A Single-Stranded DNA Binding Protein That Specifically Recognizes Cis-Acting Sequences in the Replication Origin and Transcriptional Promoter Region of *Tetrahymena* rDNA†

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ABSTRACT: Type I repeat sequences are evolutionarily conserved sequence elements found in the replication origin and transcriptional promoter region of the rRNA genes (rDNA) in *Tetrahymena thermophila.* An abundant single-stranded DNA binding protein, ssA-TIBF, specifically interacts with the A-rich strand of the Type I repeat sequence. Quantitative binding competition experiments performed with purified ssA-TIBF demonstrate that the binding site for ssA-TIBF includes sequences both within the conserved 33 nt element and in a 3′ flanking region: addition of the 3′ flanking sequence to the Type I repeat oligonucleotide increases the binding affinity of ssA-TIBF by nearly 100-fold (apparent $K_d = 3.0 \times 10^{-10}$ M). A mutation in the ssA-TIBF binding site previously shown to be the determinant of an rDNA replication defect *in vivo* results in a 25-fold decrease in ssA-TIBF binding affinity *in vitro.* ssA-TIBF also binds with high affinity to a copy of the Type I repeat sequence within the essential promoter region defined by *in vitro* transcription assays. The affinity of ssA-TIBF for the promoter repeat, which differs from other copies of the repeat at 8 out of 33 positions, is at least equal to its affinity for the Type I repeat sequences in the origin region. The biochemical properties of ssA-TIBF *in vitro* suggest that it could play a role in both replication and transcription of *Tetrahymena* rDNA *in vivo.*

Several abundant sequence-specific DNA binding proteins have been shown to be involved in multiple aspects of chromosome function [reviewed in Diffley (1992)]. For example, ABF1[1] in yeast is an essential protein that can function as either an activator or a repressor of transcription in addition to its role as an accessory factor in DNA replication, where binding of ABF1 to sites near autonomously replicating sequences (ARSs) enhances replication origin function [Walker *et al.*, 1989; Diffley & Stillman, 1988; Shore & Nasmyth, 1987; Buchman *et al.*, 1988; reviewed in Diffley (1992); Campbell & Newlon, 1991]. Another multifunctional protein, the yeast origin recognition complex (ORC), binds the essential ARS core consensus sequence and not only is required for DNA replication but also is involved in transcriptional silencing (Bell & Stillman, 1992; Foss *et al.*, 1993; Li & Herskowitz, 1993; Bell *et al.*, 1993; Micklem *et al.*, 1993). In addition, the TATA-element binding protein, TBF, has been implicated in the activation of yeast origins of replication (Lue & Kornberg, 1993). Although these are proteins that preferentially recognize duplex DNA, single-stranded DNA binding proteins also appear to carry out multiple functions in eukaryotic cells. The best characterized of these is RPA (also known as RF-A and HSSB), which is essential for SV40 replication *in vitro* (Wobbe *et al.*, 1987; Wold & Kelly, 1988; Fairman & Stillman, 1988), and has been shown to interact with transcriptional activators such as GAL4, VP16, and the tumor suppressor p53 (Li & Botchan, 1993; He *et al.*, 1993; Dutta *et al.*, 1993). RPA displays a strong preference for pyrimidines in single-stranded DNA (Kim *et al.*, 1992), but has also been reported to mediate both transcriptional repression and activation in yeast by binding to specific regulatory sites in duplex DNA (Luche *et al.*, 1993). Similarly, the yeast MCM1 protein binds to a single strand of its duplex recognition site (Grayhack, 1992) and is required for the function of at least some yeast ARS sequences in addition to serving as a transcription factor (Jarvis *et al.*, 1989; Passmore *et al.*, 1989).

In the ciliated protozoan *Tetrahymena thermophila*, both duplex and single-stranded DNA binding proteins that interact with Type I repeat sequences upstream of the rRNA genes (rDNA) have been identified (Umthun *et al.*, 1994). The rDNA in *Tetrahymena* exists as a single chromosomal copy in the germline micronucleus and as highly amplified

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[1] Abbreviations: ABF1, ARS (autonomously replicating sequence) binding factor 1; ORC, origin recognition complex; TBF, TATA-element binding factor; RPA, replication protein A; RF-A replication factor A; HSSB, human single-stranded DNA binding protein; MCM, minichromosome maintenance; rDNA, ribosomal RNA genes; 5′NTS, 5′ nontranscribed spacer; ssA-TIBF, single-stranded A-rich Type I repeat binding factor(s); *rmm,* rDNA maturation and maintenance; ds-TIBF, double-stranded Type I repeat binding factor(s); K_d, equilibrium dissociation constant; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; Pefabloc, 4-(2-aminoethyl)benzenesulfonyl fluoride·HCl; CA, competition ability.

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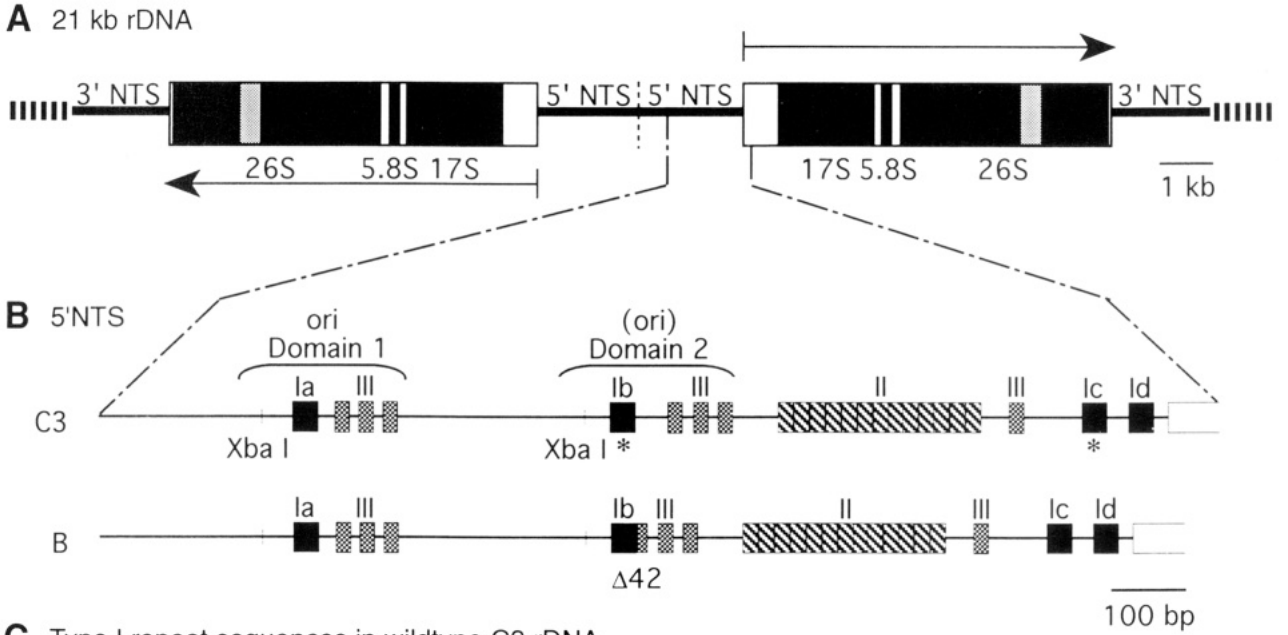

