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Type I Elements Mediate Replication Fork Pausing at Conserved Upstream Sites in the *Tetrahymena thermophila* Ribosomal DNA Minichromosome

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Two-dimensional gel electrophoresis was used to study replication of the *Tetrahymena thermophila* ribosomal DNA (rDNA) minichromosome. During vegetative growth, the rDNA is replicated exclusively from origins in the 5′ nontranscribed spacer (NTS). Whereas replication fork movement through the rest of the chromosome appears to be continuous, movement through the 5′ NTS is not. Replication forks arrest transiently at three prominent replication fork pausing sites (RFPs) located in or immediately adjacent to nucleosome-free regions of the 5′ NTS. Pausing at these sites is dramatically diminished during replication in *Escherichia coli*, suggesting that chromatin organization or *Tetrahymena*-specific proteins may be required. A conserved tripartite sequence was identified at each pausing site. Mutations in type I elements diminish pausing at proximal RFPs. Hence, type I elements, previously shown to control replication initiation, also regulate elongation of existing replication forks. Studies with rDNA transformants revealed a strong directional bias for fork pausing. Strong pausing only occurred in forks moving toward the rRNA-coding region. We propose that fork pausing in the 5′ NTS evolved to synchronize replication and transcription of the downstream rRNA genes.

DNA replication is a highly regulated process that initiates at defined chromosomal sites termed origins of replication. Eukaryotic chromosomes contain many bidirectional origins, spaced approximately 100 kb apart. Elongating replication forks typically move unabated through chromosomal DNA. However, several examples of nonuniform fork movement have been documented (3, 6, 13, 17, 41). Site-specific arrest of replication forks can be visualized by two-dimensional (2D) gel electrophoresis. Both replication fork pausing sites (RFPs) and replication fork barriers (RFBs) have been uncovered by this method. RFPs induce transient stalling of elongating replication forks (6, 15). In contrast, RFBs block further progression of an arrested fork (2, 29, 30, 44). Whereas a transiently paused fork will ultimately replicate downstream DNA sequences, sequences on opposite sides of an RFB must be replicated by two converging replication forks. Replication fork arrest may have evolved to prevent collisions between the replication and transcriptional machinery (3, 6, 19). Both sequence-specific DNA-binding proteins and the act of transcription have been shown to arrest elongating forks (6, 13, 19).

The ribosomal DNA (rDNA) of *Tetrahymena thermophila* is an attractive model system for studying DNA replication because the replication properties of this chromosome change at different stages of the life cycle. This naturally occurring minichromosome, encoding just two copies of the 17S, 5.8S, and 26S rRNA genes, is generated as part of a developmental program that produces the transcriptionally active macronucleus (Fig. 1A) (reviewed in reference 25). During macronuclear development, the single-copy rRNA genes are excised from the precursor germ line chromosome and rearranged into a 21-kb head-to-head palindrome. The resulting minichromosome is then amplified to 10,000 copies in the absence of nuclear division (45). The rDNA is replicated on average once per cell cycle during subsequent vegetative growth (8). Since macronuclear rDNA chromosomes lack centromeres, an additional copy number control mechanism has evolved to help maintain the rDNA at a high level (28).

DNA transformation studies have demonstrated that the 1.9-kb 5′ nontranscribed spacer (NTS) is necessary and sufficient to direct both amplification and vegetative replication of *Tetrahymena* rDNA (12, 37, 39). Classical genetic screens have identified important *cis*-acting regulatory determinants that control rDNA maturation in developing macronuclei or rDNA maintenance in vegetatively growing cells (24, 28; reviewed in reference 25). These include genetic determinants for vegetative replication control. Naturally occurring *B* rDNA and all known, induced rDNA maintenance mutant (*rmm*) alleles carry mutations in the 5′ rDNA NTS. These mutations are in or adjacent to phylogenetically conserved type I elements, i.e., repeated sequence elements that reside in segments of the 5′ NTS that are devoid of nucleosomes (Fig. 1A) (reviewed in reference 25). rDNA maintenance mutations are not recessive lethal, indicating that they cause only a partial loss of function. Mutant alleles manifest their defect only when placed in competition with an rDNA allele that replicates more efficiently, such as wild-type *C3* rDNA. A vegetative replication hierarchy of wild-type *C3* > *B* > *rmm* has been established (24, 28). Several lines of evidence indicate that rDNA maintenance mutations directly affect replication initiation (reviewed in reference 25). For example, the *B* rDNA mutation affects rDNA amplification as well, consistent with the mutated determinant controlling replication initiation (35).

Consistent with genetic studies, electron microscopy (EM) revealed that vegetative replication initiates in the 5′ NTS, near two of the three nucleosome-free regions designated domains 1 and 2 (Fig. 1A, D1 and D2) (4). In this study, we used 2D gel electrophoresis to confirm this finding and demonstrate that replication fork movement through the 5′ NTS is highly discontinuous. Elongating replication forks arrest transiently at several specific 5′ NTS sites, at a conserved tripartite se-

