Journal of Foraminiferal Research, v. 42, no. 2, p. 151–155, April 2012

RIBOSOMAL DNA IS DIFFERENTIALLY AMPLIFIED ACROSS LIFE-CYCLE STAGES IN THE FORAMINIFER *ALLOGROMIA LATICOLLARIS* STRAIN CSH

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ABSTRACT

Genomic content varies over the course of the life cycle in many lineages across the eukaryotic tree of life. Such variation is thought to be widespread in Foraminifera, a microbial lineage within the Rhizaria, as complex nuclear dynamics have been observed in the life cycle of diverse species. Perhaps the most striking example is the elimination of nuclear material, including DNA, which occurs prior to cell division in the uninucleate life-cycle stage of all species that have been examined. This process, termed zerfall, is hypothesized to eliminate DNA that was previously amplified during the life cycle. Here we test this hypothesis by comparing the relative copy number of ribosomal DNA and two protein-coding genes with quantitative PCR before and after zerfall in the cultured foraminifer *Allogromia laticollaris* strain CSH. Our results reveal higher relative copy number of ribosomal DNA prior to zerfall, while protein-coding loci are relatively more abundant following zerfall. This suggests 1) that like many other eukaryotic lineages, *Allogromia* differentially amplifies the ribosomal DNA locus, and 2) that zerfall enables *Allogromia* to reset copy number prior to cell division. Further, these data add to the observation that some lineages of eukaryotes can differentiate germline and somatic genetic material in the context of a single nucleus.

INTRODUCTION

Variation in genome content within an individual occurs in a multitude of eukaryotic lineages as a normal part of the life cycle (Raikov, 1982; Kondrashov, 1997; Parfrey and others, 2008; Parfrey and Katz, 2010a). Within individuals variation generally occurs within a nucleus through changes in ploidy level beyond haploid and diploid, differential amplification of portions of the genome, or recombination (Henikoff, 2005; Parfrey and others, 2008). We have argued that in healthy cells this variability reflects the ability of eukaryotes to distinguish germline and somatic genetic material through epigenetic mechanisms, even within a single nucleus (Parfrey and others, 2008; Parfrey and Katz, 2010a). This type of genomic variability is also found in stressed or abnormal cells, such as cancerous cells in humans (Storchova and Pellman, 2004; Erenpreisa and Cragg, 2007) and flax plants grown under stressful conditions (e.g., Cullis, 2005).

Amplification of ribosomal DNA (rDNA) is the most common genome modification (McGrath and Katz, 2004; Zufall and others, 2005; Parfrey and Katz, 2010a), likely because elevated rDNA copy number enables cells to rapidly shift rRNA levels in response to changes in transcriptional demand. In many lineages, including humans, high rDNA copy number is achieved via tandem arrays that are present in the genome and homogenized by concerted evolution. Another strategy is to have one copy of rDNA in the genome that is amplified extrachromosomally during development, as is the case in the slime mold *Dictyostelium*, the alga *Euglena*, and in frog embryos (Zufall and others, 2005). In some species of *Entamoeba,* rDNA is only present extrachromosomally on a plasmid and copy number varies during the life cycle (Bagchi and others, 1999). Closer inspection may reveal widespread extrachromosomal copies of many loci, as has been detected in all plants and animals examined with methods designed to detect extrachromosomal amplification (Cohen and Segal, 2009).

Foraminifera have complex life cycles characterized by an alternation between uninucleate and multinucleate life-cycle stages (Goldstein, 1999). All species whose life cycles have been studied in detail go through zerfall prior to nuclear and cell division of the uninucleate stage (Goldstein, 1997, 1999). Zerfall is characterized by marked expansion of the nucleus and the appearance of numerous dark granules of condensed nuclear material that are eventually degraded (Føyn, 1936; Dahlgren, 1964). These dark granules contain DNA in *Allogromia laticollaris* CSH (McEnery and Lee, 1976) and other taxa (Arnold, 1955; Dahlgren, 1964). Zerfall is hypothesized to eliminate DNA that was previously amplified during the uninucleate life-cycle stage (Parfrey and Katz, 2010a, b; Habura and others, 2011). The life cycle of *A. laticollaris* CSH is predominately asexual, and the uninucleate stage (called Agamont I) undergoes zerfall and multiple fission to produce multinucleated Agamont II cells (McEnery and Lee 1976; Parfrey and Katz 2010b; Fig. 1). Previously, Parfrey and Katz (2010b) observed elevated DNA content within individual nuclei of both life-cycle stages of *A. laticollaris* CSH.

