Cis-acting type I elements regulate the initiation of DNA replication, replication fork movement, and transcription of the *Tetrahymena thermophila* rDNA minichromosome and are required for cell cycle-controlled replication and developmentally programmed gene amplification. Previous studies identified three *in vitro* single-stranded type I element binding activities that were proposed to play distinct roles in replication control. Here we describe the cloning of one of these genes, TIF1, and we provide evidence for its association with type I elements *in vivo*. Furthermore, we show that TIF1 interacts (*in vitro* and *in vivo*) with pause site elements (PSE), which co-localize with replication initiation and fork arrest sites, and are shown to be essential. The *in vivo* accessibility of PSE and type I elements to potassium permanganate suggests that origin regions are frequently unwound in native chromatin. TIF1 contains sequence similarity to the *Solunum tuberosum* single strand-specific transcription factor, p24, and a related Arabidopsis protein. Antisense inhibition studies suggest that TIF1 competes with other proteins for PSE and type I element binding. TIF1 displays a marked strand bias *in vivo*, discriminating between origin- and promoter-proximal type I elements. We propose that this bias selectively modulates the binding of a different subset of proteins to the respective regulatory elements.

The *Tetrahymena thermophila* rDNA minichromosome serves as a useful paradigm for eukaryotic DNA replication control. Both the organization of the rDNA replicon and its regulation are complex. rDNA minichromosomes are formed during development of the transcriptionally active macronucleus. Site-specific chromosome fragmentation releases a 10.3-kb rDNA monomer that is converted into a 21-kb palindrome, and subsequently amplified ~5,000-fold within a single S phase (reviewed in Ref. 1). Once development is completed, rDNA replication is restricted to once per cell division in vegetatively growing progeny. Previous studies (2) revealed that the rDNA minichromosome contains two distinct sites for the initiation of DNA replication. These origins localize to 430-bp tandemly duplicated segments in the 5’-nontranscribed spacer (5’-NTS) region, designated domains 1 and 2 (D1 and D2) (Fig. 1). The 5’-NTS contains a precisely defined chromatin structure, consisting of positioned nucleosomes that bracket three nucleosome-free regions (3). The D1 and D2 replication origins are part of a single replicon composed of dispersed cis-acting regulatory determinants that localize to the nucleosome-free domains (4–6). rDNA gene amplification and vegetative replication initiation from the same replication origins (2). Thus, cell cycle control must somehow be overridden to allow for the repeated firing of replication origins during gene amplification.

Our current understanding of eukaryotic replicons comes largely from studies in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *T. thermophila*, where genetic approaches have been developed for studying the replication of artificial (yeast, *Tetrahymena*) or natural (*Tetrahymena*) minichromosomes. Replicons in all three species are composed of multiple cis-acting determinants that act in concert to control initiation. The organization of cis-acting regulatory elements differs considerably between these species. The ARS1 replicon of *S. cerevisiae*, for example, encompasses a small DNA segment (<120 bp) that is flanked by positioned nucleosomes (7). The key cis-acting element, the 11-bp ARS element, functions as a binding site for a 6-polypeptide origin recognition complex, ORC (8). Several adjacent sequence elements are required for origin activation, some of which interact with non-ORC proteins (9). *S. pombe* replicons are more complex. They can span >1,000 bp, containing multiple reiterated and partially redundant overlapping binding sites for ORC (10, 11) and enhancer elements that presumably interact with non-ORC proteins (12). Similarly, the *Tetrahymena rDNA* replicon spans >1,000 bp and contains dispersed, reiterated, and unique regulatory determinants (6, 13). The less well defined replicons of higher eukaryotes typically span thousands of base pairs (14–18).

Type I elements are phylogenetically conserved in tetrahymenid ribosomal RNA genes (rDNA) (19). They regulate at least three chromosomal processes: replication initiation, elongation of replication forks, and rRNA gene transcription. Four type I elements reside in the *T. thermophila* rDNA 5’-nontranscribed spacer (5’-NTS). Two copies (IA and IB) co-localize with replication initiation sites (Fig. 1, Domains 1 and 2), whereas the promoter-proximal IC and ID elements do not. Type I elements are essential components of the basal rDNA replicon and are responsible for both gene amplification (20) and cell cycle-regulated vegetative replication (4, 5, 21). In addition to controlling replication initiation, type I elements regulate the
transcription (24). G residue downstream of the type ID element (not shown) ablates rRNA vegetative rDNA replication are depicted for natural (B) and induced region flanked by positioned nucleosomes. Sequence changes that affect duplication, each of which contains a 230-bp nuclease-hypersensitive (22). Domains 1 and 2 correspond to an imperfect 430-bp tandem boxes C3 open boxes DNA corresponding to either the A-rich or T-rich strand of the TIF1 binds in a sequence-specific manner to single-stranded DNA amplification relative to starved (G0 arrested) cells (27). In contrast, DNA binding by TIF1 (native molecular mass 90 kDa) is essentially abolished in cells in G1 arrest (27). The most extensively studied type I element binding factors, TIF1–3, have been identified on the basis of their ability to bind to type I element sequences in vitro (27). Because the DNA-binding subunits of the TIF1–3 complexes are distinct (TIF 1, 21.5 kDa; TIF2, 85 kDa; TIF3, 32 kDa), these activities appear to be biochemically unrelated. Furthermore, their differentially regulated expression profiles suggest that these activities compete for binding to type I elements in vivo. For example, extracts from non-replicating starved cells contain dramatically elevated levels of TIF3 (65 kDa) compared with extracts from asynchronous vegetative cells or cells undergoing rDNA gene amplification (27). Concurrent with the onset of vegetative rDNA replication, TIF3 DNA binding activity is rapidly lost.2 In contrast, DNA binding by TIF1 (native molecular mass 90 kDa) and TIF2 (native molecular mass 250 kDa) is elevated 3–4-fold in vegetative cells and cells undergoing rDNA gene amplification relative to starved (G0 arrested) cells (27).

