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DNA Replication in *Tetrahymena*

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The linear ribosomal RNA gene (rDNA) minichromosome in the somatic macronucleus of *Tetrahymena thermophila* serves as a paradigm for multiple mechanisms of regulation of chromosomal DNA replication. Whereas chromosomal DNA replication in most eukaryotes is under strict cell cycle control, additional mechanisms appear to be involved in the regulation of rDNA replication in vegetatively growing *Tetrahymena* cells (Anderson 1972; Engberg et al. 1972; Truett and Gall 1977). All macronuclear chromosomes, including the rDNA minichromosome, segregate randomly and thus appear to lack centromeres. The macronucleus divides by a poorly understood amitotic mechanism, and DNA is often distributed unequally between daughter macronuclei (Doerder 1979). To explain how cells compensate for inequalities that arise from such divisions, it was proposed that a copy number control mechanism is superimposed on cell cycle control of the macronuclear DNA (Preer and Preer 1979; for review, see Larson et al. 1991). In addition, different allelic forms of the rDNA compete with each other for replication in the same macronucleus, and additional copies of the origin region confer a replication advantage to rDNA molecules. These observations are at odds with strict cell cycle control. Therefore, it was concluded that copy num-

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ber control facilitates the maintenance of rDNA levels in vegetative cells (Larson et al. 1986).

Cell cycle control of DNA replication is also suppressed during developmentally programmed rDNA amplification (Yao et al. 1974). *cis*-Acting determinants controlling the formation and propagation of the rDNA minichromosome have been identified by classic genetic and DNA transformation approaches (Larson et al. 1986; Yaeger et al. 1989; Yao et al. 1990; Yasuda and Yao 1991; Kapler and Blackburn 1994; Kapler et al. 1994). The entire minichromosome has been sequenced (Engberg and Nielson 1990), and both the vegetative replication origin (Cech and Brehm 1981; Palen and Cech 1984; R.C. Gallagher and E.H. Blackburn, unpubl.) and telomere-associated regions (Budarf and Blackburn 1986; P.D. Cohen and E.H. Blackburn, in prep.) have been shown to contain highly ordered chromatin structures.

Formation of the rDNA minichromosome exemplifies the changes in chromosome structure that are part of the process leading to nuclear dimorphism of ciliated protozoa. Ciliates contain two types of nuclei within a single cell: the germ-line micronucleus and the somatic macronucleus (for review, see Orias 1986). The transcriptionally silent micronucleus divides by normal mitosis during vegetative cell divisions and undergoes meiosis, serving as the reservoir of genetic material, during cell mating (conjugation). The macronucleus is transcriptionally active, conferring the cellular phenotype. The macronucleus is destroyed during conjugation, and one of the progeny cell micronuclei differentiates to form the new macronucleus. This process, termed macronuclear development, involves a massive rearrangement of the genome (for review, see Karrer 1986). In the developing macronucleus of *T. thermophila*, the five pairs of intact precursor chromosomes are fragmented into about 200 pieces (Altschuler and Yao 1985). Telomeres are added de novo to the newly exposed chromosome ends (Spangler et al. 1988; for review, see Yao 1989). Flanking DNA sequences are lost during fragmentation. In addition, some internal sequences are eliminated via DNA deletion and religation (Godiska and Yao 1990). Macronuclear chromosomes are replicated to about 50 copies and the rDNA minichromosome, encoding the 17S, 5.8S, and 26S ribosomal RNA genes, is amplified to 10^4 copies (Yao et al. 1974). Cultures of *Tetrahymena* can be induced to undergo macronuclear development as relatively synchronous cell populations. Consequently, the reorganization of the macronuclear genome can be monitored by molecular analyses. During subsequent vegetative growth, macronuclear chromosomes are replicated on average once per cell cycle (Engberg et al. 1972); see above.

