1766–1774 Nucleic Acids Research, 1995, Vol. 23, No. 10

Modular structural elements in the replication origin region of *Tetrahymena* rDNA

Chunying Du[1,2], Rita P. Sanzgiri[1,3], Wen-Ling Shaiu[1], Joong-Kook Choi[2], Zhen Hou[1,2], Robert M. Benbow[2,3,4] and Drena L. Dobbs[1,2,3,*]

Department of Zoology and Genetics, [1]Signal Transduction Training Group, [2]Molecular, Cellular and Developmental Biology Program, [3]Interdepartmental Genetics Program and [4]Nucleic Acid Research Facility, 2114 Molecular Biology Building, Iowa State University, Ames, IA 50011, USA

Received January 20, 1995; Revised and Accepted April 4, 1995

ABSTRACT

Computer analyses of the DNA replication origin region in the amplified rRNA genes of *Tetrahymena thermophila* identified a potential initiation zone in the 5′NTS [Dobbs, Shaiu and Benbow (1994), *Nucleic Acids Res.* 22, 2479–2489]. This region consists of a putative DNA unwinding element (DUE) aligned with predicted bent DNA segments, nuclear matrix or scaffold associated region (MAR/SAR) consensus sequences, and other common modular sequence elements previously shown to be clustered in eukaryotic chromosomal origin regions. In this study, two mung bean nuclease-hypersensitive sites in supercoiled plasmid DNA were localized within the major DUE-like element predicted by thermodynamic analyses. Three restriction fragments of the 5′NTS region predicted to contain bent DNA segments exhibited anomalous migration characteristic of bent DNA during electrophoresis on polyacrylamide gels. Restriction fragments containing the 5′NTS region bound *Tetrahymena* nuclear matrices in an *in vitro* binding assay, consistent with an association of the replication origin region with the nuclear matrix *in vivo*. The direct demonstration in a protozoan origin region of elements previously identified in *Drosophila*, chick and mammalian origin regions suggests that clusters of modular structural elements may be a conserved feature of eukaryotic chromosomal origins of replication.

INTRODUCTION

Sequence analyses of higher eukaryotic chromosomal origin regions have typically failed to identify simple consensus sequences analogous to those involved in site-specific initiation in prokaryotes, the yeast *Saccharomyces cerevisiae* and mammalian viruses (for reviews, see 1–5). Consequently, we and others have proposed that initiation of replication in higher eukaryotic chromosomes occurs within initiation zones that are more complex than previously envisaged (1,3,6–8). To gain further insight into the genetic and structural determinants for initiation of DNA replication in eukaryotic chromosomes, we devised an algorithm to identify putative initiation zones in eukaryotic DNA sequences based on the hypothesis that clusters of redundant modular sequence elements may provide origin function when arrayed in a proper context (9). To begin to characterize elements that may determine the localization of initiation events in eukaryotic chromosomal origins of replication, we have focused on the origin region of the well-characterized rRNA genes (rDNA) in the ciliated protozoan, *Tetrahymena thermophila*.

In *Tetrahymena*, the rDNA exists as a single chromosomal copy in the germline micronucleus and as highly amplified, autonomously replicating molecules in the somatic macronucleus (reviewed in 10–14). The amplified macronuclear rDNA consists of linear 21 kb palindromes in which a central non-transcribed spacer region (NTS) separates two divergently transcribed 35S pre-rRNA coding regions (Fig. 1). Physical mapping, genetic analyses and functional studies exploiting macronuclear transformation have demonstrated that sequences in the 5′NTS are involved in regulation of macronuclear rDNA replication (reviewed in 10,13,14). During vegetative growth, the macronuclear rDNA is replicated bidirectionally from an origin of replication in the 5′NTS. The vegetative origin has been mapped by electron microscopy to a region ~650 ± 300 bp from the center of the palindrome (15). Several specific mutations that affect rDNA replication and/or maintenance in the macronucleus have been mapped to the 5′NTS (16,17; G. Kapler, R. Gallagher and E. Blackburn, personal communication; W.-L. Shaiu and D. L. Dobbs unpublished data). Moreover, DNA fragments encompassing the 5′NTS promote autonomous replication of plasmids in the macronucleus (18–20) and plasmids containing multiple copies of the origin region have a replication advantage over those with fewer copies (18,20). Transformation experiments using partial deletions of the rDNA suggest that the minimal sequences required for rDNA replication/maintenance in the macronucleus include a large portion of the 5′NTS (P. Blomberg and M.-C. Yao, personal communication). Finally, two dimensional gel electrophoretic analyses are consistent with a

* To whom correspondence should be addressed

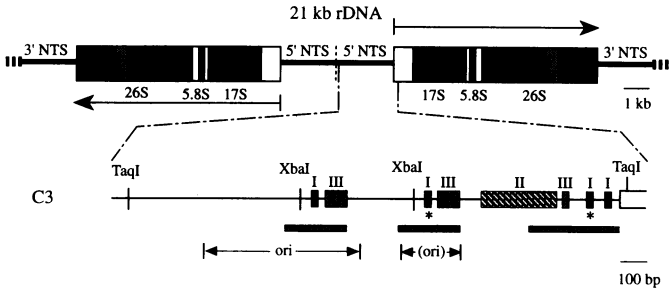

Figure 1. Structure of *Tetrahymena* macronuclear rRNA genes. Schematic diagram of the 21 kb palindromic rDNA. Arrows indicate rRNA transcription units. rRNA coding sequences are shown as solid boxes, transcribed spacers as open boxes, and the intron within the 26S gene as a grey box. Hatched lines represent telomeric sequences. The lower line is an enlarged diagram of the 5'NTS region of the C3 rDNA allele (16). Putative replication origin(s) are indicated (15,85). Shaded bars indicate nuclease hypersensitive regions in chromatin (86). I, II and III refer to Type I, II and III repeated sequence elements conserved among several ciliate species (87,88). Repeated elements are not drawn to scale. The Type I repeat closest to the coding region is required for rRNA transcription *in vitro* (81; R. Pearlman, personal communication). Type I repeats in which mutations that affect rDNA replication/maintenance have been identified are indicated by asterisks (*) (16,17; W. -L. Shaiu and D. L. Dobbs, unpublished data). Type III repeats are binding and cleavage sites for DNA topoisomerase I (89).

relatively large origin region and also indicate that replication initiation events in vegetative cells occur exclusively within the 5'NTS (14; G. Kapler, personal communication).

To begin to characterize features of this initiation zone that may contribute to origin function, we identified specific DNA sequences and structural elements within the *Tetrahymena* rDNA that were shared with five other mapped eukaryotic chromosomal replication origin regions (9). Computer analyses revealed that all six origin regions contain modular sequence elements, including predicted DNA unwinding elements (DUEs), bent DNA, nuclear matrix or scaffold-associated regions (MARs/SARs), pyrimidine tracts, yeast autonomously replicating sequences (ARSs) and transcriptional regulatory sequences (1,9). DUEs are inherently unstable duplex DNA segments associated with origins of replication (21). Mutations that raise the helical stability of DUEs reduce or eliminate origin function in *Escherichia coli* (21), SV40 (22) and yeast (23–25). Bent DNA sequences, short DNA segments that display local curvature of the double helical axis, are a conserved feature of prokaryotic replication origins (26) and have been identified within several eukaryotic origin regions (e.g., 27–31). Stably bent DNA structures are usually associated with dA tracts (2–6 bases) that occur with a 10–11 bp periodicity (32). MARs and SARs are sites at which looped domains of eukaryotic chromosomal DNA are attached to the nuclear matrix or scaffold. Several studies have provided evidence for close proximity of origin regions to MARs/SARs (reviewed in 33–35). Pyrimidine tracts, which have been shown to be preferred start sites for DNA polymerase-primase (36,37), ARS consensus sequences (38) and a variety of transcription factor binding sites are also found in association with eukaryotic replication origin regions (1,8,27,39,40).

