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Developmental Regulation of DNA Replication: Replication Fork Barriers and Programmed Gene Amplification in *Tetrahymena thermophila*

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The palindromic *Tetrahymena* ribosomal DNA (rDNA) minichromosome is amplified 10,000-fold during development. Subsequent vegetative replication is cell cycle regulated. rDNA replication differs fundamentally in cycling vegetative and nondividing amplifying cells. Using two-dimensional gel electrophoresis, we show for the first time that replication origins that direct gene amplification also function in normal dividing cells. Two classes of amplification intermediates were identified. The first class is indistinguishable from vegetative rDNA, initiating in just one of the two 5′ nontranscribed spacer (NTS) copies in the rDNA palindrome at either of two closely spaced origins. Thus, these origins are active throughout the life cycle and their regulation changes at different developmental stages. The second, novel class of amplification intermediates is generated by multiple initiation events. Intermediates with mass greater than fully replicated DNA were observed, suggesting that onionskin replication occurs at this stage. Unlike amplified rDNA in *Xenopus laevis*, the novel *Tetrahymena* species are not produced by random initiation; replication also initiates in the 5′ NTS. Surprisingly, a replication fork barrier which is activated only in these amplifying molecules blocks the progression of forks near the center of the palindrome. Whereas barriers have been previously described, this is the first instance in which programmed regulation of replication fork progression has been demonstrated in a eukaryote.

Chromosomal DNA replication is a highly regulated process that ensures faithful transmission of genetic material. According to the replicon model, replication is regulated by sequence-specific DNA-protein interactions (25, 54). Initiation has been shown to occur at specific chromosomal sites termed origins of replication. Our understanding of eukaryotic chromosomal origins has increased significantly in recent years. In the yeast *Saccharomyces cerevisiae*, replication initiates at defined sites that, within the limits of resolution, colocalize with the genetic determinants that control initiation (4, 40). A replicator complex, ORC, is stably associated with DNA at the origin (3). Long-distance DNA-protein interactions can also control replication initiation. For example, *cis*-acting replication determinants in *Schizosaccharomyces pombe* and *Tetrahymena thermophila* map hundreds of base pairs away from the origins that they regulate (7, 28, 42), and the human β-globin locus control region is ~50 kb away from the origin that it controls (1, 32).

Cell cycle control genes play critical roles in orchestrating chromosomal DNA replication. They regulate entry into and exit from S phase (reviewed in references 10 and 30) and can directly affect the initiation of replication at chromosomal origins (36). Regulatory mechanisms restrict chromosomal replication to once per S phase. The acquisition of additional gene copies can be detrimental. Amplification of oncogenes and drug target genes can lead to tumorigenesis or the resistance of tumors to chemotherapeutic agents (reviewed in references 52 and 53). Origins of replication have been identified within amplified mammalian DNAs (31, 55). However, their contribution to the amplification process cannot be directly addressed because these amplification events are relatively rare and spontaneous. In contrast, gene amplification is developmentally programmed in several organisms. Replication initiates at a single site during amplification of the *Sciara coprophila* puff II/9A region (35) and at several sites in one of the *Drosophila melanogaster* chorion gene clusters (11, 22). In these examples, gene amplification occurs in polyploid terminally differentiated cells. Whether these origins control replication in cycling mitotic cells is not known. Gene amplification in *Xenopus laevis* oocytes is markedly different. Amplification of *Xenopus* rRNA genes occurs by random initiation in the coding region and nontranscribed spacer (NTS) (23). As development proceeds, initiation events are restricted to the NTS.

T. thermophila is a particularly attractive system for studying gene amplification because amplification events occur in cells that then undergo normal cellular divisions. The *Tetrahymena* rRNA genes (ribosomal DNA [rDNA]) are extensively amplified in the absence of division during macronuclear development (reviewed in reference 28). During vegetative growth, the rDNA is replicated, on average, once per cell cycle (13). Consequently, replication can be studied in the presence or absence of cell cycle regulation. Unlike loci that undergo programmed amplification in higher eukaryotes, *Tetrahymena* rDNA exists as an autonomous minichromosome. *Tetrahymena* cultures can be induced to undergo development synchronously, providing the opportunity to study amplification events at the molecular level.

rDNA amplification is intimately linked to the nuclear dimorphism that typifies ciliated protozoa. *T. thermophila* contains two nuclei within a single cell: the transcriptionally silent, diploid, germ line micronucleus and the transcriptionally active, polyploid, somatic macronucleus (reviewed in reference 29). During conjugation, a copy of the new germ line micronucleus differentiates to become the new macronucleus of the progeny cell. This process, termed macronuclear development,

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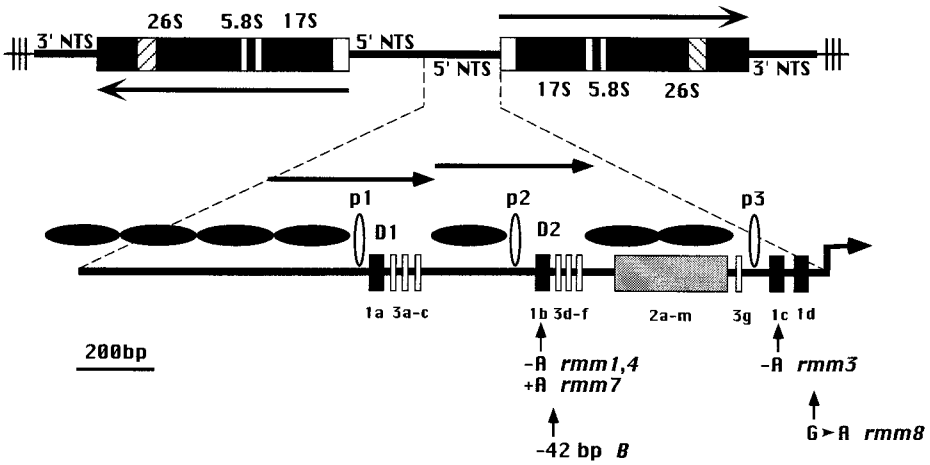

FIG. 1. Structural and functional features of rDNA minichromosomes. (Top) Macronuclear rDNA minichromosomes consist of two copies of the rRNA coding region and adjacent 5′ and 3′ NTS regions in an inverted orientation. The 35S rRNA precursor (arrow and large rectangular box) encodes the 17S, 5.8S, and 26S rRNAs (black areas, mature RNA coding regions; unshaded areas, processed RNA precursor regions; hatched area, self-splicing 26S rRNA intron). Telomeric DNA repeats (thin lines with vertical bars) are present at the rDNA termini. (Bottom) Enlargement of the 1.9-kb 5′ NTS region from the wild-type C3 rDNA allele (terminal arrow, rRNA promoter; black ovals, positioned nucleosomes in vegetative rDNA minichromosomes [47]; black boxes, type I repeats [1a to 1d]; shaded box, tandem array of type II repeats [2a–m]; open boxes, type III repeats [3a–c, 3d–f, and 3g] [9]. Domains 1 and 2 (D1, D2) are 230-bp nuclease hypersensitive regions present in rDNA isolated from vegetative cultures. D1 and D2 reside within a 430-bp sequence (long tandem arrows) that was tandemly duplicated and has undergone subsequent sequence divergence. The positions of sequence changes that affect vegetative rDNA maintenance are depicted for the naturally occurring B rDNA mutant (−42 bp) and for *rmm* mutants isolated following in vivo mutagenesis of cells carrying the C3 rDNA allele (33, 56; reviewed in reference 28). The positions of the three replication fork pausing sites identified in vegetative rDNA RIs are also depicted (open ovals p1, p2, and p3 [39]).

involves massive genomic reorganization, including site-specific chromosome fragmentation (excision) (59, 60), DNA rearrangement (19), and de novo telomere addition (51). Whereas other chromosomes attain a ploidy of ~45, the single germ line rDNA copy is amplified ~10,000-fold (58). Genetic experiments indicate that the rDNA amplification pathway is distinct from the endoreplication pathway for non-rDNA chromosomes (26).

