Origin Usage during *Euplotes* Ribosomal DNA Amplification

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The macronuclear genome of the ciliate *Euplotes* is comprised of millions of small linear DNA molecules that have telomeres on each end. These molecules are generated during the sexual stage of the life cycle, when the new macronucleus is formed by a series of DNA processing events and multiple rounds of DNA amplification. We have used two-dimensional gels to compare the location of the replication origins used during vegetative growth and the two periods during macronuclear development when DNA amplification takes place. When we examined the pattern of ribosomal DNA (rDNA) replication intermediates, we observed almost identical Y arcs regardless of when in the *Euplotes* life cycle the DNA was isolated. No bubble or bubble-to-Y arcs could be detected. This indicates that replication of the macronuclear rDNA initiates at or near the telomere even when these molecules are being differentially amplified. Since replication rarely initiated from both ends of the rDNA, we examined the direction of replication fork movement to determine which end of the rDNA served as the origin. Fork movement gels indicated that replication initiated at the 5′ end. As transcription also starts near the telomere at the 5′ end, our findings suggest that the telomere and the promoter region cooperate to recruit *Euplotes* replication initiation complexes.

In most eukaryotes, chromosomal DNA replication is highly regulated to ensure that only one round of replication takes place each time a cell passes through S phase. This tight regulation is essential because random gene amplification can lead to tumorigenesis and resistance to chemotherapeutic reagents (20, 33). However, in some organisms, gene amplification is developmentally regulated and is an essential feature of the life cycle. For example, amplification of the chorion gene clusters occurs in *Drosophila melanogaster* follicle cells during oogenesis and in puff regions of *Sciara coprophila* polytene chromosomes during late larval development (12). In both insects, amplification seems to result from an increase in the frequency of origin firing within the amplified region. Gene amplification also takes place during formation of the new macronucleus in ciliated protozoa such as *Tetrahymena*, *Euplotes*, and *Stylonychia* (15, 26). Amplification of the *Tetrahymena* ribosomal DNA (rDNA) is the result of multiple initiation events occurring at the origins that are used during vegetative growth (38). These origins are located in two adjacent 5′ nontranscribed spacer regions (5′ NTS) near the center of the palindromic rDNA minichromosome (5, 22). During vegetative growth, initiation occurs in only one 5′ NTS, but during rDNA amplification, it can occur simultaneously in both 5′ NTS. Moreover, during amplification, reinitiation can occur within a single 5′ NTS.

Ciliated protozoa have two functionally distinct nuclei, the germ line micronucleus and the vegetative macronucleus, where transcription takes place (15, 26). The macronucleus is formed from a copy of the micronucleus by a developmentally programmed reorganization that takes place after mating. In *Euplotes*, macronuclear development takes >100 h and involves extensive DNA deletion and chromosome fragmentation in addition to four separate periods of DNA replication (14, 26). The resulting macronuclear genome is comprised of highly amplified short linear DNA molecules that usually encode one gene and have telomeres at each end. The two initial periods of DNA replication occur prior to chromosome fragmentation and result in the formation of polytene chromosomes (9, 10). The third period of replication occurs after chromosome fragmentation and telomere addition but well before the round anlagen (developing macronuclei) elongate into the horseshoe shape characteristic of mature macronuclei (7). The final period occurs late in macronuclear development at the time when the anlagen start to elongate (34, 36). Differential amplification of the individual DNA molecules starts during the third period of replication (7; this paper) and results in copy numbers of 200 to 100,000 molecules per macronucleus (3, 7, 8).

It is unclear how replication of *Euplotes* macronuclear DNA molecules is regulated either during vegetative growth or macronuclear development. Electron microscopy (EM) of macronuclear DNA isolated from vegetatively growing *Euplotes* and *Stylonychia* suggested that the replication origins must lie very close to the telomere because >97% of the replication intermediates had a single replication fork (Y structure), whereas only ~1% had a replication bubble, and these bubbles were always located close to one end of the molecule (1, 24). However, sequencing of *Euplotes* and *Stylonychia* macronuclear DNA molecules has not revealed a conserved sequence that might function as an origin (2, 17, 23). Moreover, *Stylonychia* molecules that have had the 5′ untranslated region (UTR), 3′ UTR, or coding region replaced by an unrelated sequence can be maintained at a normal copy number (35). These findings
have raised the possibility that the telomere itself may serve as the origin during vegetative growth.