Here, we test the hypothesis that rDNA is differentially amplified in the uninucleate life-cycle stage that precedes zerfall by comparing relative copy number of SSU-rDNA to protein-coding genes at two life-cycle stages in the cultured foraminifer *Allogromia laticollaris* CSH. Life-cycle-stage specific genomic DNA was isolated by taking advantage of the differing numbers of nuclei characteristic of Agamont I (one nucleus) and Agamont II (many nuclei; Fig. 1). The relative copy number of SSU-rDNA, actin, and beta-tubulin was assessed by quantitative polymerase chain reaction (qPCR) in these two life-cycle stages.

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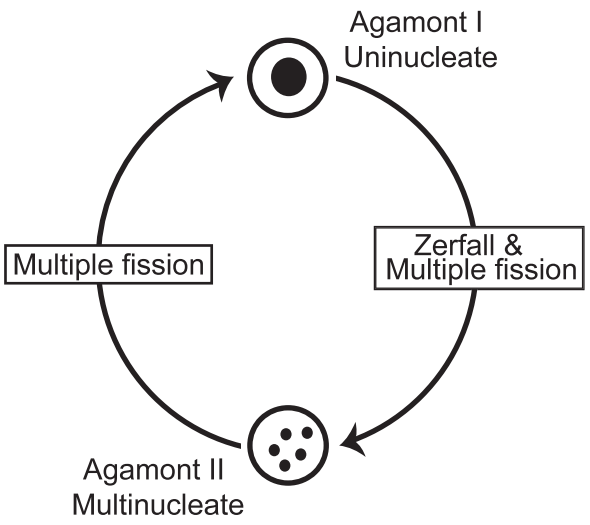

FIGURE 1. Cartoon diagram of the life cycle of *A. laticollaris* CSH adapted from Parfrey and Katz (2010b).

MATERIALS AND METHODS

CULTURES

Allogromia laticollaris CSH was originally isolated off Cold Spring Harbor, New York, in Long Island Sound in 1960 (Lee and others, 1969), and its life cycle has been studied and characterized by multiple groups (Lee and McEnery, 1970; McEnery and Lee, 1976; Parfrey and Katz, 2010b). For the present study, the isolate was cultured on bacteria with wheat extract without an eukaryotic food source to facilitate molecular analyses as described previously (Parfrey and Katz, 2010b). Note that *A. laticollaris* CSH is distinct from the type specimen of *Allogromia laticollaris* that was isolated in Florida and has a sexual life cycle more typical of Foraminifera (Arnold, 1955).

LIFE-CYCLE STAGE IDENTIFICATION

Agamont I and II cells were distinguished by their number of nuclei (Agamont I cells have one and Agamont II have multiple; Fig. 1) in DAPI stained cell populations and then separated for DNA extraction. Cultures were pelleted in a tabletop centrifuge and then fixed by adding 95% ethanol to a final concentration of 70% ethanol and a final volume of roughly 1 mL. Cells were then DAPI stained by adding 2 uL of 4′, 6-diamidino-2-phenylindole (DAPI; 2.5 mg/mL) for 30 minutes in the dark and washed twice in phosphate buffered saline (PBS). Individual cells were then arrayed 10 at a time on a microscope slide divided into 10 slots, using a 10 uL pipette with the aid of a dissecting microscope. The number of nuclei (and, therefore, life-cycle stage) of individual cells was determined under UV light (excitation filter BP330–385 and barrier filter BA420) on an Olympus BX70 epifluorescent microscope. Cells were then picked into separate tubes containing PBS with the aid of a dissecting microscope. Only unambiguous Agamont II cells were picked (e.g., cells with two or three nuclei were discarded). In total, 122 Agamont II cells and 200 Agamont I cells were picked. DNA was extracted as described below and eluted in 100 μL of water.