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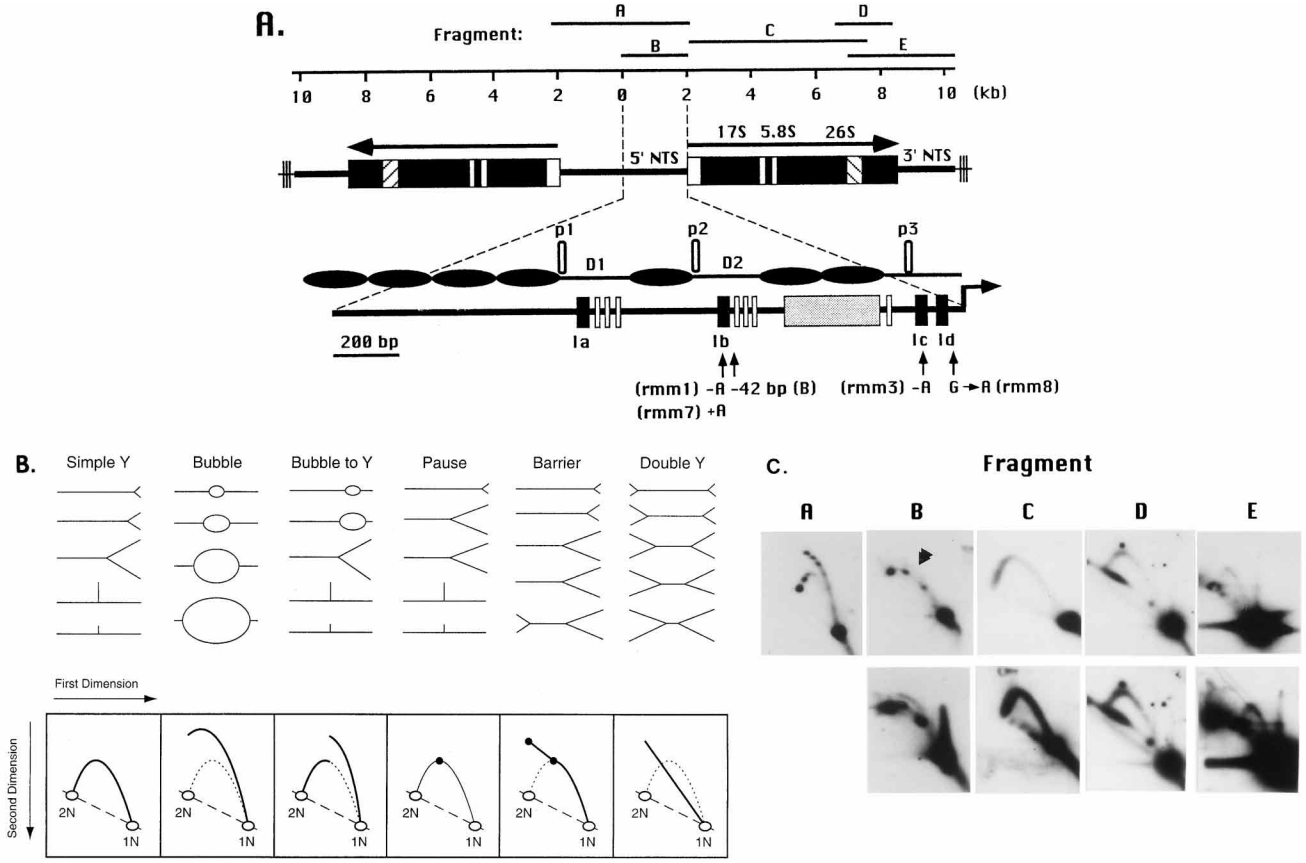

FIG. 1. Neutral-neutral 2D analysis of the *T. thermophila* rDNA minichromosome. (A) Map of the rDNA minichromosome. The macronuclear rDNA of *T. thermophila* encodes the 17S, 5.8S, and 26S rRNA genes (black areas, mature RNA-coding regions; unshaded areas, processed RNA precursor regions; hatched area, self-splicing 26S rRNA intron) (9). This 21-kb minichromosome contains two copies of a 10.3-kb sequence which are arranged in a head-to-head palindrome and capped by terminal telomeric repeats (thin bar with vertical lines). Vegetative replication initiates within the 5′ NTS (expanded region), near two nuclease-hypersensitive regions (domains 1 and 2 [D1 and D2]) (4). These regions and promoter-proximal sequences are devoid of nucleosomes (black ovals) which are precisely positioned in the remainder of the 5′ NTS (36). The positions of phylogenetically conserved type I (Ia to Id; dark filled boxes), type II (IIa to IIm; shaded box), and type III (open boxes) elements are shown. The known vegetative replication determinants reside in or adjacent to type I elements (*B* and *rmm1*, *-3*, *-7*, and *-8* mutations). The locations of replication fork pausing sites (p1, p2, p3; open ovals) mapped in this study are shown. Upper line, restriction fragments analyzed by 2D gel electrophoresis. Fragment A, 4.2-kb *Hin*dIII fragment spanning both 5′ NTS copies; fragment B, *Hph*I-*Hin*dIII (nt 154 to 2132 of the rDNA sequence); fragment C, *Cla*I-*Cla*I (nt 2169 to 7621); fragment D, *Hin*dIII-*Hin*dIII (nt 6742 to 8358); fragment E, *Sph*I-telomere (nt 7041 to 10302 plus 200 to 400 bp of telomeric sequence). (B) Schematic of replication intermediates resolved by neutral-neutral 2D gel electrophoresis (1). Simple Y, passive replication by a single fork entering from one end of the restriction fragment; bubble, bidirectional replication from an origin positioned in the center of the fragment; bubble to Y, bidirectional replication from an origin positioned asymmetrically in the fragment; pause, replication by a single fork which pauses transiently at a specific site in the restriction fragment (filled spot on simple Y arc); barrier, replication of a fragment by converging forks, in which the first fork entered and terminated at a barrier prior to entry of the second fork (the lower and upper spots correspond to intermediates that accumulate at the barrier after the first and second forks, respectively, arrested); double Y, passive replication from two converging forks initiating outside the restriction fragment. Diagonal dashed line, migration of linear duplex DNA fragments; dotted arc, reference pattern for simple Y arc intermediates. (C) Southern blot analysis of vegetative rDNA RIs resolved by neutral-neutral 2D gel electrophoresis. Probes typically spanned the entire examined fragment. Short and long exposures are shown for fragments B to E.

quence. We show that type I elements mediate replication fork arrest at pausing sites, indicating that they regulate both initiation and elongation of replication forks.

MATERIALS AND METHODS

Cell culture and enrichment for DNA replication intermediates (RIs). Vegetative cultures of the wild-type *T. thermophila C3* strain and mutant *B*, *rmm1*, *rmm7*, and *rmm8* strains were propagated at 30°C in 2% protease peptone supplemented with 10 μM $FeCl_3$, penicillin (250 μg/ml), streptomycin (250 μg/ml), and amphotericin B (25 μg/ml). *Tetrahymena* rDNA transformants were generated by electroporating mating *B* rDNA strains with plasmid prD4-1 (11). prD4-1 contains two tandemly arrayed copies of the *C3* rDNA 5′ NTS and one copy of the macronuclear rRNA coding region and 3′ NTS. The upstream 5′ NTS copy encodes a spontaneous point mutation that inactivates transcription from the adjacent promoter (38, 46). This allows for the propagation of plasmids bearing tandem 5′ NTS repeats by eliminating transcription through downstream origins of replication (38). prD4-1 transformants were selected for resistance to paromomycin, encoded within the plasmid 17S rRNA gene. prD4-1 will outcompete endogenous *B* rDNA, either replicating as a circular DNA or integrating into endogenous linear rDNA minichromosomes (47). Parmomycin-resistant cells were propagated continuously in drug to select for subpopulations in which the number of introduced 5′ NTS repeats per molecule increased due to unequal crossing over between tandem 5′ NTS copies (47).

For 2D gel studies, DNA was isolated from log-phase *Tetrahymena* cultures harvested at a density of $<2 \times 10^5$ cells/ml, using a modification of a previously described protocol (4). Briefly, cells were collected, washed once with 10 mM Tris (pH 8.0), and lysed by the addition of an equal volume of NDS (10 mM Tris, 0.5 M EDTA, 2% sodium dodecyl sulfate [SDS] [pH 9.5]) at a final density of $\sim 10^7$ cells/ml. After incubation for 20 min at 37°C, proteinase K (Boehringer Mannheim) was added to a final concentration of 1 mg/ml, and samples were incubated for 3 to 4 h at 37°C. Samples were then 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, and precipitated 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 RNase A (1 μg/ml), and RIs were enriched by binding to benzoylated naphthoylated DEAE-cellulose (BND cellulose; Sigma Chemical) (23). Plasmid DNA was prepared from log-phase *Escherichia coli* cultures as previously described (21).