As previous studies examined only replication intermediates from vegetatively grown cells (1, 24), we set out to determine whether origin usage changes when the newly formed macronuclear DNA molecules are amplified during Euplotes macronuclear development. We chose to examine the rDNA origins because in Euplotes this molecule is amplified to a copy number of ~100,000 molecules per macronucleus (8, 26). This is ~100-fold higher than the average copy number of other macronuclear DNA molecules (3), so the rDNA replication intermediates should be easier to detect if this differential amplification occurs during macronuclear development. Previous sequence analysis of the nontranscribed regions of the rDNA molecules from different euglenid species did not identify conserved sequences beyond the 50 bp immediately upstream or 30 bp downstream of the rDNA transcription unit (8). Thus, potential replication control elements were not identifiable (8).

RESULTS

Characterization of replication intermediates from vegetatively growing cells. As Euplotes replication intermediates had previously been examined by EM but not by 2D gels, it was important to determine whether DNA from vegetative cells would give the expected pattern of intermediates in a 2D gel analysis. The EM studies had revealed that ~97% of all replication intermediates have a single Y fork (1, 24). Thus, if the Euplotes rDNA is replicated from an origin at or near the telomere like the main population of macronuclear DNA molecules, this should give rise to a simple Y arc when replication intermediates are resolved in 2D gels under neutral pH conditions (neutral-neutral gels) and hybridized with an rDNA-specific probe (Fig. 1).

To obtain vegetative DNA containing a maximum number of replication intermediates, cells were starved for 3 days, refed with algae, and harvested ~17 h later. This procedure gave rise to cultures that had replication bands in ~30% of the macronuclei (data not shown). DNA was purified, treated with BND-cellulose to enrich for replication intermediates, and separated in two dimensions using neutral-neutral gels. Following transfer to nylon membrane, the rDNA molecules were identified by Southern hybridization. The Euplotes rDNA is an ~7.6-kb molecule that encodes the 17S, 5.8S, and 26S rRNAs (8). Figure 2A shows its organization and the positions of the probes used in this study, while Fig. 2B shows the pattern of rDNA replication intermediates identified by probe A. The majority of the replication intermediates lie on a simple Y arc (compare Fig. 2B to Fig. 1A), indicating that replication had initiated towards one end of the rDNA molecule. No bubble arcs or bubble-to-Y arcs could be detected, even on longer exposures. Thus, the 2D gel analysis corroborates the EM data (1, 24), as it indicates that the majority of the rDNA molecules are replicated from origins located in the vicinity of the telomere rather than from a more central position.

Location of replication origins during late macronuclear development. During macronuclear development, the final phase of DNA amplification starts well after the newly synthesized telomeres are trimmed to the mature size but just before the round anlagen elongate into the mature horseshoe-shaped macronucleus (34, 36). To determine the timing of this fourth phase of amplification for the X1 and X2 strains used in this study, mated cells were stained with DAPI (4′,6-diamidino-2-phenylindole) at various times after mixing and the number of round versus elongating anlagen were determined (data not shown). This analysis revealed that at 98 h postmixing, some of
the anlagen had begun to elongate and hence that the final phase of amplification had started.

Based on these results, DNA was isolated from *Euplotes* cells 98 h after mating and enriched for replication intermediates by using BND-cellulose. In initial experiments, undigested BND-treated DNA was resolved in neutral-neutral 2D gels and transferred to a nylon membrane and the rDNA molecules were identified by Southern hybridization using probe A (Fig. 2A). As shown in Fig. 2C, the pattern of replication intermediates was quite similar to that observed with the replication intermediates from vegetative cells. Most of the intermediates formed a simple Y arc, and as before, no bubble arcs or bubble-to-Y arcs could be detected. Thus, origin usage seems to be essentially the same during vegetative growth and the final phase of macronuclear development.

