 Nucleic Acids Research, 1999, Vol. 27, No. 15 **3079–3089**

Long range cooperative interactions regulate the initiation of replication in the *Tetrahymena thermophila* rDNA minichromosome

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Received April 16, 1999; Revised and Accepted June 16, 1999

ABSTRACT

The *Tetrahymena thermophila* rDNA exists as a 21 kb palindromic minichromosome with two initiation sites for replication in each half palindrome. These sites localize to the imperfect, repeated 430 bp segments that include the nucleosome-free domains 1 and 2 (D1 and D2). To determine if the D1 and D2 segments act independently or in concert to control initiation, stable DNA transformation assays were performed. Single domain derivatives of the plasmid prD1 failed to support autonomous replication in *Tetrahymena*. Instead, such constructs propagated exclusively by integration into endogenous rDNA minichromosomes and displayed weak origin activity as detected by 2D gel electrophoresis. D1/D1 and D2/D2 derivatives also transformed *Tetrahymena* poorly, showing similar replication defects. Hence, the D1 and D2 segments are functionally non-redundant and cooperate rather than compete to control initiation. The observed replication defect was greatly reduced in a plasmid derivative that undergoes palindrome formation in *Tetrahymena*, suggesting that a compensatory mechanism overcomes this replication block. Finally, using a transient replication assay, we present evidence that phylogenetically-conserved type I elements directly regulate DNA replication. Taken together, our data support a model in which cooperative interactions between dispersed elements coordinately control the initiation of DNA replication.

INTRODUCTION

DNA replication initiates at specific sites in chromosomes, termed origins of replication. The genetic determinants that control the initiation of replication are poorly understood in higher eukaryotes (1,2). The limited information available indicates that higher eukaryotic origins are complex, consisting of *cis*-acting determinants that can be thousands of base pairs away from the initiation site (3–6). This organization sharply contrasts the situation in the yeast *Saccharomyces cerevisiae*, where essential *cis*-acting replication determinants are positioned around the initiation site, typically spanning an ~120 bp DNA segment at the origin (7,8).

We are using the naturally occurring rDNA minichromosome of *Tetrahymena thermophila* as a model for a complex eukaryotic origin of replication. This molecule is attractive for studying the regulation of DNA replication because the replication properties of this chromosome change at different stages of the life cycle. *Tetrahymena thermophila* contains two nuclei within a single cell: the transcriptionally silent germline micronucleus and the transcriptionally active somatic macronucleus. Following conjugation, a new micronucleus forms and duplicates in the absence of cell division. Macronuclear development ensues in one daughter nucleus, in which the five germline chromosomes undergo site-specific chromosome fragmentation (9), *de novo* telomere addition (10), DNA rearrangement (11) and replication to approximately 50 copies (reviewed in 12). During this period the single copy ribosomal RNA genes (rDNA) are excised from the 'germline' chromosome, rearranged into a 21 kb palindrome and amplified to 10 000 copies (13; reviewed in 14). During subsequent vegetative divisions, replication of the rDNA and other macronuclear chromosomes is under cell cycle control. DNA molecules are replicated once (on average) per cell division. Since macronuclear chromosomes lack centromeres and segregate randomly during nuclear division, additional mechanisms have evolved to maintain the genic balance of rDNA and non-rDNA chromosomes (15,16).

A previous electron microscopy study demonstrated that vegetative (cell cycle-controlled) replication of the rDNA initiates in the 5' non-transcribed spacer (5' NTS; 17). Two initiation sites were subsequently localized by two-dimensional (2D) gel electrophoresis. They map to the tandemly duplicated 430 bp segments that include the 230 bp nucleosome-free regions, Domain 1 and Domain 2 (D1 and D2) (Fig. 1; 18). These duplicated segments contain phylogenetically conserved type I and type III elements, which are flanked by sequences that have diverged considerably (19). Replication origins in the D1/D2 region mediate rDNA gene amplification as well, indicating that cell cycle control of these origins is overridden during development (18).

Classical genetic studies provide insight into the control of rDNA replication. Mutant screens have identified *cis*-acting elements that regulate both the formation and propagation of rDNA minichromosomes (reviewed in 14). Vegetative rDNA 'maintenance' mutations reside within or immediately adjacent to

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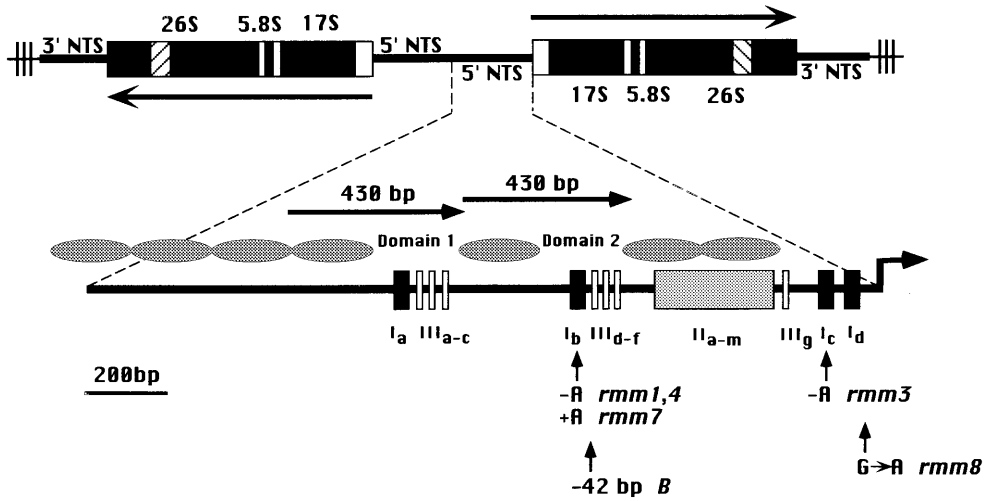

Figure 1. Structural and functional features of the *Tetrahymena* rDNA minichromosome. Top, macronuclear rDNA minichromosomes consist of two copies of the rRNA coding region and adjacent 5' and 3' NTS regions in an inverted orientation. The 35S rRNA precursor (arrow and large rectangular box) encodes the 17S, 5.8S and 26S rRNAs (mature RNA coding regions, black areas; processed RNA precursor regions, unshaded areas; self-splicing 26S rRNA intron, hatched area). Telomeric DNA repeats (thin lines with vertical bars) are present at the rDNA termini. Bottom, blowup of the 1.9 kb 5 NTS region from the wild-type *C3* rDNA allele [rRNA promoter, terminal arrow; positioned nucleosomes in vegetative rDNA minichromosomes, ovals (44); type I elements, black boxes; tandem array of type II elements, shaded box; type III elements, open boxes (19)]. The 230 bp nuclease hypersensitive Domains 1 and 2 reside in 430 bp tandemly duplicated segments that have undergone subsequent sequence divergence. The position of sequence changes that affect vegetative rDNA maintenance are depicted for the naturally-occurring *B* rDNA allele (–42 bp), and for *rmm* mutants isolated following *in vivo* mutagenesis of cells carrying the *C3* rDNA allele (reviewed in 14).

the dispersed, conserved, type I elements (Fig. 1; 16,20,21; D.D.Larson and E.H.Blackburn, personal communication; G.M.Kapler, unpublished results). As type IA and IB elements co-localize with the physically mapped origins of replication (18), it was proposed that these maintenance mutations affect the initiation of DNA replication. Mutations in the type IB element diminish rDNA gene amplification as well, supporting the idea that they regulate a replication-based process (22). Mutations in the type IC and ID elements have also been identified (21; D.D.Larson and E.H.Blackburn, personal communication). These promoter-proximal elements map well downstream of the D1 and D2 segments (1000 and 600 bp, respectively) and do not co-localize with an initiation site for DNA replication (18). A recent DNA transformation study identified segments of the 5' NTS which when deleted led to a decrease in autonomous propagation of plasmid-derived rDNA molecules (23). These experiments confirm the importance of 5' NTS sequences in rDNA minichromosome maintenance.

In this report, we use two different DNA transformation approaches and 2D gel electrophoresis to directly examine the role of conserved repeated elements in rDNA replication control. Our data indicate that type I elements are required specifically for the initiation of DNA replication, and that the segments encompassing D1 and D2 contain functionally non-redundant replication determinants. Thus, the D1 and D2 segments, each of which contains as an initiation site for DNA replication, cooperate rather than compete with one another to control rDNA replication.