EVENTS IN THE DEVELOPING MACRONUCLEUS

Formation of the rDNA Minichromosome

The rRNA gene is present as a single copy gene in the germ-line micronucleus. To form the macronuclear rDNA minichromosome, the rDNA region is excised from the germ-line chromosome (Fig. 1 and Table 1). This process is directed by at least one known class of *cis*-acting elements: the 15-bp chromosome breakage sequence (Cbs) elements that flank each end of the rDNA, as well as each of the regions that will become the other macronuclear chromosomes (Yao et al. 1987). Cbs-mediated chromosome breakage has been studied most extensively for the rDNA minichromosome. The dependence of rDNA excision on Cbs elements was demonstrated directly by transformation of developing macronuclei by microinjection of rDNA constructs with and without Cbs elements (Yao et al. 1990). A genetic screen for mutants impaired in rDNA amplification (Kapler and Blackburn 1994) also identified a point mutation in the Cbs at one end of the micronuclear rDNA (the *rmm 11/6* mutation). This Cbs mutation caused an approximately 75% reduction in the frequency of excision of the affected rDNA allele (see below). Some aspect of the Cbs-mediated chromosome fragmentation process appears to be required for the de novo addition of telomeric repeats to newly ex-

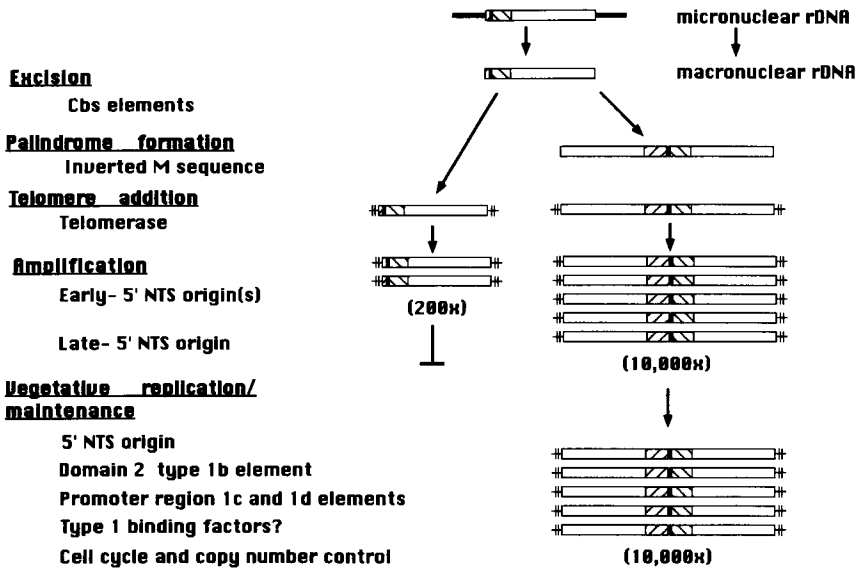

Figure 1 Model for the formation of the replication-competent palindrome. See text for explanations.

Table 1 Developmental pathway for amplification and rDNA minichromosome maintenance

Process	rDNA form	Control elements
Excision	Excised without telomeres	Cbs elements
Palindrome formation	21-kb minichromosome	inverted M repeats
Telomere addition	21-kb and 11-kb with telomeres	(telomerase)
Amplification	11 kb (10^2 copies)	
	21 kb (10^4 copies)	early-5′ NTS origin(s) (complex initiation pattern) late-5′ NTS origin
Vegetative replication	21 kb (10^4 copies)	5′ NTS origin (single initiation event/molecule)

posed chromosome ends in vivo: Introducing a restriction endonuclease-cut DNA end by microinjection into the developing macronucleus was not sufficient to promote de novo telomere addition onto this cut end (Yao et al. 1990). This finding suggests a possible interaction between the chromosome breakage machinery and the specialized DNA polymerase, telomerase, during "chromosome healing." Telomerase has been shown to be responsible for de novo telomere addition onto rDNA and other macronuclear DNA ends during macronuclear development (Yu and Blackburn 1991), as well as for telomere maintenance during vegetative cell divisions (Yu et al. 1990; see Greider et al., this volume).

During macronuclear development, the rDNA monomer is rearranged into a 21-kb head-to-head palindrome (Fig. 1) (Karrer and Gall 1976). It is not known whether this occurs during or after excision of the rDNA from its flanking micronuclear chromosomal sequences. Free, linear monomeric rDNA minichromosomes, with telomeres at both ends, are formed in the developing macronucleus, but they do not normally persist during vegetative growth (Pan and Blackburn 1981; Challoner and Blackburn 1986). It is not known whether these linear 11-kb rDNA monomers are precursors to palindromic rDNA, or whether they are by-products of rDNA excision. Transformation of developing macronuclei with monomeric rDNA constructs has shown that palindrome formation is directed by the pair of inverted 42-bp elements (M sequences) present at the 5′ end of the rDNA monomer (Yasuda and Yao 1991). The inverted M sequences are separated by a 29-bp spacer. It has been proposed that they form a hairpin structure by intra-strand base-pairing in