MOLECULAR METHODS

Two approaches were used to isolate DNA for PCR. First, to develop specific primers for qPCR, bulk genomic DNA or complementary DNA (cDNA) was prepared from roughly 100 individually washed cells that were not sorted by life-cycle stage, and second, for qPCR experiments, cells were separated by life-cycle stage as described above. For all DNA and cDNA preparations (bulk and life-cycle-stage specific) cells were washed in sterile media three times, then transferred to a sterile microfuge tube that could be used with a pestle (Kimble Chase Kontes, catalogue no. 749520-0000). Cells were then pelleted in a bench top centrifuge and the media removed. The microfuge tube was submerged in liquid nitrogen and the pellet ground into a powder. DNA was extracted from the ground pellet with the DNeasy plant kit according to standard protocols (Qiagen, catalogue no. 69106), or cDNA was generated with SuperScript III Cells Direct kit (Invitrogen, catalogue no. 18080-200), following manufacturer's instructions. We used cDNA for initial amplification of protein-coding genes with degenerate primers and genomic DNA (gDNA) to verify sequences identified from cDNA and to detect introns (none were found in actin or beta-tubulin). Genomic DNA was also used for all qPCR experiments.

We took a two-step approach to characterizing loci. Degenerate primers were initially used to amplify and determine the sequence of protein-coding genes from cDNA. Subsequently, these gene sequences were used to design specific primers for qPCR that amplify two small (150–200 bp) and non-overlapping regions of each gene (Table 1). Actin was amplified from cDNA using primers Act73D (5′ GGTGAYGAYGCNCCAMGAGC 3′) and 1354rCe (5′ GGWCCDGATTCATCRTAYTC 3′) from Flakowski and others (2005). Tubulin genes were amplified from cDNA using the beta-tubulin primers Btub_303+ (5′ GGTGCTGGTAAYAAYTGRGC 3′) and FBtub_1135− (5′ GTTGTRTTTGCHACAAADGTWCC 3′) and the alpha-tubulin primers Atub_94+ (5′ GGCAAGGAGGACGCNGCNAAYAAYTWYGC 3′) and Atub_439− (5′ CATGCCTTCNCCNACRTACC3′). These were derived from universal primers (Yoon and others, 2008) with the exception of the newly designed reverse beta-tubulin primer. SSU-rDNA was amplified with S14F1 and B (Pawlowski, 2000) and sequenced to confirm that it matches the published sequence for this isolate (AJ311218); it is identical. At least eight clones were sequenced for the protein-coding genes to capture the paralog diversity, essential for designing paralog-specific qPCR primers. Only paralogs confirmed by PCR with specific primers were used for qPCR, and only one paralog each of actin and beta-tubulin were confirmed. Paralogs were confirmed in alpha-tubulin, but qPCR failed after repeated attempts and thus was not included in the experiments presented here. Gene sequences for actin, alpha-tubulin, and beta-tubulin are deposited in GenBank (JN241682-5).

QUANTITATIVE PCR

Quantitative PCR was performed with SYBR Green® PCR master mix (Applied Biosystems, CA). Samples were run on 96-well optical plates on the ABI Prism 7300 instrument at Smith College and analyzed using SDS

TABLE 1. Quantitative PCR primers and fragment lengths.

Gene	Primer name	Primer sequence 5′-3′	Fragment	Length
Actin type 2	Allo_actP2_4+	TATGGTTGGTATGGGACAAAAAG	Act-1	58
	Allo_act_62−	GATGAAGCACAAGCAAAAAGAG		
	Allo_actP2_315+	GTACAACAGGTATAGTATTAGATATG	Act-2	107
	Allo_actP2_422−	GTATTACGTTTAGATTTAGCTGGTA		
Btub P1	Allo_btubP1_519+	ACCAGGTGAAGGTGAAGGAA	Btub-1	131
	Allo_btubP1_650−	GCATCATGTGGTTATCGTGG		
	Allo_btubP1_5+	GCCATTATACAGAAGGTGCAGA	Btub-2	91
	Allo_btubP1_95−	TGATTGTCCACAAGGTTTTCAAT		
SSU rDNA	Allo_SSU_444+	TTTCATGGTGGGGACTGACC	SSU-1	99
	Allo_SSU_542−	AAACCACCAGGAATATGTCCC		
	Allo_SSU_F2	CTTTGTACACACCGCCCG	SSU-2	145
	Allo_SSU_Rev	TAGGTGAACCTGCAGAAGGA		