The computer analyses of *Tetrahymena* rDNA identified clusters of modular sequence elements in the 5'NTS region of the rDNA, coincident with the previously mapped origin of replication, and in the 3'NTS (9). In this study, predicted DUE-like elements, intrinsically bent DNA segments, and nuclear matrix-associated DNA fragments in the origin region were experimentally verified. The demonstration that these modular structural elements are found in the origin region, but are lacking in the coding region of the rDNA, suggests that they may play a determinative role in initiation of DNA replication.

MATERIALS AND METHODS

Computer analyses of *Tetrahymena* rDNA sequences

The complete sequence of *Tetrahymena* rDNA (strain B) from the GenBank/EMBL database [accession number X54512; (see 41)] was analyzed previously (9). Briefly, putative DUEs were identified using the program Oligo 4.0 (National Biosciences, Wolfgang Rychlik, 1992) which calculates duplex stability based on nearest neighbor dinucleotide free energy values (42). A modified version of the program (provided by Dr Rychlik) was used to transfer output to Microsoft Excel for graphic display. The window size for helical stability analyses was 100 bp; each plotted data point corresponds to the free energy (ΔG in kcal/mol) required to unwind a helical segment consisting of the 100 bp immediately downstream. DUE-like elements predicted using the Oligo 4.0 program were identical to those identified using the Thermodyn program provided by Kowalski and colleagues (43). A major DUE-like element was defined as ≥200 contiguous nucleotides with an average ΔG at least 15 kcal/mol less negative than the mean for the sequence analyzed. A minor DUE-like element was defined as a region with an average ΔG at least 10 kcal/mol less negative than the mean.

Putative bent DNA segments were identified using ENDS ratio plots generated by the GentlBen program provided by Dr J. N. Anderson (44). The ENDS ratio is a measure of DNA bending calculated as the ratio of the contour length of a given axis to the shortest distance between its ends (45). A window size of 120 bp and step size of 10 bp were used.

Consensus sequences were localized using the Genetics Computer Group (GCG) Sequence Analysis Software Package (46). The program Findpatterns was used to identify the following sequence elements: *Drosophila melanogaster* SAR consensus sequences, AATAAAYAAA, TTWTWTTWTT, WADAWAYAWW, TWWTDTTWWW (47,48); murine MAR consensus sequence AATATTTTT (49): *S.cerevisiae* ARS consensus sequence, WTTTAYRTTTW (38); and pyrimidine tracts (Y_{12}) (36,37,50). In these sequences, Y = C or T, W = A or T, D = A, C or T, and R = A or G.

Plasmids

Plasmids containing the replication origin region of macronuclear rDNA from *T.thermophila* were constructed. The 1.9 kb *Taq*I restriction fragment encompassing the entire 5'NTS (see Fig. 1) was cloned into the *Acc*I site of pUC119 to generate plasmid pUC.5'NTS (see Fig. 4A). A *Hin*dIII–*Kpn*I restriction fragment containing the insert was subsequently transferred into the plasmid pBluescriptII SK (Stratagene) to generate the plasmid pSK.5'NTS (see Fig. 5A). Plasmid prD4-1, containing a tandem duplication of the 5'NTS region and one complete copy of the rRNA coding region and 3'NTS cloned into pBR322 (51), was provided by E. Blackburn. Supercoiled plasmid DNAs were prepared by alkaline lysis and purified on CsCl–EtBr gradients (52).

Mapping of mung bean nuclease-hypersensitive sites

Mung bean nuclease hypersensitivity assays were performed essentially as described (21,23). Supercoiled plasmids (4 μg) were incubated at 37°C in 32 μl of 10 mM Tris–HCl, pH 7.0, 1 mM EDTA. Mung bean nuclease (3 U, Boehringer Mannheim) was added and 1 μg samples were removed at 0, 10, 20 and 30 min. The cleaved circular DNA samples were pooled, purified by phenol–chloroform extraction and ethanol precipitation, digested with *Hin*dIII, *Eco*RI or *Sca*I, and 5′ end-labeled with ^{32}P using T4 polynucleotide kinase (52). Unincorporated label was removed using G-25 columns and samples were denatured with glyoxal before electrophoresis in 1.0% agarose gels in 10 mM Na_2HPO_4/NaH_2PO_4 buffer, pH 7.0. After electrophoresis, gels were dried and exposed to XAR-5 film or phosphor screens for analysis using a Molecular Dynamics PhosphorImager. The sites of mung bean cleavage were established by determining the distance from the labeled end to the nick in a given fragment as previously described (23).

Electrophoretic analysis of bent DNA segments

To localize stably bent DNA segment(s) in the 5′NTS of the rDNA, three sub-fragments of the 5′NTS (corresponding to the *Taq*I–*Xba*I, *Xba*I–*Xba*I and *Xba*I–*Taq*I fragments shown in Fig. 1) were generated by digestion of plasmid pSK.5′NTS with *Xba*I (see Fig. 5A below). The mobilities of the *Xba*I restriction fragments were compared after electrophoresis in 1.5% agarose gels at room temperature versus electrophoresis in 5% polyacrylamide gels at 4°C (53).

To investigate whether the predicted strongly bent DNA segment located in the 430 bp *Xba*I–*Xba*I fragment could promote DNA bending in another sequence context, the fragment was subcloned from plasmid pUC.5′XX430 into the *Xba*I site of vector pBend2 (54), kindly provided by Dr S. Adhya. The derivative plasmid, pBend.5′XX430, was sequenced through the polylinker and across one *Xba*I site to determine the orientation of the insert. pBend.5′XX430 contains two repeats of a multiple cloning region surrounding the insert, allowing generation of restriction fragments of uniform length that contain the predicted bent DNA segment at different positions relative to the ends of the fragment (see Fig. 6A below). After restriction digestion of pBend.5′XX430 with the indicated enzymes, the migration of the insert-containing fragments was compared on 1.2% agarose gels at room temperature versus 6% polyacrylamide gels at 4°C.

Assay of DNA binding to nuclear matrices

To identify regions of the rDNA that contain nuclear matrix binding sequences, nuclear matrices were prepared from isolated *Tetrahymena* macronuclei and incubated with rDNA restriction fragments in an *in vitro* binding assay based on that of Cockerill and Garrard (49). 200 ml cultures of *T.thermophila* strain C3V cells were grown in 2% PPYS medium [2% proteose peptone, 0.2% yeast extract, 0.03% Sequestrene (Ciba-Geigy)] to a density of 2.5×10^5 cells/ml at 30°C. Cells were collected by centrifugation and washed once in 10 mM Tris–HCl, pH 7.5. *Tetrahymena* macronuclei were isolated in a sucrose gradient according to the procedures of Higashinakagawa *et al.* (55), and nuclear matrix isolation was performed essentially according to Dijkwel *et al.* (56). Briefly, *Tetrahymena* cell pellets were resuspended in 0.2 M sucrose/0.25 mM $MgCl_2$, and lysed with 0.2% NP-40. Macronuclei were pelleted onto a 2.1 M sucrose cushion in a Beckman Ti 70.1 rotor at 40 000 *g* for 2 h. Macronuclear pellets were washed with cell washing buffer [10 mM Tris–HCl, pH 7.4, 20 mM KCl, 0.05 mM spermine, 0.125 mM spermidine, 1% thiodiglycol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)], and resuspended in the same buffer at 100 A_{260} U/ml. After the addition of $CuSO_4$ to 1 mM, 100 μl aliquots were incubated for 20 min at 37°C. Nine ml of lithium diiodosalicylate (LIS) extraction buffer [10 mM LIS (Sigma), 100 mM LiOAc, 20 mM Hepes–NaOH, pH 7.4, 0.1% digitonin, 1 mM EDTA] was added slowly and samples were incubated for 10 min at room temperature. The histone-depleted macronuclear matrices were pelleted and washed four times with matrix washing buffer (cell washing buffer, but with 25 mM Tris–HCl, pH 7.4, 70 mM NaCl, 0.1% Digitonin) followed by a final wash with restriction digestion buffer (10 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT). Matrices were resuspended in restriction buffer and incubated with 1000 U each of *Dra*I and *Hae*III overnight at 37°C. Reactions were stopped by addition of 10 mM EDTA.