excised monomeric rDNA (Fig. 1). This small hairpin structure could promote the formation of a full-length monomeric rDNA hairpin by intramolecular recombination or by serving as a primer for replication. The giant hairpin molecule would then be converted into an rDNA palindrome during the next round of DNA replication. Transformation experiments with *T. thermophila* revealed that a variety of sequences could substitute for the M repeat sequences in this species, so long as they were present in the correct palindromic arrangement (Yasuda and Yao 1991). However, the M repeat sequences are evolutionarily conserved among a variety of *Tetrahymena* species (Engberg et al. 1972) and *Glaucoma chattoni* (Challoner and Blackburn 1986). Therefore, the M sequences may have other, currently unknown, roles.

Regulation of rDNA Amplification

The palindromic rDNA minichromosome is amplified to 10^4 copies in the developing macronucleus (Fig. 1). By comparison, other macronuclear chromosomes attain a copy number of about 45. In contrast to non-rDNA chromosomes, which arrest at 8–16 copies if starved throughout macronuclear development, macronuclear rDNA is replicated to high levels under these conditions (Kapler and Blackburn 1994). This implies that the control of rDNA replication in the developing macronucleus is not subject to the same checkpoint controls as non-rDNA chromosomes. The *rmm11* mutation revealed that rDNA excision is necessary, but not sufficient, for correct amplification (Kapler and Blackburn 1994). This *cis*-acting mutation resides within the 3′Cbs element and has only a weak effect on rDNA excision (a 75% reduction in the frequency of cells excising the rDNA). Surprisingly, amplification of correctly excised mutant alleles is severely impaired, so that the frequency of cells which amplify the mutant rDNA allele sufficiently for viability is about one in 10^5. Because the Cbs element containing this point mutation becomes separated from the rDNA molecule upon its excision, the downstream effect on amplification exerted by the *rmm11* mutation must be indirect. The available genetic and molecular evidence suggests that rDNA amplification is restricted to a specific developmental window, such that in this mutant even correctly excised rDNA molecules are unable to replicate to a high level in the developing macronucleus (Kapler and Blackburn 1994).

rDNA amplification in developing *Tetrahymena* macronuclei involves a minimum of 12 rounds of replication to reach the level of 10^4 molecules, if amplification is entirely geometric. Amplification occurs

within a 12- to 14-hour window. The *cis*-acting sequences that directly regulate rDNA amplification are not yet well characterized. A natural genetic variant rDNA allele found in strain *B* of *T. thermophila* has a small deletion in domain 2 of the 5′ nontranscribed spacer (5′ NTS; see below and Fig. 2) relative to the strain *C3* rDNA allele. PCR experiments on individual cells undergoing macronuclear development revealed that this deletion sometimes results in decreased amplification of this rDNA allelic form (Orias and Bradshaw 1992). As described below, mutations in this element also affect vegetative replication of the rDNA (Larson et al. 1986), suggesting that amplification and vegetative replication share some common *cis*-acting determinants. Neutral-neutral two-dimensional gel revealed a complex pattern of replication intermediates in cells undergoing rDNA amplification (G.M. Kapler and E.H. Blackburn, unpubl.). Late in amplification, the predominant class of replication intermediates is identical to that of vegetative cells. This pattern is indicative of replication initiation in the central domain 1/domain 2 region of the 5′NTS (Table 1). Despite the presence of two copies of the 5′NTS, initiation occurs on only one side of the rDNA palindrome in these molecules. Since initiation from this origin is seen throughout most of the amplification period, cell cycle control of the 5′NTS origin must be suppressed in amplifying cells. A second pattern of replication intermediates is detected in amplifying cells. These novel intermediates generate

