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Delinking of S phase and cytokinesis in the protozoan parasite *Entamoeba histolytica*

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Summary

The alternation of DNA replication in S phase and chromosome segregation in M phase is a hallmark in the cell cycle of most well-studied eukaryotes and ensures that the progeny do not have more than the normal complement of genes and chromosomes. An exception to this rule has been described in cancer cells that occasionally become polyploid as a result of failure to restrain S phase despite the failure to undergo complete mitosis. Here, we describe the cell division cycle of the human pathogen, *Entamoeba histolytica*, which routinely accumulates polyploid cells. We have studied DNA synthesis in freshly subcultured cells and show that, unlike most eukaryotes, *Entamoeba* cells reduplicate their genome several times before cell division occurs. Furthermore, polyploidy may occur without nuclear division so that single nuclei may contain 1–10 times or more genome contents. Multinucleated cells may also accumulate several genome contents in each nuclei of one cell. Thus, checkpoints that normally prevent DNA reduplication until after cytokinesis in most eukaryotes are not observed in *E. histolytica.*

Introduction

A majority of eukaryotes have developed an intricate machinery for precisely replicating their genome only once per cell cycle. 'Checkpoints' or the surveillance action of proteins, ensuring the successful completion of one event before another can begin, control the progression of the cell division cycle in these organisms (Hartwell and Weinert, 1989). Thus, DNA reduplication is not permitted until mitosis has been completed. Failure to restrain S phase even though mitosis has not been completed leads to polyploidy and is often observed in cancer cells (Pathak *et al*., 1994). Polyploidy is also observed in several organisms when they undergo endoreduplication before differentiation (Grafi, 1998).

Entamoeba histolytica is a protozoan parasite, identified as a medically important pathogen, which, together with the morphologically identical, non-pathogenic species *Entamoeba dispar,* infects at least 500 million people worldwide. Its life cycle oscillates between the proliferating trophozoite and the quiescent cyst. Although previous studies have described different phases of the cell cycle of *E. histolytica* (Orozco *et al*., 1988; Dvorak *et al*., 1995; Gangopadhyay *et al*., 1997a; Vohra *et al*., 1998) and fluctuation in the DNA content of *Entamoeba* trophozoites (Lopez Revilla and Gomez, 1978), very little is known about the inherent molecular mechanisms that regulate the cell division cycle of the trophozoite or its differentiation to the cyst. An important difference from other eukaryotes is that vegetatively growing *E. histolytica* cells contain varying amounts of DNA, ranging from 1× to 8× genome contents and that polyploid cells accumulate in cultures over time (Gangopadhyay *et al*., 1997a). We have undertaken the present study to evaluate whether polyploidy in *E. histolytica* cells occurs as a result of delays in cytokinesis resulting from depletion of extracellular factors or whether polyploidy is an inherent feature of the cell cycle of *E. histolytica*.

Results and discussion

Progressive deregulation of DNA synthesis and cell division in E. histolytica *with time in culture*

Axenic cultures of *E. histolytica* are made up of a heterogeneous population of cells with varying DNA contents, and polyploid cells are more prevalent in late log phase cultures, whereas freshly subcultured cells are mostly euploid (Gangopadhyay *et al*., 1997a). Routinely, a fresh subculture was started from a 72-h-old asynchronous culture by adding about 20 million trophozoites to 50 ml of fresh medium (5×10^4 cells ml^{-1}). After 24 h, the cell count was 5×10^5 ml^{-1}, after 48 h the cell count was 3×10^6 ml^{-1} and, after 72 h, the cells reached a density of 7×10^6 ml^{-1}. At this density, the cultures are completely confluent and do not show an appreciable increase in number subsequently. At 96 h, the cells start to detach from the plastic surface and begin to die.