Several aspects of programmed rDNA gene amplification parallel spontaneous events in mammalian cells. During macronuclear development, the 10.3-kb rDNA region is excised by site-specific breakage (59, 60) and rearranged into a 21-kb palindromic minichromosome by homologous recombination between inverted repeated sequences (Fig. 1) (6, 61). By analogy, DNA excision and rearrangement are frequently observed in spontaneously amplified mammalian DNAs (reviewed in reference 52). The rDNA is then amplified extensively in the absence of nuclear division (58). Classical genetic screens have identified mutants that fail to amplify the rDNA in the developing macronucleus (26, 27). Studies on the *rmm11* mutant revealed that excision is required for proper rDNA amplification and suggested that amplification competence is temporally restricted in the developing macronucleus (26). More recent experiments with this mutant suggest that there are at least two phases of rDNA amplification (56). Little is known about direct initiators of replication in amplifying rDNA. However, DNA transformation studies suggest that amplification determinants reside in the 5′ NTS (18).

During vegetative growth the rDNA is maintained at an amplified level, being replicated on average once per cell cycle (13). DNA transformation studies revealed that the 5′ NTS is necessary and sufficient for vegetative replication (18, 48, 49). Electron microscopy (EM) identified vegetative replication bubbles whose centers were asymmetrically positioned in the 5′ NTS, suggesting that there was a single vegetative origin and just one initiation event per rDNA palindrome (8). Vegetative rDNA maintenance mutations also map to the 5′ NTS, in or adjacent to phylogenetically conserved type I elements. These repeated sequence elements reside in regions that are devoid of nucleosomes (Fig. 1) (9, 47). Two of the three nucleosome-free regions, domains 1 and 2, are part of a 430-bp imperfect tandem duplication proximal to the EM initiation site (Fig. 1). *cis*-acting maintenance mutations do not completely block vegetative replication. Instead, they manifest defects when placed in competition with other rDNA alleles, such as wild-type C3 rDNA (27, 33). Several independent lines of evidence argue that maintenance mutations directly affect rDNA replication. For example, the naturally occurring B rDNA allele is "outcompeted" by wild-type C3 rDNA during vegetative growth (33) and is also underrepresented in amplifying C3/B heterozygotes, suggesting that the mutated determinant controls replication initiation (43). Whereas a copy number control mechanism helps maintain the rDNA's genic balance (33), these determinants appear to reside elsewhere in the rDNA (48). Like other macronuclear chromosomes, rDNA does not contain a centromere.

Using two-dimensional (2D) gel electrophoresis, we previously showed that vegetative replication initiates in the 5′ NTS. These studies also revealed that replication fork movement in the 5′ NTS is not uniform (39). Three orientation-dependent replication fork pausing sites (p1, p2, and p3) were mapped (Fig. 1). Fork pausing was shown to be mediated by type I elements—genetic determinants that were previously implicated in replication initiation. In this study we used 2D gel electrophoresis to study replication during rDNA gene amplification. We demonstrate that replication origins that are active in normal dividing vegetative cells also direct rDNA gene amplification. We also provide evidence for an increased frequency of initiation in amplifying molecules. Novel replication intermediates (RIs) derived from two or more initiation events were observed, suggesting that onionskin replication occurs at this stage. A developmentally regulated replication fork barrier is active in these molecules, blocking the progression of replication forks near the center of the rDNA palindrome.

TABLE 1. *Tetrahymena* strains[a]

Strain[b]	Micronucleus	Macronucleus
SF137	C3 rDNA/hemi-2L, 4L (δ rDNA)	C3 rDNA
SB1915	B rDNA/B rDNA	B rDNA
CU427	B rDNA/B rDNA	B rDNA
SB1934	C3 rDNA/C3 rDNA	B rDNA
CU374	nulli-2L, 4L/nulli-2L, 4L (δ rDNA)	B rDNA
SB1934 × CU374	C3 rDNA/nulli-2L, 4L	C3 rDNA

[a] C3 rDNA, wild-type rDNA allele; B rDNA, mutant rDNA allele carrying a 42-bp deletion in the 5′ NTS that affects vegetative replication (33). CU374 is deleted for the left arm of chromosomes 2 and 4 in the germ line micronucleus, including the rDNA locus.

[b] SB1934 and CU374 are heterokaryon strains generated by an alternate conjugation pathway such that the macronucleus and micronucleus are genetically unrelated. SF137 is an F1 progeny line from a mating between SB1934 and CU374. The micronucleus is hemizygous for chromosome arms 2L and 4L and therefore contains only one copy of the rDNA (C3 rDNA).

MATERIALS AND METHODS

Cell culture and *Tetrahymena* strains. *Tetrahymena* strains are listed in Table 1. Vegetative *Tetrahymena* cultures were grown in 2% proteose peptone supplemented with 10 μM $FeCl_3$, 250 μg of penicillin per ml, 250 μg of streptomycin per ml, and 25 μg of amphotericin B per ml. The mated parental strains used to study rDNA amplification are heterokaryons. They contain B rDNA in the macronucleus and are homozygous for the C3 rDNA allele (strain SB1934) or are deleted for the rDNA locus (strain CU374) in the micronucleus. Consequently, only C3 rDNA is amplified in the developing macronucleus of progeny cells. Restriction site polymorphisms were used to distinguish between B and C3 rDNA. Standard genetic procedures have been described previously (44, 45).

DNA isolation and enrichment for RIs. Vegetative RIs were studied in log-phase cultures harvested at a density of $<2 \times 10^5$ cells/ml. rDNA amplification was studied in starved cultures that were mated at a density of 2×10^5 cells/ml in 10 mM Tris (pH 7.5) for 12 to 24 hours. Prior starvation of parental strains synchronizes mating and subsequent macronuclear development. DNA was isolated at various time points during macronuclear development. For several mating experiments a single replication pattern was detected at the earliest amplification time point. In other experiments, the composite pattern characteristic for later amplification time points was observed at the early amplification time point. The basis for this variation is not known.

Total genomic DNA was isolated from *Tetrahymena* cultures by a modification of a previously described protocol (8). Cells were washed in 10 mM Tris (pH 8.0) and lysed by addition of an equal volume of NDS (10 mM Tris, 0.5 M EDTA, 2% sodium dodecyl sulfate [pH 9.5]) prewarmed to 37°C. After incubation for 20 min at 37°C, 0.3 volumes of 5 mg of proteinase K (Boeringer Mannheim) per ml was added and the samples were incubated for 3 to 4 h at 37°C. The samples were diluted with an equal volume of TE (10 mM Tris, 1 mM EDTA [pH 8.0]), extracted once with phenol-chloroform (1:1) and once with chloroform before precipitation with 2.5 volumes of ethanol at room temperature. DNA samples were digested with restriction enzymes for 4 h at 37°C in the presence of 1 μg of RNase A per ml, and RIs were enriched by benzoylated naphthoylated DEAE (BND)-cellulose chromatography (Sigma) as previously described (37). tRNA was used to facilitate precipitation of the BND-cellulose eluate. Total DNA recovery was estimated to be ~5% of input.

2D gel electrophoresis. Neutral-neutral 2D agarose gel electrophoresis was performed essentially as previously described (4). Typically, 2 to 20 μg of DNA was loaded in the first dimension. Restriction fragments of >3 kb were resolved in 0.4% agarose in the first dimension and in 1.0% agarose containing 1 μg of ethidium bromide per ml in the second dimension. Smaller restriction fragments were separated in 0.7 to 1.0% agarose in the first dimension and 1.8 to 2.0% agarose in the second dimension. Typical running conditions were 1.5 V per cm for 18 h at room temperature for the first dimension and 3.0 V/cm for 24 h at 4°C for the second dimension. DNA was transferred by capillary blotting to Hybond N+ (Amersham) filters and probed with cloned DNA fragments radiolabeled by random priming with [^{32}P]dATP. The filters were exposed to Kodak XAR-5 film for several hours to >1 week at −70°C with an intensifying screen (DuPont).