In addition to the simple Y arc, a faint signal corresponding to random termination products could be seen extending upward from the 2N spot and out from the top of the Y arc (compare Fig. 1B and 2C). This implies that for a few of the rDNA molecules, a second origin located at the opposite end from the first origin fires and gives rise to a small replication fork after the bulk of the DNA has been replicated from the first origin. Random termination would then occur when the two replication forks meet. Some random termination was also observed with the DNA from vegetative cells, but this was less consistent.

Closer comparison of the replication intermediates from mated and vegetatively growing cells (Fig. 2B and C) did reveal one interesting difference. With DNA from mated cells, the intensity of the Y arc signal was fairly even across the whole arc. However, the signal from the vegetative cell DNA was routinely more intense over the first (upward) part of the arc. This suggests that the replication fork moves quite evenly along the rDNA when the molecules are being amplified during macronuclear development. However, during vegetative growth the replication fork seems to travel more slowly as it replicates the first half of the rDNA but accelerates when it reaches the middle of the molecule.

Since the rDNA molecule is relatively large, Y arcs from full-length molecules might mask small bubbles arising from origins that are within ~1 kb of the telomere, as they would be rapidly converted to Y forks. To look more carefully for origins that originate within 500 to 1,000 bp of the telomere, we digested the rDNA with *Sma*I to obtain fragments of 4.8 and 2.8 kb (Fig. 2A). We then identified replication intermediates using probes that hybridized specifically to one fragment or the other (Fig. 2A, probe B or C). As shown in Fig. 2D and E, the pattern of replication intermediates remained unchanged, with simple Y arcs as the main intermediate and no visible bubble arcs. An origin located 500 to 1,000 bp from the telomere that is replicated bidirectionally would give rise to a bubble arc of 1 to 2 kb. As this is quite large relative to the 4.8- and 2.8-kb rDNA fragments, such intermediates should have been resolved by the 2D gels shown in Fig. 2C and D. We therefore conclude that the replication origins used at this stage in macronuclear development are located very close to the telomere.

During the last stage of anlagen development, the newly generated macronuclear DNA molecules are subject to multiple closely spaced rounds of DNA replication over a period of 10 to 15 h (30, 36). To ensure that the same origins are used throughout this time period, we next analyzed the replication intermediates from cells isolated 105 and 115 h after mating. At 105 h, many but not all of the anlagen had started to elongate into a horseshoe shape, while by 115 h, almost all the

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**FIG. 1.** Schematic representation of replication intermediates detected by using neutral-neutral 2D gel electrophoresis. The expected signal (lower panel) is diagramed below the corresponding replication intermediate (upper panel). The dashed line in the lower panel marks the arc of linear double-stranded molecules of various sizes. The dotted line marks the position of the Y arc in situations where it would not appear experimentally. 1N, the 1N spot, containing linear unreplicated molecules; 2N, the 2N spot, containing linear, fully replicated molecules. The spikes extending from the 2N spot and the top of the Y arc in panel B correspond to random termination products caused by a small Y fork meeting a large Y fork migrating in the opposite direction.
anlagen had elongated and replication was almost complete. DNA isolated from cells at the two time points was loaded directly on 2D gels without BND-cellulose treatment. This allowed the relative amount of replicating DNA to be compared between samples and ensured that there was no enrichment for one specific type of replication intermediate. The lack of BND treatment made the replication intermediates harder to detect (Fig. 3); however, despite the weak signals, it was clear that the overall patterns of intermediates were very similar for all the time points and in each case corresponded to a simple Y arc. This indicates that the replication origins used to amplify individual rDNA molecules remain the same throughout the final stage of macronuclear development.

**Location of replication origins during differential amplification.** During macronuclear development in *Tetrahymena*, the rDNA is amplified to a copy number of ∼10,000 while the regular macronuclear chromosomes only reach a copy number of ∼45 (18, 37). Since this differential amplification of the rDNA is accompanied by an increase in origin reinitiation and the ability to fire two origins simultaneously (38), it seemed likely that similar changes in origin usage might also take place in *Euplotes*. To determine whether this is the case, we set out to map the *Euplotes* rDNA origins at the time when newly formed macronuclear DNA molecules have been shown to undergo differential amplification (7).