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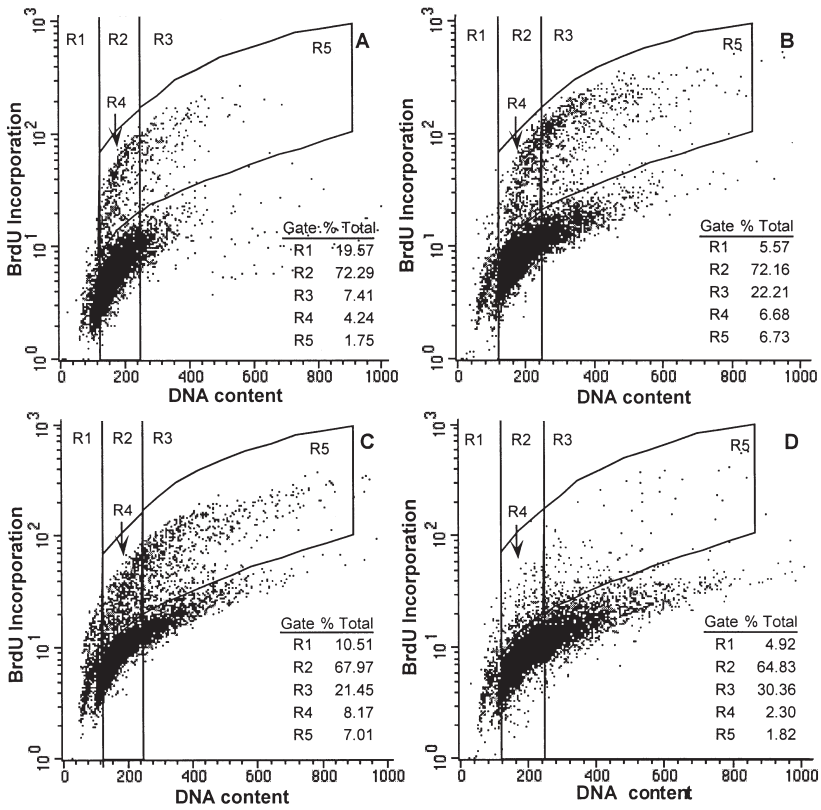

Fig. 1. DNA synthesis leading to the accumulation of polyploid cells in *E. histolytica* cultures. *E. histolytica* cultures were harvested 24 h (A), 48 h (B) and 72 h (C and D) after subculture. BrdU (100 µM) was added to the cells for 15 min at 37°C (A–C), and cells were fixed and stained with FITC-conjugated anti-BrdU antibody, followed by PI and RNase. The negative control (D) was treated in the same way, except that BrdU was not added. For simultaneous measurement of DNA synthesis with DNA content, cells were excited with 488 nm light, and emission was measured through 525 DF 20 (FITC fluorescence; FL1) and 575 DF20 (PI fluorescence; FL2). Data from 10 000 cells were recorded for each experiment and analysed using CELLQUEST software. Electronic gates were set up to demarcate G1 cells (R1), cells with 2× genome content (R2) and cells with multiple genome contents (R3). BrdU-incorporating cells containing up to 2× genome contents were demarcated in R4 (shown with arrow), and BrdU-incorporating cells with >2× genome contents were demarcated in R5. Statistics showing the percentage of cells in each gate are shown adjacent to each plot.

We have compared DNA synthesis in asynchronous cultures of *E. histolytica* trophozoites at different times after subculture by adding the thymidine analogue, 5 bromo-2′ deoxyuridine (BrdU). DNA-synthesizing or S phase cells were identified as those cells that incorporated BrdU. Incorporation of BrdU was measured by the addition of fluorescein isothiocyanate (FITC)-conjugated anti-BrdU monoclonal antibody to fixed cells and subsequent increase in FL1 fluorescence on the *y*-axis (>10^1 fluorescence channels; Fig. 1). In the negative control (Fig. 1D), BrdU was not added, but FITC-conjugated anti-BrdU antibody was added to 72-h-old cultures to estimate the non-specific binding of anti-BrdU antibody to *E. histolytica* cells. The total DNA content of the cells was measured by staining with propidium iodide (PI) and increase in fluorescence (FL2) on the *x*-axis. Cells stained with PI alone showed fluorescence values up to 10^1 on the *y*-axis, whereas unstained cells showed negligible fluorescence on both axes (data not shown).

Electronic gates R1, R2 and R3 were set up to identify cells with 1×, 2× and >2× fluorescence intensities, respectively, on the *y*-axis. Thus, G1 cells with 1× genome content were demarcated in R1. Cells with double the DNA content of G1 cells were demarcated in R2, and cells containing more than double the DNA content of G1 cells were shown in R3. Within R2 and R3, two additional gates, R4 and R5, were set up, which demarcate BrdU-incorporating or DNA-synthesizing cells whose total DNA content is twice and more than twice the genome contents respectively. The percentage of cells within each of the electronic gates was calculated, and the statistics are shown in each figure.