software (Applied Biosystems, CA). Specific primers were then designed for qPCR analysis that 1) distinguished between paralogs, as we aimed to amplify a single genomic locus; 2) were <150 bp in length, and 3) had an annealing temperature of 58–60°C as calculated by OligoCalc (http://www.basic.northwestern.edu/biotools/oligocalc.html). Two primer pairs were designed for each locus to control for biases in the amplification efficiency of primer sets (Table 1). The qPCR primers were verified by traditional PCR against bulk *Allogromia* gDNA to assess the presence of introns, as well as against cDNA. Only qPCR primer sets that yielded the correct fragment size were used in the qPCR experiments. Primers were also tested against minipreps of all paralogs to assess paralog specificity. The identity of the fragments was confirmed by sequencing.

Two separate qPCR experiments were run, each with triplicate reactions. Quantitative PCR reactions occurred in a total volume of 25 μL with 1 μL each of 10 μmole primers (from Table 1) and gDNA. Miniprep dilutions were also run to enable calculation of gene copy number. Results were analyzed in Microsoft excel. Copy number was calculated by generating a standard curve of the miniprep dilutions. Averages and standard deviations of copy number/nucleus were calculated from triplicate PCRs across both experiments.

INTERPRETATION OF COPY NUMBER

In order to interpret differences in copy number between life-cycle stages, the varying nuclear number must be taken into account: Agamont I cells have one and Agamont II cells have between 4–12. For Agamont I, gDNA was extracted from 200 cells, and thus 200 nuclei. Agamont II gDNA was extracted from 122 cells. While the nuclear number of individual cells was not recorded, the total number of nuclei is likely between 900–1100 based on previous detailed observations of these cells where the mean nuclear number was 8.5 (Parfrey and Katz, 2010b). Calculations of copy number/nucleus thus assumed 200 nuclei for Agamont I and 1000 nuclei for Agamont II. An estimate of extraction efficiency is required to determine the absolute copy number but not to assess the relative copy number between life-cycle stages, which is the goal of this study. We could not directly assess the efficiency of DNA recovery in this study because copy number is not known for any of the genes in *Allogromia*; thus there is no standard for comparison to qPCR values.

The efficiency of DNA extraction from published studies of other organisms provides guidelines of expected efficiency, but efficiency varies widely depending on cell type, methods for lysing cells, and the extraction protocol used. Estimated extraction efficiency in other studies ranges from 2–99% (e.g., Zhou and others, 1996; Mumy and Findlay, 2004; Hospodsky and others, 2010). In general, less DNA is recovered from organisms with tough cells walls, as found in *Allogromia*. For such organisms extraction efficiency increases when procedures that mechanically break open the cell wall are included in the extraction protocol, such as grinding in liquid nitrogen or sonication. For instance, efficiency of DNA extraction from *Giardia* and *Cryptosporidium* cysts increased from ~20% to 80% with the inclusion of a sonication step (Guy and others, 2003), and liquid nitrogen grinding resulted in a 25% recovery rate for DNA of the fungus *Aspergillus* spiked into soil (Kabir and others, 2003). We took measures to increase efficiency of DNA extraction from *Allogromia*, which has a tough cell wall resistant to chemical and enzymatic lysis as well as to freeze-thaw cycles. We mechanically broke cells open by grinding them in liquid nitrogen in individual microfuge tubes and pestles. Given the results from other studies with similar methods, it is reasonable to assume that liquid nitrogen cell grinding in our protocol also yielded moderate to high efficiencies of DNA. However, considering the variation introduced from many sources, we did not directly account for DNA extraction efficiency in calculations of copy number, but acknowledge that our values likely underestimate the true copy number by 2–5-fold.