Gel electrophoresis and Southern blotting. Neutral-neutral 2D gel electrophoresis was performed essentially as previously described (1). Typically, 10 μg of BND cellulose-enriched DNA was loaded in the first dimension. Restriction

fragments of >3 kb were resolved in 0.4% agarose Tris-acetate-EDTA gels in the first dimension and 1.0% agarose Tris-borate-EDTA gels containing ethidium bromide (1 μg/ml) in the second dimension. Smaller restriction fragments were separated in higher-percentage gels, ranging from 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/cm for 20 h at room temperature for the first dimension and 3.0 V/cm for 18 h at 4°C for the second dimension. For neutral-alkaline gel electrophoresis (23), the first-dimension gel conditions were identical to those described above except that twice the amount of DNA was loaded. Samples were electrophoresed in the second dimension in 50 mM NaOH at 1.5 V/cM for 20 h at 4°C with buffer recirculation to resolve nascent-strand replication intermediates. To facilitate mapping of pausing sites, nascent-strand intermediates were also resolved in one dimension under identical alkaline conditions. Accumulated 1D nascent-strand intermediates migrated at the same position in the alkali dimension as resolved neutral-alkaline replication intermediates (data not shown). Whereas accumulated 2D intermediates migrate as spots, the corresponding 1D intermediates migrate as discrete bands.

Following electrophoresis, DNA was transferred to Hybond N+ (Amersham) by capillary blotting and hybridized to cloned rDNA restriction fragments or PCR products radiolabeled with [α-^{32}P]dATP, using the Klenow fragment of DNA polymerase I and random or sequence-specific primers. Filters were hybridized overnight at 60 to 65°C in 0.5 M sodium phosphate–7% SDS, washed repeatedly with 0.1 M phosphate–2% SDS at the same temperature (5), and exposed to Kodak XAR-5 film. Short exposure times were used to accurately measure the migration positions of stalled nascent strands in neutral-alkaline 2D and alkaline 1D gels. Size determination errors were calculated based on a measurement accuracy of ±1 mm. Linear regression analysis of radiolabeled 123-bp and 1-kb DNA ladders (Gibco/BRL) was performed to determine the sizes of accumulated nascent-strand intermediates.

RESULTS

The 5′ NTS contains a vegetative replication origin and RFPs. Experiments were initiated to localize origins of replication and examine replication properties of the actively transcribed rDNA minichromosome in vegetatively growing *Tetrahymena*. Neutral-neutral 2D gel electrophoresis was first used to study rDNA RIs. RI patterns can distinguish between initiation in a DNA segment and initiation from an outlying origin (Fig. 1B). Digestion with *Hin*dIII generates a restriction fragment bearing both 5′ NTSs in their palindromic configuration (Fig. 1A, fragment A). A bubble-to-Y arc intermediate pattern was observed, indicating that replication initiates in this fragment, well within one of the 5′ NTS copies, rather than at the center of the rDNA palindrome. This result is consistent with a previous EM study of vegetative rDNA replication (4). The absence of complete simple Y arcs suggested that initiation events are restricted to the 5′ NTS. Digestion with *Hph*I plus *Hin*dIII separates the two 5′ NTS copies from one another (Fig. 1A, fragment B). Two RI patterns are generated: a bubble arc (arrow) and a complete, simple Y arc (Fig. 1C, fragment B). The large bubble arc intermediates that we observed indicates that the replication origin is at a somewhat central position in this fragment. More precise localization of 5′ NTS origins is described elsewhere (48). The complete simple Y arc results from passive replication of the second 5′ NTS copy in the rDNA minichromosome, either from initiation in the active 5′ NTS copy or from elsewhere in the rDNA. The latter possibility was unlikely, since simple Y arcs were not detected in fragment A, which contains both actively and passively replicated 5′ NTS copies. Additional experiments support the conclusion that replication initiation is restricted to the 5′ NTS (Fig. 1B, fragments C to E and reference 48). First, bubble arc intermediates were not detected in fragments spanning the rRNA coding region or 3′ NTS, consistent with initiation in the 5′ NTS only (Fig. 1C, fragments C to E, and data not shown). Second, replication forks proceed through the coding region in one direction, from the 5′ NTS toward the telomere (48). Finally, DNA transformation studies indicate that coding region and 3′ NTS fragments do not support autonomous replication (39).

Three prominent accumulated intermediates were detected on the *Hph*I-*Hin*dIII simple Y arc (Fig. 1C, fragment B). This finding indicates that replication fork movement through the 5′ NTS is discontinuous. In contrast, replication through the coding region and 3′ NTS was more or less uniform (Fig. 1C, fragments C to E). We conclude that replication fork pausing is primarily, if not exclusively, restricted to the origin-containing 5′ NTS region. Site-specific arrest of a single replication fork is sufficient to generate an accumulated intermediate on a Y arc. However, both forks must arrest concurrently to detect accumulated intermediates on a bubble arc. Specific bubble arc intermediates accumulate to high levels in restriction fragments that contain the two inverted 5′ NTS copies (Fig. 1C, fragment A). In contrast, no accumulated intermediates were detected on the bubble arc for fragment B, which contains just one 5′ NTS copy. This finding raised the possibility that pausing sites arrest only forks moving in one direction.

Neutral-alkaline 2D gel electrophoresis was used to characterize nascent strands produced during vegetative replication. In this approach, nascent DNA strands are released from RIs immediately prior to electrophoresis in the second dimension, producing a diagonal arc of RIs (Fig. 2A). The direction of fork movement can be determined by hybridization to probes from opposite ends of the restriction fragment being examined (23). Furthermore, the positions of stalled forks can be mapped accurately by comparing the migration of accumulated RIs to radiolabeled markers. Samples digested with *Hph*I plus *Hin*dIII were resolved and hybridized to probes from opposite ends of the 5′ NTS (Fig. 2B, probes 1 and 2). Three paused RIs were detected with the upstream probe (Fig. 2C; probe 1, paused intermediates, p1, p2, and p3). These accumulated intermediates have one endpoint fixed at the *Hph*I restriction site, with the other being produced by stalling of the replication fork at a pausing site. In contrast to probe 1, no paused intermediates were detected with the downstream, promoter-proximal probe 2. The size of each of the accumulated nascent-strand intermediates was determined on underexposed autoradiograms to maximize measurement accuracy. The nascent strands arrest 480 (±15), 920 (±35), and 1,620 (±50) nucleotides (nt) downstream of the *Hph*I site located at nt 154. Nascent-strand measurements were corroborated by alkaline gel electrophoresis in a single dimension (data not shown; see Materials and Methods). To more accurately map pause site 3, an *Xba*I-*Sac*II fragment was examined (Fig. 2B, probe 3). We detected a single paused intermediate, terminating 630 ± 20 nt downstream of the *Xba*I site located at position 1152 (Fig. 2C). The three pausing sites map to nt 640, 1075, and 1730 of the published *B* rDNA sequence (9), with the promoter-proximal pause site 3 being the most prominent. The map position of pause site 3 takes into account a 42-bp insertion in *C3* rDNA at position 1229 (14, 28).

