

The same phenomenon occurs in the replication of *C. fasciculata* kinetoplast DNA networks. Log phase cells were prelabeled with [^{14}C] thymidine for 18 h and were then pulsed with [^{3}H] thymidine for 10 min and chased with unlabeled thymidine. The ethidium bromide-CsCl equilibrium banding patterns of purified *Crithidia* kinetoplast DNA networks at several times during the chase are shown in Fig. 2. The pulse-labeled networks assumed a broad intermediate position in the gradient and then moved entirely to the covalently closed position by about 2 h into the chase. Another wave of nicking and subsequent covalent closure, due to the movement of the pulse-labeled cells through one entire cell cycle, can be seen in Fig. 2f and 2g. The labeled networks from stationary phase cells in Fig. 2h are completely covalently closed. The absence of contaminating nuclear DNA was demonstrated both by the ^{14}C

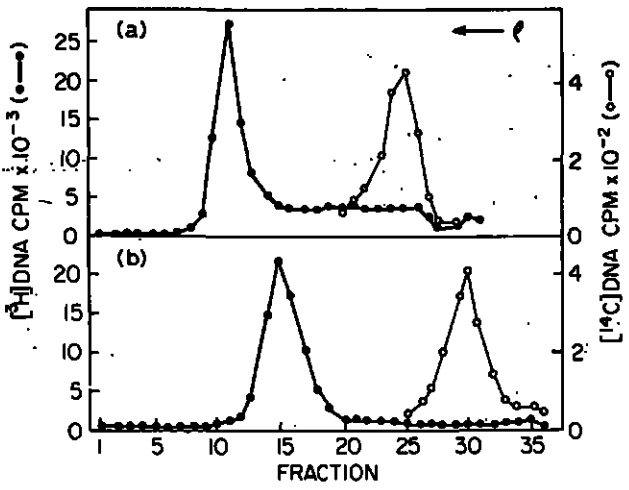

Fig. 1. Ethidium bromide—CsCl equilibrium gradients of purified ^{3}H-labeled kinetoplast DNA from *L. tarentolae* cells at two times in the growth curve. ^{14}C-labeled *L. tarentolae* nuclear DNA was added to each tube as an internal upper band marker. Centrifugation conditions: $n_D^{25°} = 1.3876$, 330 μg ethidium bromide/ml, 40 h at 40000 rev./min, 20°C, No. 50 rotor. Four drop fractions were collected from the bottom onto Whatman No. 3MM filter discs, which were trichloroacetic acid-processed and counted. (a) 8.5 h cells ($28 \cdot 10^6$ cells/ml), (b) 2 day cells ($82 \cdot 10^6$ cells/ml).

profile in each case, and also by analytical CsCl equilibrium centrifugation of each sample (data not shown).

Similar results were obtained when this experiment was repeated with *L. tarentolae* (data not shown).

Pulse-labeling minicircles

We examined the specific activities of the various molecular components of kinetoplast DNA directly after pulsing *L. tarentolae* cells with [^{3}H]-thymidine. In order to increase the amount of label taken up, the cells were partially synchronized with hydroxyurea prior to pulsing with 10 μCi/ml [^{3}H]-thymidine for 18 min. As shown in Table I, essentially no label was incorporated into closed minicircles, either "free" [7] or isolated from sonicated networks. Open "free" monomeric minicircles had a specific activity of 30 900 cpm/μg. The somewhat lower specific activities of the open minicircles, open catenanes and fragments derived from sonicated networks could be explained on the basis of the isolation technique, which yields peaks that are derived from broken or nicked closed minicircles as well as from originally open minicircles. The data are consistent with the conclusions that only apparently open minicircles take up label in a pulse and that there is no difference between the labeling of network open minicircles and "free" open minicircles. Since we do not known whether these pulse-labeled minicircles are identical in all respects to true open (Form II) minicircles, we shall designate pulse-labeled minicircles as Form II* molecules.

As shown in Fig. 3, sedimentation of pulse-labeled "free" II* minicircles in alkaline sucrose gradients demonstrated that the radioactivity was only incorporated into linear strands, and that the labeled fragments were mostly smaller than unit length minicircle DNA.