C Type I repeat sequences in wildtype C3 rDNA

Ia: ATTAGATA**TTTTTTTTGGCAAAAAAAAAAACAAAAATAGTAA**AAAATCACTTTTTTTTGAGAGTTGAAAA

Ib: ATTAGAAA**TTTTTTTTGGCAAAAAAAAAAACAAAAATAGTAA**ACCTTCCGAACTTTTGCAACTTTTGAG

Ic: CAGAGTCT**TTTTTTTTGGCAAAAAAAAAAACAAAAATAGTAA**ACCTTCCGAACTTTTTTGACTTTGAGA

Id: TGAGAAAA**ATTCTTTGGCAAAAAAAATAAAAATAATATCAG**GGGGGTAAAAATGCATATTTAAGAAGG

D Comparison of Type Ib repeat and 3' flanking sequences in C3 versus B rDNA

C3: ATTAGAAA**TTTTTTTTGGCAAAAAAAAAAACAAAAATAGTAA**ACCTTCCGAACTTTTGCAACTTTTGAG

B: ATTAGAAA**TTTTTTTTGGCAAAAAAAAAAACAAAAATAGTAA**ACTTAGAAAAATTTTTGAAAAATGAAA

FIGURE 1: Structure of the amplified macronuclear rRNA genes in *Tetrahymena thermophila*. (A) A 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 gray box. The 5′ and 3′ nontranscribed spacer regions are shown; hatched lines indicate telomeric sequences. (B) Organization of repeated sequence elements in the 5′NTS of the rDNA in inbred strains C3 and B. Putative replication origins are shown (Cech & Brehm, 1981; Cech, 1986). Domains 1 and 2 are nuclease-hypersensitive regions that lie within an ~420 bp imperfect duplication (Palen & Cech, 1984). Conserved sequence elements designated Type I, II, and III repeats are indicated (Challoner *et al.*, 1985; Niles *et al.*, 1981). Asterisks denote Type I elements altered in C3-*rmm* mutants defective in rDNA replication and/or maintenance in the macronucleus (Larson *et al.*, 1986; Yaeger *et al.*, 1989). The 42 bp deletion associated with the replication defect in B-rDNA is indicated. (C) Comparison of the four Type I repeats and flanking sequences in C3-rDNA. a−d correspond to the Type I repeats depicted in part B. The open box encloses the conserved Type I repeat. Note that three Type I repeats (Ia, Ib, Ic) are identical within the conserved region. The shaded box encloses 3′ flanking nucleotides that are identical in the Type Ib and Ic repeats and are included in the extended Type I repeat (see text). (D) Comparison of the Type Ib repeat and 3′ flanking sequences in C3-versus B-rDNA. Underlined nucleotides correspond to Type III repeat sequences that overlap the Type I repeat as a result of the 42 bp deletion in B-rDNA relative to C3.

21 kb linear palindromic molecules in the somatic macronucleus (Yao, 1986; Kapler, 1993) (Figure 1). Type I repeat sequences are evolutionarily conserved sequence elements found within the 5′ nontranscribed spacer region (5′NTS) of the rDNA, both in the replication origin region mapped by electron microscopy (Cech & Brehm, 1981) and in the transcriptional promoter region (Challoner *et al.*, 1985; Niles *et al.*, 1981). Mutations in or flanking Type I repeat sequences have previously been shown to cause defects in the replication and/or maintenance of amplified rDNA in the macronucleus (Larson *et al.*, 1986; Yaeger *et al.*, 1989; Kapler *et al.*, 1994, W.-L. Shaiu and D.L.D., unpublished data). Two *rmm* (*r*DNA *m*aturation and *m*aintenance) mutations alter the same Type I repeat (Ib) in the origin region, whereas a third alters a Type I repeat (Ic) located less than 100 bp from the transcription initiation site (asterisks in Figure 1B). In addition, the replication disadvantage exhibited by a naturally occurring variant rDNA allele in inbred strain B, relative to that in strain C3, is the result of a 42 bp deletion that removes sequences immediately downstream from the Type Ib repeat. Whereas these observations implicate Type I repeat sequences in the regulation of rDNA replication in the macronucleus, other studies have demonstrated that the Type I repeat (Id) nearest the transcriptional start site is an essential element of the rRNA gene promoter in both *T. pyriformis* and *T. thermophila* (Miyahara *et al.*, 1993; R. Pearlman, personal communication). Thus, Type I repeat sequences may be dual transcription/replication control elements, such as have been identified in several eukaryotic viral genomes and in yeast [reviewed in DePamphilis (1993), Heintz *et al.* (1992), and Rivier and Pillus (1994)].

Two classes of Type I repeat binding proteins were previously identified in *Tetrahymena* cellular extracts: ds-TIBF, which binds preferentially to duplex DNA but exhibits only moderate specificity for the Type I repeat sequence; and ssA-TIBF, a single-stranded DNA binding protein that binds specifically to the A-rich strand of the Type I repeat (Umthun *et al.*, 1994). Since Type I repeat sequences have been implicated in the regulation of both DNA replication and transcription, we wished to investigate the binding affinity of ssA-TIBF for the Type I repeat and flanking sequences. In this study, we performed quantitative binding competition experiments with purified ssA-TIBF to test (i) whether contacts with sequences outside the conserved Type I repeat contribute to the affinity of ssA-TIBF binding, (ii) whether ssA-TIBF has altered affinity for sequences that correspond to those in the B-rDNA allele, concordant with its reduced replication ability *in vivo*, and (iii) whether ssA-TIBF also binds the Type I repeat previously shown to be essential for the initiation of rRNA transcription *in vitro*. Although no physiological role has been established for ssA-TIBF, the results of these biochemical analyses support the hypothesis that interactions between ssA-TIBF and Type I repeat sequences could function in the regulation of both transcription and replication *in vivo*.

EXPERIMENTAL PROCEDURES

Materials

Enzymes and Inhibitors. T4 polynucleotide kinase was purchased from New England Biolabs. Protease inhibitors (PMSF, Pefabloc SC, leupeptin, pepstatin) were from Boehringer-Mannheim.

Chromatography Media. Micro Select-D spin columns were from 5′ Prime-3′ Prime, Inc. Phosphocellulose (P11) was purchased from Whatman. Streptavidin-conjugated agarose resin was from GIBCO BRL.

DNA Oligonucleotides. Biotinylated (5′) DNA oligonucleotide (Type I ssA45) was synthesized by the Midland Certified Reagent Co. Type I ssA33, Type I ssA35, Type I ssA37, Type I ssA38, and Type I ssA53 (see Figures 3A, 4A, 5A, and 6A) were synthesized by the Nucleic Acid Research Facility at Iowa State University. Type I ssA45 is an extended version of Type I ssA37, having four additional T's on each end (as occurs in its natural context, see Figure 1C).

Methods

Tetrahymena Cell Culture. T. thermophila strain C3V cells were grown using a rotary shaker at 30 °C in 2% PPYS (2% proteose peptone, 0.2% yeast extract, and 0.003% sequestrine) to mid-logarithmic phase (2.5×10^5 cells/mL) before lysis and S100 extract preparation.