A conserved tripartite sequence element is present at RFPs. During vegetative growth, the 5′ NTS is arranged into a precisely defined chromatin structure containing seven phased nucleosomes and three nucleosome-free regions (36). All three pausing sites map well within or at the border of these nucleosome-free regions (Fig. 1A, p1, p2, and p3; see the legend to Fig. 3 for details on nucleosome positions). DNA sequence comparisons uncovered a tripartite conserved sequence at each pausing site (Fig. 3, boxed regions). The conserved blocks at each pausing site are 7, 12, and 7 bp long, separated precisely by 18- and 8-bp spacers. The sequence at pause site 3 is in the opposite orientation to those at sites 1 and 2. Overall identities of the conserved blocks to the tripartite consensus are 100%, (26 of 26), 96% (25 of 26), and 92% (24 of 26) for pause sites 1, 2, and 3, respectively. Not surprisingly, the pause site 1 and 2 spacers are very similar (18-of-18- and 5-of-8-bp

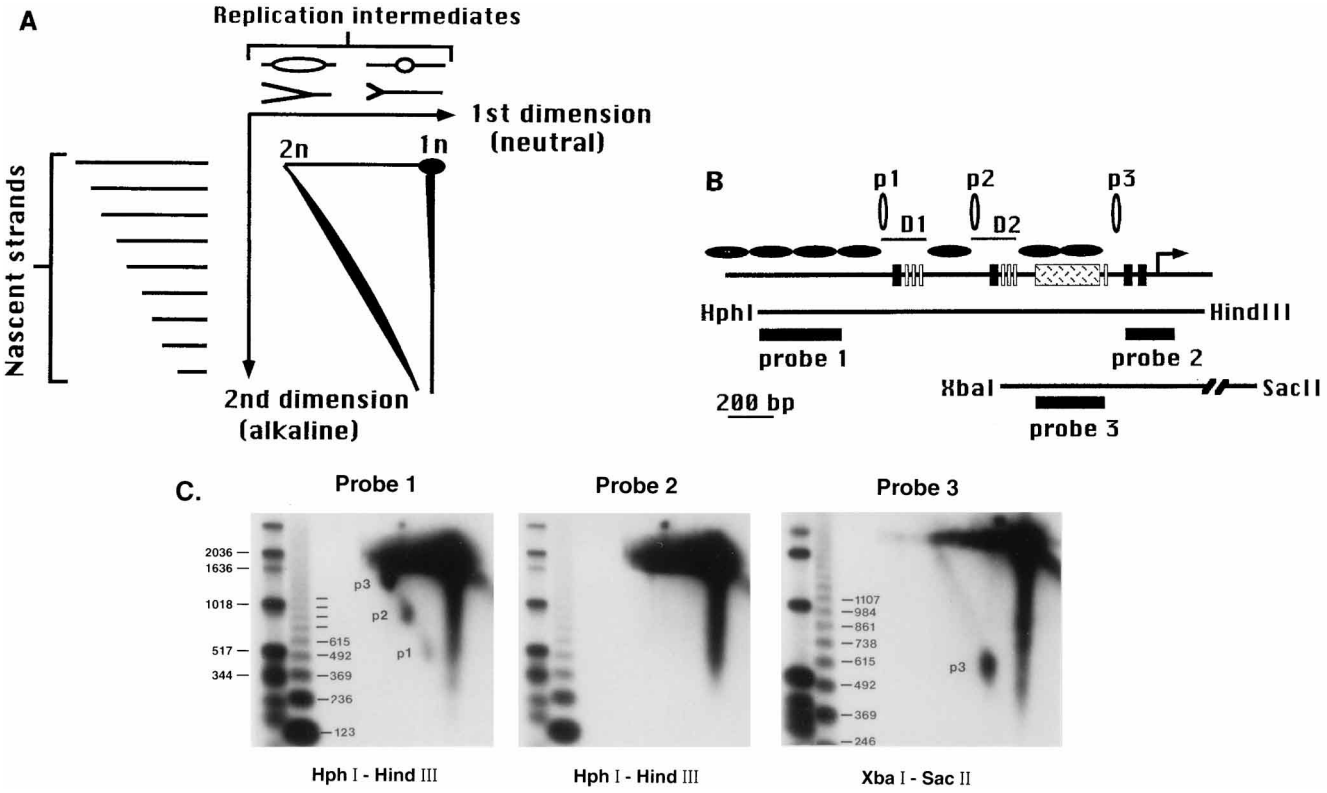

FIG. 2. Pause site mapping by neutral-alkaline 2D gel electrophoresis. (A) Schematic of nascent-strand replication intermediates resolved by neutral-alkaline 2D gel electrophoresis. The 1n spot corresponds to nonreplicating DNA. The vertical smear is derived from nicked, nonreplicating DNA, whereas the horizontal smear represents parental strands in RIs of different sizes. The diagonal arc corresponds to nascent-strand replication intermediates liberated from the parental strand by alkali denaturation prior to electrophoresis in the second dimension. (B) Schematic of restriction fragments and probes used for neutral-neutral 2D gel electrophoresis. Symbols designations are described in the legend to Fig. 1A. *Hph*I-*Hin*dIII, nt 154 to 2132 of the 5′ NTS; *Xba*I-*Sac*II, nt 1152 to 3102. Probe 1, nt 1 to 500; probe 2, nt 1667 to 1950; probe 3: nt 1323 to 1701. (C) Southern blot analysis of neutral-alkaline 2D gels. Markers, 1-kb and 123-bp ladders (Gibco/BRL) phosphorylated with [γ^{32}P]rATP. DNA from log-phase vegetative cultures was digested with *Hph*I and *Hin*dIII or *Xba*I and *Sac*II prior to 2D gel electrophoresis.

identities), as they reside in an imperfect 430-bp tandem duplication that includes domains 1 and 2 (Fig. 1A). In contrast, the two spacers at pause site 3 are dissimilar to those at sites 1 and 2, with 6-of-19- and 0-of-8-bp identities to the site 1 and site 2 spacers. All three pausing sites map <100 bp upstream of a type I element (Fig. 1A).