MATERIALS AND METHODS

Strains and culture methods

Tetrahymena strains were grown at 30°C in 2% PPYS (2% proteose peptone, 0.2% yeast extract, 10 μM $FeCl_3$), supplemented with 250 μg/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B (24). SF137 contains the wild-type macronuclear *C3* rDNA allele. CU427 and CU428 are homozygous for *B* rDNA in both the micronucleus and macronucleus. SF137 is homozygous for *C3* rDNA in the macronucleus. Previous studies demonstrated that *B* rDNA is lost from the macronucleus of heterozygous *C3/B* progeny during vegetative divisions (16).

rDNA plasmids

All plasmids used here are *C3* rDNA derivatives. Plasmids used for the transient transformation assays contain a +G mutation that inactivates transcription from the rRNA promoter (25,26). Transcription from this promoter inhibits stable transformation of circular rDNA derivatives (26). The pUC118-based plasmids, pUC1x1.9 and pUC2x1.9, contain one or two copies of the 1.9 kb 5' NTS, respectively, and no additional rDNA sequences. Various 5' NTS deletion derivatives of pUC1x1.9 were generated by PCR amplification and cloning into pUC118, followed by DNA sequencing. The D1/D2 plasmid contains the two imperfect, tandemly duplicated 430 bp segments that include D1 and D2 (nt 570–1370) (27). D1/D2/T2 extends further downstream into the tandemly arrayed type II elements (nt 570–1701). The δPromoter plasmid (δPro) is missing sequences at the rRNA promoter (nt 1764–1909), including the type IC and ID elements, but contains the remainder of the 5' NTS. JK154 contains a 2.1 kb *Hin*dIII fragment from the rRNA coding region (nt 4610–6742) cloned into pUC118.

Stable DNA transformation vectors are derivatives of the plasmid prD1 (28). They contain one intact copy of the 5' NTS, coding region and 3' NTS cloned into pBr322. This *C3* allele carries a coding region mutation that confers resistance to the antibiotic paromomycin (pm). prD1 lacks DNA sequence elements that mediate rDNA excision and palindrome formation.

prD1-δD2 is a prD1 derivative in which the 430 bp segment containing D2 (nt 935–1370) was deleted. Similarly, prD1-δD1 is missing the 430 bp segment encompassing D1 (nt 503–934), but is otherwise identical to the prD1 parental plasmid. prD1-D1+D1 contains two copies of the 430 bp D1 region and is deleted for corresponding D2 segment. Similarly, prD1-D2+D2 has two copies of the D2 region and no D1 copies. These 5' NTS derivatives were generated by cloning PCR products into the pBluescript KS$^-$ vector (as described above) and then inserting the reconstructed 5' NTS fragment into the plasmid prD1-δ5'NTS. prD1-δ*Xba*I is missing the 5' NTS *Xba*I fragment (nt 720–1152). *Xba*I cleaves at the same position in the tandemly duplicated D1 and D2 segments, ~50 bp upstream of the respective type IA and type IB elements. The resulting δ*Xba*I plasmid contains a single chimeric domain, with the 5' and 3' portions coming from D1 and D2, respectively. It contains the intact 230 bp nuclease-hypersensitive D2 domain. The plasmid Tt947-01 carries a single copy of the micronuclear rDNA gene (29). This plasmid undergoes excision and palindrome formation when introduced into the developing macronucleus. Tt947-δ*Xba*I was created by partial digestion of Tt947-01 with *Xba*I, followed by recircularization. Like prD1-δ*Xba*I, it is deleted for nt 720–1152 of the 5' NTS.

Transient and stable DNA transformation assays

Transient transformation. A transient transformation assay was developed to test the ability of pUC2x1.9 and its derivatives to replicate in *Tetrahymena*. Strains CU427 and CU428 were mated and transformed with 20 µg of purified plasmid DNA (Qiagen Inc.) by electroporation as previously described (30). Electroporated cells were resuspended in 50 ml of starvation medium (10 mM Tris, pH. 7.5) and incubated at 30°C in a 250 ml flask without shaking. Twelve hours later, the cultures were adjusted to 1% PPYS and incubated for an additional 24 h at 30°C with gentle shaking. The entire culture was harvested and washed twice with 10 mM Tris to remove plasmid DNA that was not taken up by cells. The final cell pellet was lysed and plasmid DNA was then purified on a Qiagen tip 20 column according to the manufacturer's recommendations (Qiagen Inc.). Alternatively, total genomic DNA was prepared from *Tetrahymena* as previously described (16). Following purification, the DNA sample was split in half and subjected to digestion with either *Dpn*I or *Mbo*I. Restriction-digested DNA was electrophoresed on a 1.5% agarose gel, transferred to a nylon membrane (Hybond N$^+$, Amersham) and hybridized to a radiolabeled pUC probe to assay for replication of plasmid sequences in *Tetrahymena*.

Stable transformation. prD1 and its derivatives were tested for the ability to support replication in stable *Tetrahymena* transformants. *Tetrahymena thermophila* strains CU427 and CU428 were subjected to electroporation and stable rDNA transformants were selected for resistance to pm (30). Transformants were propagated in the absence of pm and DNA was isolated at various time points between 20 and 90 vegetative divisions (31).

Pulse field and 2D gel electrophoresis

Undigested DNA from *T.thermophila* transformants was electrophoresed on a BioRad DRIII pulse field gel electrophoresis unit for 17 h at 6 V/cm in 45 mM Tris-borate (pH 8.3), 1 mM EDTA buffer (initial switch time, 1 s; final switch time, 6 s). DNA was transferred to Hybond N$^+$ and hybridized with either a 1.9 kb 5' NTS fragment or pUC vector probe to determine whether the introduced plasmid replicated autonomously in *Tetrahymena* (32).

Replication intermediates from vegetatively growing transformants were examined by 2D gel electrophoresis to determine if mutant 5' NTS copies contained active origins of replication. DNA was prepared from log phase cultures, digested with the restriction enzymes *Sac*II and *Pst*I, and replication intermediates were then enriched for by benzoylated naphthoylated DEAE cellulose chromatography (BND cellulose, Sigma) (33). Digestion with these enzymes generates a 6.2 kb palindromic fragment containing the two copies of the 5' NTS for wild-type rDNA homopalindromes. For the different prD1-derivative cointegrants examined, a 2.8–3.9 kb *Pst*I–*Sac*II fragment is produced (prD1-δD1, 2.8 kb; prD1-δD2, 3.5 kb; prD1 D2+D2, 3.2 kb and prD1-D1+D1, 3.9 kb). The 0.7 kb difference in size between the two single domain derivatives and the two double domain derivatives results from the inclusion of a *Sac*II site during cloning of the δD2 and D1+D1 derivatives. Neutral–neutral two 2D gel electrophoresis was performed as previously described (31,34). Southern blot analysis with a 5' NTS probe was used to evaluate origin activity in wild-type and mutant 5' NTS derivatives.

RESULTS

Stable transformation identifies non-redundant determinants for replication control

The importance of the 5' NTS for propagation of rDNA minichromosomes has been established by classical genetic and DNA transformation studies (reviewed in 14,23). Several indirect lines of evidence suggest that the type I elements, dispersed throughout the 5' NTS, are genetic determinants for the initiation of DNA replication (16,18,21,22). Here we use a combination of DNA transformation assays and 2D gel electrophoresis to directly examine the role of type I elements and the tandemly duplicated regions that encompass D1 and D2 in DNA replication control. Previous 2D gel studies of endogenous rDNA minichromosomes localized origins of replication to the duplicated 430 bp segments that include D1 and D2 (18). Whereas mutations in D2 and promoter-proximal type I elements have been identified in rDNA maintenance mutant screens [Fig. 1 *B* and *rmm1* rDNA alleles (16), *rmm4* (20), *rmm8* (21), *rmm3* (D.D.Larson and E.H.Blackburn, personal communication), *rmm7* (G.M.K., unpublished results)], no D1 mutations have been identified to date. This raised the possibility that the tandemly duplicated D1 and D2 segments might not be functionally equivalent.