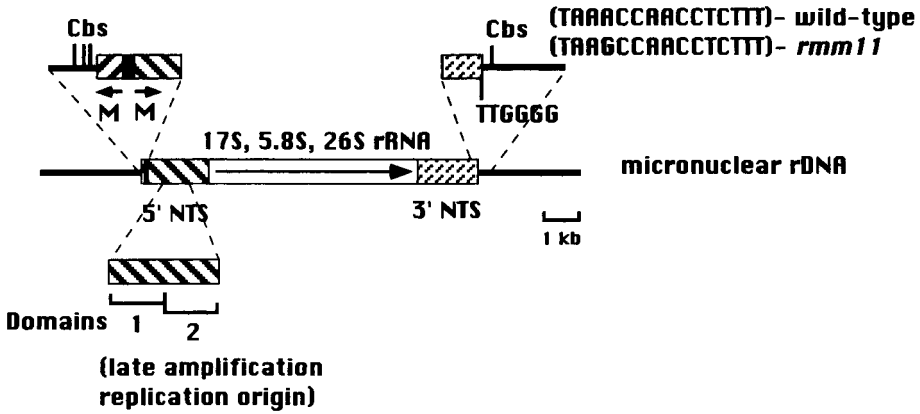

Figure 2 Micronuclear rDNA locus in *T. thermophila*. *cis*-Acting elements specifically required for rDNA maturation steps. (Cbs) Chromosome breakage sequence; (M) M repeats; (TTGGGG) telomeric repeat unit present at the site of new telomere addition. Sequences of wild-type (wt) and mutant (*rmm11*) Cbs element at 3′ end of rDNA are shown at upper right. See text for explanations.

the sole pattern seen at the earliest stage of amplification. Preliminary studies suggest that initiation is also occurring in the vicinity of the 5′NTS; however, multiple initiation events can be seen in these rDNA palindromes. It remains to be determined whether these intermediates initiate at the central region or at new sites in the rDNA.

MAINTENANCE OF THE rDNA MINICHROMOSOME IN THE VEGETATIVE MACRONUCLEUS

Replication of Palindromic and Monomeric rDNA

In contrast to rDNA palindromes, which amplify to 10^4 copies during new macronuclear development, rDNA monomers replicate to about 200 copies in the developing macronucleus and do not persist in vegetative cells (Fig. 1) (Pan and Blackburn 1981). It is possible that proximity of the 5′NTS to telomeric DNA confers a replication disadvantage to rDNA monomers. In *Saccharomyces cerevisiae*, proximity to a telomere delays the activation of an early-firing replication origin; the efficiency of firing is not necessarily affected (Ferguson et al. 1991). In *Tetrahymena*, it is possible that rDNA palindrome formation provides a mechanism for distancing the rDNA replication origin from a repressive chromatin environment at the telomere. However, rDNA constructs defective in palindrome formation replicate stably as linear monomers in strains partially defective for maintenance of the endogenous palindrome (Yasuda and Yao 1991). Therefore, palindromic configuration is not required for origin activity. In the related holotrichous ciliate *Glaucoma chattoni*, and in hypotrichous ciliates, macronuclear rDNA exists exclusively as monomeric linear molecules (Katzen et al. 1981 and references therein). Thus, although palindrome formation may confer a replication and/or maintenance advantage in *T. thermophila*, it is not required for long-term rDNA maintenance in the ciliate macronucleus.

Sequence and Structural Features of the rDNA Origin Region

As shown in Figure 3, in the 21-kb palindromic rDNA molecule the two rRNA transcription units are separated by about 3.8 kb of noncoding sequence (two inverted identical 1.9-kb sequences) spanning the center of symmetry of the molecule. Several approaches—physical mapping, genetic analyses, and macronuclear transformation—have shown that the 1.9-kb 5′NTS contains the vegetative replication origin, *cis*-acting sequences involved in the vegetative replication and/or maintenance of the