Replication fork pausing is markedly diminished in *E. coli*. Replication of the 5′ NTS region in *E. coli* was examined to determine if fork pausing is intrinsic to the primary DNA sequence or occurs by another mechanism. If pausing was induced solely by a DNA secondary structure, such as a hairpin, then strong fork pausing should occur during replication in *E. coli*. Plasmids bearing a complete copy of the 5′ NTS were propagated in *E. coli*, and RIs were isolated (21). Since the ColE1 origin is unidirectional, plasmids with the 5′ NTS inserted in opposite orientations were analyzed (Fig. 4, schematics [arrows denote the direction of replication]). Passive replication of 5′ NTS sequences in *E. coli* will generate a simple Y arc pattern in the *Hph*I-*Msp*I fragment which spans the entire 5′ NTS region (Fig. 4B and C). Similarly, passive replication of one of the two 5′ NTS copies from endogenous *Tetrahymena* rDNA will generate a simple Y arc pattern. In DNA isolated from *Tetrahymena*, the vast majority of RIs accumulate at pause sites 1 to 3 (Fig. 4A). In contrast, a relatively consistent continuum of intermediates was detected when the 5′ NTS was replicated in *E. coli* (Fig. 4B and C). No accumulated intermediates were detected when these sequences were replicated from the promoter toward the start of the 5′ NTS (Fig. 4C). A slight increase in hybridization was detected in higher-molecular-weight intermediates during replication in the opposite direction (Fig. 4B). This finding suggests that a very low level of pausing may occur at sites 2 and 3 during replication in *E.*

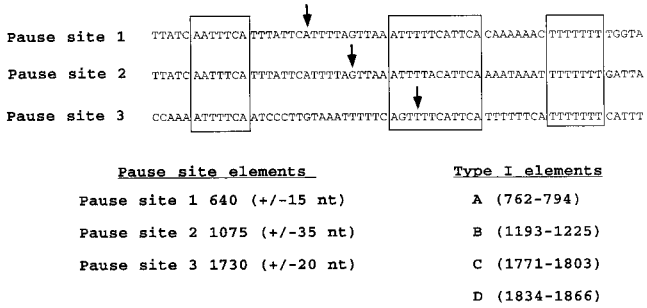

FIG. 3. Pause site sequence alignments. Boxed regions correspond to the three conserved sequences present at the 5′ NTS RFPs. The mapped position of each pausing site is marked by a vertical arrow. The nucleotide position of each mapped pausing site and measurement errors are shown below, along with the map positions of proximal type I elements, based on the numbering for the published *B* rDNA sequence (9). The map position of pause site 3 takes into account a 42-bp insertion in *C3* rDNA at position 1229 relative to the published *B* rDNA sequence. The orientation of the pause site 3 conserved elements is opposite those found at sites 1 and 2. Type Ia to Ic elements have the sequence TTTTTTGGCAAAAAAAAAAACAAAAATAG. The type Id element has seven substitutions (28). Nucleosome 4 spans nt 490 to 640 (±10 bp), and nucleosome 5 spans nt 935 to 1087 (±10 bp) (18).

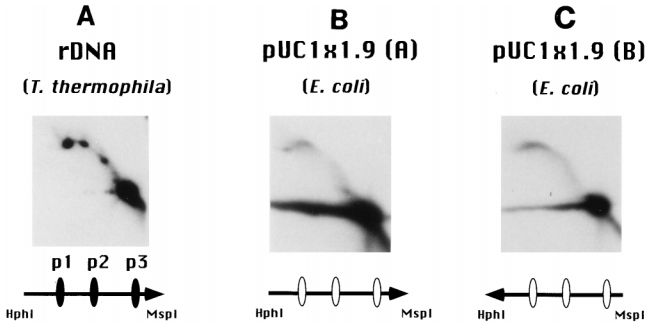

FIG. 4. Replication of the 5′ NTS region in *T. thermophila* and *E. coli*. Plasmids pUC1x1.9(A) and pUC1x1.9(B) contain one copy of the 5′ NTS (nt 30 to 1909) cloned in opposite orientations in the vector pUC118. They carry a single nucleotide insertion in the rRNA promoter that inactivates transcription but has no effect on pausing (Fig. 5). As the ColE1 plasmid origin is unidirectional, RIs from the respective plasmids are derived from passive replication of the 5′ NTS in opposite directions (arrows denote the direction of replication). DNA prepared from *T. thermophila* and *E. coli* was examined by neutral-neutral 2D gel electrophoresis after digestion with *Hph*I plus *Msp*I (nt 150 to 1906). Southern blots were probed with a fragment spanning the entire 1.9-kb 5′ NTS region.

coli. We conclude that strong fork pausing is induced only in *Tetrahymena*, possibly dependent on sequence-specific binding proteins or chromatin organization.

Replication fork pausing is primarily unidirectional. The rDNA minichromosome contains two inverted copies of the 5′ NTS with six potential pausing sites, three on each side of the palindrome. Whereas accumulated bubble arc intermediates were detected in restriction fragments that span both 5′ NTS copies (Fig. 1C, fragment A), no accumulated bubble arc intermediates were detected in fragments that contained just one 5′ NTS copy (Fig. 1C, fragment B). Recall that bubble arc intermediates of a defined size will accumulate only if both forks have arrested simultaneously at a pausing site. The absence of stalled bubble arc intermediates in fragment B, which contains three potential pausing sites, raised the possibility that one or more of the pausing sites was orientation dependent.

This prediction was tested by examining rDNA derivatives that contained additional tandem copies of the 5′ NTS copies. Plasmid prD4-1 was introduced into *T. thermophila*, and transformants were selected for resistance to paromomycin. The input plasmid contained two copies of the 5′ NTS in a head-to-tail configuration and can replicate as a circular extrachromosomal DNA or recombine with endogenous rDNA. A single nucleotide mutation in the upstream 5′ NTS copy inactivates that promoter, allowing for efficient replication of this plasmid in *Tetrahymena* (38). Transformants were propagated in paromomycin to select for subpopulations that contained higher levels of this plasmid. These cells carry rDNA molecules with additional copies of the upstream 5′ NTS sequence due to unequal crossing over (47). Southern blot analysis of transformant cell lines after 60 fissions revealed significant heterogeneity in the number of 5′ NTS repeats per rDNA molecule (data not shown). The majority contained a minimum of six repeats, with over 12 copies per molecule being commonly observed (limit resolution of standard agarose gels).