The direction of movement of replication forks was assessed by electrophoresing *Cla*I-digested RIs in the first dimension and redigesting RIs in situ with *Sst*I prior to electrophoresis in the second dimension. For in situ digestion, the first-dimension gel slices (6 by 1 by 1 cm) were washed twice for 30 min each with 10 mM Tris–0.1 mM EDTA at room temperature. Slices were then equilibrated twice for 1 h each at room temperature in *Sst*I restriction enzyme buffer (Gibco/BRL) supplemented with 100 μg of bovine serum albumin per ml. The buffer was carefully removed and 20 to 40 μl of *Sst*I (20 U/μl) was pipetted directly onto the gel slice. After incubation at 37°C for 5 h, the gel slice was washed for 30 min in 10 mM Tris–1 mM EDTA (pH 8.0) and electrophoresed in the second dimension.

RESULTS

Localization of vegetative replication origins in palindromic 5′ NTS fragments. A previous EM study suggested that vegetative rDNA replication initiates at one 5′ NTS site for the B rDNA allele, proximal to domain 1 (8). In an effort to increase our understanding of the regulation of DNA replication, we used 2D gel electrophoresis to study RIs in log-phase vegetative *Tetrahymena* cells and in nondividing cultures undergoing programmed gene amplification. First, we localized vegetative replication origins in strains containing B or C3 rDNA in the macronucleus (Table 1). C3 rDNA has a vegetative replication advantage over B rDNA due to a 42-bp deletion in domain 2 of the B rDNA 5′ NTS (33, 57). DNA from asynchronous vegetative cultures was digested with restriction enzymes, and RIs were resolved in two dimensions under neutral pH conditions (4). Characteristic RI patterns are diagnostic for initiation within or outside a DNA fragment (Fig. 2A).

The 21-kb rDNA minichromosome contains two copies of a 10.3-kb sequence in a head-to-head palindromic configuration. Digestion with *Msp*I generates a 3.9-kb fragment containing the two inverted 5′ NTS copies (Fig. 2B). Southern blot analysis revealed a "bubble-to-Y" arc pattern for B and C3 rDNA alleles (Fig. 2C). Complete, simple Y arcs were not detected, even in overexposed autoradiograms, suggesting that initiation is restricted to the 5′ NTS during vegetative replication. Additional experiments demonstrated this to be the case (see below and Fig. 5 and 6). Whereas a bubble-to-Y arc profile is typically diagnostic for an origin positioned asymmetrically within a fragment (Fig. 2A), this conclusion could not be drawn in this case because accumulated intermediates were detected on both arcs (Fig. 2C), indicating that the two diverging forks were not moving continuously.

Digestion with *Hha*I generates a 2.7-kb palindromic fragment in which 550 bp has been removed from each end. A composite pattern containing a bubble arc and complete Y arc was detected for both rDNA alleles (Fig. 2C). Consistent with the *Msp*I data, the incomplete bubble arc represents molecules that initiate at an internal site and convert to Y arc intermediates as replication proceeds. However, the *Hha*I Y arc is complete, containing molecules with very short nascent strands. The simplest interpretation is that replication initiates at more than one 5′ NTS site, one significantly upstream of the *Hha*I site and another close to or downstream of this site but well upstream of the *Msp*I site (Fig. 2B). Alternatively, replication might initiate at one site close to the end of the fragment, with some forks being trapped transiently at a pausing site (producing bubbles) and others moving unabated (producing simple Ys). The absence of double Y intermediates or other novel species (which might be indicative of a bubble-plus-Y intermediate) suggests that there is a single initiation event per rDNA palindrome despite the presence of two 5′ NTS copies per molecule. This interpretation is consistent with conclusions from the previous EM study (8) and 2D gel experiments performed on nonpalindromic 5′ NTS fragments (see below).

Physical methods for mapping bidirectional origins typically rely on the assumption that the diverging forks move at constant and equal rates. Transient pausing of one fork can affect the accuracy of origin mapping. To more precisely localize vegetative replication origins, we used the three fork pausing sites that we previously identified as landmarks for examining RIs (Fig. 2B) (39). These sites are polar, strongly arresting forks that move from the center of the rDNA towards the telomere. Whereas site-specific arrest of the single fork in a Y arc will cause a specific intermediate to accumulate, both forks