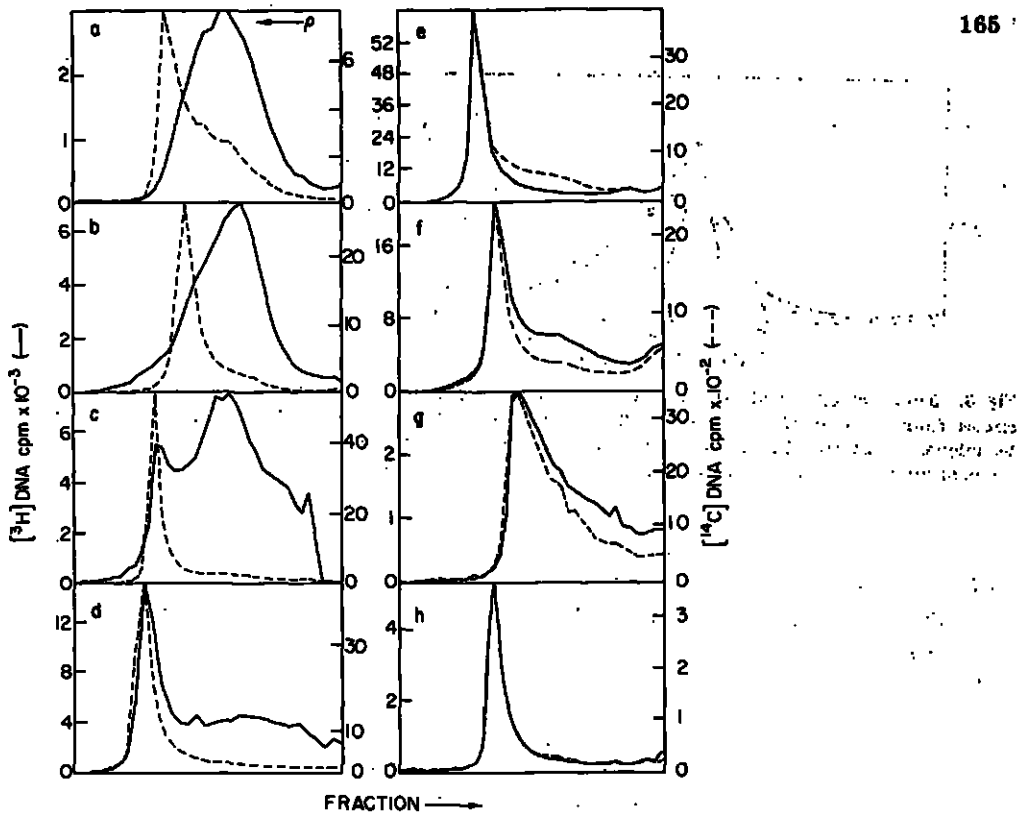

Fig. 2. Ethidium bromide—CsCl equilibrium gradients of purified kinetoplast DNA from pulsed and chased log phase *C. fasciculata* cells. Cells were prelabeled in Kidder and Dutta's medium for 18 h with [^{14}C] thymidine (1 μCi/ml, 36.6 Ci/M) and were then washed and pulsed with [^{3}H] thymidine for 10 min (50 μCi/ml, 18 Ci/mM). The cells were then washed in SBG with 100 μg/ml of unlabeled thymidine and were resuspended in BHI medium with 50 μg/ml of unlabeled thymidine. The kinetoplast DNA was purified as described elsewhere [13]. Centrifugation conditions: $n_D^{25°}$ = 1.3880, 40 000 rev./min, 20°C 48 h, No. 50 rotor. Four-drop fractions were collected from the bottom onto Whatman 3MM discs which were trichloroacetic acid-processed and counted. The data were corrected for crossover and computer plotted. — — —, ^{14}C-prelabeled DNA; ———, ^{3}H pulse-labeled DNA. The chase times were as follows: (a) 0, (b) 0.5 h, (c) 1.0 h, (d) 1.5 h, (e) 2.0 h, (f) 5.0 h, (g) 6.0 h, (h) 3.0 days.