Sequence-Specific DNA Chromatography Media. Sequence-specific DNA chromatography media were prepared essentially as described (Ostrowski & Bomsztyk, 1993); 2.5 mL of streptavidin−agarose resin was washed 3 times in TEG buffer [10 mM Tris/HCl, pH 7.5, 1 mM EDTA, and 10% (v/v) glycerol]. The agarose beads were collected by centrifugation at 500*g* for 2 min after each wash. The TEG-equilibrated resin was resuspended in an equal volume of TEG buffer, to which 500 μg of biotinylated DNA oligonucleotides (Type I ssA45) was added. The biotin−streptavidin conjugation was carried out at room temperature with gentle rotation of the mixture for 90 min. The UV absorbance (260 nm) of the supernatant was measured to monitor the efficiency of the conjugation process.

Preparation of S100 Extracts and Purification of ssA-TIBF. S100 extracts were prepared essentially as described (Greider & Blackburn, 1985). *Tetrahymena* cells were collected by centrifugation at 2600*g* for 7 min, washed twice in 10 mM Tris-HCl (pH 7.5), and resuspended in 5 pellet-volumes of HMG buffer [20 mM HEPES (pH 7.9), 1 mM $MgCl_2$, 10% (v/v) glycerol, 0.2 mM PMSF, 2 μg/mL leupeptin, 1 μg/mL pepstatin, and 1 mM DTT]. Cells were lysed by adding NP40 to a final concentration of 0.2% in HMG and stirring on ice for 30 min. The whole cell extract was centrifuged at 100000*g* for 60 min at 4 °C in a Beckman TLA 100.3 fixed-angle rotor. Aliquots of the supernatant (S100) were frozen immediately in liquid nitrogen, and stored at −80 °C. Approximately 13 mL of S100 extract was routinely obtained from a 1 L culture of mid-log phase *Tetrahymena* cells (2.5×10^5 cells/mL).

All subsequent manipulations were carried out at 4 °C. Typically, 30 mL of S100 extract (4 mg/mL protein) was loaded onto a phosphocellulose column (2.5 × 10 cm) equilibrated with HMG buffer with 50 mM NaCl. The column was washed with 2.5 column volumes of the same buffer at a flow rate of 20 mL/h. ssA-TIBF was eluted with HMG buffer containing 250 mM NaCl. This partially purified fraction (11 mg of total protein, 10-fold purification) was loaded onto a sequence-specific DNA (Type I ssA45) affinity column (1.0 × 2.5 cm) in HMG buffer containing 100 mM NaCl. The column was washed with 10 volumes of the same buffer at a flow rate of 10 mL/h and eluted using a linear gradient of NaCl (100 mM to 1 M) in HMG buffer. The fractions containing ssA-TIBF (400 mM NaCl) were pooled (0.15 mg of protein, 45-fold purification), diluted to 100 mM NaCl with HMG buffer, reloaded onto the same column, and eluted, resulting in an additional 22-fold purification. The overall yield of ssA-TIBF was ~4 μg of ssA-TIBF (33% yield). The overall purification obtained was 9900-fold.

Protein Determination. Protein concentration was measured by the method of Bradford using bovine serum albumin (BSA) as standard (Bradford, 1976). For very dilute column fractions, protein concentration was estimated on the basis of the intensity of stained bands after SDS−polyacrylamide gel electrophoresis (Laemmli, 1970) and silver staining (Merril *et al.*, 1981).

Oligonucleotide Probes and Competitors. Synthesized DNA oligonucleotides were purified by reverse-phase HPLC or by preparative polyacrylamide gel electrophoresis (Ausubel *et al.*, 1987). Concentrations of oligonucleotides were determined spectrophotometrically. Oligonucleotides were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Sambrook *et al.*, 1989) and purified using Micro Select-D spin columns.

Gel Mobility Shift Assays. Gel mobility shift experiments were performed essentially as described (Umthun *et al.*, 1994). Typically, 0.1 ng of ^{32}P-labeled oligonucleotide was incubated with 0.01−0.15 ng of affinity-purified ssA-TIBF for 15 min on ice. Standard reaction mixtures contained 20 mM HEPES (pH 7.9), 200 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, 0.1 mM Pefabloc SC, 2 μg/mL leupeptin, 1 μg/mL pepstatin, and 10% glycerol (v/v) in a total volume of

20−40 μL. For binding competition assays, 1 μL of an unlabeled oligonucleotide at the appropriate concentration in STE [100 mM NaCl, 10 mM Tris/HCl (pH 7.5), and 1 mM EDTA] was added prior to the addition of ssA-TIBF. Samples were subjected to electrophoresis in 5% polyacrylamide gels in 0.6 × Tris−borate−EDTA buffer (Ausubel *et al.*, 1987) for 60 min at 15−20 V/cm at room temperature. After electrophoresis, gels were dried and used to expose Kodak XAR-5 film or phosphor screens. Radioactivity in individual bands was quantitated using a Molecular Dynamics PhosphorImager.

Calculation of Binding Parameters. Calculations and curve-fitting were carried out using Microsoft Excel or DeltaGraph on a Macintosh IIci computer. Bound and unbound oligonucleotides were quantitated in gel mobility shift assays as described above.

An apparent equilibrium dissociation constant (K_d) was calculated from data obtained in two Types of experiments: (1) A constant amount of Type I ssA37 oligonucleotide was titrated with increasing amounts of ssA-TIBF (Figure 4B). An apparent K_d was estimated from the resultant hyperbolic binding isotherm (Figure 4C; K_d = [ssA-TIBF] at 50% maximal binding) (Ausubel *et al.*, 1987). (2) A constant amount of ssA-TIBF was titrated with increasing amounts of Type I ssA37 oligonucleotide (Figure 4D). An apparent K_d was calculated from the resultant linear double-reciprocal plot (Figure 4E), according to the equation $1/r = 1/n + K_d/n[A]$, where r = the moles of Type I ssA37 bound per mole of ssA-TIBF, n = the number of Type I ssA37 binding sites per ssA-TIBF molecule, and [A] = the concentration of unbound Type I ssA37 (Freifelder, 1982).

The number of binding sites (n) per ssA-TIBF molecule was derived from the y-intercept of the double-reciprocal plot ($1/n$) and from the y-intercept of a Scatchard plot (n/K_d). Binding cooperativity was examined by assessing the linearity of these plots and by calculation of a Hill coefficient (n_H).

Relative binding affinities of oligonucleotides were determined in binding competition assays, using a subsaturating concentration of ssA-TIBF. The concentration of competitor required to reduce the bound complex by 50% was assumed to be proportional to the affinity of ssA-TIBF for that competitor and was used to calculate the relative competition ability (CA) of the oligonucleotide. The competition ability of the oligonucleotide with the same sequence as the ^{32}P-labeled reference DNA was defined as 100%.