For *in vitro* binding assays, 50 ng of ^{32}P-labeled restriction fragments of plasmid prD4-1 and 6 μg of sonicated calf thymus DNA were incubated with aliquots of the digested nuclear matrices for 3 h at room temperature with gentle agitation. An equal volume of ice-cold matrix washing buffer was added and the reaction mixtures were centrifuged at 8000 *g* for 10 min at 4°C. The pellets contained plasmid fragments that remained attached to the nuclear matrix. The pellets were washed once with the same buffer and resuspended in 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.1% SDS and digested with 200 mg/ml proteinase K for 4 h at 50°C. DNA samples were recovered by phenol extraction and ethanol precipitation, and loaded on 0.8% agarose gels. Gels was dried, exposed to phosphor screens and analyzed using a Molecular Dynamics PhosphorImager.

RESULTS

Common modular sequence elements in *Tetrahymena* rDNA

The macronuclear rDNA sequence from *T.thermophila* was analyzed for modular sequence elements shared with chromosomal origins of replication in other eukaryotes (9). Two large clusters of elements were identified, one in the 5′NTS, in the region containing the previously mapped origin of replication (see Fig. 1), and another in the 3′NTS (Fig. 2). DUE-like elements, regions of intrinsically bent DNA, SAR consensus sequences, pyrimidine tracts and ARS consensus sequences were aligned within these regions. In contrast, no predicted DUE-like elements or bent DNA, and relatively few consensus sequences were identified in the transcribed region (Fig. 2). Three structural elements that may contribute to origin function—putative DUEs, bent DNA and matrix attachment sequences—were identified by the computer analyses. The predicted distributions of these structural elements in the 5′NTS and the adjoining 2 kb coding region are shown in Figure 3; in this study the presence of these elements in the rDNA origin region was verified experimentally.

The 5′NTS has an overall AT content of ~80% (Fig. 3B). Although AT-rich regions are often found in association with replication origins and would be expected to facilitate unwinding of duplex DNA, the free energy required for DNA unwinding does not strictly correlate with AT-richness. Instead, it is

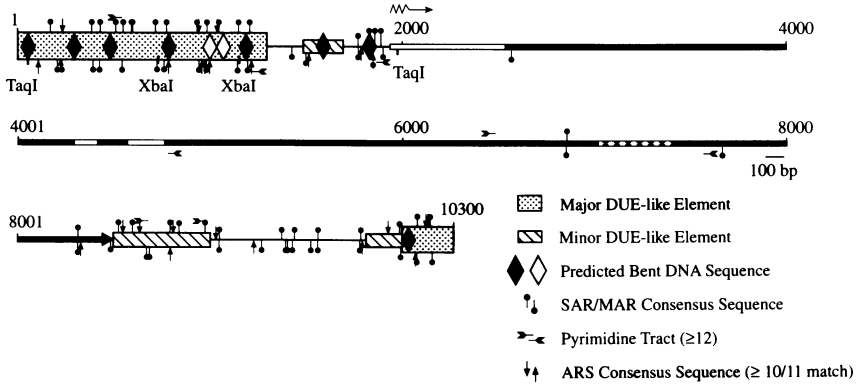

Figure 2. Arrangement of common modular structural elements in *Tetrahymena* rDNA. Schematic diagram of the location of common modular sequence elements in *Tetrahymena* rDNA identified by computer analyses (9). One half of the palindromic rDNA is shown. Nucleotide position 1 corresponds to the center of the palindrome and the beginning of the 5′NTS. The transcribed region is indicated by the thin box extending from position 1887 to 8522; rRNA coding sequences are shown as solid boxes, transcribed spacers as open boxes, and the intron within the 26S gene as a cross-hatched region. Sequence and structural elements depicted by symbols are described in the text. Major and minor DUE-like elements were delimited as described in Materials and Methods. Predicted bent DNA segments with ENDS ratios ≥1.2 are indicated by solid diamonds; two additional bent segments with ENDS ratios >1.19 are indicated by open diamonds (see text). Perfect matches to SAR consensus sequences, uninterrupted tracts of ≥12 pyrimidines and 10/11 or 11/11 matches to the ARS core consensus sequence are indicated.

dependent on the frequencies of nearest-neighbor dinucleotide sequences (42). Work of Kowalski and colleagues has shown that the propensity for a duplex DNA segment to unwind can be predicted using algorithms that calculate the free energy difference between the duplex and single-stranded states for that segment, and that easily unwound regions predicted by such thermodynamic analyses can be validated by monitoring their hypersensitivity to single strand-specific nucleases (23,43). Previously, we used the Oligo 4.0 program to analyze the helical stability of sequences in the rDNA and localize DUE-like elements (Fig. 3C). One predicted major DUE extends from position 1 to 1300 within the 5′NTS, encompassing a total of 2600 bp in the center of the palindromic rDNA molecule.

The predicted locations of intrinsically bent regions in the 5′NTS are shown in Figure 3D. Two of these with ENDS ratios >1.4, and two with ENDS ratios ≥1.3 are located within the major DUE-like element. Finally, the distribution of SAR/MAR consensus sequences (33,47–49) is shown in Figure 3E. These sequences are much more abundant in the 5′NTS than in the adjacent transcribed region: one broad cluster coincides with the DUE-like element, and a smaller cluster is located in the promoter region immediately upstream from the transcription start site.

Identification of a DUE-like element in the 5′NTS: mapping of mung bean nuclease-hypersensitive sites

DNA sequences that promote unwinding of duplex DNA molecules can be detected as sites that are hypersensitive to single strand-specific nucleases in negatively supercoiled plasmid DNA (21,23). To corroborate the DUE-like element predicted by thermodynamic analysis of the rDNA replication origin region, we mapped mung bean nuclease-hypersensitive sites in pUC.5′NTS, a plasmid containing a 1.9 kb *Taq*I–*Taq*I restriction fragment corresponding to the 5′NTS of the rDNA (Fig. 4A). Supercoiled pUC.5′NTS DNA was incubated with mung bean nuclease and subsequently linearized with the indicated restriction enzymes. The products were ^{32}P-labeled, denatured in glyoxal and separated by agarose gel electrophoresis. As shown in Figure 4B, each restriction enzyme digestion produced five labeled bands: a 5.1 kb fragment corresponding to unit length DNA (i.e. not cleaved by mung bean nuclease) and two pairs of smaller bands that provide a measure of the distance from the restriction enzyme sites to the predominant mung bean nuclease cleavage sites (thick arrows in Fig. 4A). The size of the restriction fragments in each band was estimated from the migration position of the center of each band. It should be emphasized the bands are broad, suggesting that each band corresponds to a population of fragments of similar size that presumably result from nicking within ~100 bp regions by mung bean nuclease. The pairs of bands, 1.1 and 4.0 kb from the *Eco*RI digest, 2.5 and 2.6 kb from the *Sca*I digest, and 0.9 and 4.2 kb from the *Hin*dIII digest, taken together, unambiguously assign one nuclease hypersensitive site to the 5′NTS at a location 0.9 kb downstream from the *Hin*dIII site (Fig. 4A, right arrow). Similarly, the remaining pairs of bands, 1.6 and 3.5 kb from *Eco*RI, 2.1 and 3.0 from *Sca*I, and 0.4 and 4.7 kb from *Hin*dIII, map another nuclease-hypersensitive site to the 5′NTS at a location 0.4 kb downstream from the *Hin*dIII site (Fig. 4A, left arrow). These two sites (indicated by black bars in Fig. 3C) correspond precisely with the energy minima within the broad DUE predicted by thermodynamic analyses. No other major nuclease hypersensitive sites were observed, either in rDNA or vector sequences.