A schematic representation of one such molecule bearing nine 5′ NTS copies is shown in Fig. 5A. Digestion with *Msp*I, which cuts once in the 5′ NTS (at nt 1906), will release one palindromic fragment, analogous to endogenous rDNA and several monomeric copies derived from the tandem 5′ NTS arrays. When replication initiates at a downstream 5′ NTS copy (Fig. 5A, replication bubble), the two copies present in the palindromic *Msp*I fragment will be passively replicated. If pausing sites are orientation independent, six abundant paused intermediates should be detected in this palindromic fragment, three derived from each 5′ NTS copy. In contrast to normal rDNA minichromosomes (Fig. 1C, fragment A), the palindromic prD4-1 fragment was passively replicated, producing a simple Y arc pattern of replication intermediates (Fig. 5B,

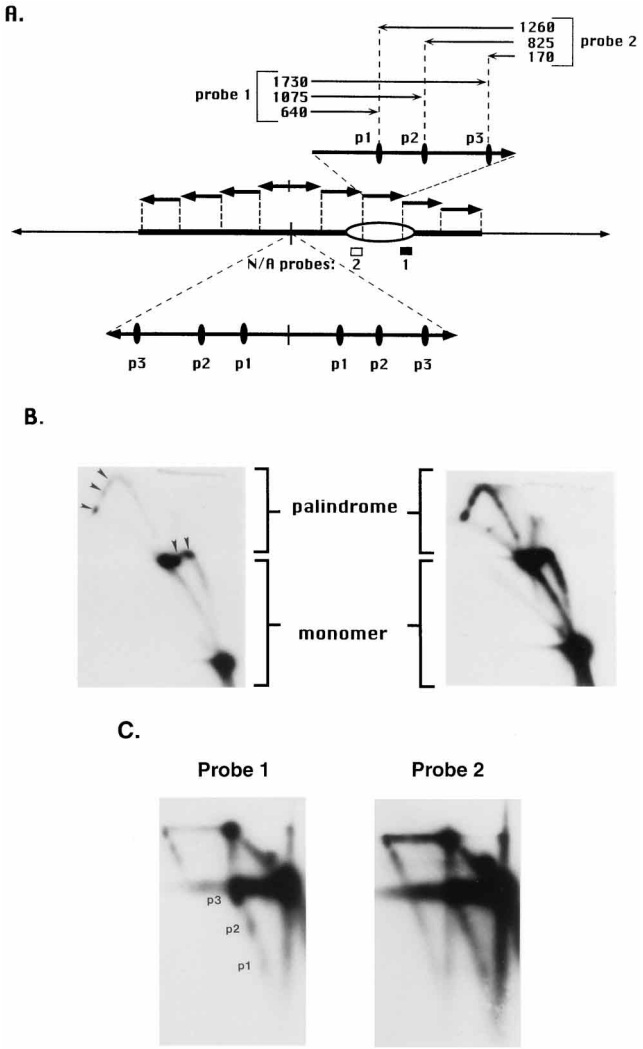

FIG. 5. Replication fork pausing in *T. thermophila* transformants. prD4-1 transformants were analyzed by 2D gel electrophoresis after prolonged propagation in paromomycin to select for cells carrying 5′ NTS expansions. (A) Schematic representation of an rDNA molecule carrying nine copies of the 5′ NTS. Initiation from a 5′ NTS copy in the tandem array is depicted (bubble). Digestion with *Msp*I (vertical hatched lines at nt 1906) will release the single palindromic 5′ NTS fragment (bottom) and monomeric fragments (top) derived from the tandem 5′ NTS copies. Probes 1 (filled box; nt 1 to 500) and 2 (open box; nt 1667 to 1950) were used to study fork movement in neutral-alkaline 2D gels. For rDNA monomers, pausing at sites p1, p2, and p3 could generate as many as six distinct accumulated intermediates of the designated sized, depending on whether pausing occurred during replication toward the promoter end (neutral-alkaline [N/A] probe 1), toward beginning of the 5′ NTS fragment (probe 2), or in both directions. (B) DNA from transformants was digested with *Msp*I, resolved on neutral-neutral 2D gels, and hybridized to a 1.9-kb probe spanning the entire 5′ NTS. Arrowheads point to prominent accumulated intermediates in monomeric and palindromic 5′ NTS copies. (C) Neutral-alkaline 2D gel analysis of *Msp*I-digested DNA. Hybridization to probes 1 and 2 depicted in panel A is shown. Paused intermediates in the monomeric 5′ NTS fragments are designated p1, p2, and p3.

palindrome). Consequently, the palindromic 5′ NTS copies were replicated primarily, if not exclusively, from downstream origins in the prD4-1 transformant. Three paused intermediates were detected in the larger RIs derived from the palindrome, when replication was proceeding from the center of the palindrome toward the promoter (left panel). Longer exposures showed comparatively modest pausing in lower-molecular-weight intermediates (right panel), suggesting that there is a strong but not absolute directional bias for fork pausing.

Monomeric rDNA copies were also examine by both neutral-neutral and neutral-alkaline gel electrophoresis. Passively replicated monomeric fragments will be replicated in different directions, depending on their locations relative to the copy that initiated replication. Tandem 5′ NTS copies that are downstream of the initiating fragment will be replicated in the same direction as the passively replicated 5′ NTS copy in endogenous rDNA (Fig. 5A; see fork orientation in *Msp*I fragments that flank the initiating 5′ NTS copy). Upstream tandem copies will be replicated by forks moving in the opposite direction. In total, six stalled intermediates should be resolved in monomeric rDNA fragments if pausing sites arrest forks moving in both directions: three derived from replication toward the promoter end and three derived from replication toward the beginning of each 5′ NTS copy. Neutral-neutral analysis detected only simple Y arc intermediates in 5′ NTS monomers, indicating that most of the arrayed 5′ NTS copies were passively replicated (Fig. 5A). Similar to the normal rDNA minichromosome (Fig. 1C, fragment B), the strongest paused intermediates were in high-molecular-weight RIs (Fig. 5B, monomer, arrowheads). This result is consistent with pausing in forks that moved in just one direction—from the beginning of the 5′ NTS toward the promoter.

Neutral-alkaline analysis of the tandemly arrayed 5′ NTS monomers verified the strong directional bias for fork pausing. Probe 1 (Fig. 5A) assesses pausing in forks moving towards the coding region, whereas probe 2 detects pausing in forks moving in the opposite direction. Hybridization was detected on the nascent strand arc with both probes, demonstrating that forks moved in both directions through monomeric 5′ NTS copies (Fig. 5C). Three paused intermediates were detected with probe 1, consistent with the arrest of forks moving toward the coding region (Fig. 5C, p1, p2, and p3). As in endogenous rDNA, pausing was most prominent at the promoter-proximal pause site 3. If pausing sites function in both orientations, a prominent 200-nt intermediate should be detected with probe 2, in addition to two fainter products of 800 and 1,300 nt, corresponding to pausing at sites 3, 2, and 1, respectively. No such intermediates were detected; instead, a consistent smear of nascent strands was observed, with faint hybridization above the smear (Fig. 5C). This increased hybridization occurred at the exact positions as the paused intermediates detected with probe 1 and is probably due to hybridization of repeated sequences in probe 2 (which includes two type I elements) to intermediates replicating toward the promoter. From these experiments, we conclude that the three pausing sites show a directional bias, arresting strongly when forks proceed from the beginning of the 5′ NTS towards the promoter. Since replication forks pause in tandemly arrayed 5′ NTS copies that lack a functional promoter (38), pausing is independent of transcription.