When the culture is fresh (Fig. 1A; 24 h), the number of cells with a DNA content is >2× (R3) is about 7.4% of the total population. After 48 h and 72 h, the number of cells with a DNA content >2× (R3) is about 22%. The number of cells in R1 (1×) decreased from 19.57% (Fig. 1A; 24 h) to 5.57% (Fig. 1B; 48 h). This decrease in number represents those cells that transited from G1 to S phase between 24 h and 48 h. After 72 h (Fig. 1C), the number of cells in R1 showed an increase compared with those at 48 h, suggesting that cells from R2 and/or R3 had completed cell division and returned to 1× genome content. The values for the R1, R2 and R3 populations of the negative control (Fig. 1D) do not exactly match those of the 72 h cells (Fig. 1C), but the DNA content profile (Fig. 1D) shows an accumulation of cells in R3, similar to that observed in Fig. 1B and C. It may be noted that variation in cell numbers in different flasks of asynchronous cultures is commonly observed.

Furthermore, Fig. 1A–C shows that BrdU-incorporating cells (R4 and R5) synthesized DNA beyond 2× genome content (cells demarcated in R5) without undergoing cell division. Thus, the percentage of cells in R5 increased

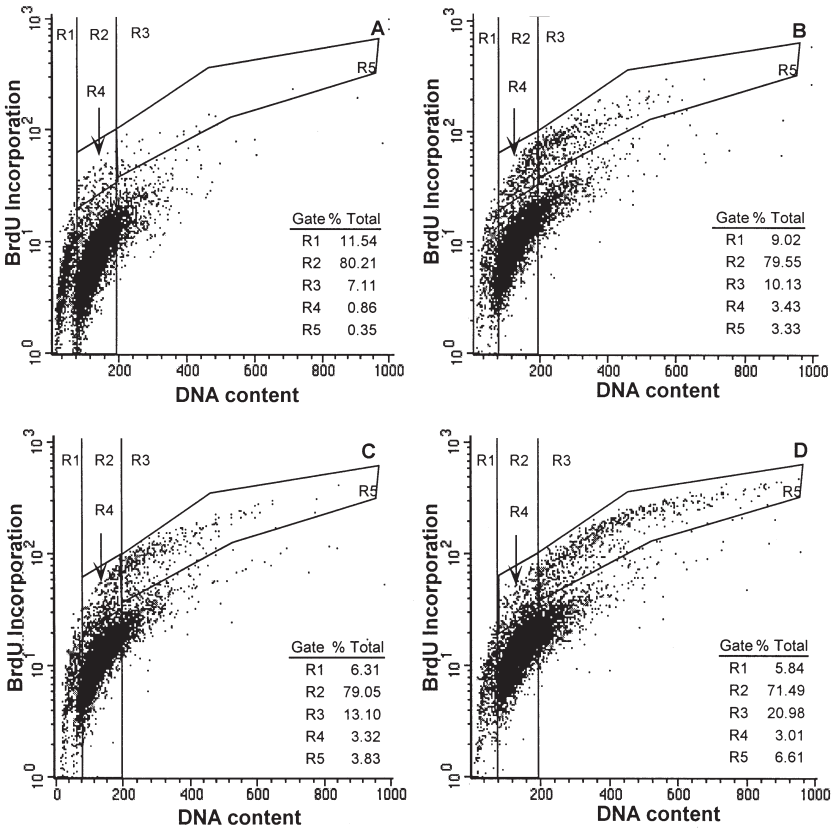

Fig. 2. Genome reduplication without cell division. *E. histolytica* cells were continuously subcultured every 24 h and serum starved for 12 h. Serum was added again, and DNA synthesis was measured in serum-starved (A) and serum-added cells (B–D). BrdU was added for 15 min (B) and chased with complete medium for 30 min (C) and 90 min (D). Electronic gates were set up to demarcate G1 cells (R1), cells with 2× genome content (R2) and cells with multiple genome contents (R3). BrdU-incorporating cells containing up to 2× genome contents were demarcated in R4 (shown with arrow), and BrdU-incorporating cells with >2× genome contents were demarcated in R5. Statistics showing the percentage of cells in each gate are shown adjacent to each plot.