RESULTS AND DISCUSSION

The results of quantitative PCR, which were consistent across two sets of primers for each locus, demonstrate that relative copy number differs by gene and across life-cycle stages (Fig. 2). The important observation is the change in the ratio of copy number across life-cycle stages between ribosomal DNA and protein-coding genes. Ribosomal DNA is relatively more abundant in Agamont I cells compared to Agamont II cells, and the same pattern is not observed for the protein-coding genes actin and β-tubulin. The copy number/nucleus of SSU-rDNA is significantly greater in Agamont I nuclei compared to Agamont II ($p<0.001$; Fig. 3), and the ratio between these values is >10 (Fig. 2). However, the relationship is reversed for both protein-coding

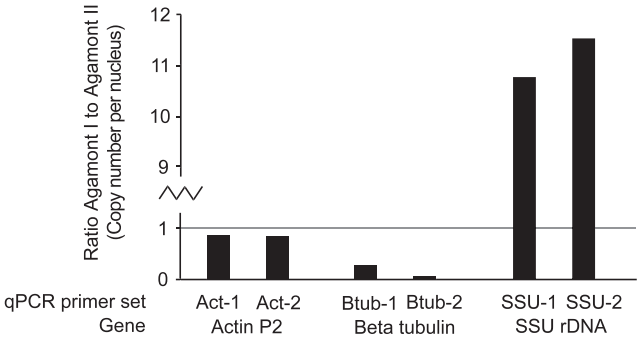

FIGURE 2. Relative copy number of Agamont I compared to Agamont II reveals higher amplification of SSU-rDNA prior to zerfall. Values are the ratio of copy number/nucleus in Agamont I (pre-zerfall) versus Agamont II (post-zerfall) for each qPCR primer set. The grey line is at 1, which would indicate parity. Ratios >1 indicates that there are more copies in Agamont I, while a ratio <1 indicates that there are more copies in Agamont II. See Table 1 for qPCR primers.

genes, which both have fewer copies in Agamont I nuclei relative to Agamont II nuclei (Fig. 3) and thus ratios <1 (Fig. 2). The difference in copy number/nucleus between life-cycle stages is significant for beta-tubulin ($p<0.05$) but not for actin (Fig. 3). These results support the hypothesis that rDNA is amplified relative to protein coding genes in the Agamont I life-cycle stage, and these extra rDNA copies are likely eliminated during zerfall.

As a null hypothesis, we expect that gene copy number should be equal to or correlated with nuclear number if ploidy level is constant. This null hypothesis can be rejected for SSU-rDNA, which was present at much higher copy number/nucleus in Agamont I than Agamont II ($p<0.001$; Fig. 3). The protein-coding genes show either a reduction in copy number/nucleus in Agamont I cells or equivalent numbers for both life-cycle stages, though change in ratio is less than for SSU-rDNA. The results presented are calculated using the average number of nuclei in Agamont II cells from a previous study (8.5 nuclei/cell, see Methods). When the minimum (4) or maximum (12) number of nuclei are instead used to calculate copy number per nucleus the results are generally consistent. The result for SSU-rDNA and the difference in the ratio of copy number/nucleus across life-cycle stages for SSU-rDNA and protein-coding genes remain unchanged by the different methods of calculation, lending confidence to these results.

Disentangling the relative contribution of polyploidization and differential amplification is essential to understand fully the nuclear dynamics of *A. laticollaris* CHS. We previously observed that DNA content increases with cell size in both life-cycle stages of *A. laticollaris* CSH, and calculated that if the observed increase in DNA content was due primarily to increasing ploidy levels during cell growth, then ploidy levels reach 300N in Agamont I and 100N in Agamont II (Parfrey and Katz, 2010b). However, the present results suggest that rDNA amplification is responsible for some of the observed increase in DNA content because the qPCR results reveal that SSU-rDNA is differentially amplified in the Agamont I life-cycle stage (Fig. 2). A rough estimation of the relative contribution of rDNA based on the copy number estimated here and the genome size and ploidy estimates from our previous work (Parfrey and Katz, 2010b) suggests that during growth rDNA is responsible for at most ~10% of the total increase in DNA content. Thus, it is likely that both differential amplification of rDNA (and possibly other loci) combined with some level of polyploidization contribute to the nuclear dynamics.