TABLE I

PULSE-LABELING OF *L. TARENTOLAE* KINETOPLAST DNA MINICIRCLES WITH [^{3}H] THYMIDINE

L. tarentolae cells in Medium CA were partially synchronized with hydroxyurea and immediately after release from hydroxyurea inhibition were pulsed with [^{3}H] thymidine (10 μCi/ml, 18 Ci/mM) for 18 min. Kinetoplast DNA networks were isolated, sonicated and separated into closed and open fractions by ethidium bromide—CsCl equilibirum centrifugation. The various molecular species were resolved by band velocity sedimentation in 5—20% sucrose gradients in SSC. The absence of nuclear DNA in the upper ethidium bromide—CsCl band was demonstrated by analytical CsCl equilibrium centrifugation. "Free" minicircles were isolated by alkaline lysis Method II of Wesley and Simpson [7], and were separated into closed and open molecules by band velocity sedimentation in 5—20% alkaline sucrose gradients.

DNA components from sonicated networks	Specific activity (cpm/μg)	"free" minicircles	Specific activity (cpm/μg)
Closed minicircles	180	closed	530
Half minicircle fragments	20240	open	30900
Open minicircles	24680		
Higher open catenanes	23250		

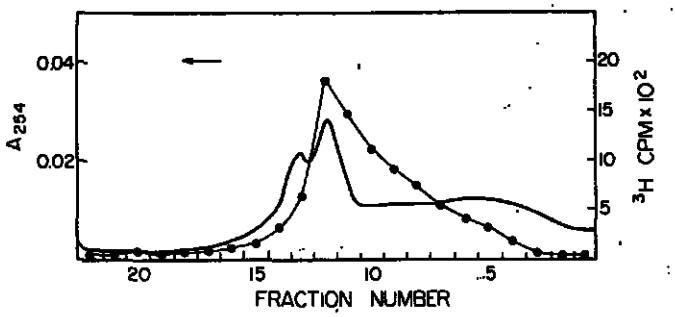

Fig. 3. Band velocity sedimentation of [^{3}H]thymidine pulse-labeled "free" monomeric II* minicircles from *L. tarentolae* in 5—20% alkaline sucrose gradient. The experiment and the preparation techniques are described in Table I. Centrifugation conditions: 60 000 rev./min, SW 50 rotor, 10 h, 4°C. ——, absorbance at 254 nm and the filled circles the radioactivity profile.

Isolation of networks from partially synchronized cells

The actual division process of kinetoplast DNA networks in thin sections has been described for several species (See ref. 1 for review). This process implies a doubling of the surface area of the kinetoplast DNA network, assuming a uni-minicircular thickness at all stages of replication.

As shown in Figs 4 and 5, networks isolated from hydroxyurea-synchronized [4] *L. tarentolae* and *C. fasciculata* cells at different points in the cell cycles showed the expected increase in surface area when spread on glass slides, stained with Giemsa and examined in the light microscope. The differences between the size distribution of early S phase networks, intermediate S phase networks, and late S phase networks are readily apparent. The networks in Figs 4d and Fig. 5e were taken from cells at the end of S phase and

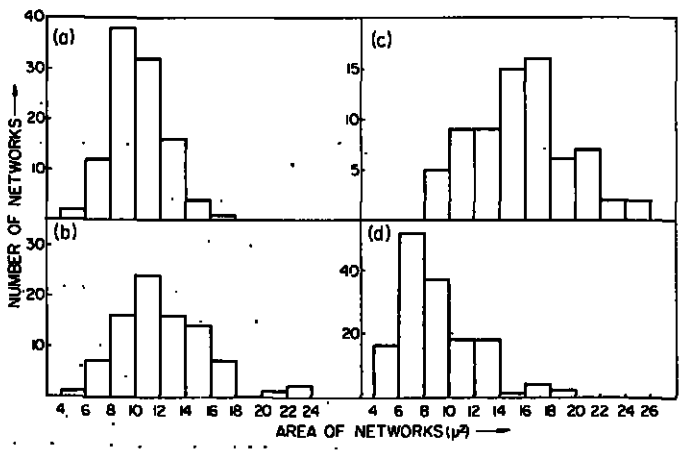

Fig. 4. Size distribution histograms of *L. tarentolae* kinetoplast DNA networks isolated from hydroxyurea synchronized cells [4] at several times in the cell cycle. The networks were processed for light microscopy as described in Methods. (a) Early S phase, 1 h after release from inhibition. (b) Intermediate S phase 2 h after release from inhibition. (c) Late S phase, 3 h after release from inhibition. (d) End of S phase, beginning of cell division, 4 h after release from inhibition.