progressively from 1.75% at 24 h to 7% after 72 h of culture. In typical eukaryotes, DNA synthesis would have stopped after one genome duplication, and mitosis would have occurred (Stillman, 1996). In contrast, our results demonstrate: (i) DNA synthesis beyond 2× genome content; and (ii) progressive accumulation of polyploid cells with increasing time in culture. Accumulation of polyploid cells with increasing time has been demonstrated earlier (Gangopadhyay *et al*., 1997a), and it suggests a progressive deregulation of DNA synthesis from cell division. This may result partly from depletion of growth factors from the culture medium with time, when cell division appears to be inhibited but DNA synthesis is not affected and continues without check, leading to the accumulation of polyploids. On the other hand, it is also possible that the amoeba cell cycle is programmed to synthesize multiple copies of the genome before cell division and is not dependent on extracellular factors for its control.

Reduplication of the Entamoeba *genome without cell division*

To examine whether, as suggested above, *E. histolytica* is inherently capable of reduplicating its genome without cell division, we minimized the effect of depletion of the culture medium using cells that were continuously subcultured every 24 h, so that nutrients and growth factors were not depleted from the culture. We observed that, in such cultures, the DNA content of cells was restricted to 1× and 2× genome contents (Fig. 2). We next studied the DNA-synthesizing profile of these cells within one generation time to see whether these cells underwent genome reduplication or whether DNA synthesis arrested at 2× genome content. To do this, we synchronized the cells in two stages – first, by subculturing them every 24 h in fresh medium for 7 days and, secondly, after 1 week of continuous subculture in fresh medium, these cells were synchronized further by serum starvation for 12 h at 37°C (Gangopadhyay *et al*., 1997a).

Electronic gates were set up to demarcate cells with 1× (R1), 2× (R2) and >2× (R3) genome contents (Fig. 2). Gates R4 and R5 demarcate BrdU-incorporating cells with 2× and >2× genome contents respectively. We reported earlier that serum starvation inhibits DNA synthesis in *E. histolytica* trophozoites (Gangopadhyay *et al*., 1997a). A 15 min pulse of BrdU to serum-starved cells shows 0.86% of total cells in gate R4 and 0.35% of total cells in R5 (Fig. 2A), showing inhibition of DNA synthesis. DNA synthesis was initiated in these cells by adding 10%

serum. Two hours after the readdition of serum, the cells were incubated for 15 min with BrdU (Fig. 2B). Incorporation of BrdU was measured at this time, and it can be seen that R4 contains 2.5% of the total cells and R5 contains 3.75% of the total cells. Thus, DNA synthesis was initiated after the addition of serum (Fig. 2B).

BrdU incorporation was chased for 30 min (Fig. 2C) and 90 min (Fig. 2D) after the initial pulse of 15 min by dilution with complete medium. It can be seen that the number of cells increased in R5 (in Fig. 2C and D), with cells attaining almost 10× genome contents in 90 min, suggesting that genome reduplication occurs without cell division. Furthermore, as the S phase of *E. histolytica* lasts for 5–6 h (Dvorak *et al*., 1995; Gangopadhyay *et al*., 1997a), it appears that genome reduplication occurs within the S phase. Thus, unlike other eukaryotes, genome reduplication without cell division may be an inherent property of *E. histolytica.*

Genome reduplication occurs without nuclear division

It has been questioned previously whether polyploid *E. histolytica* cells are necessarily multinucleated or whether uninucleated cells may also be polyploid. To determine this, we have used the CompuCyte laser scanning cytometer to measure the DNA content of individual nuclei in ethanol-fixed *E. histolytica* trophozoites stained with PI. The fluorescence intensity of PI was quantified to represent the DNA content of the nuclei. After scanning $>10^3$ nuclei for DNA content, the minimum PI integral values (411 000–494 646; average 460 615) were taken to represent one genome content. Table 1 summarizes the DNA content of cells with one, two or three nuclei. For uninucleated cells, it was observed that 10% of the cells contained 1×, 48% contained 1–2×, 26% contained 2–3×, 0.06% contained 3–4×, and a few isolated cells contained >4× genome contents (≈ 36×). In cells containing two or three nuclei, each of the nuclei contained 1–4× genome contents. Thus, nuclear division is not a prerequisite for genome reduplication, and cells may accumulate multiple genome contents in a single nucleus before undergoing karyokinesis.