Although the differential amplification seen for non-rDNA molecules appears to take place soon after the micronuclear chromosomes are fragmented to form the new macronuclear DNA molecules (7), the precise timing of the event had not been determined for the rDNA molecule. We therefore isolated anlagen DNA from mated *Euplotes* cells at various time points after chromosome fragmentation (this takes place ∼48 h after mating of the X1 and X2 strains) and looked for differential rDNA amplification by both ethidium staining and Southern hybridization. As shown in Fig. 4A, DNA isolated 64 h postmating gave a homogeneous smear when separated in an agarose gel, whereas by 88 h a banding pattern that displayed clear differences in staining intensity between bands had become visible. The smear of DNA fragments observed at 64 h probably reflects the presence of both newly formed macronuclear DNA molecules and residual non-macronuclear-destined sequences in the developing macronucleus. Although the differential amplification seen for non-rDNA molecules appears to take place soon after the micronuclear chromosomes are fragmented to form the new macronuclear DNA molecules (7), the precise timing of the event had not been determined for the rDNA molecule. We therefore isolated anlagen DNA from mated *Euplotes* cells at various time points after chromosome fragmentation (this takes place ∼48 h after mating of the X1 and X2 strains) and looked for differential rDNA amplification by both ethidium staining and Southern hybridization. As shown in Fig. 4A, DNA isolated 64 h postmating gave a homogeneous smear when separated in an agarose gel, whereas by 88 h a banding pattern that displayed clear differences in staining intensity between bands had become visible. The smear of DNA fragments observed at 64 h probably reflects the presence of both newly formed macronuclear DNA molecules and residual non-macronuclear-destined sequences in the developing macronucleus. Although the residual non-macronuclear-destined sequences may partially mask the discrete banding pattern of the individual macronuclear DNA molecules, the homogeneity of the staining suggests that differential amplification of the macronuclear DNA had not yet begun. In contrast, the different intensities of the bands observed at later time points indicates

![Fig. 2. Mapping the rDNA replication origins used during vegetative growth and late macronuclear development. (A) Map of the *E. crassus* macronuclear rDNA molecule. Grey boxes, coding regions for 17S, 5.8S, and 26S rRNA; black lines, noncoding sequence; black boxes, telomeric repeats. Restriction sites: X: XbaI; C: ClaI; Sa: SacII; Sn: SnaBI; Sm: SmaI; H: HindIII; E: EcoRI. Fragments used as probes are shown below the rDNA map. (B to E) DNA samples were separated in neutral-neutral 2D gels, and the rDNA replication intermediates were identified by Southern hybridization. (B) DNA isolated from vegetatively growing cells hybridized with probe A. (C to E) DNA isolated from developing cells 98 h after mating. (C) rDNA replication intermediates from undigested DNA hybridized with probe A. (D) SmaI digested DNA hybridized with rDNA probe B. (E) SmaI digested DNA hybridized with rDNA probe C. The sizes of the 1N spots and positions of molecular weight markers run in the first dimension are shown at the bottom of each gel.](image-url)

![Fig. 3. Comparison of rDNA replication intermediates formed at various times during the final stages of macronuclear development. Undigested, non-BND-treated DNA was separated in 2D gels and hybridized to rDNA probe A at 98 (A), 105 (B), and 115 (C) h postmating.](image-url)
When Southern hybridization was used to identify the rDNA, that specific DNA molecules had been differentially amplified. When Southern hybridization was used to identify the rDNA, this amplification was clearly visible, as the rDNA signal showed a continuous increase between the 64- and 84-h time points (Fig. 4B). In contrast, hybridization of the same blot with probes for the actin and histone H4 sequences, by later time points it was visible as a discrete band in the ethidium-stained gel that was brighter than most of the other bands.