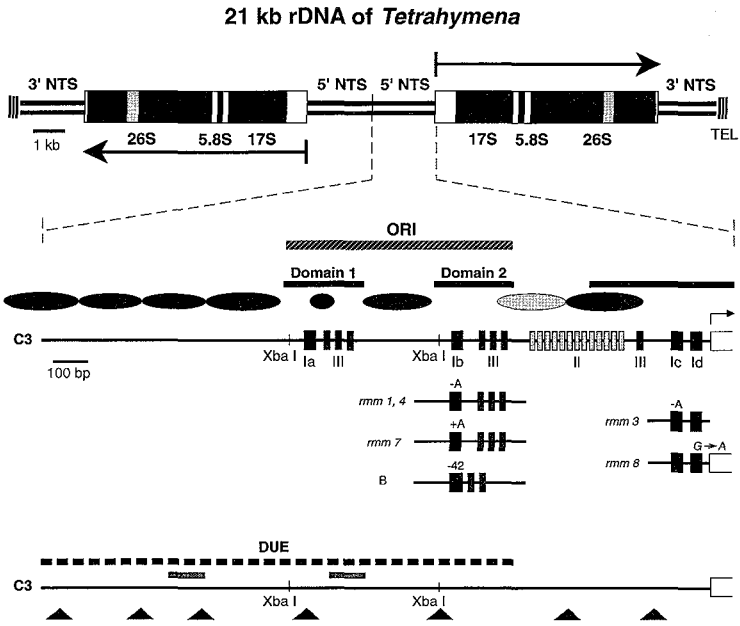

Figure 3 Structural and functional features of the macronuclear palindromic rDNA. (*Top*) Thick bar, rRNA transcription unit and coding regions (*black*); thin open bars, 5′NTS and 3′NTS; vertical lines, telomeric DNA repeat region. (*Middle*) Line, expanded version of 5′NTS of *C3* strain rDNA. Open bar at right, 5′ end of rRNA transcription unit; vertical black rectangles, type I and type III repeats; tall stippled rectangles, type II repeats; base sequence changes in various mutants (*rmm1, 3, 4, 7, 8*), or *B* strain rDNA (*B*) are shown below the line; ovals, nucleosomes; black horizontal bars, DNase I hypersensitive regions, as indicated; origin, region to which functional origin has been mapped. (*Bottom*) Structural and sequence features of bare rDNA. Hatched bar, DUE (calculated); gray bars, mung bean hypersensitive regions; filled triangles, positions of bent DNA. See text for explanations.

rDNA minichromosome, and the late-amplification replication origin described above (for review, see Larson et al. 1991; Kapler 1993). The vegetative origin has been physically mapped both by electron microscopy and by two-dimensional gel electrophoresis. Based on the assumptions that initiation occurs in the center of replication bubble structures and proceeds bidirectionally at equal rates, the electron microscopic mapping places the origin within an approximately 600-bp interval located about 650 bp from the center of the palindrome in the *B* rDNA strain (Cech and Brehm 1981). Analysis of rDNA replicative intermediates using two-dimensional gel electrophoresis shows that all detectable

initiation events (i.e., > 95% of initiation events) occur within the 5′ NTS (G.M. Kapler and E.H. Blackburn, unpubl.).

The rDNA replication origin region contains two approximately 270-bp nuclease hypersensitive domains, designated domain 1 and domain 2 (see Fig. 3), that flank an array of seven precisely positioned nucleosomes centered at the axis of symmetry of the palindrome. A third nuclease hypersensitive domain and a distinctive DNase I footprint encompass the rRNA promoter and transcription initiation region (Bonven and Westergaard 1982; Palen and Cech 1984; Pan et al. 1995; R. Gallagher and E. Blackburn, unpubl.). Domains 1 and 2 are each contained within an approximately 400-bp sequence that is in direct tandem repeat; there is some sequence divergence between the two repeats (see Fig. 3). In another *Tetrahymena* species, *T. pyriformis*, the corresponding sequence and its hypersensitive domain are present only once in the 5′ NTS (Niles et al. 1981; Palen and Cech 1984). Although domain 1 (located between 640 and 935 nucleotides from the palindromic center) most closely coincides with the vegetative origin mapped by electron microscopy, domain 2 also lies within the broad region of the origin defined by two-dimensional gel electrophoresis of replicating intermediates. The 5′ NTS also contains three families of evolutionarily conserved repeated sequences referred to as type I, type II, and type III repeats (Fig. 3) (Niles et al. 1981; Challoner et al. 1985). Each domain contains one type I and three type III repeats in a spatial arrangement along the DNA that is highly conserved between the domains and between holotrichous ciliate species (Challoner et al. 1985). The type I repeats were identified in genetic studies (Larson et al. 1986; Yaeger et al. 1989) as *cis*-acting replication control elements that are also implicated in the regulation of transcription (see below): The type I repeat immediately upstream of the transcription start site has been shown to be essential for rRNA transcription in vitro (Miyahara et al. 1993; R.R. Pearlman, pers. comm.). The type III repeats are binding and cleavage sites for DNA topoisomerase I (Bonven et al. 1985). Type II repeats are located between the promoter region and repeat 2. Two of these are required for maintenance of the rDNA as an episome (M.-C. Yao, pers. comm.).