The continued synthesis of DNA without alternation with cell division suggests that *E. histolytica* trophozoites undergo endoreduplication. Unlike other eukaryotes, in which mitosis is inhibited when they switch to endoreduplication before differentiation (Grafi, 1998), *E. histolytica* trophozoites continue to proliferate as they undergo endoreduplication. Furthermore, the occurrence of multinucleated cells suggests that more than one nuclear division may occur before cell division. Our results support the DNA fluctuation observed earlier by Lopez Revilla and Gomez (1978). Thus, trophozoites may contain from 1× to 10× genome contents during their growth phase.

Table 1. Genome content of *E. histolytica* nuclei.

One nucleus per cell	Two nuclei per cell	Three nuclei per cell
1 (14)	2.2/1.6	3.7/2/1.2
1.1 (2)	4.3/3	2.5/2/1.5
1.2 (5)	1.9/2.1	1.3/1.5/2
1.3 (4)	1.5/1.7	
1.4 (7)	1/1.1	
1.5 (11)	1.2/1.4	
1.6 (7)	2.6/2.1	
1.7 (8)	1.4/1.2	
1.8 (5)	1.4/1.2	
1.9 (7)	0.9/1.1	
2 (6)	1.9/1.9	
2.1 (5)	1.3/1.3	
2.2 (3)	2.8/1	
2.3 (7)		
2.4 (3)		
2.5 (6)		
2.7 (1)		
2.8 (5)		
2.9 (2)		
3 (1)		
3.1 (3)		
3.3 (1)		
3.5 (3)		
3.8 (1)		
4.2 (3)		
5.5 (4)		
6.6 (2)		
7.1 (1)		
12 (1)		
15 (1)		
24 (1)		
36 (1)		

Numbers 1–36 in column 1 represent genome contents. 1× genome content was calculated from an average of 15 nuclei showing the minimum value of PI fluorescence (from 411 000 to 494 646; average 460 615). Values >1× were calculated by normalizing the PI integral values against 1×. Numbers in parenthesis denote number of cells for the same PI content. Columns 2 and 3 show the genome contents of each nucleus in a cell with two or three nuclei.

Activation of mitotic cyclin–Cdc2 kinase complex (Stern and Nurse, 1996) is normally required for entry into mitosis. Inhibition of this mitotic CDK activity has been shown to cause cells to undergo multiple rounds of DNA replication (Stillman, 1996). Cyclin E has been reported to play a central role in regulating the initiation of S phase in both mitotic and endocycles (Sauer *et al*., 1995). A homologue of *cdc2* (Lohia and Samuelson, 1993) has been cloned from *E. histolytica,* but cyclin genes have not yet been identified in this organism. Therefore, at this time, it is not possible to analyse the role of different cyclins and CDKs in the regulation of the *E. histolytica* cell cycle. Mcm proteins are known to act as licensing factors in regulating the initiation of DNA replication (Stillman, 1996), and their presence in *E. histolytica* (Gangopad-

hyay *et al*., 1997b; Das and Lohia, 2000) suggests that they may be involved in the regulation of DNA replication. However, preliminary results show that Mcm homologues in amoeba are structurally altered (Gangopadhyay *et al*., 1997b). Therefore, their role in regulating initiation of DNA replication in *E. histolytica* may also be modified.