Mutations in type I elements affect replication fork pausing. Phylogenetically conserved type I elements are located 50 to 100 bp downstream of each pausing site (Fig. 1A and 3). Mutations in and adjacent to the type Ib to Id elements impair but do not eliminate replication during vegetative growth (reviewed in reference 25). They manifest a replication phenotype only when placed in competition with another rDNA allele. A replication hierarchy of *C3* > *B* > *rmm* has been established by genetic experiments (24, 28). Type Id elements have also been implicated in transcription initiation, as they are required for faithful in vitro transcription (34). To test whether type I elements played a role in fork pausing, mutant alleles were examined by 2D gel electrophoresis. Neutral-alkaline analysis indicated that pausing is more pronounced at site 2 than at site 1 for wild-type *C3* rDNA (Fig. 6A). In contrast, in the *B*, *rmm1*, and *rmm7* mutants, pausing at site 2 was markedly diminished. *rmm1* and *rmm7* mutants carry a single nucleotide deletion and insertion, respectively, in the type Ib element immediately downstream of pause site 2 (Fig. 1A). *B* rDNA has a 42-bp deletion that fuses the type Ib element and adjacent type III element. By comparison, the *rmm8* mutation (G-to-A substitution downstream of the type Id element) had no detectable effect on pausing (Fig. 6A).

Neutral-neutral analysis of the palindromic *Hin*dIII fragment (Fig. 1A, fragment A) confirmed these results for the mutant *B*, *rmm1*, *rmm7*, and *rmm8* alleles. The bubble-to-Y pattern is indicative of initiation in only one 5′ NTS copy, at an asymmetric position in the molecule (Fig. 6B). In contrast to the monomeric 5′ NTS fragment (Fig. 1A, fragment B; Fig. 1C), accumulated intermediates were seen on both the bubble and Y arc. Three prominent and two minor intermediates were observed on the bubble arc (Fig. 6B, *C3*). For bubble arc RIs, both forks must arrest to detect a stalled intermediate. Based on their sizes, the three abundant high-molecular-weight bubble arc intermediates are predicted to result from the arrest of forks on opposite sides of the palindrome at p3 + p3, p3 + p2, and p3 + p1, respectively for the higher- to lower-molecular-weight species. Relative to wild-type *C3* rDNA, diminished pausing was reproducibly detected in second-highest bubble arc intermediate (arrow) for the *B*, *rmm1*, and *rmm7* mutants but not for the *rmm8* mutant, consistent with our prediction that this bubble arc intermediate contains at least one fork arrested at pause site 2. Similarly, the second of three accumulated Y arc RIs was also diminished in these mutants, as expected for pausing in this intermediate at site 2. None of these mutations completely abolish pausing. Instead, they cause only a partial loss of function, similar to their effect on vegetative replication. As the *rmm8* mutation has no effect on pausing, we conclude that replication initiation and pausing phenotypes are not obligately coupled.

DISCUSSION

Replication of the *T. thermophila* rDNA minichromosome is dynamically regulated. During macronuclear development, the rDNA is amplified in the absence of nuclear division. Subsequently, the rDNA is replicated on average once per cell cycle during vegetative growth (reviewed in reference 25). Whereas a basic understanding of replication initiation is beginning to emerge, little is known about other mechanisms that might regulate replication of this chromosome. In this study, we used 2D gel electrophoresis to study vegetative replication origins and replication properties of the rDNA minichromosome. Bubble arc intermediates were detected in the 5′ NTS region only, confirming EM studies (4) and demonstrating that vegetative replication initiates solely in the 5′ NTS. Furthermore, we discovered that replication fork movement in the 5′ NTS region is highly irregular. Accumulated replication intermediates were identified, resulting from the arrest of replication forks at specific positions in the 5′ NTS.

The accumulated 5′ NTS intermediates could result from transient fork pausing or terminal arrest at a replication fork

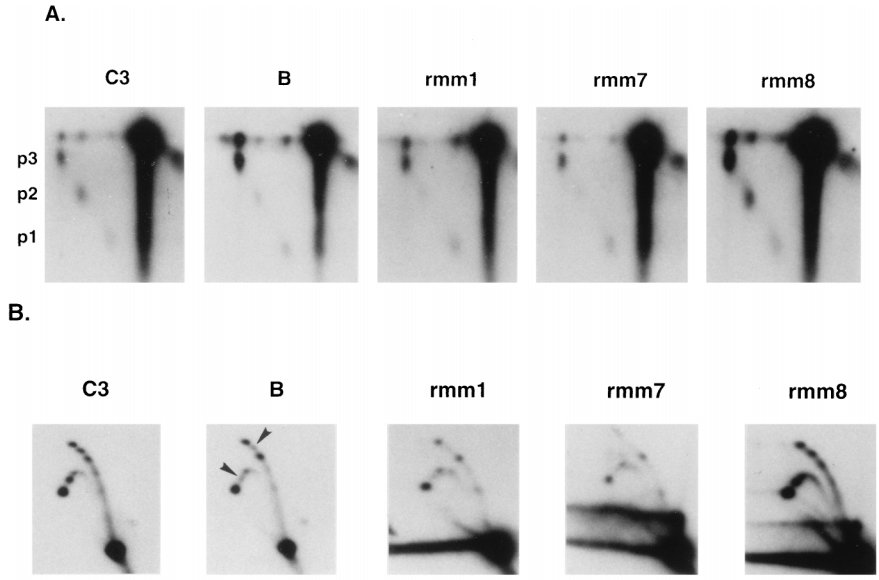

FIG. 6. Replication fork pausing in rDNA maintenance mutants, determined by 2D analysis of wild-type *C3* and mutant *B*, *rmm1*, *rmm7*, and *rmm8* alleles (see Fig. 1A for mutation sites). (A) Neutral-alkaline 2D analysis of DNAs digested with *Hph*I plus *Hind*III (Fig. 1A, fragment B), hybridized to a probe spanning the entire 5′ NTS. Pause sites 1 to 3 are designated p1, p2, and p3. (B) Neutral-neutral 2D analysis of *Hin*dIII-digested DNA (Fig. 1A, fragment A) hybridized to a probe that spans the entire 5′ NTS. This 4.2-kb *Hin*dIII fragment contains both copies of the 5′ NTS. Arrows point to RIs whose abundance decreases in the mutant rDNA alleles *B*, *rmm1*, and *rmm7*.