Additional specific DNA sequence motifs and structural elements shared with other eukaryotic chromosomal origins of replication have been identified in *Tetrahymena* rDNA (Dobbs et al. 1994). The rDNA origin comprises a large region of easily unwound DNA (a putative DNA unwinding element, DUE), coincident with predicted bent DNA segments, nuclear matrix attachment consensus sequences (MARs/SARs), and yeast ARS consensus sequences. Three predicted structural features

have been verified experimentally: (1) Two mung bean nuclease-hypersensitive sites in supercoiled plasmid DNA are located within the major DUE-like element predicted by thermodynamic analyses; (2) three restriction fragments of the 5′ NTS region which are predicted to contain bent DNA segments do exhibit anomalous migration characteristic of bent DNA during electrophoresis on polyacrylamide gels; (3) restriction fragments of the 5′ NTS region bind nuclear matrices in an in vitro binding assay, consistent with an association of the replication origin region with the nuclear matrix in vivo (Du et al. 1995). Although functional roles for DUE-like elements, bent DNA, and matrix-associated sequences in *Tetrahymena* rDNA replication have not been directly demonstrated, the association of these three structural elements with other eukaryotic replication origins is in accordance with the hypothesis that they may contribute to origin function in vivo (Benbow et al. 1992; Dobbs et al. 1994).

Studies of rDNA Replication by Genetics and Transformation

A classic genetic approach has been used to identify *cis*-acting sequences involved in rDNA origin function (Larson et al. 1986; Yaeger et al. 1989; Kapler and Blackburn 1994; Kapler et al. 1994; R.C. Gallagher et al., unpubl.). These studies exploited the observation that the rDNA allele of inbred strain *C3* has a replication and/or maintenance advantage over that of inbred strain *B* when both alleles reside in the same macronucleus (Pan et al. 1982). Thirteen *rmm* (*r*DNA *m*aturation and *m*aintenance) mutations that abolish the advantage of *C3* rDNA have been characterized (Larson et al. 1986; Yaeger et al. 1989; Kapler and Blackburn 1994; Kapler et al. 1994; R.C. Gallagher et al., unpubl.). These mutations fall into two major classes: (1) maturation mutants (*rmm6, 10, 11, 12,* and *13*) that show defects during excision, palindrome formation, and amplification in the developing macronucleus (see above) and (2) maintenance mutants (*rmm1, 2, 3, 4, 5, 7, 8,* and *9*) that appear to affect rDNA replication and/or maintenance during vegetative growth. Several mutations of the second class have been mapped to the 5′ NTS (Larson et al. 1986; Yaeger et al. 1989; R.C. Gallagher et al., unpubl.) both within the physically mapped origin region and in the rRNA promoter (Fig. 3). The replication disadvantage exhibited by the *B* rDNA allele relative to *C3* rDNA is the result of a 42-bp deletion that removes sequences immediately downstream from the type Ib repeat in domain 2. Three mutations (*rmm1, 4,* and *7*) alter the same type I repeat (Ib) in the origin region, whereas a fourth (*rmm3*) alters a type I repeat (Ic) located

less than 100 bp from the transcription initiation site (Fig. 2). On the basis of these studies, it was proposed that competition for limiting *trans*-acting factors that interact with the type I repeats could explain the differential replication properties of rDNA alleles (Larson et al. 1986). In addition, the *rmm8* mutation has recently been shown to alter another conserved promoter sequence (R.C. Gallagher and E.H. Blackburn, unpubl.). The *rmm8* and *rmm3* promoter changes are about 0.6 kb from the mapped origin region, suggesting the possibility of long-range interactions between these regions.

The importance of the 5′NTS region in rDNA replication and/or maintenance has been directly demonstrated in experiments using rDNA constructs to transform macronuclei by microinjection or electroporation (Tondravi and Yao 1986; Yu et al. 1988; Yu and Blackburn 1989, 1990; Gaertig and Gorovsky 1992; Pan and Blackburn 1995). Circular constructs containing only one half of the palindrome or one copy of the 5′NTS alone do transform macronuclei, but they do so by homologous recombination with the endogenous rDNA (Yu et al. 1988). In contrast, constructs containing a tandem duplication of the 5′NTS adjacent to the rest of the rRNA gene exhibit both autonomous replication properties and a replication advantage over the endogenous palindromic molecules: Intermolecular homologous recombination events between the transformed construct and the endogenous rDNA give rise to rDNA replicons containing progressively larger numbers of tandem 5′NTS repeats (Yu and Blackburn 1990; Romero and Blackburn 1995). Cells eventually accumulate linear rDNA minichromosomes carrying the rRNA coding sequences linked to more than 30 copies of the 5′NTS repeats. These rDNA replicons are maintained at high copy number and often completely replace the endogenous rDNA allele. Similarly, constructs containing the same tandem duplication of the 5′NTS unlinked to rRNA gene sequences also replicate autonomously and give rise to plasmids containing long tandem arrays of solely 5′NTS repeats, lacking the original bacterial vector sequence (Pan and Blackburn 1995). These tandem 5′NTS arrays exhibit a strong replication advantage in the absence of selection and eventually accumulate to about 10% of the total cellular DNA.