As differential amplification took place between the 72- and 80-h time points, we examined the rDNA replication intermediates from DNA isolated from mated cells 72 and 75 h postmixing. Surprisingly, the 2D gel analysis revealed a pattern of replication intermediates that was almost identical to that observed with DNA from vegetatively growing cells and cells in the final stage of macronuclear development (Fig. 5). As before, a strong Y arc was observed but no bubble or bubble-to-Y arcs. This indicates that the origins remained telomeric and new internally located origins were not activated. In addition to the Y arc, a faint spot was observed at a position that corresponds to a size greater than that of the 2N (~15 kb) replication intermediate. This >2N spot probably corresponds to molecules that have reinitiated replication from an origin before the first round of replication is complete. However, equivalent >2N signals were sometimes seen with DNA isolated in the final stages of macronuclear development. Thus, contrary to the situation in *Tetrahymena*, origin usage does not appear to change significantly during differential amplification of *Euplotes* macronuclear DNA molecules.

**Determining the direction of replication fork movement.** The presence of random termination products in DNA isolated from both mated and vegetatively growing cells indicates that it is possible for replication to initiate at both ends of a macronuclear DNA molecule. However, full double Y arcs extending from the 1N spot to the position of the random termination products comprised a very small fraction of the total replication intermediates (compare Fig. 1E, Fig. 2, and Fig. 5). This observation fits with the paucity of double Y molecules that were observed during EM studies of vegetative replication intermediates (1), and it indicates that replication routinely initiates at one or other end of a macronuclear DNA molecule but not at both ends simultaneously.

Given this situation, we next asked whether the choice of end for replication initiation is random or whether there is a preference for initiation at the end with either the 5' or the 3' UTR. To answer this question we used a modified neutral-neutral 2D gel analysis to determine the direction of replication fork movement (6, 11). Directional information can be obtained by performing an "in-gel" restriction digestion after separation in the first dimension. The restriction digestion produces one of two different Y arcs depending on the direction in which replication is proceeding (Fig. 6A and B). Both Y arcs originate below the original arc of linear double-stranded molecules of various sizes because the fragment size is reduced by the restriction digestion. However, if the replication fork originates at the end of the molecule that is being identified by the probe (Fig. 6A), then the Y arc originates directly below the original arc of linear double-stranded molecules.

To examine the direction of replication fork movement on rDNA molecules, undigested DNA from mated cells was sep-
The DNA was then digested in the gel with enzymes that cut either towards the 5' end (SnaBI and SacII) or towards the 3' end (HindIII and Smal), separated in the second dimension, and probed with either probe A or B (Fig. 2A). Unfortunately, SnaBI and SacII worked poorly for in-gel digestions, so usable information was obtained only with HindIII and Smal. These enzymes generate 5' rDNA fragments of 5.7 and 4.8 kb, respectively (Fig. 2A). Since initiation of replication within or near the 5' UTR would result in movement of the main replication fork towards the 3' end of the molecule, hybridization of probe B to the HindIII- or Smal-digested DNA would give rise to a Y arc that rises directly from the unreplicated 1N spot (Fig. 6A). However, if initiation is in or near the 3' UTR, so that the replication fork moves towards the 5' end of the molecule, the start of the Y arc would be displaced laterally from the 1N spot (Fig. 6B). As shown in Fig. 6C to E, digestion with either HindIII or Smal resulted in a Y arc that rose directly from the restriction-digested 1N spot. This is particularly obvious in the Smal digest, where digestion was incomplete, so that both the original undigested and new Smal-digested patterns of replication intermediates can be seen. The observed pattern of Y arc indicates that the direction of replication fork movement is from the 5' end of the rDNA towards the 3' end. Thus, the bulk of DNA replication initiates at the end of the rDNA molecule where the promoter is located.}

**FIG. 6.** Determining the direction of replication fork movement. (A and B) Schematic representation of the rDNA replication intermediates that would be detected by probe B following in-gel digestion with Smal or HindIII and electrophoresis in the second dimension. (A) Signal expected if replication starts in or near the 5' UTR; (B) signal expected if replication starts in or near the 3' UTR. (C to E) Replication intermediates detected by probe B with undigested DNA (C), in-gel digestion with HindIII (D), or in-gel digestion with Smal (E). The positions of molecular weight markers run in the second dimension are shown on the right.