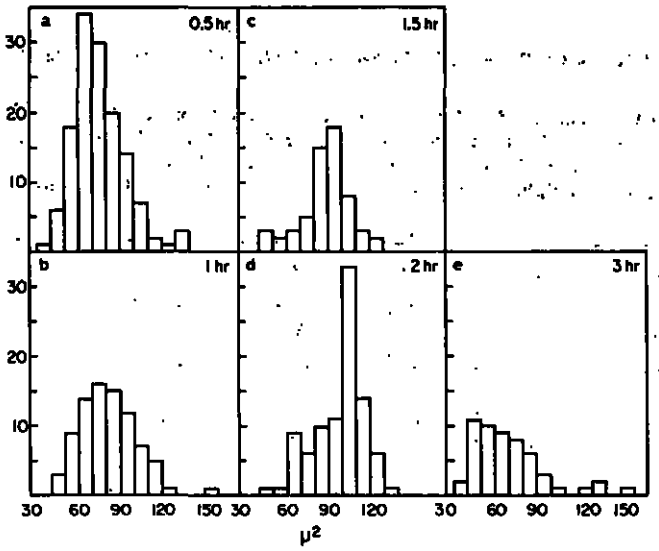

Fig. 5. Size distribution histograms of *C. fasciculata* kinetoplast DNA networks isolated from hydroxyurea synchronized cells at several times in the cell cycle. The networks were processed for light microscopy as described in Methods. (a) Early S phase, 30 min after release from inhibition. (b) Intermediate S phase, 1 h after release from inhibition. (c) Intermediate S phase, 1.5 h after release from inhibition. (d) Late S phase, 2.0 h after release from inhibition. (e) Cell division, 3 h after release from inhibition.

the beginning of cell division, and the decrease in the average network size is consistent with kinetoplast DNA division. The larger size of *Crithidia* networks as compared to *Leishmania* networks is a striking difference between the species.

Localization of the sites of DNA replication by autoradiography of [³H] thymidine labeled networks

Due to the large surface area of isolated networks when spread on glass slides, it was possible to localize the sites of DNA replication within the enlarging network by means of light microscope autoradiography. This was performed with both *L. tarentolae* and *C. fasciculata*, although the kinetoplast DNA networks of *Crithidia* are larger and less fragile and therefore more suitable for this technique. Log phase *C. fasciculata* cells were pulsed for 10 min with [³H]thymidine and then chased with unlabeled thymidine. Kinetoplast DNA networks were isolated and processed for light microscope autoradiography. Labeling patterns were classified into several basic types and the relative numbers of labeled networks showing these patterns were scored. The data from one representative experiment are shown in Table II and micrographs of representative labeled patterns observed are presented in Fig. 6. It should be emphasized, however, that the schematic classes of patterns given in Table II represent an artificial classification superimposed on a continuum of observed patterns. For example, the "gapped peripheral" networks had great variations in the size of the gap, and the "gapped central" networks merged into the "central" networks.

TABLE II

VARIATION IN PATTERNS OF PULSE-LABELED KINETOPLAST DNA NETWORKS OF *C. FASCICULATA* DURING A CHASE

Log phase cells were pulse-labeled with 50 μCi/ml of [^{3}H] thymidine in Medium CA for 10 min and then washed and chased in Medium CA containing 100 μg/ml of unlabeled thymidine. Kinetoplast DNA networks were isolated from the cells at the indicated times and spread on glass slides for autoradiography. Labeling patterns: (a) peripheral, (b) gapped peripheral, (c) centripetal, (d) gapped centripetal, (e) central, (f) random, (g) miscellaneous.