RESULTS

Analysis of Purified ssA-TIBF by SDS−Polyacrylamide Gel Electrophoresis. Gel mobility shift assays using extracts or partially purified fractions from *Tetrahymena* cells established that ssA-TIBF specifically recognizes the A-rich strand of the Type I repeat sequence (Umthun *et al.*, 1994). To more rigorously examine the binding characteristics of ssA-TIBF, we first purified ssA-TIBF ~10 000-fold using a combination of phosphocellulose and sequence-specific DNA affinity chromatography (see Experimental Procedures). The purified fractions were pooled and analyzed by SDS−polyacrylamide gel electrophoresis followed by silver staining. One major band was observed migrating as a polypeptide of 24 kDa (Figure 2). Three less prominent bands were observed at 32, 35, and 70 kDa. All four bands were sensitive to proteinase K and resistant to DNase I

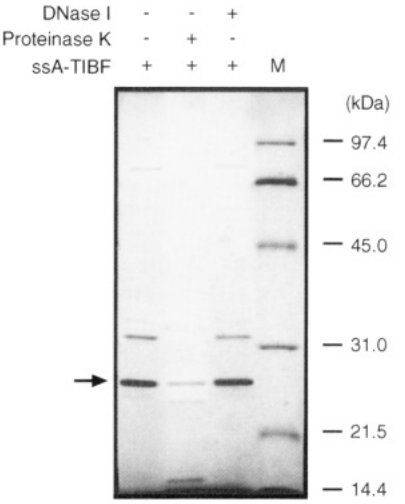

FIGURE 2: SDS−polyacrylamide gel electrophoresis of purified ssA-TIBF. 50 ng of ssA-TIBF fraction was analyzed on a 13% SDS−polyacrylamide gel, with or without prior treatment with proteinase K or DNase I as indicated. The arrow indicates the predominant 24 kDa polypeptide. The migration positions of molecular size markers are shown on the right.

treatment. The abundance of ssA-TIBF was estimated from the purification to be ~10^5 copies per cell.

In our previous study, multiple DNA−protein complexes were observed in gel mobility shift assays when ^{32}P-labeled Type I repeat oligonucleotides were employed as probes in cell extracts or partially purified fractions (Umthun *et al.*, 1994). Using the ssA-TIBF purified in this study, only one major complex was observed in every gel mobility shift assay (see Figures 3−6 below). This is consistent with the interpretation that the multiple bands observed previously were the result of partial proteolysis of ssA-TIBF (Umthun *et al.*, 1994).

The ssA-TIBF Binding Site Extends Beyond the Conserved Type I Repeat Sequence into 3′ Flanking DNA. Previous work established that sequences in the 3′ end of the conserved 33 nt Type I repeat sequence are critical for ssA-TIBF binding: deletion of these sequences results in a ~50% decrease in the amount of complex formed with ssA-TIBF (Umthun *et al.*, 1994). To determine whether additional sequences downstream from the Type I repeat contribute to binding, we quantitated the affinity of ssA-TIBF for oligonucleotides containing the Type Ib repeat and 3′ flanking DNA (Figure 1). This region was of special interest because it contains the determinant of the replication disadvantage of B-relative to C3-rDNA (Larson *et al.*, 1986; Yaeger *et al.*, 1989). Moreover, in wild-Type C3-rDNA, these sequences are identical for the Type Ib and Ic repeats (Figure 1C), and C3-*rmm* mutations that affect rDNA replication and/or maintenance in the macronucleus are due to single base pair deletions within one of these two repeats (asterisks in Figure 1B) (Larson *et al.*, 1986; Yaeger *et al.*, 1989, W.-L. Shaiu and D.L.D., unpublished data). Competition experiments using a subsaturating concentration of ssA-TIBF were carried out to assess the contribution of 3′ flanking sequences to ssA-TIBF binding in gel mobility shift assays (Figure 3). Sequences of the oligonucleotides used in these experiments are shown in Figure 3A. Type I ssA33 (A33) corresponds to the A-rich strand of the 33 nt conserved Type I repeat. Type I ssA37 (A37) contains most of the Type I repeat plus 11 nt derived from sequences immediately downstream from

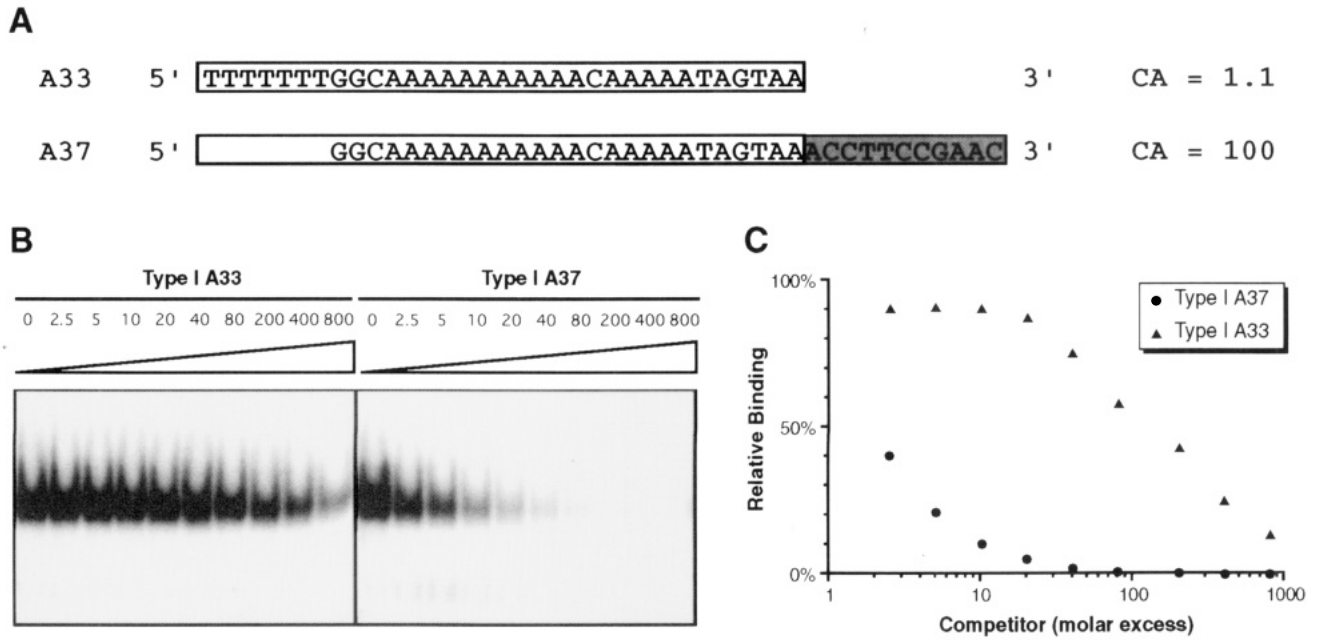

FIGURE 3: ssA-TIBF binding site extends beyond the conserved Type I repeat sequence into 3′ flanking DNA. (A) Sequences of single-stranded Type I repeat-containing oligonucleotides used in binding competition experiments. A33 (Type I ssA33) corresponds to the A-rich strand of the conserved 33 nt Type Ib repeat in the 5′NTS (Figure 1). A37 (Type I ssA37) contains an 11 nt 3′ extension corresponding to sequences adjacent to the Type Ib repeat in C3-rDNA and lacks seven 5′ T residues previously shown to be dispensable for binding. Nucleotides within the open box and shaded area are as described in Figure 1. CA refers to the relative competition ability of each oligonucleotide determined in part C (see Experimental Procedures). (B) Binding of ssA-TIBF to the extended Type I repeat (Type I ssA37). Competition assays were carried out as described under Experimental Procedures. Each reaction contained 0.1 ng of ^{32}P-labeled Type I ssA37 oligonucleotide and a subsaturating concentration (0.05 ng) of ssA-TIBF, and was challenged with the unlabeled competitor indicated above each gel. Triangles indicate increasing concentrations of competitors: left side, 0−800-fold molar excess of Type I ssA33; right side, 0−800-fold molar excess of Type I ssA37. (C) Competition ability of oligonucleotides. The radioactivity in shifted complexes was quantitated as described under Experimental Procedures. Values are expressed relative to the amount of complex formed in the absence of competitor. Each data point represents the average of two independent experiments.