We first examined a rDNA vector, prD1, which contains one intact copy of the wild-type *C3* rDNA 5' NTS, coding region and 3' NTS (Fig. 2A). This plasmid lacks the sequence elements that mediate rDNA excision and palindrome formation. Consequently, we anticipated that prD1 might be maintained as a circular episome in *Tetrahymena*. Transformants were generated by electroporating mating *Tetrahymen*a cultures and selecting for paromomycin-resistant (pm^r) progeny. Recipient cells contain the *B* rDNA allele in their macronucleus. Since palindromic *C3* rDNA out-propagates *B* rDNA palindromes in vegetatively growing cells, plasmid-derived *C3* rDNA molecules might behave similarly (16). On average, the prD1 plasmid generated

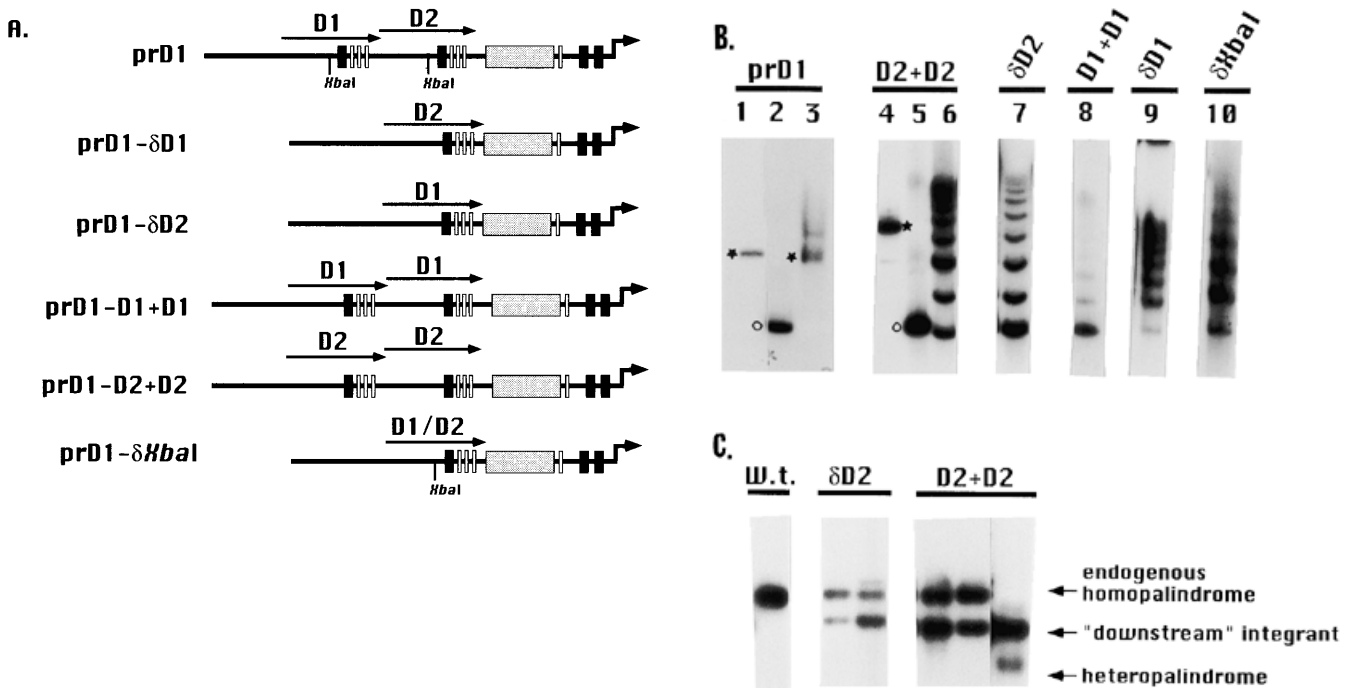

Figure 2. Molecular analysis of *Tetrahymena* cultures following stable transformation with rDNA plasmid derivatives. (**A**) Schematic diagram of the 5' NTS for the plasmid prD1 and various prD1 derivatives tested for stable transformation of *Tetrahymena*. prD1 contains a single copy of the macronuclear rDNA 5' NTS linked to the pm^r coding region and 3' NTS. This plasmid lacks DNA sequence elements that mediate rDNA excision and palindrome formation during macronuclear development. D1 and D2, 430 bp tandemly duplicated segments that include the nucleosome-free domains 1 and 2. Black, shaded and open boxes correspond to the conserved type I, II and III elements, respectively. Terminal arrow, rRNA promoter. prD1 derivatives contain either one intact domain (δD1, δD2), two identical domains (D1+D1, D2+D2), or a single chimeric domain (δ*Xba*I). The position of the *Xba*I sites in the wild type prD1 and mutant prD1-δ*Xba*I plasmids are shown. (**B**) Pulse field gel electrophoresis of DNA from *Tetrahymena* cells stably transformed with various rDNA vectors (probe, rDNA 5' NTS fragment). Lanes 1 and 4, input prD1 and prD1-D2+D2 plasmid DNA (star, supercoiled plasmid DNA). Lanes 2 and 5, *Tetrahymena* rDNA from untransformed cells (open circle, endogenous homopalindromic rDNA). Lanes 3, 6–10, DNA from *Tetrahymena* cultures transformed with prD1 and various prD1 derivatives. (**C**) Southern blot of *Bgl*II-digested DNA from prD1-derivative transformants that showed cointegration into endogenous rDNA (probe, 5' NTS fragment, nt 1–500). The physical position of wild-type and mutant 5' NTS copies in transformant cointegrants is dependent on the site of recombination. This *Bgl*II polymorphism can distinguish between recombination within the 5' NTS (generating heteropalindromes) versus recombination at a downstream position (which retains the endogenous wild-type homopalindrome and places the plasmid-derived origins downstream). W.t., untransformed *Tetrahymena*. δD2, D2+D2, cells transformed with prD1-δD2 and prD1-D2+D2, respectively.

~800 pm^r transformants per experiment (Table 1). This frequency was comparable to the plasmid prD4-1 which contains an additional, tandemly arrayed 5' NTS copy (25). Whereas prD4-1 can be maintained as an episome, it is not ideal for studying DNA replication. prD4-1 frequently recombines with itself or with endogenous rDNA minichromosomes. Long term propagation of prD4-1 transformants can generate rDNA molecules with more than 10 copies of the 5' NTS due to unequal crossing over in the tandemly arrayed 5' NTS copies (25).

To determine whether prD1 replicated autonomously in *Tetrahymena*, undigested genomic DNA from prD1 transformants was examined by conventional (data not shown) and pulse field gel electrophoresis (Fig. 2B). Similar to the input plasmid (Fig. 2B, lane 1), unit length extrachromosomal circular DNA was detected in DNA isolated from *Tetrahymena* transformants (Fig. 2B, lane 3). Although prD1 showed evidence for integration in some transformants (data not shown), autonomous replication was sufficient to cause the plasmid to completely replace endogenous rDNA [Fig. 2B, lane 2 (untransformed) and lane 3 (transformed)].

To determine if prD1 derivatives carrying only a single origin domain could support autonomous replication, plasmids deleted for either of the 430 bp segments, spanning D1 (prD1-δD1) or D2 (prD1-δD2), were introduced into *Tetrahymena* (Fig. 2A). In contrast to prD1, these deletion derivatives transformed *Tetrahymena* poorly (Table 1). Pulse field gel analysis of transformants revealed an extensive ladder of molecules that hybridized to either a pUC vector (data not shown) or 5' NTS probe (Fig. 2B, lanes 7 and 9). The size of molecules in these ladders ranged from ~20 to >150 kb, increasing in ~15 kb intervals. Their migration properties were consistent with that of linear DNAs, suggesting that the plasmids had recombined into endogenous rDNA. Transformation with the plasmid prD1-δ*Xba*I (Fig. 2A) gave similar results (Table 1; Fig. 2B, lane 10). This plasmid contains a single chimeric D1/D2 domain that includes the entire nuclease-hypersensitive D2 region (including the conserved type I and type III elements). We conclude that the presence of just one origin-containing segment severely impairs autonomous propagation of prD1 derivatives.