Time (h)	(a)	(b)	(c)	(d)	(e)	(f)	(g)	Total counted
0	93%	3%	0%	0%	0%	0%	4%	370
3	11	46	14	14	13	1	1	304
4.5	1	26	14	24	24	10	1	201
6	1	10	2	17	8	60	3	301

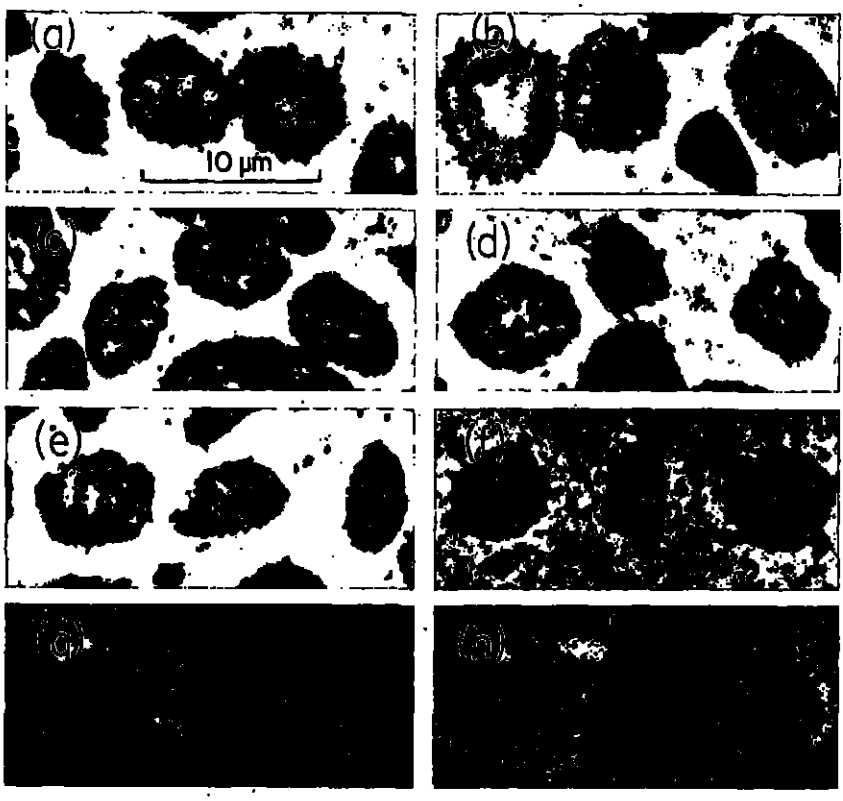

Fig. 6. Light microscope autoradiogaphs of kinetoplast DNA networks, isolated from *C. fasciculata* cells pulsed with [^{3}H] thymidine and chased with unlabeled thymidine. The experimental conditions are described in Table II. (a) and (b) pulse-labeled networks. (c) through (h) pulsed and chased networks. The networks were selected to demonstrate the various types of labeling patterns observed in the chase situation.

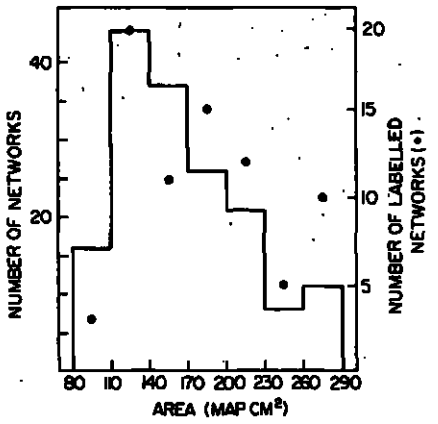

Fig. 7. Size distribution histograms of kinetoplast DNA networks isolated from *C. fasciculata* cells pulse-labeled with [^{3}H]thymidine. The experimental details are given in Table II. •, number of labeled networks present in each size class. This represents only the zero time of Table II.

Directly after the pulse, more than 90% of the labeled networks showed a definite peripheral labeling pattern, a fact which implies that networks in all portions of the S phase exhibited a peripheral replication pattern. This conclusion was strengthened by scoring the frequency distribution of peripherally labeled networks at zero time after the pulse as a function of network size as shown in Fig. 7.

With a progression of the chase the labeling patterns showed definite changes. As shown in Table II, the silver grains moved centripetally as the chase progressed to 4.5 h. Often gaps in the circular ring of grains were apparent. After 6 h of chase a significant portion of labeled networks exhibited a scattered or random grain distribution pattern, implying a redistribution of labeled DNA throughout the network structure.