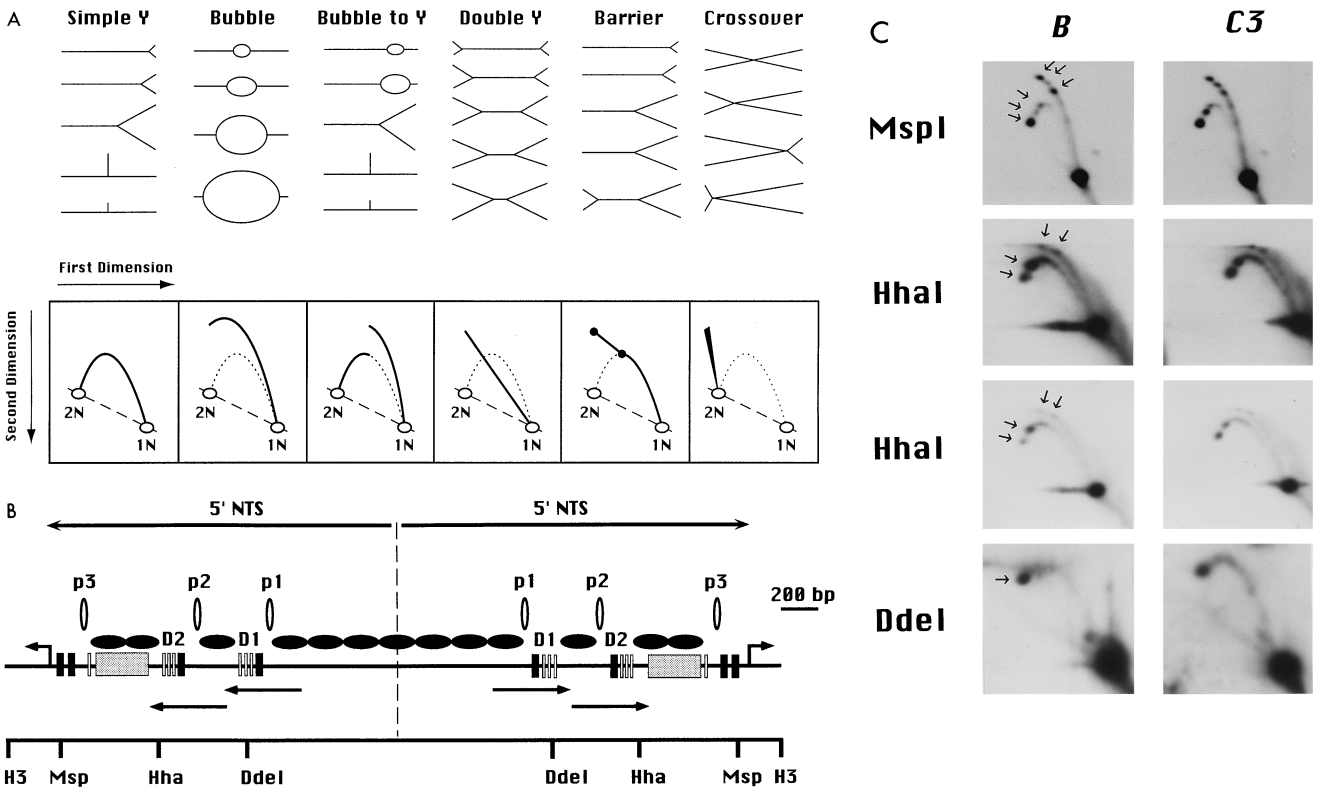

FIG. 2. Vegetative origin mapping in palindromic rDNA fragments. (A) Schematic representation of RIs resolved by neutral-neutral 2D gel electrophoresis (3). Simple Y arc, passive replication by a single fork entering from one end of the restriction fragment; bubble arc, bidirectional replication from an origin positioned in the center of the fragment; bubble-to-Y arc, bidirectional replication from an origin positioned asymmetrically in the fragment; double Y arc, passive replication from two converging forks initiating outside of the restriction fragment; barrier arc, replication of a fragment by converging forks, in which the first fork enters and terminates at a barrier prior to entry of the second fork (the spots on the barrier arc correspond to intermediates that accumulate at the barrier); crossover, recombination between nonreplicating DNA molecules. The 2-N spike arc results from branch migration of the crossover junction, which generates 2-N recombination intermediates with different migration properties. Diagonal dashed line, arc of nonreplicating linear restriction fragments; solid arc, migration pattern of the particular replication intermediate class; dotted arc, migration pattern of simple Y arc intermediates relative to other RIs. (B) Partial restriction map of the 5′ NTS, including restriction sites used for the analysis of palindromic fragments generated by cleavage at the *Hin*dIII (H3, nucleotide [nt] 2132), *Msp*I (Msp, nt 1906), *Hha*I (Hha, nt 1347), or *Dde*I (Dde, nt 828) site. Vertical dashed line, 29-bp nonpalindromic spacer forming the axis of symmetry for the oppositely oriented copies of the 5′ NTS (long arrows); short arrows, 430-bp tandem duplications that include the nucleosome-free regions domain 1 and domain 2. Other symbols are as described for Fig. 1. (C) DNA samples from asynchronous, log-phase, vegetative cultures were subjected to 2D gel analysis following digestion with a single restriction enzyme and enrichment for RIs on BND cellulose (see Materials and Methods). Macronuclear C3 (SF137) and B (SB1915 or CU427) rDNA were examined. The probe used was a 1.9-kb fragment spanning the entire 5′ NTS (nt 1 to 1909) or the central 500-bp fragment (*Dde*I analysis only).

in a bubble arc intermediate must arrest for a molecule of defined size to accumulate.

Three prominent accumulated intermediates and several minor ones were detected on the bubble arc for the palindromic *Msp*I fragment (Fig. 2C). The B rDNA mutation does not change the shape of the RI pattern relative to C3 rDNA. It does, however, cause a decrease in the accumulation of specific stalled bubble and Y arc intermediates due to diminished pausing of replication forks at pause site 2 (Fig. 2B and C) (39). This is consistent with at least one of the forks in the diminished *Msp*I bubble arc intermediate being trapped at pause site 2. Consequently, some or possibly all of the rDNA molecules initiate replication upstream of pause site 2 and therefore upstream of domain 2. No attempt was made to determine the composition of all of the stalled *Msp*I bubble arc intermediates. Instead, a smaller palindromic fragment (*Hha*I) which retains just pause sites 1 and 2 was examined (Fig. 2B).

Two accumulated bubble arc intermediates were detected in the *Hha*I 5′ NTS fragment (Fig. 2C) in addition to two paused Y arc intermediates. As fork pausing sites are polar (39), only two accumulated bubble arc intermediates should be observed if replication initiated between pause sites 1 and 2 rather than further upstream. The accumulated bubble arc intermediates should arrest at pause sites 1 (left) plus 2 (right) and 2 (left) plus 2 (right) (Fig. 2B). A third stalled bubble arc intermediate should have been detected if replication initiated upstream of pause site 1, arresting at pause sites 1 (left) plus 1 (right). This was not observed, even with longer exposures (Fig. 2C). Like the *Msp*I digest, the relative abundance of paused bubble versus Y arc intermediates differed between the two rDNA alleles. These differences are consistent with the most-upstream initiation events occurring between pause sites 1 and 2 rather than further upstream. Finally, bubble arcs were not evident in the 1.6-kb palindromic *Dde*I fragment (Fig. 2B and C). If the upstream initiation events occurred well within this segment, prominent bubble arcs should have been visible since bubbles could get trapped by the two p1 pausing sites. We can detect bubble arcs in fragments of similar size (see below), indicating that we had not reached the limit of resolution. These data suggest that vegetative replication does not initiate near the center of the palindrome.

Localization of 5′ NTS origins in nonpalindromic fragments. Restriction fragments containing just one 5′ NTS copy were examined to determine if replication initiated at more

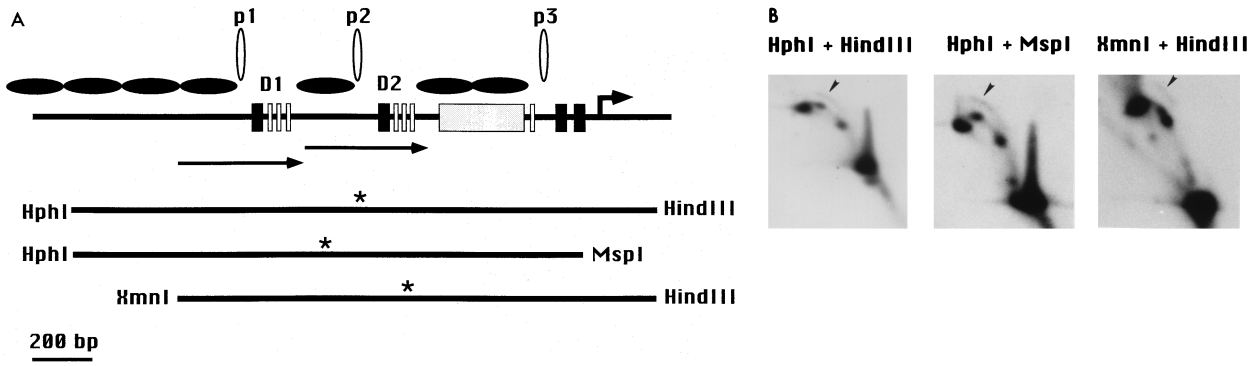

FIG. 3. Vegetative origin mapping in nonpalindromic rDNA fragments. (A) Schematic of the 5′ NTS and restriction fragments used to map origins in nonpalindromic rDNA fragments. Long arrows, 430-bp tandem duplications that include the nucleosome-free regions domain 1 and domain 2. Other symbols are as described for Fig. 1. The center of each restriction fragment is indicated by an asterisk. (B) DNA from asychronous log-phase vegetative *Tetrahymena* cells was subjected to 2D gel analysis following digestion with *Hph*I plus *Hin*dIII, *Hph*I plus *Msp*I, or *Xmn*I plus *Hin*dIII. The 5′ NTS was cut twice in each digest, separating the two 5′ NTS copies from one another. Restriction sites: *Hph*I, nucleotide (nt) 154; *Xmn*I, nt 500; *Msp*I, nt 1906; *Hin*dIII, nt 2132. The probe used was a 1.9-kb fragment spanning the entire 5′ NTS (nt 1 to 1909).

than one 5′ NTS site. Vegetative DNA preparations were digested with restriction enzymes that cleave at two 5′ NTS sites, producing nonpalindromic fragments in which the two inverted 5′ NTS copies were no longer joined (Fig. 3A). Complete Y arcs are predicted if only one 5′ NTS copy is active, whereas double Y arcs are expected if both 5′ NTS copies initiate in a single molecule. A prominent simple Y arc containing paused RIs was detected for each nonpalindromic fragment (Fig. 3B). No double Y arcs were observed, indicating that initiation events are restricted to one side of the palindrome.