barrier. If the later were true, these products would represent aborted replication events, since there are no downstream origins in the coding region or 3′ NTS. Several lines of evidence indicate that replication forks are pausing transiently rather than aborting replication. Whereas aborted RIs could still serve as substrates for initiation in the second 5′ NTS copy, only single initiation events were detected in vegetative rDNA minichromosomes (Fig. 1C, fragment A) and in prD4-1 derivatives bearing additional 5′ NTS copies (Fig. 5B). In both instances, stalled forks were detected in the passively replicated 5′ NTS copies. However, no double Y intermediate were observed, indicating that the arrested forks were pausing transiently rather than terminating at a barrier. In contrast, rDNA amplification occurs by initiation in both 5′ NTS copies, with converging forks arresting at a bona fide RFB (48). Thus, multiple initiation events and replication fork barriers are detectable under the gel conditions that we used.

RFPs map within or at the border of the three nucleosome-free regions of the 5′ NTS (36). Conserved DNA sequences were identified at the three pausing sites, consisting of 7-, 12-, and 7-bp blocks of near perfect homology, separated precisely by 18- and 8-bp spacers. This tripartite sequence element shows no similarity to RFBs in *Saccharomyces cerevisiae*, pea, and *Xenopus* rRNA gene clusters (3, 16, 27, 44) nor to bacterial, viral, and eukaryotic chromosomal fork arrest sites present at other genetic loci (7, 15, 17). The conserved *Tetrahymena* sequences at pause site 3 are in the opposite orientation to those at sites 1 and 2; however, they all show the same polar bias for pausing. Consequently, the orientation of these sequences cannot confer the strong directional bias for pausing that we observed. In all cases, phylogenetically conserved type I elements were found just downstream of rDNA pausing sites. In contrast to the tripartite sequence elements at p1-3, all type I elements have the same orientation.

A functional role for type I elements in rDNA replication was originally assigned based on the diminished replication efficiency of mutant alleles when placed in competition with other rDNA alleles (28). Whereas several type I mutations map to the origin-proximal type Ib element, the *rmm3* and *rmm8* mutations are hundreds of base pairs away, near the rRNA promoter. Distal type I elements may modulate replication initiation through long-range interactions with origin-proximal elements, similar to several replication determinants in bacterial plasmids, animal viruses, and yeasts (32, 33, 39).

Our experiments indicate that type I elements also mediate replication fork pausing. In contrast to their effect on DNA replication (48), these elements affect pausing only locally, regulating fork arrest only at the immediate adjacent site. Diminished fork pausing was observed in the *rmm1*, *rmm7*, and *B* rDNA mutants, bearing mutations in and adjacent to the type Ib element. Consistent with type I elements regulating pausing, Gallagher and Blackburn have detected altered fork pausing in the *rmm3* mutant (1-bp deletion in the type Ic element [14]). Finally, we show here that the *rmm8* mutation, downstream of the Id element, has no effect on pausing. It is conceivable that the type Ic element alone regulates pausing at site 3. Alternatively, type Id could be involved, with the *rmm8* mutation residing outside of the pausing determinant domain. Minimally, the *rmm8* mutation indicates that replication initiation and pausing phenotypes are separable.

Replication fork pausing sites and barriers have been proposed to prevent head-on collisions between the replication machinery and transcriptional apparatus (2, 6, 18). Presumably, these mechanisms evolved to assure the fidelity of one or both processes. Contrary to these examples, replication forks pause only when they are moving in the same direction as transcription in *Tetrahymena* rDNA. We propose that replication fork pausing in the 5′ NTS coordinates replication and transcription, in this case preventing rear-end collisions between the two polymerases. In vivo experiments demonstrated that replication forks moving in either direction can displace transcription complexes in an *E. coli* rRNA gene array (10). This result indicates that both head-on and rear-end collisions can be disruptive. In contrast, phage T4 can apparently accom-

modate simultaneous replication and transcription, as RNA polymerase sidesteps the replication machinery, in vitro, without being displaced (31). The existence of specific sites that arrest replication forks in rRNA genes of several eukaryotes suggests that replication and transcription must be temporally and spatially separated for some highly transcribed genes.

The physical basis for pausing in *Tetrahymena* remains to be determined. Our experiments rule out several possibilities and suggest other intriguing ones. First, strong fork pausing was not observed when the *Tetrahymena* sequences were replicated in *E. coli*. Hence, pausing is not dictated solely by the primary DNA sequence. The absence of significant secondary structure in this fragment suggests that there are additional requirements for pausing. In contrast to yeast tRNA genes (6), pausing in *Tetrahymena* is not dependent on the act of transcription. Experiments with prD4-1 transformants indicate that replication forks pause at promoter-proximal sites in 5′ NTS derivatives that lack a functional promoter.

In *E. coli* and *Bacillus subtilis*, binding of a terminator protein to a cognate DNA-binding element mediates replication fork arrest (20, 26). Similarly, protein binding is necessary to arrest forks at the Epstein-Barr virus origin repeat sequence and yeast centromere (7, 15). Stable in vivo interactions are readily detected at termination sites and centromeric sites that mediate fork arrest in bacteria and *S. cerevisiae*, respectively (15, 20). Since these interactions can be detected in asynchronous cultures, these protein barriers are likely to be in place prior to replication. In vivo footprinting experiments do not support this kind of mechanism for *Tetrahymena*. Although a stable in vivo footprint has been detected over the two type I elements proximal to the rRNA promoter, similar DNA-protein interactions are not seen at the three pausing sites or at the upstream type Ia and Ib elements (14, 38). Furthermore, mutations that eliminate the promoter-proximal footprint do not ablate pausing at the proximal p3 site, indicating that these particular interactions are not required for fork pausing. These results suggest that putative proteins that mediate pausing might interact only transiently with *cis*-acting determinants in the rDNA.

Recently, a strand-specific type I element binding protein, ssA-TIBF, was identified by an in vitro gel shift assay (22, 43). It is plausible that this single-stranded binding site is generated only during DNA replication. In one model, ssA-TIBF somehow promotes replication initiation and remains associated with the replication machinery at the elongating replication fork, analogous to simian virus 40 T antigen (42). An associated helicase unwinds DNA at the replication fork. Strand separation would expose downstream ssA-TIBF binding sites and induce pausing upon binding of this factor. Strand-specific binding of this factor could potentially generate the strong fork arrest polarity that we observed, as the four binding sites have the same polarity. Future experiments will distinguish between this and other models.

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