Localization of intrinsically bent DNA segments in the rDNA origin region

Sequence-directed DNA curvature (bent DNA segments) can be localized by comparing the electrophoretic mobilities of DNA fragments in agarose versus polyacrylamide gels (53). In polyacrylamide gels, fragments containing bent DNA segments migrate with higher apparent molecular weights than expected; these anomalous migrations are not observed in agarose gels where each fragment migrates as predicted from its molecular weight. Restriction fragments encompassing the entire 5′NTS of *Tetrahymena* rDNA have previously been shown to exhibit anomalous migration in polyacrylamide gels, suggesting one or more bent DNA segments in the 5′NTS (57,58). In contrast, various restriction fragments of the rDNA coding region do not exhibit anomalous migration in polyacrylamide gels (data not shown).

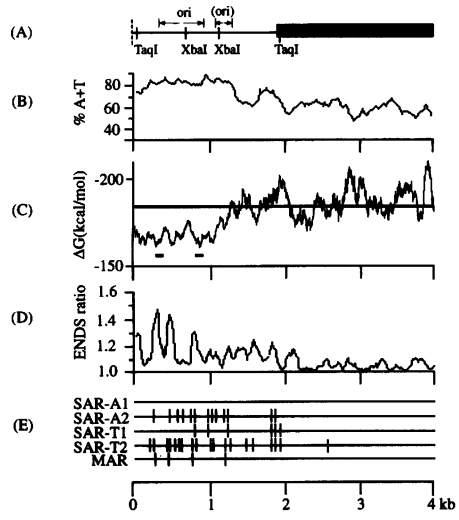

Figure 3. Predicted regions of helical instability, intrinsic DNA bending and nuclear matrix attachment in the rDNA origin region. (**A**) Diagram of the 5′NTS region of macronuclear rDNA and a portion of the adjacent transcribed region. The dashed line at the left end of the 4 kb segment shown corresponds to the center of the 21 kb palindromic rDNA. The 5′NTS is shown as a thin line and the transcribed region as a solid box. (**B**) A + T content of the sequence depicted in (A). Each point represents % A+T of a 120 bp segment as a function of its position in the rDNA sequence. (**C**) Predicted regions of helical instability. Helical stability of duplex DNA segments was calculated and graphed as a function of position (9; see Materials and Methods). Each point represents the free energy (ΔG in kcal/mol) required to unwind a 100 bp window of duplex DNA. The bold line indicates the average ΔG value for the complete 21 kb rDNA sequence. Valleys represent inherently unstable duplex DNA segments. The positions of the two major mung bean nuclease-hypersensitive sites in the 5′NTS region in supercoiled plasmids (see Fig. 4) are indicated by solid bars. (**D**) Predicted regions of intrinsic DNA bending. ENDS ratios were determined using a 120 bp window and graphed as a function of position (9; see Materials and Methods). The peaks represent regions predicted to display most pronounced DNA bending. (**E**) Predicted sites of nuclear matrix attachment. Perfect matches to SAR and MAR consensus sequences (9; see Materials and Methods) are indicated. Many additional imperfect matches also present in the region are not shown.

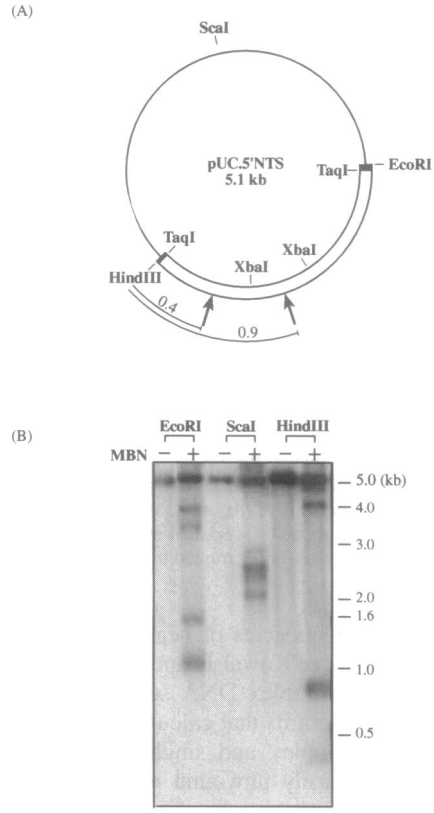

Figure 4. Identification of mung bean nuclease-hypersensitive sites in the rDNA origin region. (**A**) Map of the 5′NTS plasmid pUC.5′NTS showing the locations of mung bean nuclease-hypersensitive sites and pertinent restriction enzyme sites. The 5′NTS region (nucleotides 62–1971) of the palindromic rDNA sequence is represented by an open box, the pUC119 cloning vector is shown as a thin line with polylinker sequences represented by filled boxes. The arrows on the outer circumference indicate the nuclease-hypersensitive sites mapped in (B). *Hin*dIII and *Eco*RI sites in the polylinker and the *Sca*I site in the cloning vector are indicated on the outer circumference. *Taq*I and *Xba*I sites in the 5′NTS are indicated on the inner circumference. (**B**) Mapping the mung bean nuclease-hypersensitive sites in the 5′NTS plasmid. Supercoiled plasmid DNA was nicked with mung bean nuclease, linearized with the indicated restriction enzyme, ^{32}P-labeled and denatured prior to agarose gel electrophoresis as described in Materials and Methods. MBN +, treated with mung bean nuclease; MBN –, untreated control. Sizes of single-stranded DNA markers are indicated.

The rDNA replication origin region was surveyed for stably bent DNA by examining the electrophoretic behavior of restriction fragments of the 5′NTS on 5% polyacrylamide gels (Fig. 5). Cleavage of the plasmid pSK.5′NTS with *Xba*I is expected to generate one large vector DNA fragment (2.9 kb) and three fragments of 0.67, 0.43 and 0.82 kb, corresponding to the *Taq*I–*Xba*I, *Xba*I–*Xba*I and *Xba*I–*Taq*I segments of the 5′NTS (Figs 1 and 5A). As shown in Figure 5B, a band migrating at 2.9 kb was observed for the vector DNA (fragment A), but all three remaining bands—B, C and D—migrated anomalously, with apparent molecular weights of 2.3, 1.9 and 0.5 kb, respectively. To assign these bands to the appropriate fragments of the 5′NTS, each band was excised from the polyacrylamide gel and re-analyzed by electrophoresis in a 1.5% agarose gel (Fig. 5C), along with a control sample of the *Xba*I-digested plasmid. In the agarose gel, each of the three bands migrated as expected (B = 0.8 kb, C = 0.7 kb, D = 0.4 kb). These results indicate that all three restriction fragments of the 5′NTS contain segments of bent DNA, in agreement with computer predictions based on ENDS ratio calculations (Fig. 3D).

The central *Xba*I–*Xba*I fragment, which coincides with the origin mapped by electron microscopy, contains several segments with high ENDS ratios, yet exhibited only a slight migration anomaly in the polyacrylamide gel shown in Figure 5B. Therefore, we analyzed this region in more detail by testing whether it could promote DNA bending in another sequence context. The *Xba*I–*Xba*I fragment was subcloned into plasmid pBend2, which contains a set of circularly-permuted cloning sites (54). Digestion of the derivative plasmid, pBend.5′XX430 with six different restriction enzymes generated a set of fragments of uniform length, but containing the predicted bent DNA segments at different positions relative to the fragment ends (Fig. 6A). Fragments containing more centrally located bent segments are more retarded in polyacrylamide gels than those with bent segments near their ends (54). As shown in Figure 6B, all fragments migrated to the same position on a 1.2% agarose gel (right panel); in contrast, they formed a ladder of anomalously