The control experiment for the random distribution of label over totally labeled networks was impossible to perform with *C. fasciculata* since it has been shown that any attempt at continuous labeling with [^{3}H]thymidine leads to an apparent pulse-chase situation due to a cell-mediated conversion of thymidine to thymine in the medium [13]. However, electron microscopic evidence has been presented by Renger and Wolstenholme [3] for a homogeneous distribution of DNA molecules throughout the network structure of a related species, *Crithidia acanthocephali*.

Qualitatively identical results were obtained with *L. tarentolae*, but the localization of silver grains over kinetoplast DNA networks during the chase was difficult to quantitate due to the small size and fragility of the networks from this species.

Replication of L. tarentolae minicircles

The apparent limitation of DNA replication to the periphery of the network structure leads to an interesting prediction concerning molecules within the interior of the network, which apparently would not be replicated. In a

density transfer experiment using a heavy isotope to label the newly replicated DNA, one would expect to see a large amount of light/light (LL) parental molecules remaining after one generation and the simultaneous appearance of an equal amount of heavy/heavy (HH) daughter molecules.

The minicircle is the major molecular species of the network. Therefore, a density transfer experiment was performed using minicircles isolated from networks of *L. tarentolae;* this species was employed due to the availability of techniques to isolate high yields of minicircles from the kinetoplast DNA [7].

Bromodeoxyuridine was employed as a density label. Cells were grown for three days in Medium CA with [^{3}H]thymidine and then transferred to Medium CA with $^{32}P_i$ and bromodeoxyuridine. After 1.0 (19.7 h) and 1.9 (29.5 h) cell divisions, cells were harvested and kinetoplast DNA networks were isolated. Closed minicircles were isolated from sonicated networks by band velocity sedimentation in alkaline sucrose and then centrifuged to equilibrium in neutral CsCl as shown in Fig. 8a and 8b. Nuclear DNA was isolated from the cell lysates as an internal control and also centrifuged to equilibrium in neutral CsCl (Fig. 8c and 8d).

The small LL minicircle shoulders in both gradients of Fig. 8a and 8b are probably derived from non-viable cells rather than from any non-replicated