the Type Ib repeat in C3-rDNA. To minimize differences in lengths of the competitor oligonucleotides, seven T's in the 5′ end of the Type I repeat were omitted from Type I ssA37, since previous work had shown that removal of these nucleotides has minimal (<10%) effect on ssA-TIBF binding affinity (Umthun *et al.*, 1994). Complexes formed between affinity-purified ssA-TIBF and either Type I ssA33 or Type I ssA37 exhibited identical mobility in gel mobility shift assays (data not shown). To determine the relative binding affinity of ssA-TIBF for the two substrates, a constant amount of ^{32}P-labeled Type I ssA37 reference DNA was challenged with increasing amounts of unlabeled competitor DNA (Figure 3B). A 2.5-fold molar excess of the Type I ssA37 competitor caused a 60% reduction in ssA-TIBF binding to ^{32}P-labeled Type I ssA37, whereas a 200-fold molar excess of Type I ssA33 was required to achieve approximately the same level of competition (Figure 3B, C). These results established that ssA-TIBF has nearly 100-fold higher affinity for Type I ssA37 than for Type I ssA33 (Figure 3C). Therefore, the ssA-TIBF binding site extends beyond the conserved Type I repeat into 3′ flanking DNA.

ssA-TIBF Binds the Extended Type I Repeat Sequence with a K_d of 3 × 10^{-10} M. To quantitate the affinity of ssA-TIBF for the extended Type I repeat sequence, an apparent equilibrium dissociation constant (K_d) for binding of ssA-TIBF to the Type I repeat sequence was determined in gel mobility shift titration assays using ^{32}P-labeled Type I ssA37 as substrate (Figure 4). In the experiment shown in Figure 4B, increasing concentrations of purified ssA-TIBF were incubated with a constant concentration of the Type I ssA37 probe. When the fraction of oligonucleotide bound was plotted as a function of ssA-TIBF concentration, a hyperbolic curve was obtained, suggesting that ssA-TIBF binds to Type I ssA37 with no cooperativity (Figure 4C). Saturation occurred with >97% of the oligonucleotide bound. In the experiment shown in Figure 4D, increasing concentrations of the Type I ssA37 probe were incubated with a constant concentration of ssA-TIBF. A double-reciprocal plot of these data gave a straight line, also characteristic of noncooperative binding. The apparent K_d derived from the slope was 3.0 × 10^{-10} M, in agreement with the value estimated from the experiment shown in Figure 4B, C (K_d = 3.9 × 10^{-10} M). Similar values were obtained from a Scatchard plot (K_d = 3.4 × 10^{-10} M) and a Hill plot (K_d = 3.6 × 10^{-10} M). The number of binding sites (n) was determined from the double-reciprocal plot (n = 3.9; see Figure 4E), as well as from a Scatchard plot (n = 4.2; not shown). These binding sites appeared to be identical and independent, as indicated by the linearity of the double-reciprocal and Scatchard plots. The lack of cooperativity was confirmed in a Hill plot (Hill coefficient, n_H = 1; not shown).

The Binding Affinity of ssA-TIBF for C3 and B Type I Repeats Parallels the Difference in Replication Efficiencies between C3- and B-rDNA in Vivo. To explore the possibility that a difference in the affinity of ssA-TIBF for C3- versus B-rDNA sequences could explain the replication disadvantage B-rDNA manifests in the presence of C3-rDNA *in vivo*, we quantitated the affinity of ssA-TIBF for oligonucleotides, reflecting the sequence differences between the two rDNA alleles. Competition experiments monitoring the binding of ssA-TIBF to a ^{32}P-labeled 53 nt oligonucleotide (C3 Type I ssA53), consisting of the C3 Type Ib repeat and 3′ flanking