Since the tandemly duplicated D1+D2 segments were required for autonomous replication of prD1, we next asked whether these partially degenerate segments were functionally redundant. *Tetrahymena* was transformed with the plasmids

prD1-D1+D1 and prD1-D2+D2, each of which carries two 430 bp segments that are identical in sequence. Both plasmids transformed *Tetrahymena* as poorly as their single domain counterparts (Table 1). Similar to single domain derivatives, integration into endogenous rDNA was observed, producing an array of molecules with multiple plasmid copies (Fig. 2B, lanes 6 and 8). Since wild-type function was not restored in plasmids carrying two identical domains, we conclude that the segments encompassing D1 and D2 are functionally non-redundant and must provide unique genetic determinants for rDNA minichromosome maintenance.

Table 1. Stable transformation of rDNA plasmid derivatives

	Transformation frequency[a] (%)	Range (%)
prD1	100	–
prD1-δD1	0.5 (n = 4)	0–2.5
prD1-δD2	3 (n = 5)	0–8
prD1-D1+D1	0.4 (n = 6)	0–1
prD1-D2+D2	1.2 (n = 5)	0–2.5
prD1-δ*Xba*I	5 (n = 3)	1–9
Tt947	100	–
Tt947-δ*Xba*I	100 (n = 2)	85–110

[a]Frequency relative to wild-type plasmid controls (prD1 or Tt947). Average transformants per experiment: prD1, ~800; Tt947, ~1000.

Origin activity in plasmid cointegrants

The observed integration of mutant rDNA plasmids into endogenous rDNA minichromosomes indicated that these plasmids could not propagate autonomously. Restriction analysis revealed that intact unit length plasmids had inserted tandemly into endogenous rDNA chromosomes (data not shown). To examine the structure of cointegrant molecules, we mapped the site of recombination between plasmid and endogenous rDNA. *Bgl*II digestion of endogenous rDNA produces a palindromic 8.8 kb fragment that hybridizes to a probe spanning nt 1–500 of the 5' NTS (Fig. 2C, lane 1, wild-type). A novel *Bgl*II site engineered into the 5' NTS of prD1 derivatives was used to determine whether the central palindromic copies of the 5' NTS consisted of two wild-type copies (8.8 kb homopalindromes) or a wild-type and mutant copy in cointegrants (4.9–5.3 kb heteropalindromes, the exact size of which is dependent on the plasmid used to transform *Tetrahymena*). Recombination events distal to the mutated origin region would produce cointegrants in which the mutant origin was positioned downstream of the central palindromic origins [6.2–6.6 kb *Bgl*II fragment containing the rDNA 3' NTS (3.0 kb), plasmid vector sequences (2.7 kb) and 5' portion of the mutant 5' NTS (0.5–0.9 kb)]. Southern blot analysis indicated that virtually all clonal transformants contained wild-type homopalindromes and integrated downstream copies of the mutant origin (Fig. 2C, lanes 2–5 and data not shown). Heteropalindromes, although rarely observed, were easily detected (Fig. 2C, lane 6). In such cases, downstream cointegrant molecules were also detected due to additional plasmid insertion events. Only transformant lines carrying wild-type homopalindromes and downstream mutant origins were analyzed further.

2D gel electrophoresis was used to determine if the integrated downstream mutant origins were active (34). In this approach, replication intermediates are separated by size and shape. Initiation within a restriction fragment will produce a signature bubble arc pattern, resulting from replication forks that move bidirectionally away from the origin (Fig. 3B). Passive replication of that same fragment will generate a complete simple Y arc pattern, as a single replication fork passes through the region. Untransformed *Tetrahymena* cells contain a 6.2 kb *Sac*II fragment, carrying the two wild-type 5' NTS copies located at the center of the rDNA palindrome (Fig. 3A). Consistent with a previous analysis of a smaller palindromic fragment (18), wild-type cells generated a bubble-to-Y arc pattern in this *Sac*II fragment. The partial bubble (Fig. 3C, wild-type; solid arrow) is diagnostic for initiation at an asymmetric position within this fragment (Fig. 3B, bubble-to-Y). Because complete simple Y arcs, initiating from the unreplicated duplex (1N) spot were not observed (Fig. 3B and C; wild-type, open arrow), we conclude that all detectable initiation events occurred within the central *Sac*II fragment.

To assess origin activity in the various prD1 derivatives, 2D gel electrophoresis was performed on transformants in which the mutant origins had integrated downstream of the palindromic, wild-type copies. Double digestion with *Pst*I and *Sac*II allowed us to simultaneously visualize replication within the wild-type (Fig. 3A; central 6.2 kb *Sac*II fragment) and mutant (Fig. 3A; 2.8–3.9 kb *Pst*I–*Sac*II fragment; see Materials and Methods) origin fragments. In contrast to wild-type cells, cointegrants carrying a single domain displayed two classes of replication intermediates in the central *Sac*II fragment. The majority of replicating molecules produced replication bubbles, indicative of initiation from the wild-type origins within this fragment (Fig. 3D, prD1-δD2; Fig. 3F, prD1-δD1; *Sac*II, upper solid arrow). A simple Y arc was observed in the prD1-δD2 derivative (Fig. 3D; *Sac*II, upper open arrow), indicating that the wild-type origins are passively replicated in a small percentage of molecules. Similarly, low molecular weight replication intermediates were evident on the Y arc for the prD1-δD1 transformant (Fig. 3F, upper open arrow). We conclude that the mutant origins are partially functional in these single domain cointegrants.

Cointegrants carrying two identical 430 bp segments also showed a composite pattern of replication intermediates (Fig. 3E, prD1-D1+D1; Fig. 3G, prD1-D2+D2), consisting of replication bubbles (upper solid arrows) and simple Y arcs (upper open arrows). The ratio of simple Y arcs to bubble arcs was greater for the prD1-D1+D1 transformant compared to the other mutant derivatives, suggesting that the replication efficiency of the prD1-D1+D1 mutant origin may be higher. However, this difference must be small, since the prD1-D1+D1 plasmid failed to transform *Tetrahymena* with a high frequency (Table 1) and did not replicate autonomously in transformants (Fig. 2B). The ratio of simple Y arc to bubble arcs was not elevated in the prD1-D2+D2 transformant relative to the single D2 domain derivative prD1-δD1 (Fig. 3F and G, *Sac*II fragment), suggesting that second D2 copy did not improve the efficiency of DNA replication.

Replication intermediates within the mutant rDNA fragment were also examined. The 2.8–3.9 kb *Pst*I–*Sac*II fragment spans