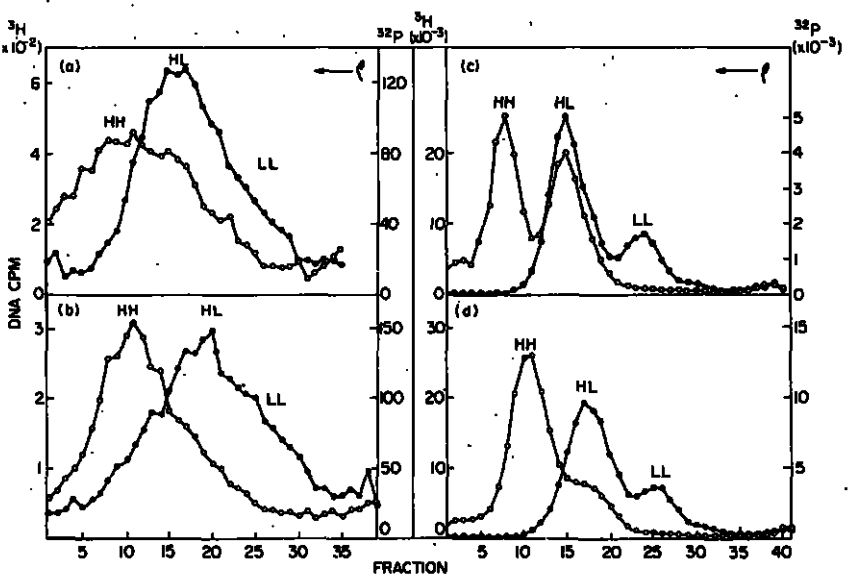

Fig. 8. Preparative CsCl equilibrium gradients of closed monomeric minicircles and nuclear DNA from *L. tarentolae* cells in a density transfer experiment. Cells were grown for 3 days in Medium CA with [^{3}H]thymidine (10 μCi/ml, 2 μg/ml) and were then transferred to Medium CA with $^{32}P_i$ (10 μCi/ml) and bromodeoxyuridine (200 μg/ml). After 1.0 cell divisions (19.7 h) and 1.9 cell divisions (29.5 h) cells were removed and lysed in hot sarkosyl-pronase. After removal of the kinetoplast DNA networks by centrifugation, the nuclear DNA was recovered from the lysate by alcohol precipitation and purified as described previously [2]. Closed monomeric minicircles were isolated from sonicated networks by alkaline sucrose velocity gradients as described previously [7]. Centrifugation conditions: $n_D^{25°} = 1.4010$, 70 h at 39 000 rev./min, 20°C, No. 50 rotor. The tube was fractionated by dripping 5 drop fractions onto Whatman No. 3MM filter discs which were trichloroacetic acid-processed and counted. ●, parental ^{3}H-labeled DNA; ○, new ^{32}P-labeled DNA. (a) Closed monomeric minicircles, 1.0 cell divisions. (b) Closed monomeric minicircles, 1.9 cell divisions. (c) Nuclear DNA, 1.0 cell divisions. (d) Nuclear DNA, 1.9 cell divisions.

internal network minicircles, as shown by the identical appearance of the nuclear DNA patterns in gradients (c) and (d). The relative amounts of the HL and HH peaks do not accurately reflect the expected ratios calculated from the number of cell divisions, but this is probably due to the abnormally long period required for the cell number to double (19.7 h as compared to the normal log phase doubling time of 9—12 h). The fact that the LL—LH—HH transitions seen in the case of the minicircles were identical to those seen in the case of the nuclear DNA suggests strongly that all of the minicircles in dividing cells replicated and that they replicated in an apparently normal semi-conservative fashion. This conclusion is obviously dependent on a random recovery of minicircles from all parts of the network structure. That this is likely is indicated by the recovery of approx. 23% of the total network DNA in the form of closed monomeric minicircles by the technique employed [7].

Discussion

We have described several aspects of the replication of the kinetoplast DNA networks of two related species of hemoflagellates, *L. tarentolae* and *C. fasciculata*. Several salient points have emerged. Networks containing replicating minicircles and pulse-labeled minicircles isolated both from networks and from the "free" class of molecules exhibit an equilibrium density in ethidium bromide—CsCl which is less than that of non-replicating covalently closed networks. These results could be explained either by the presence of nicked minicircles replicating by the rolling circle model [14] or by the presence of closed minicircles replicating by the model of Sebring et al. [15]. Replicating closed circular duplexes have been reported for SV40 DNA [16,17] and also polyoma DNA replication [18]. Apparently similar replicative forms have also been reported to occur at high frequency in the kinetoplast DNA of *Trypanosoma cruzi* cells that were grown in the presence of the drug, berenil [19]. In the cases of *L. tarentolae* and *C. fasciculata*, the available evidence is not sufficient to distinguish between these modes of replication.

The topographical limitation of DNA replication in the kinetoplast DNA network to the periphery of the structure is a striking but unexplained phenomenon. We speculate that this is an expression of the localization of membrane binding sites. Whatever the mechanism, this phenomenon, together with the density transfer results, suggest that there is lateral mobility of molecules within the network structure which allows interior minicircles to pass through the peripheral locus of replication. The redistribution of pulse-labeled DNA throughout the network as observed in the autoradiography chase experiments is another indication of lateral mobility. One possible mechanism could be the existence of a large amount of recombination of minicircles that leads to linear molecules composed of broken and rejoined minicircles. These interdigitated linear molecules would then allow the necessary lateral mobility, and eventually would undergo another round of breakage and rejoining to reform covalently closed minicircles. Steinert has independently proposed a similar mechanism to explain the appearance of linear molecules during an electron microscopic pulsechase experiment with *Crithidia luciliae* (personal communication). Evidence for extensive recombination between kinetoplast DNA mini-

circles of *C. acanthocephali* has recently been obtained by Manning and Wolstenholme [20].

The kinetoplast DNA of *L. tarentolae* and *C. fasciculata* have some basic differences in molecular structure and organization, but show many similarities in the mode of replication. An investigation of the precise differences and similarities between these species and others in the order, *Kinetoplastida*, may lead to a better understanding of the evolution of the mitochondrial DNA in this group of protozoa and perhaps even shed some light on the selective forces involved in such an unusual gene amplification.

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