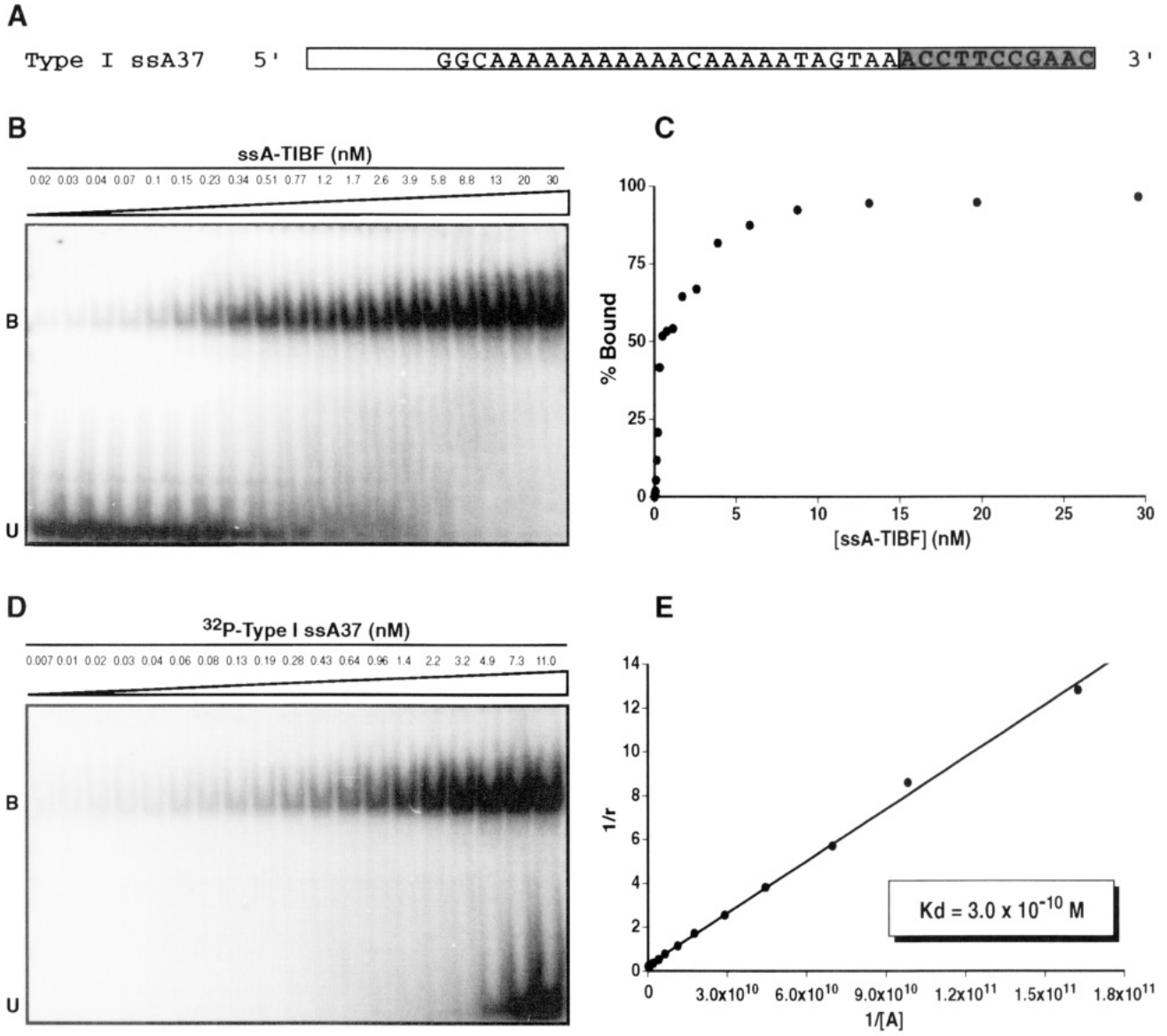

FIGURE 4: Determination of an apparent equilibrium dissociation constant for the interaction of ssA-TIBF with the extended Type I repeat sequence. (A) Sequence of the 3′ extended Type I repeat oligonucleotide (Type I ssA37). Nucleotides within the open box and shaded area are as described in Figure 1. (B) Representative gel mobility shift assay in which 0.1 ng of ^{32}P-labeled Type I ssA37 oligonucleotide (0.3 nM) was incubated with the indicated concentrations of ssA-TIBF (0.02−30 nM). B and U show the positions of bound and unbound oligonucleotides. (C) Binding of ssA-TIBF to the extended Type I sequence. Bound and unbound Type I ssA37 oligonucleotides in (B) were quantitated as described under Experimental Procedures, and the percent oligonucleotide bound was plotted as a function of ssA-TIBF concentration. In these experiments, saturation of binding occurred with >97% of the input DNA bound. An apparent K_d was estimated as described under Experimental Procedures. (D) Representative gel mobility shift assay in which 0.6 ng of ssA-TIBF (1 nM) was incubated in the presence of the indicated amounts of ^{32}P-labeled Type I ssA37 oligonucleotide (0.007−11 nM). (E) Double-reciprocal plot of the data in (D). Values for $1/r$ and $1/[A]$ (where r = moles of oligonucleotide bound per mole of ssA-TIBF and [A] = concentration of unbound oligonucleotide), the apparent K_d, and the number of binding sites per ssA-TIBF molecule were calculated as described under Experimental Procedures.

sequences, are shown in Figure 5. Competitors were either C3 A53 or the corresponding 53 nt sequence from B-rDNA, or 35 nt oligonucleotides from C3- and B-rDNA that lacked the 5′ end and most of the central A-rich portion of the Type I repeat (Figure 5A). Both C3-rDNA oligonucleotides were much stronger competitors than their B-rDNA counterparts: there was at least a 25-fold difference in the affinity of ssA-TIBF for C3- versus B-rDNA sequences (Figure 5B, C). These striking differences in binding affinity parallel the difference in replication efficiency of the C3- and B-rDNA alleles *in vivo* (see Discussion).

ssA-TIBF Also Binds with High Affinity to the rRNA Gene Promoter. The Type Id repeat adjacent to the rRNA transcription initiation site has been shown to be an essential promoter element in *in vitro* transcription studies (Miyahara *et al.*, 1993; R. Pearlman, personal communication). The promoter Type I repeat differs from the three upstream Type I repeats at 8 out of 33 positions in the conserved element, and there are additional sequence differences in the 3′ flanking DNA (see Figure 1). To examine the affinity of ssA-TIBF for this variant Type Id repeat sequence, we compared its competition ability to that of the Type Ib repeat in the origin region. Type I ssA38, an oligonucleotide corresponding to the Type Id repeat and its 3′ flanking sequence, competed effectively for ssA-TIBF binding, demonstrating that ssA-TIBF has high affinity for the

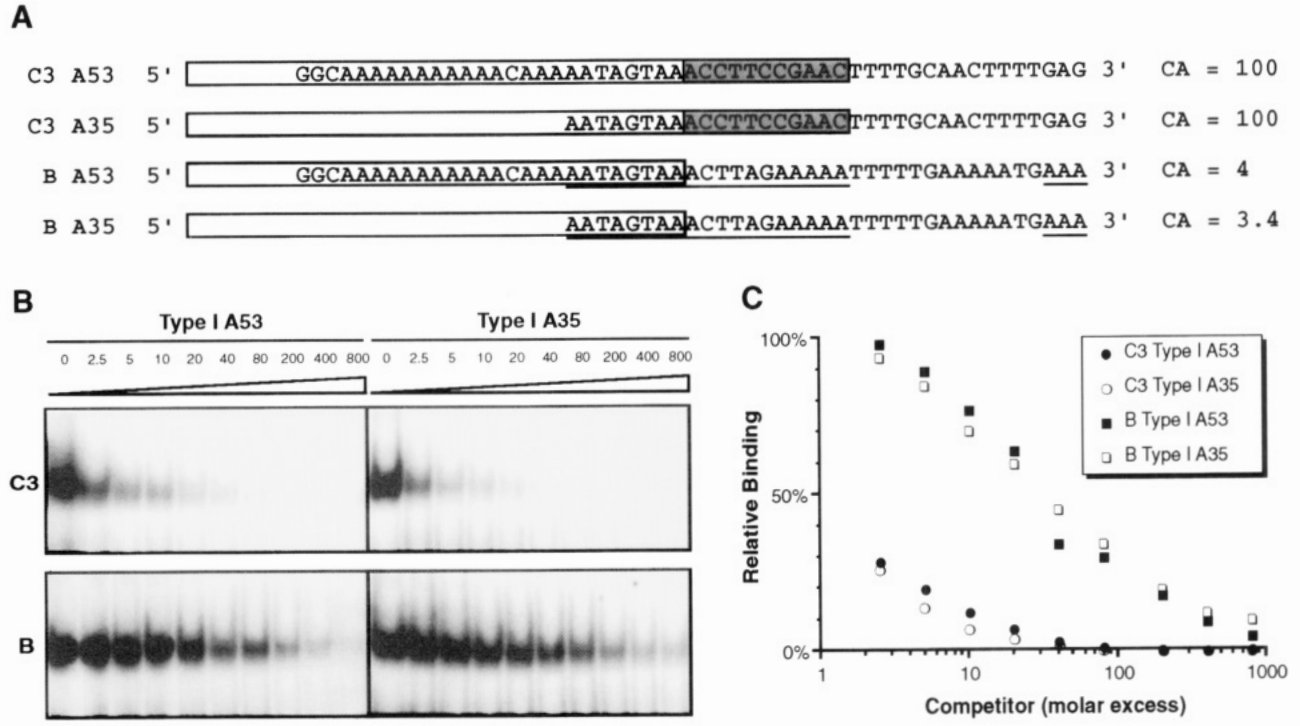

FIGURE 5: Affinity of ssA-TIBF for C3- versus B-rDNA sequences *in vitro* parallels their relative replication efficiencies *in vivo*. (A) Sequences of oligonucleotides used in binding competition experiments. C3 A53, C3 A35, B A53, and B A35 correspond to extended (53 nt Type I ssA53) and deleted (35 nt Type I ssA35) Type Ib repeat sequences derived from C3- and B-rDNA, respectively. Nucleotides within the open box and shaded area are as described in Figure 1. Underlined nucleotides correspond to the Type III repeat sequence that overlaps the Type I repeat as a result of the 42 bp deletion in B-rDNA. (B) Binding of ssA-TIBF to C3- versus B-rDNA sequences. Competition assays were carried out as described under Experimental Procedures. Reactions contained 0.1 ng of ^{32}P-labeled C3 A53 oligonucleotide and 0.05 ng of ssA-TIBF, incubated in the presence of the unlabeled competitor indicated above each gel. Experiments in which competitors were C3-rDNA sequences are shown in the top panels; B-rDNA competitors are shown in the bottom panels. Triangles indicate increasing concentrations of competitors: left side, 0−800-fold molar excess of A53; right side, 0−800-fold molar excess of ssA35. (C) Competition ability of oligonucleotides, as in Figure 3C. Data from a single representative experiment are shown; essentially identical results were obtained in duplicate experiments.

promoter Type I element as well as for the upstream copies of the Type I repeat (Figure 6B,C).