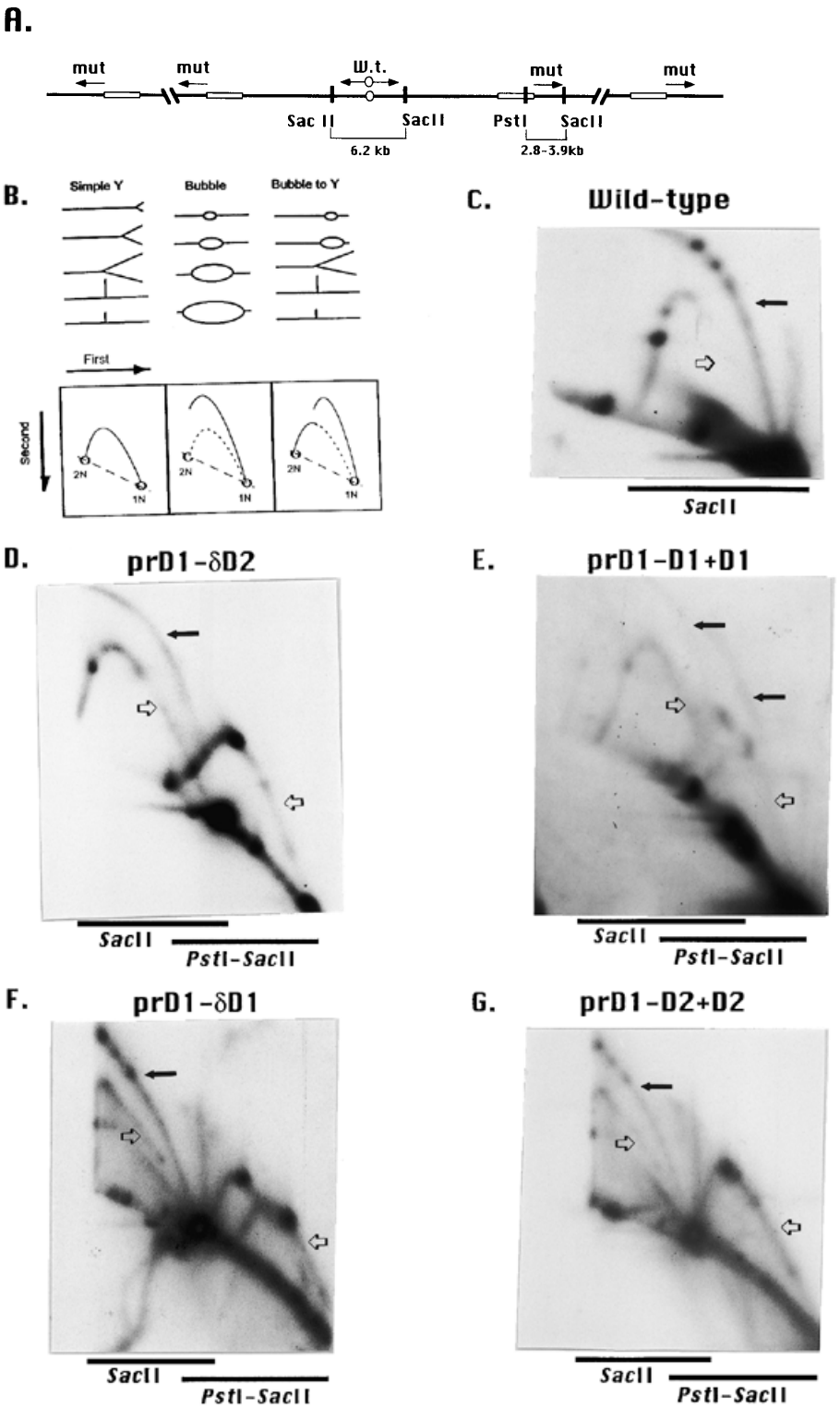

Figure 3. 2D gel electrophoresis of rDNA replication intermediates from prD1 derivative transformants. (**A**) Partial restriction map of rDNA minichromosomes for plasmid cointegrants (not drawn to scale). 2D gel electrophoresis was performed on transformants in which mutant 5' NTS copies had integrated downstream of the inverted wild-type copies at the center of the rDNA palindrome [wild-type, open circle, center of rDNA palindrome with inverted 5' NTS copies (wt double arrow); mutant, pBr322 vector segment + mutant 5' NTS copy (open box + mut arrow)]. (**B**) Schematic representation of replication intermediates resolved by neutral–neutral 2D gel electrophoresis. 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. Diagonal dashed line, arc of non-replicating linear restriction fragments. Solid arc, migration pattern of depicted replication intermediate class. Dotted arc, migration pattern of simple Y arc intermediates relative to other replication intermediates. (**C**) 2D gel analysis of the central 6.2 kb *Sac*II restriction fragment in untransformed cells. (**D**–**G**) 2D gel analysis of prD1 derivative transformants following digestion with *Pst*I+*Sac*II. Replication of the wild-type palindromic copies (6.2 kb *Sac*II fragment) and mutant 5' NTS copies (2.8–3.9 kb *Pst*I–*Sac*II fragment; see Materials and Methods) (A) are visualized. Solid arrows, bubble arcs; open arrows, position of low molecular weight replication intermediates in complete, simple Y arcs.

the pUC vector and 5' NTS region of the integrated mutant plasmid molecules (Fig. 3A). A simple Y arc was detected in all transformants (Fig. 3D–G, lower open arrow), indicating that the mutant origins were primarily replicated passively. A faint bubble arc was detected in the mutant origin fragment for prD1-D1+D1 transformant (Fig. 3E; *Pst*I–*Sac*II, lower solid arrow), consistent with this origin being partially active. Longer exposures also showed faint bubble arc intermediates in this mutant fragment for the prD1-δD2 transformant (data not shown). However, no bubble arc intermediates were detected in this fragment for the remaining transformants (prD1-δD1 and prD1-δD2). The collective data indicate that replication initiation is significantly compromised, but not completely abolished in all prD1 derivatives carrying just one or two identical D1- or D2-containing segments.

A single 5' NTS domain can support replication of rDNA palindromes

The experiments described above revealed that deleting either D1 or D2 causes a defect in rDNA replication. In contrast, a previous study concluded that single domain derivatives can support autonomous replication in *Tetrahymena* (23). Our experiments used a rDNA vector that replicates as a circular episome, while the previous study utilized a rearrangement vector, pMND1, which undergoes excision and palindrome formation in the developing macronucleus. Additionally, the rRNA coding region and 3' NTS were replaced with the ribosomal protein L29 gene in the pMND1 plasmid. To further investigate the role of the D1 and D2 segments in rDNA replication control, we carried out transformation experiments with the rearrangement vector, Tt947-01, and a derivative carrying a single 5' NTS domain (Tt947-δ*Xba*I). These plasmids are analogous to prD1 and prD1-δ*Xba*I, except that they undergo excision and palindrome formation. Efficient transformation was observed for both rearrangement plasmids (Table 1). This result ruled out the possibility that autonomous replication in the previous study was facilitated by sequences in the L29 gene. Thus, a single domain is sufficient to support replication in molecules that have undergone excision and palindrome formation, but not in molecules that propagate as circular monomers.

To assess autonomous replication directly, DNA was prepared from clonal wild-type (Tt947-01) and mutant (Tt947-δ*Xba*I) transformants. Digestion with *Sph*I was used to distinguish between plasmid-derived (*C3*) and endogenous (*B*) rDNA molecules. Both the wild-type and mutant plasmids generated autonomously replicating *C3* rDNA minichromosomes (Fig. 4A, P1; 4 out of 12 clonal lines shown), indicating that a single domain can support replication in palindromic minichromosomes. Roughly equivalent amounts of *C3* and *B* rDNA were detected at the earliest time point analyzed for the δ*Xba*I transformants (P1 = ~20 generations). This ratio was maintained over 90 generations in the absence of selection for this plasmid (Fig. 4, Tt947-δ*Xba*I, P10). In contrast, virtually all of the rDNA in wild-type transformants was derived from the *C3* rDNA plasmid (Fig. 4; Tt947-01, P1 and P10). We conclude that palindromic molecules carrying a single domain can support autonomous replication. They do, however, manifest a subtle defect, similar *rmm* mutants isolated by classical genetic approaches (reviewed in 14), in that they do not completely replace endogenous *B* rDNA.

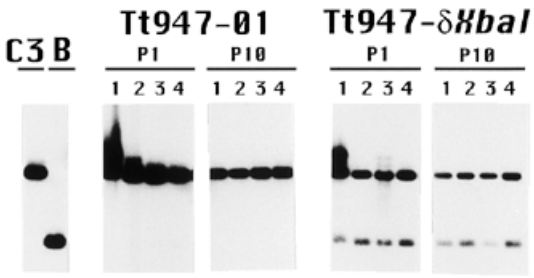

Figure 4. Southern blot analysis of *Tetrahymena* transformed with rDNA rearrangement vectors. The wild-type rearrangement vector Tt947-01 and deletion derivative Tt947-δ*Xba*I were transformed into *T.thermophila* cells. pm^r transformants were analyzed by Southern blotting with a 5' NTS probe. DNA analyses from passages 1 (approximately 20 fissions) and 10 (approximately 90 fissions) of four clonal transformant lines are shown. Digestion with *Sph*I was used to distinguish between the endogenous *B* and plasmid-derived *C3* rDNA alleles.

Transient transformation reveals that type I elements regulate rDNA replication

Point mutations in type I elements have been shown to cause mutant *C3* rDNA alleles to lose their advantage over *B* rDNA in the macronucleus of heterozygous *C3/B* cells (reviewed in 14). This loss occurs gradually (20–60 generations), making it difficult to assess whether type I elements regulate replication or some other chromosomal process (i.e. segregation or copy number control). To address this issue directly, we developed a transient DNA transformation assay for studying replication of rDNA plasmids with intact or truncated copies of the 5' NTS (Fig. 5A). Plasmids purified from the *Escherichia coli* strain DH5α were introduced into mating *Tetrahymena* cells by electroporation. Transformation cultures were propagated for approximately five fissions in the absence of selective pressure. Plasmid DNA was then re-isolated from the entire transformant culture and subjected to digestion with the restriction enzyme *Dpn*I or *Mbo*I. *Dpn*I and *Mbo*I cleave DNA at the same recognition sequence; however, their ability to do so is dependent on the methylation state the adenine residue in the recognition sequence, GATC. Plasmid DNA isolated from DH5α is methylated on both strands and therefore sensitive to cleavage by *Dpn*I and resistant to *Mbo*I (Fig. 5B, *E.coli*). Because adenine methylation occurs at a very low frequency in *Tetrahymena* (0.4%) (35), one round of replication will primarily generate hemimethylated DNA. These molecules are insensitive to digestion by both enzymes. A second round of replication in *Tetrahymena* will produce unmethylated duplex DNA, which can now be cleaved by *Mbo*I.