Bubble arc intermediates would be detected if an origin was more or less centrally localized in these 5′ NTS fragments. A prominent bubble arc was detected in restriction fragments (*Hph*I-*Hin*dIII [1.95 kb] and *Hph*I-*Msp*I [1.7 kb]) in which the tandemly duplicated sequences (430 bp) containing domains 1 and 2 were internally positioned (Fig. 3). High-molecular-weight bubble arc intermediates are consistent with initiation at an internal position. However, initiation near the promoter-proximal end could not be ruled out by these data, since the fork moving towards the promoter can get trapped by the p3 pausing site. Both forks cannot be trapped in replication bubbles for these fragments, since pausing sites only arrest the fork that moves towards the promoter. Digestion with *Xmn*I plus *Hin*dIII removes 350 bp upstream of domain 1 such that the upstream domain 1 duplication is near the end of this fragment and domain 2 is centrally located (Fig. 3A). Initiation from the domain 1 region should generate small bubbles that rapidly convert to Y arc intermediates. However, in addition to the simple Y arc, high-molecular-weight bubble arc intermediates were detected in this fragment (Fig. 3B, right panel). These data indicate that initiation events also occur at an internal position, possibly proximal to domain 2. Additional mapping experiments demonstrate that the downstream initiation site is not promoter-proximal (Fig. 2C and Fig. 6B). Furthermore, B and C3 rDNA alleles generated indistinguishable bubble arc profiles in these analyses (data not shown). We conclude that vegetative replication initiates at more than one site in the 5′ NTS, proximal to domain 1 and domain 2.

Replication initiation in amplifying rDNA minichromosomes. Cell cycle control of replication must be suppressed during macronuclear development because rDNA amplification occurs in nondividing cells. We investigated whether gene amplification initiates at the same 5′ NTS sites that control vegetative replication, at amplification-specific origins, or randomly, by examining RIs in developing macronuclei. The mated parental strains are heterokaryons, each containing B rDNA in their (parental) macronucleus. SB1934 is homozygous for the C3 rDNA allele in its germ line micronucleus, and CU374 is nullisomic for chromosome arms 2L and 4L and therefore deleted for the micronuclear rDNA copy (Table 1). Only C3 rDNA will be present in the developing nucleus of progeny cells. An *Sph*I polymorphism was used to resolve C3 rDNA from parental B rDNA. In standard Southern blots, C3 rDNA was first detected in progeny around 10 to 12 h after initiation of mating, with an estimated abundance of ~50 copies per cell (26). Amplification to 10,000 copies was essentially complete by 24 h.

2D gel analysis of the central palindromic *Hin*dIII fragment (Fig. 2B) revealed a complex pattern of RIs in amplifying rDNA minichromosomes (Fig. 4A). Two replication patterns are evident, with the relative abundance of the two classes changing as development proceeds. The earliest detectable time point consists solely of a new replication pattern. The bubble-to-Y arc pattern characteristic of vegetative rDNA is completely absent at this time (compare at 12 h versus vegetative [Fig. 4A]) but is detected at later stages. The appearance of the "vegetative" pattern indicates that the 5′ NTS origins which are active in normal dividing cells also function in the developing macronucleus, where cell cycle control mechanisms are not operational. This pattern corresponds to a single initiation event per molecule in one of the two 5′ NTS copies.

The novel class of RIs is present at all amplification time points (Fig. 4A). The shape of this arc is diagnostic for replication from converging forks (Fig. 2A). This pattern indicates that initiation events are occurring on both sides of the palindrome. Two abundant accumulated intermediates were detected on this replication arc, with approximate masses of 1.5*N* and 2*N*, respectively, where *N* is the length of the restriction fragment (Fig. 4A, left panel, intermediates 1 and 2). The approximate mass of these accumulated intermediates suggests that the converging forks might arrest at a common site. This pattern is consistent with replication initiating first on one side of the palindrome (Fig. 4B), the first stalled intermediate (1.5*N*) being generated when the upstream fork arrests at a 5′ NTS barrier. An independent initiation event on the other side of the palindrome would generate the second fork that converges on this barrier, to produce the second stalled intermediate (2*N*).