DISCUSSION

Evolutionarily conserved Type I repeat sequences have been implicated in the control of both replication and transcription of rDNA in *Tetrahymena thermophila* (Larson *et al.*, 1986; Miyahara *et al.*, 1993). Previously, we reported the identification in *Tetrahymena* of a sequence-specific single-stranded DNA binding protein, ssA-TIBF, that specifically recognizes the Type I repeat sequence (Umthun *et al.*, 1994). In the present study, we demonstrate that purified ssA-TIBF recognizes sequences both within and flanking the 33 nt conserved Type I repeat, and binds with high affinity (K_d = 3.0 × 10^{-10} M). In *in vitro* binding competition experiments, ssA-TIBF has 25-fold lower affinity for sequences that correspond to those in the B-rDNA allele, which has reduced replication efficiency *in vivo*. In addition, ssA-TIBF specifically interacts with both the promoter Type I repeat sequence and Type I repeat sequences in the replication origin region. The results of our *in vitro* binding studies, therefore, support the hypothesis that ssA-TIBF could be involved in control of replication and/or transcription of rDNA *in vivo*.

A predominant 24 kDa polypeptide was observed in purified ssA-TIBF by SDS−polyacrylamide gel electrophoresis. Analysis of the binding of ssA-TIBF to Type I repeat oligonucleotides suggests, however, that native ssA-TIBF contains four oligonucleotide binding sites. No binding cooperativity was indicated by either a Scatchard or a Hill plot. The native molecular mass of ssA-TIBF based on the sedimentation coefficient determined on glycerol gradients (Z.H., unpublished data) is in the range of 90−100 kDa. A simple interpretation of these data is that native ssA-TIBF is a tetramer of 24 kDa polypeptides containing identical and independent binding sites.

The differential affinity of ssA-TIBF for C3- versus B-rDNA sequences *in vitro* parallels previously documented differences in the replication and/or maintenance of the C3 and B alleles in the macronucleus (Larson *et al.*, 1986). A replication hierarchy exists among rDNA alleles in which C3-rDNA outreplicates B, and B outreplicates C3-*rmm* rDNA alleles in heterozygous macronuclei. The eventual predominance of C3- over B-rDNA could be accounted for by a 10% difference in replication efficiency of the alleles (Larson *et al.*, 1986). The differential affinity of ssA-TIBF for C3- versus B-rDNA sequences found in this study is consistent with a model in which the observed hierarchy may be the consequence of differential competition of the alleles for a common trans-acting factor (ssA-TIBF?). In a physiological context, the 25-fold affinity differences measured *in vitro* could explain the replicative differences observed between the rDNAs *in vivo*. Therefore, on the basis of the results of the binding competition experiments, we speculate that ssA-TIBF could play a role in the regulation of rDNA replication.

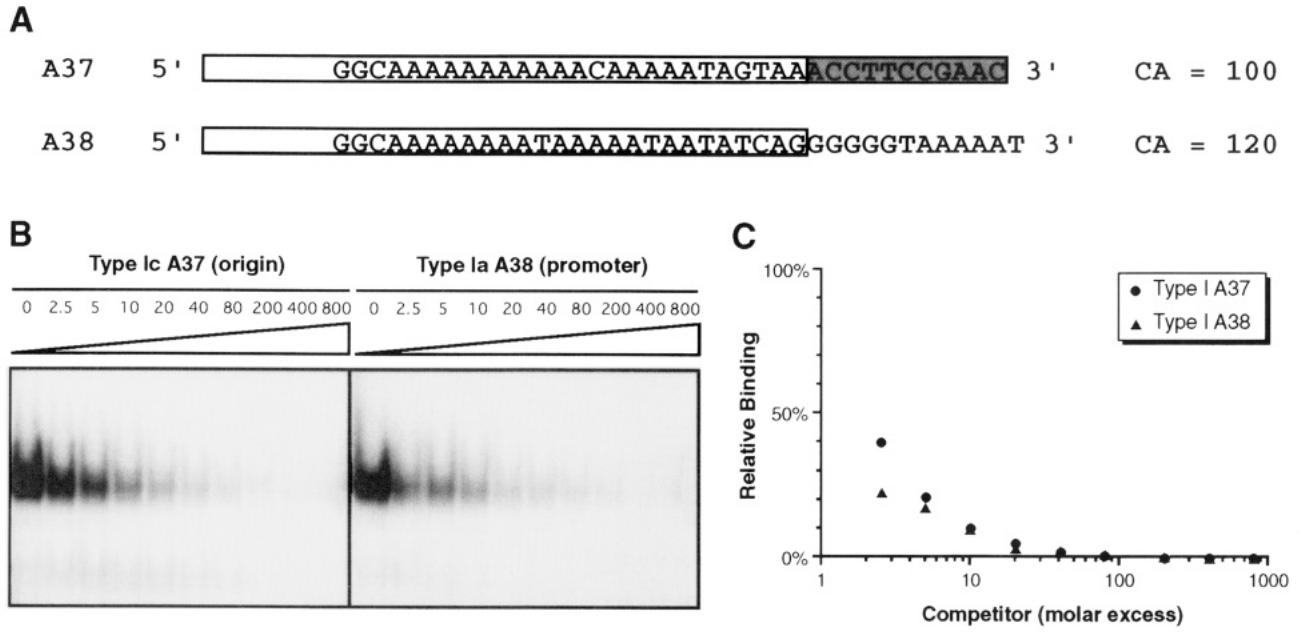

FIGURE 6: ssA-TIBF binds to the rRNA gene promoter. (A) Sequences of oligonucleotides used in binding experiments. A37 and A38 correspond to the extended Type Ib repeat in the origin region (Type I ssA37) and the extended Type Id element in the promoter region (Type I ssA38), respectively. Nucleotides within the open box and shaded area are as described in Figure 1. (B) Binding of ssA-TIBF to the promoter Type I repeat. Competition assays were carried out as described under Experimental Procedures. Reactions contained 0.1 ng of ^{32}P-labeled C3 Type I ssA37 oligonucleotide and 0.05 ng of ssA-TIBF, incubated in the presence of the unlabeled competitor indicated above each gel. Triangles indicate increasing concentrations of competitors: left side, 0−800-fold molar excess of Type I ssA37; right side, 0−800-fold molar excess of Type I ssA38. (C) Competition ability of oligonucleotides, as in Figure 3C. Data from a single representative experiment are shown; essentially identical results were obtained in duplicate experiments.