Plasmids carrying all or portions of the macronuclear rDNA were tested for the ability to replicate in *Tetrahymena* (Fig. 5A). First we asked whether two copies of the 5' NTS were sufficient for replication in this transient assay. pUC2x1.9 contains two tandemly arrayed copies of the 5' NTS and no other rDNA sequences. In contrast, prD4-1 contains two tandem copies of the 5' NTS and a single copy of the rRNA coding region and 3' NTS (Fig. 5A; 25). Transformation with either plasmid (data not shown) or co-transformation with both plasmids (Fig. 5B, 2x1.9 + 4-1) generated *Mbo*I-sensitive DNA, indicating that replication had occurred in *Tetrahymena*. Since both plasmids replicated to

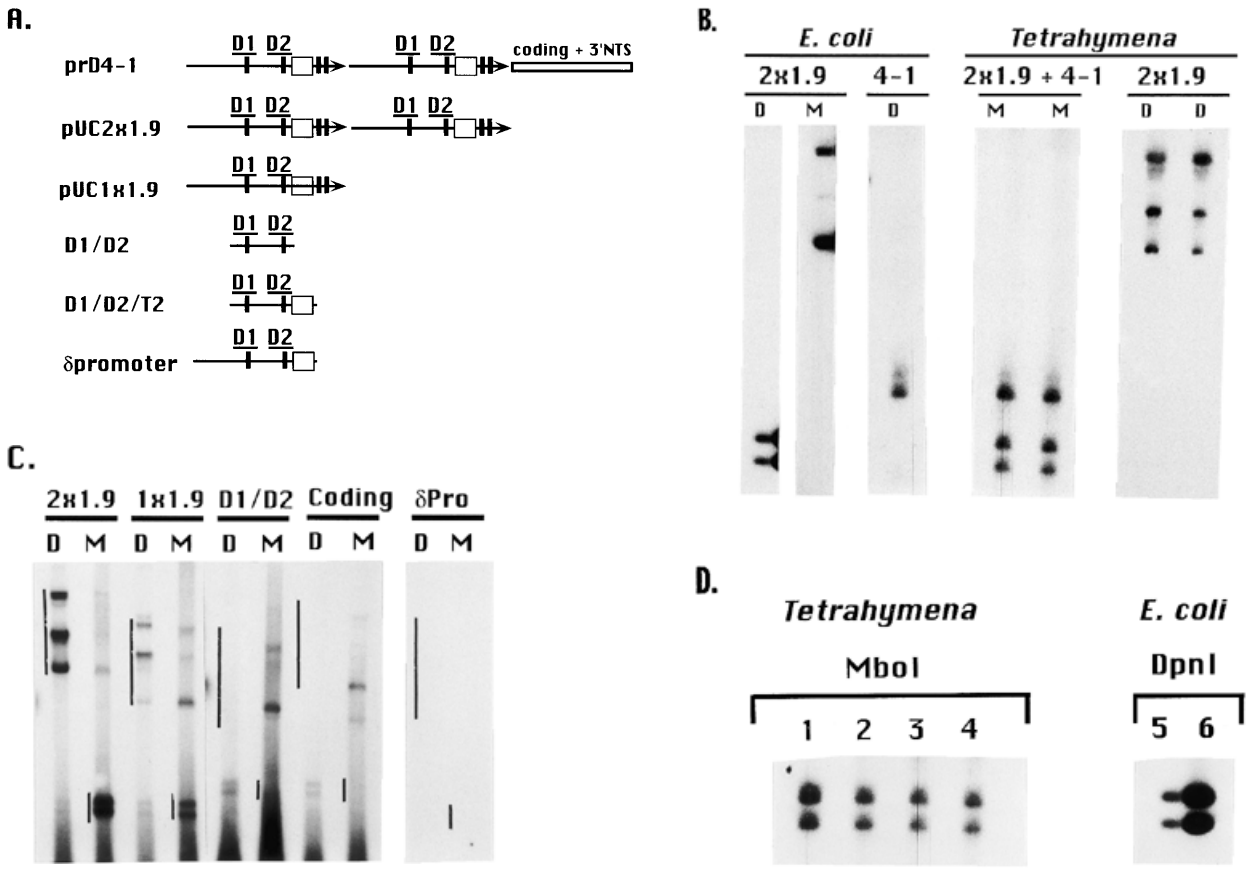

Figure 5. Transient rDNA replication assay. (**A**) Plasmid constructs tested for replication in *Tetrahymena* by transient transformation. prD4-1 contains a single copy of the rRNA coding region and 3' NTS linked to two tandem copies of the 5' NTS in the vector pBr322 (25). pUC2x1.9 and pUC1x1.9 contain two or one copy of the 5' NTS and no other rDNA sequences in the vector pUC118, respectively. The remaining plasmids are deletion derivatives of pUC1x1.9 (Materials and Methods). D1 and D2, 430 bp segments containing the nucleosome-free regions domain 1 and domain 2; arrowhead, rRNA promoter; solid boxes, type IA-ID elements; open box, type IIA-M (T2) elements. (**B**) Southern blot analysis of input plasmid DNA (*E.coli*) and DNA isolated from transformed *Tetrahymena* cultures (*Tetrahymena*) following hybridization with a pUC vector probe. D, *Dpn*I; M, *Mbo*I. Input plasmid DNA is *Dpn*I-sensitive and *Mbo*I-resistant (*E.coli*). (**C**) Transient transformation with various rDNA derivatives. The predicted positions for DNA molecules that underwent replication in *Tetrahymena* are indicated by vertical bars. Coding, 2.1 kb rRNA coding region fragment (Materials and Methods). The remaining plasmids are depicted in (A). (**D**) Sequential transformations with pUC2x1.9 plasmid DNA. Four consecutive transformations were performed with the same plasmid to address the reproducibility of this method. Products were analyzed for replication in *Tetrahymena* by digestion with *Mbo*I (1–4). *Escherichia coli*: *Dpn*I-sensitive input plasmid DNA (10 ng, lane 5, 100 ng, lane 6).

comparable levels in co-transformation assays, the coding region and 3' NTS appear to be dispensable.

Previous stable transformation studies revealed that the two tandem copies of the 5' NTS present in prD4-1 and pUC2x1.9 can recombine with other rDNA molecules, generating long tandem 5' NTS arrays (36,37). This can confer a selective advantage to recombinant molecules during long term propagation of transformants. To test whether recombination was occurring in our transient assay, DNA isolated from pUC2x1.9 transformant cultures was digested with *Dpn*I and hybridized with a pBr322 vector probe. As expected, *Dpn*I-resistant (and therefore replicated) DNA was detected (Fig. 5B; *Tetrahymena* 2x1.9). The *Dpn*I-resistant molecules co-migrated with the *Mbo*I-resistant input plasmid. Unit length supercoiled, nicked circular and linear topoisomers were observed following replication in *Tetrahymena*, indicating that these molecules had not recombined with endogenous rDNA or with one another.

We next assessed whether replication of a single copy of the 5' NTS or portions therein could be detected in this assay. pUC1x1.9 contains one copy of the 5' NTS and no other rDNA sequences (Fig. 5A). Replication of this plasmid in *Tetrahymena* was readily detected (Fig. 5C, 1x1.9), but was not as pronounced as the plasmid with two 5' NTS copies (Fig. 5C, 2x1.9). In contrast, replication of a rRNA coding region plasmid was not observed, indicating a requirement for a bona fide origin of replication (Fig. 5C, coding). The plasmid D1/D2 contains the two 5' NTS replication origins, but lacks flanking DNA sequences (Fig. 5A). *Dpn*I-resistant, *Mbo*I-sensitive DNA was not detected in *Tetrahymena* cultures transformed with this plasmid, indicating a requirement for additional 5' NTS sequences (Fig. 5C, D1/D2). Inclusion of the adjacent type II elements (Fig. 5A, D1/D2/T2) was not sufficient to support autonomous replication (data not shown). Similarly, replication was not observed for a plasmid deleted for just the

type IC and ID elements at the rRNA promoter (Fig. 5A, δPromoter; Fig. 5C). Based on the intensity of the signal obtained for the plasmid pUC1x1.9, we estimate the replication efficiency of the defective 5' NTS deletion derivatives to be at least 10–20-fold lower than that of the intact 5' NTS copy.