A "minimal" origin of replication for the rDNA minichromosome has not yet been clearly defined. The limitation in currently available systems lies in the fact that any introduced replicon has to compete with the rRNA genes required in high copy numbers for viability. However, the genetic analysis described above, together with recent deletion analyses, suggests that a large portion of the 5′NTS region is required for replication and/or maintenance of the rDNA in the macronucleus (M.-C. Yao,

pers. comm.). Hence, the functional origin of replication in *Tetrahymena* rDNA may be considerably larger than the compact chromosomal origins of *S. cerevisiae* (Marahrens and Stillman 1994). Moreover, as with many eukaryotic viral origins, replication initiation and transcription are likely to share functional controlling elements.

Inhibition of Origin Function in rDNA Constructs by Transcription through the Origin

The replication advantage displayed by the tandem duplication of the 5′NTS plus promoter segment occurs only when the upstream copies of the 5′NTS carry an inactivated rRNA promoter. Such inactivation was first proposed for a spontaneous mutation that occurred in a highly conserved sequence in the rRNA promoter (+G at position −20, see Fig. 3) (Yu and Blackburn 1989) and allowed this tandemly repeated region to be maintained. Subsequently, the +G promoter mutation was shown directly to destroy promoter function (Pan et al. 1995). Failure to maintain tandem wild-type 5′NTS-plus-promoter segments appears to be due to transcription through the origin, because the replication advantage is restored by mutating or deleting the rRNA promoter of this 5′NTS (Yu and Blackburn 1989) or by inserting a transcriptional termination signal immediately downstream from the wild-type promoter (Pan et al. 1995).

Regulation of Vegetative rDNA Replication

Although competition for limiting amounts of a *trans*-acting replication factor that binds type I repeat sequences may be an important factor in rDNA maintenance control, it cannot be the sole determinant of rRNA gene copy number. When additional copies of the rDNA origin region were introduced into macronuclei, either linked or unlinked to rRNA genes, the overall copy number of rRNA coding sequences remained constant, even though rDNA molecules with up to 30 tandem copies of the origin region accumulated over time (Yu and Blackburn 1990). The rRNA gene copy number also remained constant in the presence of a threefold excess of tandem repeats of the origin region located on separate molecules from the rRNA genes themselves (Pan and Blackburn 1995). Thus, rRNA gene dosage is not controlled solely by monitoring the copy number of the origin region, but must involve additional sequences either in the rRNA coding region or the 3′NTS (Pan and Blackburn 1995).

DNA/Protein Interactions in the rDNA Origin Region

DNA/protein interactions in the replication origin region have been characterized by DNase I and Fe-EDTA footprinting of partially purified factors on origin DNA (Umthun et al. 1994; Hou et al. 1995) and DNase I, DMS, and $KMnO_4$ footprinting of this region in nuclei (Pan et al. 1995; R.C Gallagher and E.H. Blackburn, unpubl.). These studies reveal a complex pattern of protections and hypersensitivities throughout the origin and promoter regions, with pronounced protection of nucleotides within and adjacent to the 33-nucleotide conserved type I repeat sequence. Two classes of proteins that bind the type I repeat in vitro have been identified and characterized using synthetic oligonucleotides in electrophoretic mobility shift assays (Umthun et al. 1994). One of these, ds-TIBF, binds preferentially to duplex DNA and exhibits only moderate specificity for type I repeat sequences. In contrast, an abundant single-stranded DNA-binding protein, ssA-TIBF, specifically recognizes the A-rich strand of the type I repeat sequence. Quantitative binding competition experiments demonstrate that sequences in 3′ flanking DNA, in addition to those in the 3′ end of the conserved type I repeat, are important determinants for binding (Hou et al. 1995). Recently, the most highly purified fractions (~70,000-fold enriched) of ssA-TIBF have been shown to contain an ATP-dependent DNA helicase activity, suggesting the exciting possibility that the ssA-TIBF type I repeat-binding activity and the copurifying DNA helicase activity are functionally associated in vivo (C. Du et al., unpubl.).