Replication fork barriers differ from pausing sites in that

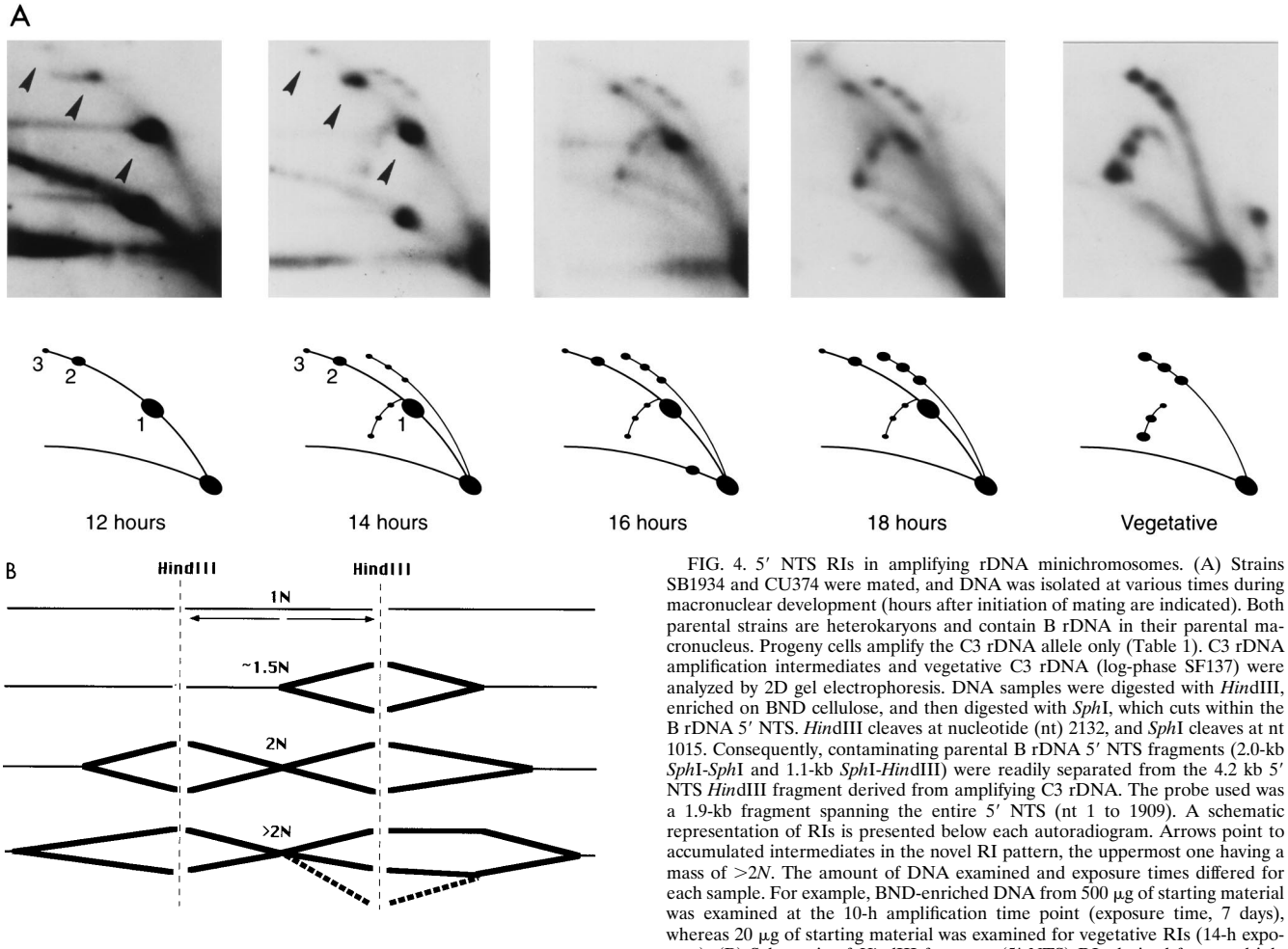

FIG. 4. 5′ NTS RIs in amplifying rDNA minichromosomes. (A) Strains SB1934 and CU374 were mated, and DNA was isolated at various times during macronuclear development (hours after initiation of mating are indicated). Both parental strains are heterokaryons and contain B rDNA in their parental macronucleus. Progeny cells amplify the C3 rDNA allele only (Table 1). C3 rDNA amplification intermediates and vegetative C3 rDNA (log-phase SF137) were analyzed by 2D gel electrophoresis. DNA samples were digested with *Hin*dIII, enriched on BND cellulose, and then digested with *Sph*I, which cuts within the B rDNA 5′ NTS. *Hin*dIII cleaves at nucleotide (nt) 2132, and *Sph*I cleaves at nt 1015. Consequently, contaminating parental B rDNA 5′ NTS fragments (2.0-kb *Sph*I-*Sph*I and 1.1-kb *Sph*I-*Hin*dIII) were readily separated from the 4.2 kb 5′ NTS *Hin*dIII fragment derived from amplifying C3 rDNA. The probe used was a 1.9-kb fragment spanning the entire 5′ NTS (nt 1 to 1909). A schematic representation of RIs is presented below each autoradiogram. Arrows point to accumulated intermediates in the novel RI pattern, the uppermost one having a mass of >2*N*. The amount of DNA examined and exposure times differed for each sample. For example, BND-enriched DNA from 500 μg of starting material was examined at the 10-h amplification time point (exposure time, 7 days), whereas 20 μg of starting material was examined for vegetative RIs (14-h exposure). (B) Schematic of *Hin*dIII fragment (5′ NTS) RIs derived from multiple initiation events. The 1.5*N*, 2*N*, and >2*N* intermediates accumulate due to the arrest of replication forks at a 5′ NTS barrier, with the preceding arrested intermediate serving as the substrate for the next initiation event.

they block any further movement of a replication fork rather than just transiently arresting its progression. Consequently, sequences downstream of a barrier must be replicated by a fork derived from another origin. All of the features of our data are consistent with the 5′ NTS arrest site functioning as a barrier rather than a transient pausing site. (i) The novel replication arc pattern is diagnostic for converging replication forks, consistent with more than one initiation event per molecule. (ii) The appearance of both 1.5*N* and 2*N* accumulated intermediates indicates that initiation events on opposite sides of the palindrome are not coupled. (iii) Neither full bubble, bubble-to-Y, nor simple Y arcs were observed at the earliest amplification timepoint. If a fork was simply pausing transiently, at least one of these patterns would have been generated. (iv) The converging forks appear to arrest at the same site, consistent with this site functioning as a barrier rather than a pause site. Based on modeling studies of *Escherichia coli* plasmids that contain unidirectional origins (41), we cannot resolve whether the two initiation events occurred within or outside of the *Hin*dIII fragment in this new class of RIs.

A faint replication arc ascending from the arrested 2*N* intermediate and terminating at a third accumulated species was detected in the early amplification time point (12 h, intermediate 3 [Fig. 4A]). Such molecules with an apparent mass of >2*N* are evident at all amplification time points (Fig. 4A) and were detected for another restriction digest (*Hph*I-*Hin*dIII), indicating that they are not a gel artifact (see Fig. 6B). One possibility is that these molecules were generated by replication on a recombination intermediate substrate, but several lines of evidence argue against this. First, the diagnostic pattern for recombination intermediates was not detected in the *Hin*dIII fragment. Recombination between nonreplicating molecules will generate Holliday junction intermediates with 2*N* masses (Fig. 2A, right panel). Branch migration of the crossover junction will produce intermediates that have the same mass but different shapes, generating a recombination arc spike that tracks continuously upwards from the 2*N* spot. This spike was noticeably absent in the *Hin*dIII amplification intermediates (Fig. 4A). Furthermore, since only a single >2*N* arc was observed, the substrate(s) that replicates further must have a similar shape. This result is inconsistent with recombination intermediates serving as these substrates. Finally, intermediates with additional mass ascend from the accumulated 2*N* spot, suggesting that the 2*N* RI is the substrate. We propose that the higher-molecular-weight intermediates are generated by onionskin replication, in which reinitiation occurs on nascent DNA prior to completion of the previous round of replication (Fig. 4B).

Mapping replication origins in early amplification intermediates. Multiple initiation events generate the new class of RIs seen in amplifying rDNA. These molecules may be produced by several mechanisms. Replication might initiate randomly in the rDNA, similar to rDNA replication during early embryonic divisions in *X. laevis* (24). Alternatively, replication may be directed by amplification-specific origins in the coding region or 3′ NTS. Finally, replication might initiate in the 5′ NTS, at novel sites, or at the vegetative origins. A coding region fragment was examined to begin to discriminate between these possibilities (Fig. 5A). Similar to vegetative rDNA, standard 2D gel analysis detected only simple Y arcs in early amplification intermediates derived from the 5.5-kb *Cla*I coding region fragment (Fig. 5B). Consequently, at least half of the rDNA minichromosome is passively replicated in amplifying cells, ruling out the random initiation model.

A modification of the neutral-neutral 2D gel method was employed to determine if replication initiated upstream or downstream of the coding region *Cla*I fragment in the novel amplification intermediates (5). The direction of fork movement through the coding region was determined by electrophoresing *Cla*I intermediates in the first dimension and then digesting them in situ with *Sst*I prior to electrophoresis in the second dimension. *Sst*I cuts asymmetrically in the 5.5-kb *Cla*I fragment, generating a 3.9-kb fragment (Fig. 5A). If the forks entered this fragment from the 3′ NTS end (Fig. 5A, right panel), the Y arc would rise directly from the unreplicated 1*N* *Cla*I-*Sst*I spot. Conversely, if the forks entered from the 5′ NTS end (Fig. 5A, left panel), the Y arc would first be displaced laterally from the unbranched 1*N* linear. Both patterns would be detected if the forks moved in both directions. Laterally displaced Y arcs were detected in both vegetative and early amplification intermediates (Fig. 5B). However, no arcs ascending directly upward from the *Cla*I-*Sst*I 1*N* spot were observed. Consequently, both vegetative and early amplification intermediates initiate replication upstream of this coding region fragment. A faint spot located at the 1*N* *Cla*I-*Cla*I position was also detected, due to incomplete in situ digestion with *Sst*I. This fragment resolved sufficiently from in situ-digested DNA and did not obscure these analyses.

Standard 2D gel analysis was performed on other restriction fragments to determine if the novel amplification intermediates initiated near the rRNA promoter or further upstream (Fig. 6A). The 4.0-kb *Xba*I-*Fok*I and 2.0-kb *Xba*I-*Sac*II fragments contain the 35S rRNA precursor region and promoter at a central position, respectively. Only simple Y arcs were detected in these fragments for both vegetative and early amplification intermediates (Fig. 6B), indicating that amplification does not initiate in these regions. Interestingly, recombination intermediates were detected in these fragments in DNA isolated from developing macronuclei. Recombination intermediates were much more abundant in the *Xba*I-*Fok*I fragment, suggesting either that recombination had occurred well within the coding region or that branch migration into the 5′ NTS was impeded. Recall that recombination intermediates were completely absent in amplification intermediates for the palindromic *Hin*dIII fragment which contains both 5′ NTS copies.