Because the transient assay analyzes DNA replication directly, we conclude that these 5' NTS deletions affect DNA replication rather than some other maintenance function. Mutations affecting segregation should be transparent in this assay, as the entire transformed culture was examined. In addition, long term propagation of transformants is not required, and a potential bias was not generated since selection for the plasmid was never imposed. One limitation that we observed was variability in the sensitivity of our assay from one mating to another. However, the reproducibility within a single experiment was consistent (compare the replicated DNA signal in four consecutive transformations with the pUC2x1.9 plasmid; Fig. 5D; *Tetrahymena*, *Mbo*I). Thus, type I elements are genetic determinants for the initiation of replication.

DISCUSSION

The rDNA minichromosome of *T.thermophila* is a useful model for studying complex origins of DNA replication. Previous physical studies identified two closely linked sites for the initiation of replication, localizing to the tandemly duplicated 430 bp 5' NTS segments that span the nucleosome-free regions D1 and D2 (Fig. 1; 18). Genetic studies showed that *cis*-acting rDNA maintenance determinants also map to the 5' NTS and are dispersed (reviewed in 14). They reside in the D2 and rRNA promoter regions, in or immediately downstream of phylogenetically-conserved type I elements. Indirect lines of evidence suggested that type I elements regulate replication rather than some other maintenance function. First, most rDNA maintenance mutations localize to the D2 origin region. Secondly, the *B* rDNA mutation affects both gene amplification in the developing macronucleus and subsequent vegetative 'maintenance' (22). Furthermore, while copy number control plays a role in rDNA maintenance, the responsible *cis*-acting regulatory determinants appear to reside outside the 5' NTS (37).

Here, we developed a transient DNA transformation assay to directly test whether type I elements play a role in replication initiation. In this approach, replication in *Tetrahymena* converts plasmid DNA from a *Dpn*I-sensitive to a *Dpn*I-resistant form. Using this assay, we provide evidence that type I elements are genetic determinants for the initiation of DNA replication. We show that the tandem 430 bp segments spanning the D1 and D2 origins are not sufficient to support replication, and that deleting the type IC and ID elements diminishes replication to an undetectable level (at least 10–20-fold below wild-type). A previous study revealed that type I elements modulate replication fork movement, causing replication forks to arrest transiently at specific adjacent sites (31). In conjunction with the work presented here, we now conclude that type I elements regulate two distinct processes: the initiation of DNA replication and elongation of replication forks. These events could be controlled by one or more *trans*-acting factors. If the former possibility proves correct, then this regulatory protein would be similar to SV40 T antigen, which initiates replication and remains associated with the replication machinery during the elongation phase (38).

Having established the role of type I elements in replication control, we set out to determine whether flanking DNA sequences were important. As mentioned above, replication initiation localizes to segments that include D1 and D2. Although two-thirds of the rDNA maintenance mutations characterized to date map to D2 type I elements, no D1 mutations have been found (Fig. 1). One possible explanation is that the D1 and D2 origin regions function independently of one another. Accordingly, mutations in D2 might be more easily obtained if D2 was the preferred initiation site. Differences in primary sequence or chromosomal context of these origins (39,40) could explain the observed mutational bias. Alternatively, the duplicated segments spanning D1 and D2 might act cooperatively to control initiation, but not be genetically equivalent. To distinguish between these possibilities, we utilized stable DNA transformation and 2D gel electrophoresis to examine mutant alleles that were reintroduced into *Tetrahymena*. These experiments demonstrated that both imperfect, tandemly duplicated segments (D1 and D2) contribute to the initiation of DNA replication and that their genetic contributions are not equivalent.

The first vector that we employed was predicted to be maintained as a circular episome. Indeed, the wild-type plasmid prD1 transformed *Tetrahymena* efficiently and replicated as an autonomous, circular monomer. In contrast, prD1 derivatives carrying a single domain (prD1-δD1, -δD2 or -δ*Xba*I) transformed *Tetrahymena* poorly. These plasmids propagated by integration into endogenous rDNA minichromosomes and displayed very weak origin activity. Thus, one domain is not sufficient to support autonomous replication as a circular episome. Importantly, plasmids carrying two identical domains (D1+D1 or D2+D2) showed similar phenotypes. These results indicated that the D1- and D2-containing segments are not functionally interchangeable and may interact genetically.

Transformation with a rDNA rearrangement vector gave different results. In this case, deleting one domain had little effect on DNA replication. The plasmid Tt947-δ*Xba*I transformed *Tetrahymena* as efficiently as the wild-type (D1+D2) control. Plasmid-derived molecules replicated as autonomous, homopalindromic minichromosomes. However, replication was modestly compromised as the plasmid-derived (*C3*) rDNA failed to overtake endogenous (*B*) rDNA during prolonged vegetative growth. This result agrees completely with data obtained for a similar rearrangement vector (23). Since the 5' NTS of Tt947-δ*Xba*I and the episomal vector prD1-δ*Xba*I are identical, we conclude that the replication defect is somehow suppressed when the mutation is incorporated into linear, palindromic minichromosomes. Thus, only under certain conditions, does replication require these two functionally distinct regions.

Several scenarios could explain why episomal vectors require two origin-containing domains and rearrangement vectors do not. Previous transformation studies demonstrated that linear rDNA chromosomes are preferentially retained over circular plasmid forms (36,41). Consequently, a given mutation might cause a severe phenotype in circular episomes, but produce a more subtle one in rDNA palindromes. Hence, prD1 may simply be more sensitive that rearrangement vectors for

identifying mutations affecting rDNA replication. The inability of prD1-derivatives to compete with endogenous palindromes might also be influenced by 'origin dosage'. For example, although palindromes and linear 11 kb monomers form during development, only palindromes are retained during vegetative divisions (42). Episomes (monomers) might similarly compete poorly with palindromes (dimers) for rate limiting factors. This possibility is supported by transformation studies showing that molecules carrying additional (3→10) 5' NTS copies are preferentially retained during prolonged culturing (36,37). In the context of the experiments described here, single domain prD1 plasmids would have just one origin-containing domain, whereas endogenous rDNA minichromosomes would have four (2× D1 and 2× D2). However, the episomal and rearrangement plasmids, prD1-D2+D2 and Tt947-δ*Xba*I, would each contain two origin domains (defined here as the initiation site domains, D1 and D2). Their capacity to support replication differed dramatically: the episomal plasmid transformed *Tetrahymena* poorly and integrated into endogenous minichromosomes, while the rearrangement plasmid replicated autonomously. Thus, origin dosage alone is not sufficient to explain the difference in observed results.

The most intriguing possibility for this difference, supported by our studies, is that the initiation of rDNA replication is mediated by long distance, cooperative interactions between dispersed, functionally distinct replication determinants. Interactions between the promoter region and initiation sites have been inferred by previous genetic studies, as mutations in the promoter-proximal type IC and ID elements (which map 1000 and 600 bp downstream of the respective D1 and D2 origin regions) cause rDNA maintenance defects (18,21; D.L.Dobbs and E.H.Blackburn, personal communication). Biochemical evidence for promoter/origin interactions was recently obtained (21). In that study, the DNA footprint in the D1- and D2-containing segments was shown to be similarly altered in the *rmm3* mutant, suggesting that these interactions are important for propagation of rDNA minichromosomes. The promoter-proximal *rmm3* mutation (type IC $^{-}$A mutation) affects the ability of this *C3* rDNA allele to propagate when placed in competition with wild-type *C3* or mutant *B* rDNA molecules, but has no detectable effect on rRNA transcription. Whether the D1 and D2 segments were genetically equivalent entities that compete for interactions with promoter-proximal type I elements (Fig. 6, left panel) was not determined.