The ssA-TIBF exhibits differential affinity for *C3* versus *B* rDNA sequences in vitro, which parallels differences in the ability of the *C3* and *B* alleles to compete for replication and/or maintenance in the macronucleus. There is at least a 25-fold higher affinity of ssA-TIBF for *C3* versus *B* rDNA sequences (Hou et al. 1995). It should be noted, however, that several induced *C3-rmm* mutations are due to a single base pair deletion or insertion in the central tract of 11 A residues in copies of the type I repeat (Larson et al. 1986; Yaeger et al. 1989; W.-L. Shaiu and D.L. Dobbs; R.C. Gallagher et al., unpubl.). DNase I footprinting experiments using purified ssA-TIBF indicate that it strongly protects nucleotides at the 3′ end of, and flanking, the type I repeat, but makes few contacts in the central A+T-rich region (Hou et al. 1995). In contrast, both strands of the A+T tract are protected in footprinting experiments performed with S100 extracts of *Tetrahymena* cells (Umthun et al. 1994). It seems likely, therefore, that additional proteins that recognize type I repeat sequences could be involved in the replication phenotypes of these *rmm* mutants. The abundance and high affinity of ssA-TIBF for type I repeats suggests

that it may have an important function in the cell. Since no reliable sequence-dependent in vitro replication system has been reported in *Tetrahymena*, it is not yet possible to directly assess the role of ssA-TIBF in rDNA replication.

The conserved 16-bp type III repeats have been identified as DNA topoisomerase I binding sites in vitro and in vivo (Bonven et al. 1985). The association of topoisomerase I with rDNA sequences was first detected in experiments in which treatment of rDNA chromatin with SDS prior to high ionic strength buffer resulted in site-specific single-strand cleavages by the endogenous topoisomerase I, which remained covalently attached to the 3′ end generated by cleavage (Bonven and Westergaard 1982). Three topoisomerase I cleavage sites map within each of the three nuclease hypersensitive domains in the 5′NTS (domains 1 and 2 and the rRNA promoter; see Fig. 3); a fourth maps near the transcription termination site (Gocke et al. 1983; Bonven et al. 1985). Since these topoisomerase I binding sites are found in the replication origin region and at both ends of the transcription unit, topoisomerase I/type III repeat interactions may play a dual role in the replication and transcription of the rDNA (Bonven et al. 1985).

SUMMARY AND FUTURE DIRECTIONS

The rDNA of *Tetrahymena* has some unique advantages as a model eukaryotic system for studying replication control. The presence of the rRNA gene as a single-copy germ-line gene, making Mendelian genetics possible, is most unusual among both eukaryotes and prokaryotes. The rDNA minichromosome is generated by a set of closely regulated, developmentally programmed events, which can be induced to occur synchronously in large cultures, allowing for molecular analysis. The small size of this DNA, its high level of amplification, and its highly ordered chromatin facilitate molecular and biochemical characterizations, making it comparable to viral replicons in these respects. However, the *Tetrahymena* rDNA represents an unusual situation in that it is a nuclear cellular replicon under both cell-cycle control and copy-number control. The copy-number control of the replicon permits competition between replicons in vivo, allowing very small differences in such competition to be detected over several vegetative divisions with high sensitivity.

Although additional determinants of rDNA origin function very likely include aspects of chromatin structure that have not yet been investigated, the biochemical, molecular, and classic genetic studies of rDNA replication suggest that the functional rDNA origin in vegetatively divid-

ing cells is contained within an approximately 1.9-kb region corresponding to the 5′NTS. *cis*-Acting sequences involved in replication control appear to be functionally redundant and dispersed throughout a region of low intrinsic helical stability. The type I repeat, for example, is present in four copies within the 5′NTS. As one of very few genetically defined replication control elements so far described in eukaryotic cellular origins, it is interesting that the type I repeat is also an rRNA gene promoter component. Hence, the involvement of transcription factor binding sites in origin function, which has been clearly demonstrated in eukaryotic viruses and yeast, also appears to be a feature of the *Tetrahymena* rDNA origin. No putative replication initiator proteins or binding sites have been identified in *Tetrahymena*.

The recent development of rDNA origin-containing vectors with selectable markers that replicate autonomously in macronuclei (Gaertig et al. 1994; Pan and Blackburn 1995), and progress toward developing an in vitro replication system (D. Dobbs, unpubl.), should facilitate further delineation of the *cis*-acting elements and other functional components in rDNA origin function.

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