Our results suggest that genome reduplication occurs during the S phase of *E. histolytica* trophozoites' cell cycle. The accumulation of polyploid cells is observed in late log phase cultures. These cells may have resulted either from depletion of extracellular factors controlling cell division or as precursors to apoptotic cells or those differentiating to cysts. Indeed, in *Entamoeba invadens*, the reptilian parasite, we have demonstrated that trophozoites undergo two rounds of genome duplication without cell division during encystation (Ganguly and Lohia, 2001). At this time, it is difficult to sort these polyploid cells and show whether they are terminal cells that cannot divide any further or whether polyploidy resulted from a temporary failure to divide because of depletion of extracellular factors from the growth medium. Results obtained from different laboratories (Lopez Revilla and Gomez, 1978; Orozco *et al*., 1988; Dvorak *et al*., 1995; Gangopadhyay *et al*., 1997a; Ganguly and Lohia, 2001) suggest that the cell division cycle of *Entamoeba* is different from that observed in other eukaryotes (Nasmyth, 1996; Stillman, 1996). It needs to be established in future whether polyploidy in *Entamoeba* cells occurs as a result of loss of regulatory processes that restrict genome duplication once and only once per cell cycle as in most eukaryotes (Stillman, 1996), or if the observed genome reduplication is similar to the endocycles demonstrated in plants and *Drosophila* (Traas *et al*., 1998), or if amoeba cells have unique regulatory mechanisms that control their cell division and maintain genome fidelity. Analysis of the molecular mechanisms that control the proliferation of this human pathogen will be extremely important in identifying growth-inhibitory compounds.

Conclusions

We have studied DNA synthesis in freshly subcultured cells and show that, unlike most eukaryotes, *Entamoeba* cells reduplicate their genome several times before cell division occurs. Furthermore, polyploidy may occur without nuclear division, so that single nuclei may contain 1–10× or more genome contents. Multinucleated cells may also accumulate several genome contents in each nuclei of one cell. Thus, DNA reduplication appears to occur several times before cell division, which suggests that hitherto unknown regulatory mechanisms control the cell division cycle of this protozoan parasite.

Experimental procedures

Materials

5′-Bromo-2 deoxyuridine (BrdU), propidium iodide and other reagents were purchased from Sigma. Monoclonal anti-BrdU antibody was purchased from Becton Dickinson.

Cell culture

Axenic cultures of *E. histolytica* (HM1:IMSS) trophozoites were routinely subcultured in TYIS-33 medium (Diamond *et al*., 1978). Cultures (24 h) were obtained by subculturing every day. Serum starvation was carried out by incubating 24 h cultures in TYI-33 medium (without serum) for 12 h at 37°C.

BrdU incorporation

To study DNA synthesis, BrdU (0.1 mM) was added to the cultures 15 min before harvesting at every time point (Gangopadhyay *et al*., 1997a). For pulse–chase experiments, after incubation with BrdU for 15 min, cells were washed and resuspended in complete medium and harvested after 30 or 90 min. Cells were then harvested and fixed in 70% ethanol on ice for 15 min. For the measurement of BrdU incorporation, fixed cells were washed with phosphate-buffered saline (PBS) and treated with 1.6 N HCl and pepsin (0.2 mg ml^{-1}) at 37°C for 30 min for simultaneous hydrolysis and denaturation of DNA. The cell suspension was then neutralized with 1 M Tris-HCl, pH 8.0, to stop hydrolysis and washed again with PBS. Next, the cells were resuspended in PBS containing 0.5% Tween 20 and stained with FITC-conjugated anti-BrdU antibody (1:100 dilution) for 30 min at room temperature in the dark. Cells were subsequently washed in PBS containing 0.5% Tween 20 and treated with RNase (0.3 mg ml^{-1} for 16 h) and PI (0.5 mg ml^{-1} for 30 min) in the dark. Cells were analysed for DNA synthesis and DNA content by flow cytometry.

Flow cytometry and laser scanning cytometry

Flow cytometric analysis was carried out using a FACSCalibur flow cytometer (Becton Dickinson) equipped with a single laser system (6 W Innova 90-6 argon ion laser). For simultaneous measurement of DNA synthesis with DNA content, cells were excited with 488 nm light, and emission was measured through 525 DF 20 (FITC-conjugated anti-BrdU antibody for FITC fluorescence; FL1) and 575 DF20 (for PI fluorescence; FL2). Data from 10 000 cells were recorded for each experiment, and all these sets were analysed using CELLQUEST software. To measure the DNA content of individual nuclei, cells were fixed with 70% ethanol, stained with RNase (0.3 mg ml^{-1} for 16 h) and PI (0.5 mg ml^{-1} for 30 min) in the dark and put on a glass slide with a coverslip. These cells were individually scanned for DNA content by a CompuCyte scanning cytometer (courtesy of Dr Leona Samson, Harvard School of Public Health, USA).

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