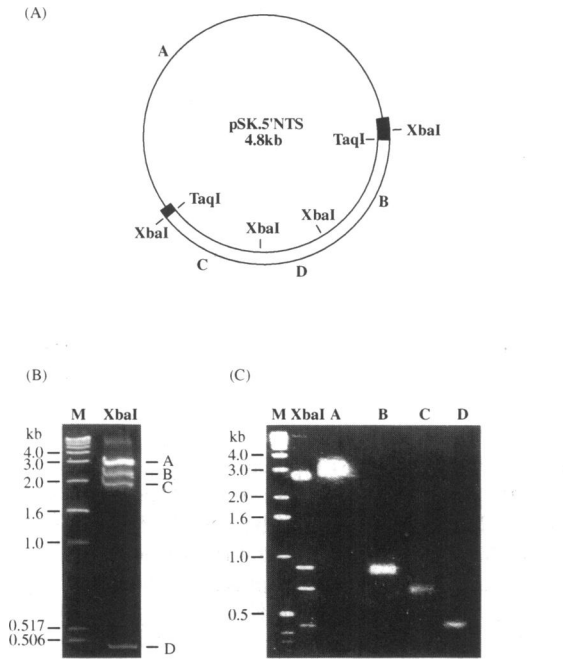

Figure 5. Identification of bent DNA segments in rDNA origin region. (**A**) Map of the 5′NTS plasmid pSK.5′NTS showing the locations of pertinent restriction enzyme sites. The 5′NTS region (nucleotides 62–1971) of the palindromic rDNA sequence is represented by an open box, the pBluescriptII SK cloning vector is shown as a thin line with polylinker sequences represented by filled boxes. Two *Xba*I sites in the polylinkers are indicated on the outer circumference (see Materials and Methods). *Taq*I and *Xba*I sites in the 5′NTS are indicated on the inner circumference. Letters indicate the identities of the fragments determined in (C). (**B**) pSK.5′NTS DNA was digested with *Xba*I and subjected to electrophoresis in a 5% polyacrylamide gel. Lane M shows molecular weight markers. (**C**) Bands isolated from the polyacrylamide gel in (B) were re-analyzed by electrophoresis in a 1.5% agarose gel. The letter above each gel lane in the agarose gel indicates the fragment excised from the polyacrylamide gel. pSK.5′NTS DNA was digested with *Xba*I and loaded directly on the agarose gel for comparison. Lane M shows molecular weight markers.

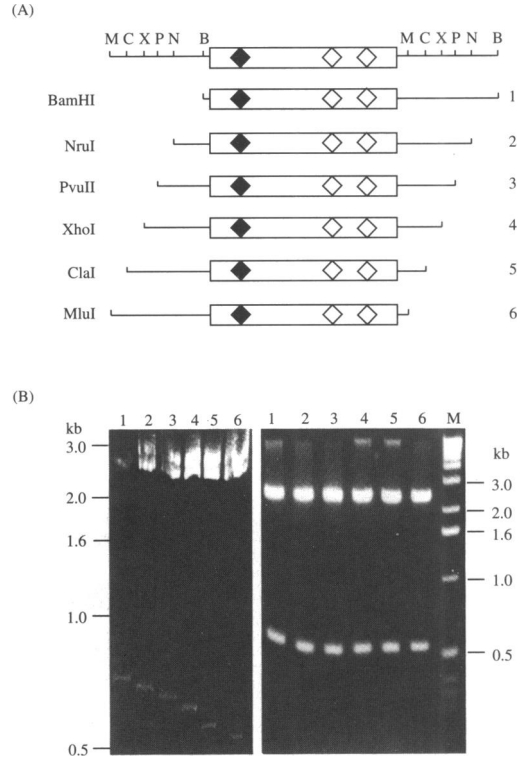

Figure 6. Electrophoretic analysis of bent DNA in the rDNA origin region. (**A**) Schematic diagram of the *Xba*I–*Xba*I fragment cloned into a circularly-permuted polylinker region in pBend.5′XX430. The box represents the 430 bp *Xba*I–*Xba*I fragment from the rDNA origin region cloned into a unique *Xba*I site in a pBend2 vector (see Materials and Methods). The positions of the predicted bent segments are indicated by diamonds (solid diamond indicates ENDS ratio ≥1.3; open diamonds indicate ENDS ratios >1.19). The thin line represents the 121 bp polylinker region of the vector, with the positions of relevant restriction sites indicated. (For clarity, the polylinker has been drawn on a 2-fold expanded scale.) Diagrams below represent the 550 bp DNA fragments generated by digestion of pBend.5′XX 430 with *Bam*HI (B), *Nru*I (N), *Pvu*II (P), *Xho*I (X), *Cla*I (C) and *Mlu*I (M). (**B**) Migration of restriction fragments of pBend5′XX430 in polyacrylamide versus agarose gels. pBend.5′XX430 DNA was digested with the restriction enzymes indicated in (A) and the digestion products were fractionated on a 6% polyacrylamide gel (left panel) or 1.2% agarose gel (right panel). Lane 1 (*Bam*HI), lane 2 (*Nru*I), lane 3 (*Pvu*II), Lane 4 (*Xho*I), lane 5 (*Cla*I), lane 6 (*Mlu*I), lane M, molecular weight markers, with sizes indicated.

migrating bands on a 6% polyacrylamide gel (left panel), confirming that the *Xba*I–*Xba*I fragment contains bent DNA. The fragment generated by *Bam*HI digestion (lane 1) exhibited the greatest retardation, indicating that the bent segment predicted by the ENDS ratio of 1.3 (Fig. 6A, solid diamond) has less effect on the migration of the fragment in polyacrylamide gels than the two closely-spaced bent DNA segments with lower ENDS ratios of ~1.2 (Fig. 6A, open diamonds).

Identification of a nuclear matrix-binding region within the 5′NTS

To test whether the 5′NTS and 3′NTS regions of the rDNA contain sequences that bind nuclear matrices (MARs), we carried out *in vitro* binding assays as described by Cockerill and Garrard (49). Mixtures of ^{32}P-labeled rDNA restriction fragments were incubated with *Tetrahymena* nuclear matrices in the presence of unlabeled non-specific competitor DNA (see Materials and Methods). After centrifugation and washing, matrix-associated fragments were purified, separated by agarose gel electrophoresis, and visualized by autoradiography. By definition, specific fragments preferentially retained by nuclear matrices after competition contain an '*in vitro* MAR' (49). In the experiments shown in Figure 7, plasmid prD4-1, which contains a tandem duplication of the 5′NTS and a single copy of the rRNA coding and 3′NTS regions (51), was digested with different combinations of restriction enzymes. Digestion with *Hin*dIII (Fig. 7B) yielded a 4.4 kb fragment (band a) that contains both copies of the 5′NTS and associated strongly with the isolated nuclear matrices (59% bound). The coding region and 3′NTS were cleaved into several fragments. The coding region fragments (bands c, d, e and h) and one of the 3′NTS fragments (band g) did not associate with the nuclear matrices (<16% of each fragment bound), whereas the other 3′NTS fragment was associated (band f; 62% bound). In the experiment shown in Figure 7C, prD4-1 was multiply digested with *Nhe*I, *Bam*HI, *Eco*RI and *Xho*I. Both 5′NTS fragments (bands c′ and d′) bound isolated nuclear matrices very strongly (>98% bound), but the coding region fragments did not (e.g., band a′, 22% bound). The upstream portion of the 3′NTS (band e′), which