Initiation at upstream 5′ NTS sites was examined in the *Hph*I-*Hin*dIII fragment, in which domains 1 and 2 are centrally positioned. As shown above (Fig. 3B), both a simple Y arc and bubble arc were detected in vegetative rDNA (Fig. 6B). The Y arc is generated by passive replication of the second 5′ NTS copy and also by the active initiating copy, once one fork passes the end of this fragment. A more complex composite pattern was seen in the early amplification intermediates, indicating that multiple classes of intermediates were present in this sample. (i) Y arcs that descend to intersect the arc of linear fragments were observed in these amplification intermediates. Whether they are due to passive replication of the second 5′ NTS copy and/or initiation close to the *Hph*I site cannot be determined. (ii) Somewhat diffuse hybridization was reproducibly detected above the Y arc. This may correspond to bubble arc intermediates initiating within this DNA fragment. The diffuse nature of these intermediates may indicate that there is heterogeneity, with some intermediates consisting solely of bubbles and others containing both a bubble and a Y fork derived from initiation in the second 5′ NTS copy. (iii) A continuously ascending barrier (double Y arc) was also observed. Two accumulated species were detected on this arc. The smaller one has a mass slightly less than 2*N*, suggesting that the fork barrier resides near one end of this fragment. Taking the *Hin*dIII results into consideration (Fig. 4A), the barrier must reside near the center of the rDNA palindrome, far upstream of the sites that induce transient replication fork pausing in vegetative rDNA (39). The larger accumulated intermediate

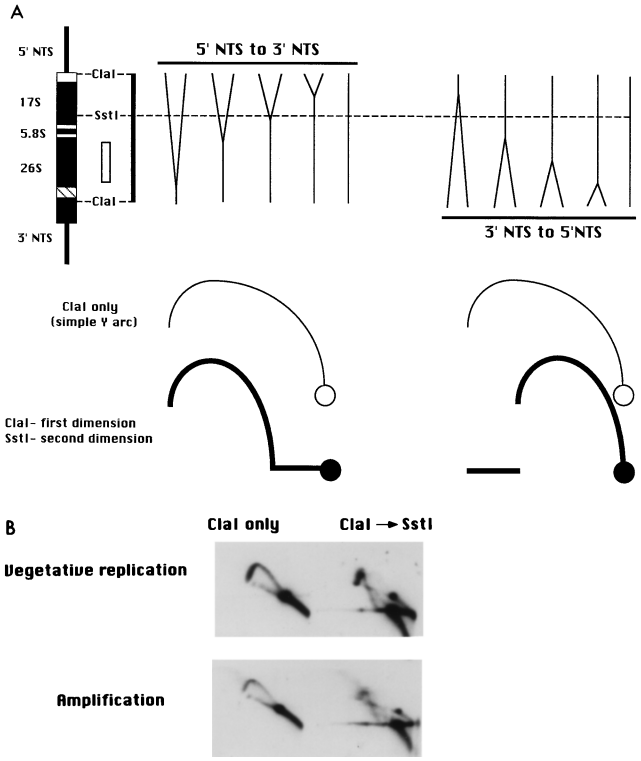

FIG. 5. Direction of fork movement analysis in rDNA minichromosomes. (A) Partial restriction map of half of the rDNA palindrome and schematic of RIs derived from passive replication of the *Cla*I coding region fragment. (Upper diagrams) Left profile, passive replication in which the replication fork moves from the 5′ NTS towards the 3′ NTS; right profile, fork movement from the 3′ NTS to 5′ NTS. Relevant restriction sites: *Cla*I, nucleotide (nt) 2169 and 7621; *Sst*I, nt 3751. *Sst*I cleaves asymmetrically within the *Cla*I fragment. The DNA probe (open rectangle, nt 5214 to 6676) hybridizes to the large *Cla*I-*Sst*I fragment in double digests. (Lower diagrams) Schematic profiles for passive replication of DNA digested with *Cla*I only (open 1*N* spot and thin-lined arc) and for *Cla*I-digested DNA that was resolved in the first dimension and digested in situ with *Sst*I prior to electrophoresis in the second dimension (filled 1*N* spot and thick-lined arc). The left and right 2D *Cla*I plus *Sst*I patterns are predicted for forks entering from the 5′ and 3′ NTS proximal sides, respectively. (B) Left panels, standard neutral-neutral 2D gel analysis of the 5.5-kb *Cla*I coding region fragment; right panels, direction of fork movement analysis of *Cla*I-digested RIs cleaved with *Sst*I prior to electrophoresis in the second dimension. Asynchronous vegetative RIs (SF137) and rDNA amplification intermediates at the 14-h time point (Fig. 4) were analyzed.

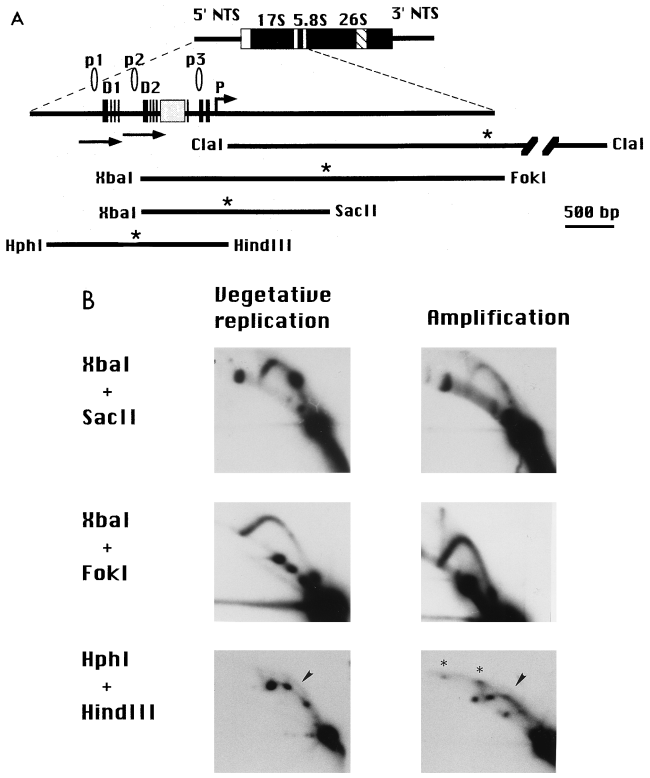

FIG. 6. 2D gel analysis of the promoter and upstream 5′ NTS regions in vegetative and amplifying rDNA minichromosomes. (A) Depiction of half of the rDNA palindrome and expansion of the relevant region. Thick black boxes, type I repeats; shaded box, tandem array of type II repeats; vertical bars, type III repeats; D1 and D2, domains 1 and 2 within the 430-bp tandem duplications (long arrows); p1, p2, and p3, replication fork pausing sites; P, promoter. Relevant restriction fragments are shown with their centers marked with asterisks (*Hph*I site, nucleotide [nt] 154; *Xba*I, nt 1152; *Hin*dIII, nt 2132; *Fok*I, nt 4957; *Sac*II, nt 3102; *Cla*I, nt 2169 and 7621). (B) 2D gel analysis of DNA from asynchronous vegetative C3 rDNA cultures (SF137) and early amplification cultures. Probes used for the analysis of the *Xba*I-*Sac*II, *Xba*I-*Fok*I, and *Hph*I-*Hin*dIII fragment were the radiolabeled *Xba*I-*Sac*II (nt 1152 to 3102), *Hin*dIII (nt 2373 to 4610), and 1.9-kb 5′ NTS (nt 1 to 1909) fragments, respectively. DNAs were subjected to 2D gel analysis following restriction digestion and enrichment for RIs. For the *Xba*I plus *Sac*II digest, a 10-h time point was used. In that particular developmental time course, the 10-h sample generated a *Hin*dIII pattern comparable to the 14-h time point examined in the other amplification samples shown here and in Fig. 4 and 5.

has a mass of >2*N*, similar to that seen in *Hin*dIII-digested amplification intermediates (Fig. 4A). Like the *Hin*dIII analysis, the discrete arc leading to the larger accumulated species traces back to the preceding stalled intermediate, suggesting that the arrested 2*N* RI is the substrate for further replication. These data are consistent with onionskin replication, in which replication reinitiates on the nascent DNA strand prior to completion of the previous round of replication.

DISCUSSION

Cell cycle-regulated replication initiates at more than one site in the 5′ NTS. The *Tetrahymena* rDNA minichromosome is an attractive model system for studying DNA replication because the replication properties of this chromosome change at different stages of the life cycle. During macronuclear development, the rDNA is extensively amplified in the absence of nuclear division. During subsequent vegetative growth, the rDNA is replicated on average once per cell cycle. Using 2D gel electrophoresis, we show that vegetative replication initiates in just one of the two 5′ NTS copies present in palindromic rDNA. Previously, we determined that replication forks pause at several sites in the 5′ NTS in vegetatively growing cells (39). Transient pausing of replication forks has been observed in other systems, including yeast and human rRNA gene repeats (5, 37, 38), yeast centromere, and the *SUP53* tRNA gene (12, 20). In contrast to these examples, several pausing sites reside in the short (~1-kb) interval that encompasses the *Tetrahymena* origin region. We exploited this information to localize one of the two initiation sites to a 400-bp segment that includes a repeated sequence element that affects rDNA replication control (domain 1 [Fig. 1]). Furthermore, we identified downstream initiation events proximal to domain 2, the second copy of this tandem duplication.