The variation in genomic content across life-cycle stages in *Allogromia* uncovered here adds to the broader debate on the extent of genome dynamics across eukaryotes. Studies of organisms that span the eukaryotic tree of life continue to uncover both extensive intraspecific variation in genome content (Raikov, 1982; Parfrey and others, 2008; Mukherjee and others, 2009; Parfrey and Katz, 2010a) and increasing complexity of genomic processes and their

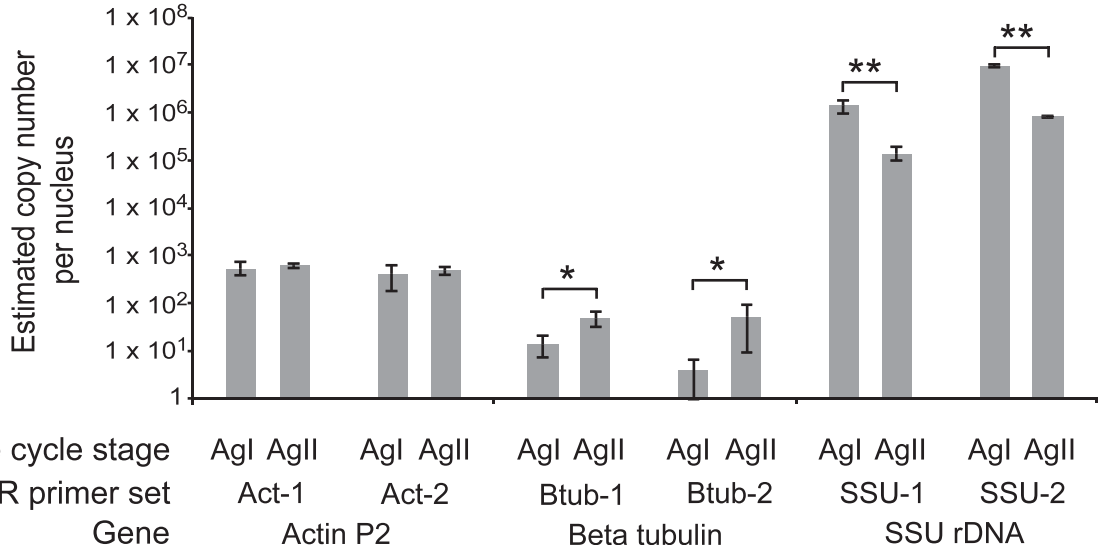

FIGURE 3. Copy number of protein-coding genes and rDNA varies between life-cycle stages in *Allogromia laticollaris* CSH. Copy number of actin does not differ significantly, and beta-tubulin (Btub) is higher in Agamont II (Ag II), while SSU-rDNA copy number is higher in Agamont I (Ag I). Student's t-test * $P<0.05$, ** $P<0.001$. See Table 1 for qPCR primer sets. Copy number estimated by qPCR of Ag I and Ag II specific gDNAs and normalized to the approximate number of nuclei. Error bars are the standard deviation across two experiments of three replicates each.

regulation (e.g., Henikoff, 2005; Redon and others, 2006; Li and others, 2011). The realization that intraspecific variation in genome content is pervasive in eukaryotes raises questions about how genomes are stably transmitted through generations in the face of this variability. Zerfall-type processes, in which DNA is eliminated from the nucleus and therefore not transmitted to future generations, present a mechanism that allows eukaryotic taxa to vary somatic genome content during the life cycle while maintaining the integrity of the inherited (germline) genome (Parfrey and Katz, 2010a).

ACKNOWLEDGMENTS

We thank Sue Goldstein and Sam Bowser for insightful discussions. This manuscript was improved following comments from Jan Pawlowski, two anonymous reviewers, and the Colorado University interdisciplinary writing group. The work was supported in part by grants from the Cushman Foundation for Foraminiferal Research and a Society of Systematic Biology miniPEET award to LWP and two NSF grants to LAK: Assembling the Tree of Life (043115) and Systematics grant (DEB RUI:0919152).

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Received 29 July 2011
Accepted 13 January 2012