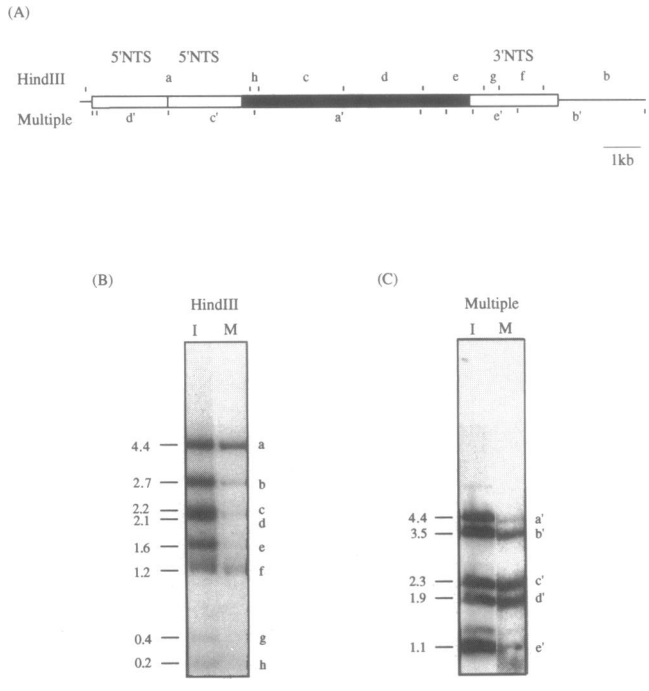

Figure 7. Identification of nuclear matrix-binding sequences in the rDNA origin region. (**A**) Diagram of the rDNA-containing plasmid prD4-1 showing relevant restriction enzyme sites. The open boxes represent the 5′NTS region (which has been duplicated in this construct) and the 3′NTS region; the solid box represents the rRNA coding region. Vertical lines indicate the positions of restriction enzyme sites for *Hin*dIII (upper) and a combination of *Nhe*I, *Xho*I, *Eco*RI and *Bam*HI (lower). The expected restriction fragments are indicated by lower case letters in descending order of size for each digestion. (**B** and **C**) Identification of matrix-associated restriction fragments of prD4-1 in an *in vitro* binding assay. Nuclear matrices prepared from *Tetrahymena* cells (see Materials and Methods) were incubated with ^{32}P-labeled input DNA fragments derived from prD4-1. DNA fragments that bound to isolated matrices were compared with input DNA fragments on 0.8% agarose gels. Input DNA fragments (I) were prepared from restriction enzyme digestion with *Hin*dIII alone (B) or with a combination of *Nhe*I, *Xho*I, *Eco*RI and *Bam*HI (C). M indicates the lanes containing matrix bound restriction fragments. Deduced restriction fragment sizes are indicated on the left. Letters on the right indicate corresponding fragments in the diagrams in (A) above.

overlaps band g (see Fig. 7A), did not associate strongly with nuclear matrices, whereas band b′, which overlaps band f, did (71% bound). This is consistent with the presence of a matrix attachment region in the downstream portion of the 3′NTS.

DISCUSSION

Both physical mapping and functional studies have localized an origin of DNA replication within the 5′ non-transcribed spacer region of the palindromic macronuclear rDNA of *T.thermophila* (see Introduction). We have begun to characterize modular structural elements within the origin region that could influence the localization of initiation events. Computer analyses of the rDNA sequence had shown that the origin region contains a large putative DUE-like element, coincident with predicted bent DNA segments, and clusters of MAR/SAR consensus sequences (9). In this study, these three predicted structural features were verified experimentally. Although functional roles for DUE-like elements, bent DNA and matrix-associated sequences in rDNA replication have not been directly demonstrated, the association of these structural elements with other eukaryotic replication origin regions is in accordance with the hypothesis that they may contribute to origin function *in vivo* (1,9).

A region of helical instability coincides with the rDNA replication origin region

DNA unwinding in replication origin regions is a prerequisite for initiation of DNA synthesis. DUEs are associated with origins of replication in *E.coli*, SV40 and *S.cerevisiae*, and are thought to contribute to origin function by facilitating the initial unwinding events (21–25). In yeast replication origins, DUEs are found in the 3′ flanking region adjacent to the ARS consensus sequence (59) and have been shown to be genetic determinants of origin activity. Mutations in DUEs that stabilize the DNA helix diminish or eliminate ARS function on plasmids (24,43) and deletions in the DUE associated with the ORI305 on chromosome III can abolish its activity (25). Dissimilar DNA sequences can be substituted for wild-type sequences in DUEs, provided that they are also easily unwound. These results support the idea that specific structural elements, in addition to specific sequences such as the ARS consensus sequence, may be essential components of eukaryotic chromosomal origins (25).

Thermodynamic analyses of the *Tetrahymena* rDNA sequence identified an usually broad region of intrinsic helical instability element in the 5′NTS. It spans the palindromic center of the rDNA molecule, encompassing a 2600 bp region with an overall mean ΔG for unwinding of 168 kcal/mol, compared to a mean of 184 kcal/mol for the entire rDNA sequence (9). This is the most extensive region of helical instability identified in a survey of eukaryotic DNA sequences that included rRNA genes and NTS regions from yeast, human and *Xenopus* (D. L. Dobbs and R. M. Benbow, unpublished observations). The significance of this finding is not yet clear, but it cannot be explained simply on the basis of the AT-richness of the *Tetrahymena* 5′NTS, since no similarly broad predicted DUEs were identified in examination of >10 randomized versions of the sequence, in which the A+T content was identical to that of the native sequence (D. L. Dobbs and R. M. Benbow, unpublished observations). In this study, we demonstrated that two sites within the major predicted DUE in the rDNA origin region are hypersensitive to mung bean nuclease in supercoiled plasmid DNA. Each site corresponds to a small region of perhaps 100 bp within the 5′NTS that is preferentially nicked and coincides with an energy minimum identified by thermodynamic analyses. Functional assays will be required to determine whether the two sites correspond to two independent DUEs or whether they represent two sites of preferred nicking within a much larger DUE-like element predicted by the thermodynamic analyses. In the rDNA repeats of yeast, an easily unwound region is required for replication origin function and exhibits hypersensitivity to mung bean nuclease nicking throughout a 100 bp region (24). This region contains a genetically-defined DUE that was predicted by thermodynamic analyses. The strong correlation between helical instability and replication efficiency of mutated ribosomal ARS derivatives in yeast (24) suggests that the easily unwound region in the 5′NTS of *Tetrahymena* rDNA may play a similar role in origin function.

A region containing several bent DNA segments coincides with the rDNA replication origin region

Bent DNA sequences are associated with origins of replication in both prokaryotic and eukaryotic genomes, e.g., bacteriophage λ (60), SV40 (61,62), yeast (29,30), the DHFR gene of CHO cells (27), the human c-*myc* gene (63) and pea rDNA (31). The role of bent DNA in replication is unclear. In yeast, a bent DNA element has been reported to enhance ARS1 function on plasmids (30), although it is not essential for function (64). It has been suggested that bent DNA functions as a structural landmark for replication or transcription proteins (30,65) or that it fosters the interaction of proteins bound at distal sites (66).

Each of three non-overlapping restriction fragments derived from the *Tetrahymena* rDNA origin region exhibited anomalous migration in polyacrylamide gels characteristic of bent DNA, as predicted by computer analyses. In one case, however, the segment which appeared to contribute most to bending did not exhibit the highest ENDS ratio (see Fig. 6). It remains to be determined whether the bent segments characterized in this study play any direct or indirect role in rDNA origin function, e.g., by facilitating initiator protein interactions, or by facilitating the association of the origin region with the nuclear matrix (67).

Association of the rDNA replication origin region with the nuclear matrix

Origins of chromosomal replication have long been proposed to be associated with the nuclear matrix (reviewed in 33–35). Newly synthesized DNA is preferentially associated with the nuclear matrix (68,69) and it is possible to enrich for replicative intermediates in DNA samples by isolation of the nuclear matrix (70). Specific DNA sequences implicated in the attachment of chromatin to the nuclear matrix have been named matrix or scaffold attachment regions (MARs or SARs). MARs are very AT-rich sequences, ~0.3–1 kb in length, that are preferentially bound and cleaved by DNA topoisomerase II (33–35). No definitive consensus sequence for MARs has been identified, but several relatively short AT-rich consensus sequences shared by some MARs/SARs have been reported (e.g., 47–49). MARs share several other sequence characteristics, such as (dA) and (dT) tracts, ATATAT boxes, bent DNA segments, a narrow minor groove, and inherent helical instability under superhelical stress (71–73, and references therein).