Experiments described here now provide genetic evidence for interactions between the origin-containing D1 and D2 segments. While 5' NTS copies deleted for D1 and D2 can function independently as weak origins of replication, efficient replication requires genetic determinants from both origin-containing segments. Thus, interactions between D1 and D2, and interactions with the promoter region are required to maximize replication from either origin (Fig. 6, right panel). Since the episomal D1+D1 and D2+D2 plasmids failed to support autonomous replication, D1 and D2 must have evolved unique functions in replication control. Cooperativity might extend further to origins in adjacent 5' NTS copies. We speculate that this might be occurring in our transient replication assays, as the signal for a plasmid carrying two 5' NTS copies is much greater than that for a plasmid with just one 5' NTS copy. In the context of our stable transformation studies, adjacent 5' NTS copies might cooperate and compensate for the absence of genetic determinants in single domain derivatives. Accordingly, deletion of a single origin-containing segment might be tolerated due to the close proximity of adjacent, similarly compromised 5' NTS copies. A mechanism of this sort has been previously described in *S.cerevisiae*, where reiteration of a non-functional ARS element can restore the capacity to support autonomous replication (43). It is tempting to speculate that the precise positioned nucleosomes in the rDNA 5' NTS (Fig. 1) might play a role in coordinating origins of replication on one or both sizes of the rDNA palindrome.

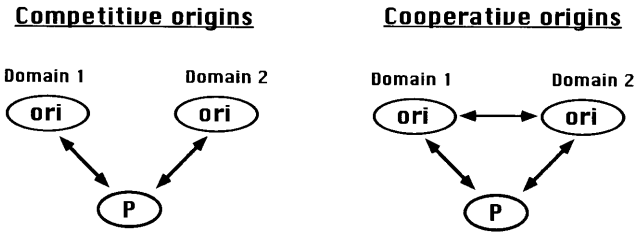

Figure 6. Model for long distance interactions in the rDNA 5' NTS. Schematic representation of genetic interactions for competitive (non-cooperative) and cooperative dispersed genetic determinants for rDNA replication. **Left**, the absence of cooperative interactions between the D1 and D2 origin-containing segments would establish a competitive situation, in which these origins independently vie for interactions with promoter-proximal rDNA replication determinants (P). **Right**, if distinct regulatory determinants reside in each of the origin-containing segments, then cooperative interactions between D1 and D2 regions might be required to promote efficient initiation from either origin. Both direct interactions between the D1 and D2 segments, and interactions with the rRNA promoter region would be required for efficient initiation of DNA replication.

ACKNOWLEDGEMENTS

We thank Audrey Nicholson and Tara Morrison for help constructing several plasmids used here. We also thank Dorothy Shippen for critical reading of this manuscript and comments. This work was support by NIH grant GM56572.

REFERENCES

1. Krysan,P.J., Haase,S.B. and Calos,M.P. (1989) *Mol. Cell. Biol.*, **9**, 1026–1033.
2. Gilbert,D.M. (1998) *Curr. Opin. Gen. Dev.*, **8**, 194–199.
3. Delidakis,C. and Kafatos,F.C. (1989) *EMBO J.*, **8**, 891–901.
4. Heck,M.M.S. and Spradling,A.C. (1990) *J. Cell Biol.*, **110**, 903–914.
5. Kitsberg,D., Selig,S. and Cedar,H. (1993) *Nature*, **366**, 588–590.
6. Aladjem,M.I., Groudine,M., Brody,L.L., Dieken,E.S., Fournier,R.E., Wahl,G.M. and Epner,E.M. (1995) *Science*, **270**, 815–819.
7. Marahrens,Y. and Stillman,B. (1992) *Science*, **255**, 817–823.
8. Bielinsky,A.-K. and Gerbi,S. (1998) *Science*, **279**, 95–98.
9. Yao,M.-C., Yao,C.-H. and Monks,B. (1990) *Cell*, **63**, 763–772.
10. King,B.O. and Yao,M.-C. (1982) *Cell*, **31**, 177–182.
11. Godiska,R. and Yao,M.-C. (1990) *Cell*, **61**, 1237–1246.
12. Karrer,K.M. (1986) In Gall,J.G. (ed.), *The Molecular Biology of Ciliated Protozoa*. Academic Press, Orlando, FL, pp. 85–110.
13. Karrer,K.M. and Gall,J.G. (1976) *J. Mol. Biol.*, **104**, 421–453.
14. Kapler,G.M., Dobbs,D.L. and Blackburn,E.H. (1996) In DePamphilis,M.L. (ed.), *DNA Replication in Eukaryotic Cells*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 915–932.
15. Preer,J.R. and Preer,L.B. (1979) *J. Protozool.*, **26**, 14–18.
16. Larson,D.D., Blackburn,E.H., Yaeger,P.C. and Orias,E. (1986) *Cell*, **47**, 229–240.

17. Cech,T.R. and Brehm,S.L. (1981) *Nucleic Acids Res.*, **9**, 3531–3543.
18. Zhang,Z., MacAlpine,D.M. and Kapler,G.M. (1997) *Mol. Cell. Biol.*, **17**, 6147–6156.
19. Challoner,P.B., Amin,A.A., Pearlman,R.E. and Blackburn,E.H. (1985) *Nucleic Acids Res.*, **13**, 2661–2680.
20. Yaeger,P.C., Orias,E., Shaiu,W.-L., Larson,D.D. and Blackburn,E.H. (1989) *Mol. Cell. Biol.*, **9**, 452–460.
21. Gallagher,R.C. and Blackburn,E.H. (1998) *Mol. Cell. Biol.*, **18**, 3021–3033.
22. Orias,E. and Bradshaw,A.D. (1992) *Dev. Gen.*, **13**, 87–93.
23. Blomberg,P., Randolph,C., Yao,C.-H. and Yao,M.-C. (1997) *Mol. Cell. Biol.*, **17**, 7237–7247.
24. Orias,E. and Hamilton,E.P. (1979) *Genetics*, **91**, 657–671.
25. Yu,G.L. and Blackburn,E.H. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 8487–8491.
26. Pan,W.-J., Gallagher,R.C. and Blackburn,E.H. (1995) *Mol. Cell. Biol.*, **15**, 3372–3381.
27. Engberg,J. and Nielsen,H. (1990) *Nucleic Acids Res.*, **18**, 6915–6919.
28. Yu,G.L., Hasson,M. and Blackburn,E.H. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 5151–5155.
29. Yao,M.-C. and Yao,C.-H. (1989) *Mol. Cell. Biol.*, **9**, 1092–1099.
30. Gaertig,J. and Gorovsky,M.A. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 9196–9200.
31. MacAlpine,D.M., Zhang,Z. and Kapler,G.M. (1997) *Mol. Cell. Biol.*, **17**, 4517–4525.
32. Kapler,G.M. (1993) *Curr. Opin. Gen. Dev.*, **3**, 730–735.
33. Linskens,M.H.K. and Huberman,J.A. (1988) *Mol. Cell. Biol.*, **8**, 4927–4935.
34. Brewer,B.J. and Fangman,W.L. (1987) *Cell*, **51**, 463–471.
35. Karrer,K.M. and Yao,M.-C. (1988) *Gene*, **74**, 105–107.
36. Yu,G.-L. and Blackburn,E.H. (1990) *Mol. Cell. Biol.*, **10**, 2070–2080.
37. Pan,W.-J. and Blackburn,E.H. (1995) *Nucleic Acids Res.*, **23**, 1561–1569.
38. Goetz,G.S., Dean,F.B., Hurwitz,J. and Matson,S.W. (1988) *J. Biol. Chem.*, **263**, 383–392.
39. Brewer,B.J. and Fangman,W.L. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 3418–3422.
40. Marahrens,Y. and Stillman,B. (1994) *EMBO J.*, **13**, 3395–3400.
41. Romero,D.P. and Blackburn,E.H. (1995) *J. Eukaryotic Microbiol.*, **42**, 32–43.
42. Pan,W.-C. and Blackburn,E.H. (1981) *Cell*, **23**, 459–466.
43. Zweifel,S.G. and Fangman,W.L. (1990) *Yeast*, **6**, 179–186.
44. Palen,T.E. and Cech,T.R. (1984) *Cell*, **36**, 933–942.