The finding that ssA-TIBF also binds to the Type I repeat nearest the transcriptional start site, even though there are numerous differences in internal and flanking sequences, suggests a possible role for ssA-TIBF in transcriptional regulation of *Tetrahymena* rRNA. The promoter Type Id repeat differs from the three identical upstream copies by 8 nt within the 33 nt conserved Type I repeat, and also lacks 3′ flanking sequences that ssA-TIBF recognizes in the Type Ib repeat in the origin region (Figure 1). Nevertheless, the affinity of ssA-TIBF for the promoter Type Id repeat equals or exceeds its affinity for the extended Type Ib repeat. Accurate initiation of rRNA transcription in both *T. pyriformis* (Miyahara *et al.*, 1993) and *T. thermophila* (R. Pearlman, personal communication) requires the Type Id repeat. Therefore, the high affinity of ssA-TIBF for the Type Id repeat demonstrated in this study suggests that it may also play a role in rRNA transcription.

The most important determinants for ssA-TIBF binding are in the 3′ end of the conserved Type I repeat and in 3′ flanking DNA. Although ssA-TIBF was originally identified as a DNA binding protein that specifically interacts with the conserved 33 nt Type I repeat (Umthun *et al.*, 1994), this study demonstrates that additional sequences which make a major contribution to ssA-TIBF binding affinity are located outside the conserved element. It should be noted, however, that although ssA-TIBF can distinguish between the C3- and B-rDNA alleles *in vitro,* several induced C3-*rmm* mutations are due to a single base pair deletion 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.D., unpublished data). We were unable to detect a significant difference in the affinity of ssA-TIBF for an oligonucleotide corresponding to this deletion (Umthun *et al.*, 1994). Taken together, these data suggest that an additional protein(s) that recognize(s) Type I repeat sequences could be involved in the replication of phenotypes of these *rmm* mutants. This idea is corroborated by footprinting experiments that show purified ssA-TIBF preferentially contacts nucleotides at the 3′ end of the Type I repeat (Z.H., unpublished data), whereas proteins in S100 extracts of *Tetrahymena* cells also strongly protect the central A-rich portion of the Type I repeat sequence (Umthun *et al.*, 1994).

Cis-acting transcriptional control sequences that function as enhancers of DNA replication have been documented in eukaryotic DNA viruses as well as in yeast, and several DNA binding proteins are known to function both as transcription factors and as activators of replication [see introduction and DePamphilis (1993) and Heintz *et al.* (1992)]. One specific cis-acting DNA sequence that has been definitively shown to be essential for the activation of a eukaryotic chromosomal origin of replication is the 11 bp ARS core consensus sequence in yeast [Rivier & Rine, 1992; Deshpande & Newlon, 1992; reviewed in Newlon and Theis (1993) and Rowley *et al.* (1994)]. Two classes of DNA binding proteins that specifically recognize the ARS core consensus sequence have been reported. The first of these are single-stranded DNA binding protein(s) that bind the T-rich strand of the ARS core consensus sequence (Hofmann & Gasser, 1991; Kuno *et al.*, 1990, 1991; Schmidt *et al.*, 1991; Zeidler *et al.*, 1993). Currently, there is no evidence that any of these proteins function directly in DNA replication (Cockell *et al.*, 1994; Zeidler *et al.*, 1993). The second class of proteins that recognizes the ARS core consensus sequence includes two multiprotein complexes that bind with high specificity to the ARS element in double-stranded DNA: core binding factor (CBF) (Estes *et al.*, 1992) and the origin recognition complex (ORC) (Bell & Stillman, 1992). Compelling genetic evidence that ORC not only is required for DNA

replication but also plays a role in transcriptional repression in yeast has been obtained recently (Bell *et al.*, 1993; Foss *et al.*, 1993; Li & Herskowitz, 1993; Micklem *et al.*, 1993).

DNA binding proteins that recognize specific sequence motifs associated with replication origin regions in other eukaryotes have also been reported. A 28 kDa protein (Pur) that recognizes a single-stranded purine-rich sequence element located within the c-*myc* replication initiation zone and associated with several other eukaryotic origins of replication has been isolated from HeLa cells (Bergemann & Johnson, 1992). Another HeLa cell protein, a 60 kDa polypeptide designated RIP60, recognizes an ATT-rich motif in a stably bent DNA segment within the DHFR replication origin region (Dailey *et al.*, 1990). As is the case for ssA-TIBF, the physiological significance of these proteins is not known. In contrast, the single-stranded DNA binding protein RPA binds preferentially to the pyrimidine-rich strand of the SV40 origin of replication (Kim *et al.*, 1992; Carmichael *et al.*, 1993) and has been shown to be essential for the initiation of SV40 replication *in vitro* (Wobbe *et al.*, 1987; Wold & Kelly, 1988; Fairman & Stillman, 1988). In addition, many single-stranded DNA binding proteins that interact specifically with DNA sequences involved in transcriptional regulation and recombination have been reported (e.g., Gaillard & Strauss, 1990; Santoro *et al.*, 1991; Jansen-Durr *et al.*, 1992; Carmichael *et al.*, 1993; Hsu *et al.*, 1993; Wang *et al.*, 1993; Ito *et al.*, 1994; Luche *et al.*, 1993; Tzfati *et al.*, 1992; Yamazaki *et al.*, 1992).

The potential importance of single-stranded DNA binding proteins that recognize specific sequences in replication origin regions is emphasized by consideration of the probable structure of eukaryotic origin regions during initiation. Extensive unwinding of origin regions has been suggested in sea urchin embryos (Baldari *et al.*, 1978), *Xenopus* embryos (Gaudette & Benbow, 1986), and the Chinese hamster ovary DHFR amplicon (Linskens & Huberman, 1990). Thus, the Type I repeat sequences in the *Tetrahymena* rDNA origin region could exist in a partially or fully single-stranded configuration during interphase. This possibility is reinforced by the observation that the rDNA origin region encompasses a large DNA unwinding element (DUE) (Dobbs *et al.*, 1994; Du *et al.*, 1995). DUEs are intrinsically unstable duplex DNA segments that have been shown to be important determinants of origin function in yeast as well as in *E. coli* and viruses such as SV40 (Umek & Kowalski, 1988; Kowalski & Eddy, 1989; Lin & Kowalski, 1993). Since the Type Ia and Ib repeats are located within the major DUE, it is not unreasonable to speculate that recognition of the Type I repeats by ssA-TIBF could occur in a previously unwound region of DNA prior to the onset of DNA synthesis.

Very few genetically defined cis-acting sequences involved in the control of chromosomal DNA replication have been identified in eukaryotes. In addition to the well-characterized ARS sequences in yeast, these are limited to sequences in the *Drosophila* chorion genes [reviewed in Orr-Weaver (1991)], the Chinese hamster ovary DHFR locus (Handeli *et al.*, 1989), the human β-globin gene region (Kitsberg *et al.*, 1993), and the *Tetrahymena* rDNA [reviewed in Kapler (1993)]. Although the precise role of Type I repeat sequences in *Tetrahymena* rDNA metabolism is not known, the abundance and high affinity of ssA-TIBF for the Type I repeat suggest that ssA-TIBF 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. Nevertheless, the availability of purified ssA-TIBF should facilitate the investigation of its role in transcription of *Tetrahymena* rDNA *in vitro*. In summary, the results of the binding competition experiments reported here raise the possibility that ssA-TIBF may be another example of a multifunctional protein that concomitantly regulates transcription and activates or enhances DNA replication.

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