In this study, a MAR was identified in the rDNA origin region on the basis of its ability to bind *Tetrahymena* nuclear matrices *in vitro*. The presence of clusters of MAR/SAR consensus sequences, bent DNA and easily unwound regions in both the 5′NTS and 3′NTS suggested that both might contain matrix binding regions. Matrix binding was exhibited by fragments containing the 5′NTS, either as a single copy or as a tandem duplication, and, to a somewhat lesser extent, by fragments derived from the 3′NTS, but not by fragments derived from the rRNA coding region. It should be noted, however, that the presence of MAR/SAR consensus sequences or bent DNA does not always correlate with matrix attachment: e.g., a restriction fragment from the initiation region of the chick histone H1 gene that strongly associates with the nuclear matrix lacks SAR and MAR consensus sequences (9), and DNA bending is not essential for matrix binding (74,75). The results of the *in vitro* binding studies reported here are in agreement with the finding that restriction fragments from the rDNA origin region are more abundant in matrix-associated DNA than in loop domain DNA directly isolated from *Tetrahymena* macronuclei (76). Since origin regions of *Tetrahymena* rDNA, *Drosophila* rDNA (77) and several other eukaryotic genes (78–80) contain nuclear matrix binding regions, it seems reasonable that matrix attachment may be an important component of origin structure.

The rDNA origin region in *Tetrahymena* is large and complex

Taken together, the analyses and experimental data in this and previous studies (reviewed in 14) lend credence to the view that the functional rDNA origin region in *Tetrahymena* is larger than replication origins of prokaryotes, eukaryotic viruses and yeast, and contains redundant functional elements. Published electron micrographs (15) and our own unpublished observations have shown only large replication bubbles. The easily unwound region in the 5′NTS spans >2.5 kb of the palindromic rDNA and encompasses multiple segments of intrinsically bent DNA and clusters of MAR/SAR consensus sequences, and ARS consensus sequences. In addition, four copies of a genetically-defined replication control element, the Type I repeat (16), are located within the 5′NTS. Interestingly, one copy of the Type I repeat is also a required component of the rRNA gene promoter (81; R. Pearlman, personal communication), suggesting that transcriptional control sequences may play a role in *Tetrahymena* origin function as they do in eukaryotic viruses and yeast (reviewed in 82–84). The identification of *rmm* (rDNA maturation or maintenance) mutations in two copies of the Type I repeat separated by >600 bp argues that *cis*-acting control sequences for rDNA replication encompass a rather broad region (16,17; W. -L. Shaiu and D. L. Dobbs, unpublished data). The Type I repeat and immediately adjacent sequences are recognized by a sequence-specific single-stranded DNA binding protein (90,91) that may play an important role in DNA replication (C. Du, Z. Hou and D. L. Dobbs, unpublished data). All of these findings are consistent with the results of recent deletion studies which suggest that a large portion of the 5′NTS region is required for replication and/or maintenance of the rDNA in the macronucleus (P. Blomberg and M. -C. Yao, personal communication). Since predicted DUE-like elements, regions of bent DNA and MARs are aligned in origin regions from diverse eukaryotes (1,8,9,27,92), the properties of the *Tetrahymena* origin region determined in this study may be representative of eukaryotic origins in general.

ACKNOWLEDGEMENTS

We thank Dave Kowalski and John Anderson for kindly providing the Thermodyn and GentlBen programs, Sankar Adhya for providing plasmid pBend2, and Elizabeth Blackburn for providing plasmid prD4-1. We are grateful to members of the DNA Replication Society for their thoughtful insights and to Aric Troll and Michelle McCurnin for their assistance in preparing the manuscript. This research was supported by grants from the National Institutes of Health and the Biotechnology Council of Iowa State University. This is Journal Paper #16143 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA (Project 3065).