A previous EM study examining the B rDNA allele suggested that replication initiates at a single 5′ NTS site, proximal to domain 1 (8). Our studies identified two 5′ NTS origins and revealed that both origins are active in B and C3 rDNA. Thus, the B rDNA (domain 2) mutation does not selectively inactivate replication from domain 2 but may instead decrease the frequency of initiation from both origins. Due to technical limitations, the closely spaced origins that we uncovered were not resolved by EM. B rDNA manifests a vegetative replication disadvantage when placed in competition with C3 rDNA in the same macronucleus (33). Other rDNA alleles show similar replication defects (27). Some of the vegetative maintenance mutations are proximal to the rRNA promoter, hundreds of base pairs downstream of the domain 1 and 2 origins (Fig. 1) (reviewed in reference 28), and the promoter region is not active as an origin (Fig. 2 and 6). Preliminary 2D gel experiments with these mutants are consistent with the data presented here, suggesting that the two 5′ NTS origins are coordinately regulated (39, 62). We propose that regulation is mediated by long-distance interactions between the dispersed, repeated type I elements. Studies of *S. pombe* (7, 63), *D. melanogaster* (11), and mammals (1, 21, 32) indicate that replication control determinants need not be clustered immediately around the origin that they regulate. Furthermore, in *Drosophila* and mammals, several origins have been identified in broad initiation zones (11, 22, 38, 50, 55). Whether they are coordinately controlled by dispersed determinants, as we propose here, remains to be determined.

The 5′ NTS origins are active during rDNA gene amplification. Whereas replication origins that direct gene amplification have been identified in other developmental systems, it is not known whether they function in normal somatic cells (11, 22, 35). To address this question in *Tetrahymena*, we examined rDNA RIs generated during new macronuclear development. A composite pattern of 5′ NTS RIs was detected in amplifying rDNA. One of the patterns was identical to that seen in vegetative rDNA, corresponding to a single 5′ NTS initiation event per rDNA palindrome (Fig. 4A). We conclude that the origins that direct vegetative replication are also active during rDNA gene amplification. As amplification occurs in the absence of nuclear division, cell cycle control of these origins must be suppressed. A previous genetic study suggested that amplification and vegetative replication utilize common genetic determinants (43). DNA transformation studies are also consistent with the 5′ NTS containing determinants for both amplification and vegetative replication (18). Our data strongly support these conclusions, demonstrating that origins in the 5′ NTS direct both rDNA gene amplification and replication during normal vegetative divisions.

Our experiments uncovered a new class of RIs responsible for a large proportion of amplified rDNA molecules. The pathway that produces these molecules predominates particularly

at the earlier stages of amplification. Several developmentally regulated mechanisms may be involved. For example, during the early stages of *Xenopus* development, replication initiates randomly in the coding and noncoding regions of the rRNA gene cluster (23, 24). Initiation is gradually restricted to the nontranscribed spacer as the length of S phase increases. Similar to *Xenopus*, the *Tetrahymena* rDNA is extensively replicated during a brief amplification period (from 2 to 10,000 copies in ~12 h), as opposed to vegetative replication (four doublings/12 h). In stark contrast to the situation in *Xenopus*, we show that amplified *Tetrahymena* chromosomes are not produced by random initiation. The rRNA coding region is passively replicated, and all detectable replication forks move in the same direction—from the center of the rDNA towards the telomere. These data argue against amplification-specific origins in the coding region or 3′ NTS.

Similar to programmed gene amplification events in *Drosophila* and *Sciara* (11, 22, 36), our mapping studies are consistent with site-specific initiation of replication in the novel *Tetrahymena* amplification intermediates. Whereas technical limitations precluded us from mapping these origins precisely in the 5′ NTS, existing data suggest that at least some of these initiation events may be proximal to the domain 1-domain 2 region. The data clearly indicate that the promoter region is not active as an amplification origin. Although the promoter region contains known replication control determinants, it does not function as an initiation site during vegetative divisions as well.

rDNA amplification involves multiple initiation events per chromosome. Global mechanisms that restrict replication to once per cell cycle are not operational during *Tetrahymena* macronuclear development. In addition to rDNA amplification, non-rDNA chromosomes replicate to ~45 copies. Cell cycle control is first imposed after development is completed. However, DNA replication is still regulated during macronuclear development. Under the conditions that we used, non-rDNA chromosomes arrest at 4 to 8 copies at approximately 10 h into macronuclear development (2). The rDNA continues to be amplified, indicating that the mechanisms that regulate the abundance of other chromosomes are distinct (26).

rDNA amplification differs from vegetative replication in several fundamental ways. First, multiple initiation events occur within single rDNA molecules during gene amplification. In addition to producing intermediates derived from two initiations, one on each side of the palindrome, molecules with a mass greater than fully replicated DNA are generated (Fig. 4A and 6B). Several lines of evidence indicate that the additional mass results from a second round of replication on an arrested RI rather than from replication on substrates undergoing recombination. We propose that onionskin replication is occurring at this stage, due to reinitiation on nascent rDNA molecules prior to completion of the previous round of replication. Onionskin intermediates have been visualized by EM for amplifying *Drosophila* chorion genes (46), their detection being facilitated by the slow elongation rate of replication forks and large replication bubbles. Onionskin intermediates have not been previously detected by 2D gel electrophoresis. The primary factor that allowed us to identify these putative intermediates was the arrest of converging replication forks at the 5′ NTS barrier. This barrier effectively trapped RIs derived from multiple initiation events.

The strong fork barrier was a prominent feature of RIs detected in amplifying rDNA. The barrier revealed that replication initiates on both sides of the palindrome. Since two prominent stalled intermediates were detected, these initiation events must be uncoupled. This barrier is noticeably absent in molecules replicated during vegetative growth, where replication is mediated exclusively by a single initiation event per molecule. This is the first example in eukaryotes in which the progression of a replication fork has been shown to be regulated. In *Bacillus subtilis*, elongating replication forks arrest downstream of the chromosomal origin during stringent response (34). Arrest is dependent on expression of replication terminator protein, a protein that binds DNA and inhibits the DNA helicase activity associated with the replication machinery. The reasons and mechanisms for regulating replication fork arrest, pausing, and initiation frequencies in *Tetrahymena* remain to be determined. rDNA origins are internally positioned in amplifying and vegetative cells, so positional context, such as proximity to a telomere, cannot account for their different replication properties, as is seen in *S. cerevisiae* (15).

During vegetative growth, the 5′ NTS is organized in chromatin that contains precisely positioned nucleosomes (47). Strikingly, all known vegetative replication mutations map to these nucleosome-free 5′ NTS regions. Chromatin organization could play an important role in controlling the frequency of initiation and/or properties of elongating complexes. It is plausible that the replication fork barrier is not functional in vegetative cells because it is sequestered in nucleosomes. The composite pattern of amplification intermediates might reflect heterogeneity in rDNA chromatin structure in the developing macronucleus. In the human β-globin gene locus, alterations in chromatin structure and gene expression correlate with changes in replication properties (early versus late replication) (14, 16, 17). Here we show a different kind of modulation of origin activity. Developmental regulation in *Tetrahymena* involves a change in the frequency of initiation of replication (one versus more than one). Postinitiation events that modulate the elongation of replication forks (pausing and arrest) may be important determinants that regulate replication of this chromosome. Interestingly, some of the *cis*-acting mutations that affect vegetative replication initiation also diminish replication fork pausing (39).

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