**DISCUSSION**

We have used 2D gels to map the replication origins that are used during the two periods in *Euplotes* macronuclear development when the newly formed macronuclear DNA molecules are amplified. Interestingly, we have found that the pattern of rDNA replication intermediates observed with DNA isolated during either stage is very similar to that observed with DNA from vegetatively growing cells. In all three cases, most of the rDNA replication intermediates migrated as a Y arc in the 2D gel analysis, indicating that origins are located in the vicinity of the telomere rather than at a more internal location. The level of replication intermediates corresponding to double Y arcs or >2N spots was also fairly similar, indicating that the level of reinitiation or initiation from both telomeres remains un-
changed. Thus, in contrast to the situation observed in Tetrahymena, there is little alteration in origin usage during Euplotes macronuclear development. Since the number of origins does not appear to change during macronuclear development, differential amplification of the rDNA must be achieved by regulating how often an origin fires. While this might occur by assembling different regulatory complexes at each origin to control firing frequency, a simpler solution might be to control the length of time during which replication can take place. This would avoid the need to reinitiate replication before the previous round is complete and might be achieved by regulating the timing of excision or telomere trimming of the individual macronuclear DNA molecules (7).

In addition to showing that origins are located near the telomere, we have demonstrated that replication initiates preferentially at the end of the rDNA molecule where the promoter is located rather than randomly at either end. This is a striking result because macronuclear DNA molecules do not have an obvious conserved sequence that might serve as an origin in or near the 5′ UTR. Since transcription from the developing macronucleus starts soon after telomere addition and continues throughout DNA amplification (28), the main distinguishing feature between the 5′ and 3′ end of the rDNA molecule is the presence or absence of a transcription initiation complex. Consequently, our finding suggests that either the promoter complex or the chromatin structure of the promoter region somehow assists in assembly of the replication initiation complex. Like previous EM studies, 2D gels do not have sufficient resolution to determine whether replication initiates right at or merely close to the telomere. However, if the telomere alone were responsible for loading of the replication initiation complex, one would not expect a preference for the 5′ versus the 3′ end of the rDNA, as all macronuclear telomeres are composed of the same DNA sequence and terminus-binding protein (27, 29). However, it is possible that the telomere (perhaps the telomere-binding protein) is responsible for recruiting the replication initiation factors, but assembly of the initiation complex is facilitated by the promoter region (34). This could explain why in both mated and vegetatively growing cells, replication does in some instances initiate at the end of the molecule where transcription terminates (seen in the molecules undergoing random termination). It could also explain why no conserved origin sequence has been found in any of the hypotrichous ciliates and why in Stylonychia the sequence of the 5′ UTR, 3′ UTR, and coding region can be completely replaced with an unrelated sequence (35). In the absence of a promoter complex, the telomere would still recruit the replication initiation factors and the replication complex would still form, but more slowly. Thus, molecules lacking a promoter region could still be maintained at a normal copy number.

One caveat of this study is that the analysis was performed with rDNA molecules because of their high copy number. Thus, one has to ask whether our results are representative of other macronuclear DNA molecules. We would argue that they are because many other molecules are also amplified to a high copy number and hence are replicated during both the time periods studied (3, 7). Moreover, although our attempts to detect replication intermediates from other macronuclear DNA molecules resulted in weak signals, Y arcs were observed with both the telomerase catalytic subunit (TERT) and another molecule of unknown function (data not shown).

Most organisms ensure that only one round of replication takes place during each cell cycle by using a complicated system where the origin is marked via binding of Orc, the origin recognition complex, and subsequent loading of CDC6, the minichromosome maintenance proteins, and other factors that “license” the origin to initiate replication (19, 21, 31). However, in Euplotes, the telomeric and promoter structures that are probably involved in origin assembly are present throughout the cell cycle, and nuclease footprinting experiments do not suggest that Orc is bound in the vicinity of the 5′ UTR (C. Price, unpublished results). This raises the question of how Euplotes limits rereplication of macronuclear DNA molecules during S phase. One interesting possibility is that replication initiation is controlled by the replication bands instead of the normal licensing system. Replication bands are specialized structures found in hypotrichous ciliates in which DNA replication takes place (25, 26). These visible structures assemble at one end of the macronucleus and gradually move towards the other end, replicating the DNA molecules in their path. Perhaps assembly of the replication initiation complex on a Euplotes macronuclear DNA molecule is restricted to the time when that molecule is actually passing through the replication band.

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