REFERENCES

1 Benbow, R.M., Zhao, J. and Larson, D.D. (1992) *BioEssays* **14**, 661–670.
2 Hamlin, J. (1992) *Crit. Rev. Eukaryotic Gene Expression* **2**, 359–381.
3 DePamphilis, M.L. (1993) *Annu. Rev. Biochem.* **62**, 29–63.
4 Rowley, A., Dowell, S.J. and Diffley, J.F.X. (1994) *Biochim. Biophys. Acta* **1217**, 239–256.
5 Newlon, C.S. and Theis, J.F. (1993) *Curr. Opin. Genet. Dev.* **3**, 752–758.
6 Linskens, M.H.K. and Huberman, J.A. (1990) *Cell* **62**, 845–847.
7 Held, P.G. and Heintz, N.H. (1992) *Biochim. Biophys. Acta.* **1130**, 235–246.
8 Gale, J.M., Tobey, R.A. and D'Anna, J.A. (1992) *J. Mol. Biol.* **224**, 343–358.
9 Dobbs, D.L., Shaiu, W.-L. and Benbow, R.M. (1994) *Nucleic Acids Res.* **22**, 2479–2489.
10 Kapler, G.M. (1993) *Curr. Opin. Genet. Dev.* **3**, 730–735.
11 Yao, M.-C. (1986) In Gall, J.G. (ed.), *The Molecular Biology of Ciliated Protozoa*. Academic Press, Inc., Orlando, FL, pp. 179–201.
12 Engberg, J. (1985) *Eur. J. Cell Biol.* **36**, 133–151.
13 Larson, D.D., Umthun, A.R. and Shaiu, W.-L. (1991) *J. Protozool.* **38**, 258–263.
14 Blackburn, E.H., Kapler, G.M., and Dobbs, D.L. (1995) In DePamphilis, M.L. (ed.), *DNA Replication in Eukaryotic Cells*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, in press.
15 Cech, T.R. and Brehm, S.L. (1981) *Nucleic Acids Res.* **9**, 3531–3543.
16 Larson, D.D., Blackburn, E.H., Yaeger, P.C. and Orias, E. (1986) *Cell* **47**, 229–240.
17 Yaeger, P.C., Orias, E., Shaiu, W.-L., Larson, D.D. and Blackburn, E.H. (1989) *Mol. Cell. Biol.* **9**, 452–460.
18 Yu, G.L. and Blackburn, E.H. (1990) *Mol. Cell. Biol.* **10**, 2070–2080.
19 Gaertig, J., Gu, L., Hai, B. and Gorovsky, M. (1994) *Nucleic Acids Res.* **22**, 5391–5398.
20 Pan, W.-J. and Blackburn, E.H. (1995) *Nucleic Acids Res.* **23**, 1561–1569.
21 Kowalski, D. and Eddy, M.J. (1989) *EMBO J.* **8**, 4335–4344.
22 Lin, S. and Kowalski, D. (1994) *J. Mol. Biol.* **235**, 496–507.
23 Umek, R.M. and Kowalski, D. (1988) *Cell* **52**, 559–567.
24 Miller, C.A. and Kowalski, D. (1993) *Mol. Cell. Biol.* **13**, 5360–5369.
25 Huang, R.-Y. and Kowalski, D. (1993) *EMBO J.* **12**, 4521–4531.
26 Eckdahl, T.T. and Anderson, J.N. (1990) *Nucleic Acids Res.* **18**, 1609–1612.
27 Caddle, M.S., Lussier, R.H. and Heintz, N.H. (1990) *J. Mol. Biol.* **211**, 19–33.
28 Linial, M. and Shlomai, J. (1988) *Nucleic Acids Res.* **16**, 6477–6492.
29 Snyder, M., Buchman, A.R. and Davis, R.W. (1986) *Nature* **324**, 87–89.
30 Williams, J.S., Eckdahl, T.T. and Anderson, J.N. (1988) *Mol. Cell. Biol.* **8**, 2763–2769.
31 Hernández, P., Martín-Parras, L., Martínez-Robles, M.L. and Schvartzman, J.B. (1993) *EMBO J.* **12**, 1475–1485.
32 Koo, H.-S., Wu, H.-M. and Crothers, D.M. (1986) *Nature* **320**, 501–506.
33 Gasser, S.M., Amati, B.B., Cardenas, M.E. and Hofmann, J.F.-X. (1989) *Int. Rev. Cytol.* **119**, 57–96.
34 Phi-Van, L. and Strätling, W.H. (1990) *Prog. Mol. Subcell. Biol.* **1**, 1–11.
35 Roberge, M. and Gasser, S.M. (1992) *Mol. Microbiol.* **6**, 419–423.
36 Suzuki, M., Savoysky, E., Izuta, S., Tatebe, M., Okajimna, T. and Yoshida, S. (1993) *Biochemistry* **32**, 12782–12792.
37 Suzuki, M., Izuta, S., Savoysky, E., Sakurai, T., Simbulan, C., Tatebe, M., Kojima, K. and Yoshida, S. (1993) *Biochemistry Mol. Biol. Int.* **29**, 645–652.
38 Campbell, J.L. and Newlon, C.S. (1991) In Broach, J.R., Pringle, J.R. and Jones, E.W. (eds), *The Molecular and Cellular Biology of the Yeast Saccharomyces: Genome Dynamics, Protein Synthesis and Energetics*. Cold Spring Harbor Press, Cold Spring Harbor, N.Y., pp. 41–146.
39 Virta-Pearlman, V.J., Gunaratne, P.H. and Chinault, A.C. (1993) *Mol. Cell. Biol.* **13**, 5931–5942.
40 Tasheva, E.S. and Roufa, D.J. (1994) *Mol. Cell. Biol.* **14**, 5628–5635.
41 Engberg, J. and Nielsen, H. (1990) *Nucleic Acids Res.* **18**, 6915–6919.
42 Breslauer, K.J., Frank, R., Blocker, H. and Marky, L.A. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 3746–3750.
43 Natale, D.A., Schubert, A.E. and Kowalski, D. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 2654–2658.
44 VanWye, J.D., Bronson, E.C. and Anderson, J.N. (1991) *Nucleic Acids Res.* **19**, 5253–5261.
45 Eckdahl, T.T. and Anderson, J.N. (1987) *Nucleic Acids Res.* **15**, 8531–8545.
46 Devereux, J., Haeberli, P. and Smithies, O. (1984) *Nucleic Acids Res.* **12**, 387–395.
47 Gasser, S.M. and Laemmli, U.K. (1986) *Cell* **46**, 521–530.
48 Rao, B.S., Zannis-Hadjopoulos, M., Price, G.B., Reitman, M. and Martin, R.G. (1990) *Gene* **87**, 233–242.
49 Cockerill, P.N. and Garrard, W.T. (1986) *Cell* **44**, 273–282.
50 Kaiserman, H.B., Marini, N.J., Poll, E.H.A. and Benbow, R. (1990) In Strauss, P.R. and Wilson, S.H. (eds), *The Eukaryotic Nucleus: Molecular Biochemistry and Macromolecular Assemblies*. Caldwell, New Jersey, pp. 783–811.
51 Yu, G.-L. and Blackburn, E.H. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 8487–8491.
52 Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
53 Marini, J.C., Levene, S.D., Crothers, D.M. and Englund, P.T. (1982) *Proc. Natl. Acad. Sci., USA* **79**, 7664–7668.
54 Kim, J., Zweib, C., Wu, C. and Adhya, S. (1989) *Gene* **85**, 15–23.
55 Higashinakagawa, T., Narushima-Iio, M., Saiga, H., Kondo, S. and Mita, T. (1992) *Chromosoma* **101**, 413–419.
56 Dijkwel, P.A., Vaughn, J.P. and Hamlin, J.L. (1991) *Mol. Cell. Biol.* **11**, 3850–3859.
57 Amin, A.A. and Pearlman, R.E. (1985) *Nucleic Acids Res.* **13**, 2647–2659.
58 Christiansen, K., Bonven, B.J. and Westergaard, O. (1987) *J. Mol. Biol.* **193**, 517–525.
59 Natale, D.A., Umek, R. and Kowalski, D. (1993) *Nucleic Acids Res.* **21**, 555–560.
60 Zahn, K. and Blattner, F.R. (1987) *Science* **236**, 416–422.
61 Deb, S., Delucia, A.L., Koff, A., Tsui, S. and Tegtmeyer, P. (1986) *Mol. Cell. Biol.* **6**, 4578–4584.
62 Ryder, K., Silver, S., DeLucia, A.L., Fanning, E. and Tegtmeyer, P. (1986) *Cell* **44**, 719–725.
63 Bergemann, A.D., Ma, Z.-W. and Johnson, E.M. (1992) *Mol. Cell. Biol.* **12**, 5673–5682.
64 Marahrens, Y. and Stillman, B. (1992) *Science* **255**, 817–823.
65 Linial, M. and Shlomai, J. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 8205–8209.
66 Stenzel, T.T., Patel, P. and Bastia, D. (1987) *Cell* **49**, 709–717.
67 Homberger, H.P. (1989) *Chromosoma* **98**, 99–104.
68 Nakayasu, H. and Berezney, R. (1989) *J. Cell Biol.* **108**, 1–11.
69 Hozak, P., Hassan, A.B., Jackson, D.A. and Cook, P.R. (1993) *Cell* **73**, 361–373.
70 Vaughn, J.P., Dijkwel, P.A., Mullenders, L.H.F. and Hamlin, J.L. (1990) *Nucleic Acids Res.* **18**, 1965–1969.
71 Bode, J., Kohwi, Y., Dickinson, L., Joh, T., Klehr, D., Mielke, C. and Kohwi-Shigematsu, T. (1992) *Science* **255**, 195–197.
72 Ludérus, M.E.E., den Blaauwen, J.L., de Smit, O.J.B., Compton, D.A. and van Driel, R. (1994) *Mol. Cell. Biol.* **14**, 6297–6305.
73 Boulikas, T. (1993) *J. Cell. Biochem.* **52**, 14–22.
74 Amati, B., Pick, T., Laroche, T. and Gasser, S.M. (1990) *EMBO J.* **9**, 4007–4016.
75 von Kries, J.P., Phi-Van, L., Diekmann, S. and Strätling, W.H. (1990) *Nucleic Acids Res.* **18**, 3881–3885.
76 Shaiu, W.-L. (1993) Ph.D. Thesis, Iowa State University.
77 Brun, C., Surdej, P. and Miassod, R. (1993) *Exp. Cell Res.* **208**, 104–114.
78 Amati, B.B. and Gasser, S.M. (1988) *Cell* **54**, 967–978.
79 Dijkwel, P.A. and Hamlin, J.L. (1988) *Mol. Cell. Biol.* **8**, 5398–5409.
80 Trempe, J.P., Lindstrom, Y.L. and Leffak, M. (1988) *Mol. Cell. Biol.* **8**, 1657–1663.
81 Miyahara, K., Hashimoto, N., Higashinakagawa, T. and Pearlman, R.E. (1993) *Gene* **127**, 209–213.
82 DePamphilis, M.L. (1993) *Trends Cell Biol.* **3**, 161–167.
83 Heintz, N.H. (1992) *Curr. Opin. Cell Biol.* **4**, 459–467.
84 Rivier, D.H. and Pillus, L. (1994) *Cell* **76**, 963–966.
85 Cech, T.R. (1986) In Gall, J.G. (ed.), *The Molecular Biology of Ciliated Protozoa*. Academic Press, Inc., Orlando, pp. 203–225.
86 Palen, T.E. and Cech, T.R. (1984) *Cell* **36**, 933–942.
87 Challoner, P.B., Amin, A.A., Pearlman, R.E. and Blackburn, E.H. (1985) *Nucleic Acids Res.* **13**, 2661–2680.
88 Niles, E.G., Sutiphong, J. and Haque, S. (1981) *J. Biol. Chem.* **256**, 12849–12856.
89 Bonven, B.J., Gocke, E. and Westergaard, O. (1985) *Cell* **41**, 541–551.
90 Umthun, A.R., Hou, Z., Shaiu, W.-L., Sibenaller, Z.A., and Dobbs, D.L. (1994) *Nucleic Acids Res.* **22**, 4432–4440.
91 Hou, Z., Umthun, A.R., and Dobbs, D.L. (1995) *Biochemistry* **34**, 4583–4592.
92 Shinomiya, T. and Ina, S. (1994) *Mol. Cell. Biol.* **14**, 7374–7403.