The most extensively studied type I element binding activity, TIF1 (previously designated ssA-TIBF), is a homotetramer with a subunit molecular mass of 21 kDa (25, 28, 29). Purified TIF1 binds in a sequence-specific manner to single-stranded DNA corresponding to either the A-rich or T-rich strand of the type I elements, raising the possibility that it stabilizes replication origins in an unwound state (25). In vitro footprinting studies demonstrated that sequences immediately downstream of the type IB element modulate TIF1 binding. These downstream sequences are responsible for the different replication properties of B and C3 rDNA alleles (4, 21, 22).

We describe the isolation and characterization of the TIF1 gene. We demonstrate that TIF1 binds to single-stranded type I elements in vivo, as well as to an additional phylogenetically conserved sequence, the pause site element (PSE), that is shown here to be essential. TIF1 contains limited sequence similarity to a sequence-specific single-stranded DNA-binding protein in plants (30), suggesting that these proteins may share structural features for target DNA recognition. Reverse genetic studies argue that Tetrahymena rDNA metabolism (replication initiation, replication elongation, and rRNA transcription) is regulated by multiple PSE and type I element binding proteins, some of which may have evolved overlapping, complementary functions. Our in vivo analysis of native chromatin suggests that TIF1 may modulate the binding of distinct subsets of trans-acting factors to different cis-acting regulatory determinants.

EXPERIMENTAL PROCEDURES

Strains and Culturing Methods—For the purification of TIF1 were prepared from T. thermophila strain CU428. Cultures were grown at 30 °C in 2% FPPS (2% proteose peptone, 0.2% yeast extract, 0.005% sequestrine) containing penicillin (250 μg/ml), streptomycin (100 μg/ml), and amphotericin (250 ng/ml) (31).

Gel Retardation and UV Cross-linking Studies—Gel retardation assays were carried out as described previously (25). Unless otherwise stated, oligonucleotides were purified by polyacrylamide gel electrophoresis prior to use. For standard binding and oligonucleotide competition studies, saturating amounts of affinity-purified TIF1 were incubated with 0.1 pmol of labeled oligonucleotide for 15 min on ice in 12 mM Hepes (pH 7.9), 0.1 mM EDTA, 30 mM KCl, 12.5% glycerol (v/v), 5 mM MgCl2, 1 mM dithiothreitol, and 5 μg of bovine serum albumin. For competition assays, unlabeled oligonucleotides were added to the binding reaction. Oligonucleotide substrates included the C3 type IB element (ssA37; A-rich strand, 5’-AAAAAATGAATGA-3’), PSE1–3 (PSE1–3 NN, ssPSE1–3; Watson strand, 5’-TTTTGAACCCATTTGTTT-3’), and the “non-specific” coding region oligonucleotide 2290rc (25).

UV cross-linking studies were performed to assess the presence of the different type I element binding activities in antisense transfectants. The 32P-5’-end-labeled C3 type IB oligonucleotide (ssA37) used in these experiments contributes a mass of ~12 kDa to DNA-protein complexes. Complexes were formed with crude S100 extracts using the gel shift conditions described above. Following UV cross-linking, covalent DNA-protein complexes were resolved by denaturing SDS-gel electrophoresis (27). Protein molecular weight standards were used to estimate the mass of covalently cross-linked protein-DNA complexes.

TIF1 Purification and Protein Sequencing—S100 extracts were prepared from strain CU428 as described (29). Approximately 60 ml of extract (protein concentration 8–10 mg/ml) were obtained from a 4-liter culture of cells grown to a density of 2 × 106/ml. The final yield of purified TIF1 protein was 4–6 μg. TIF1 was purified by sequential fractionation on conventional (Bio-Gel-HTP (Bio-Rad) and double-stranded DNA cellulose (Sigma)) and type I element oligonucleotide affinity resins as described previously (25). TIF1 and TIF3 elute as overlapping peaks during stepwise sodium chloride elution (100–1400 mM NaCl) of the final oligonucleotide affinity column. Affinity-purified fractions containing just TIF1 were concentrated by filtration on polyvinylidene difluoride membranes (Millipore), prior to amino-terminal sequencing or subjecting to partial proteolysis with endo-Lys-C or endo-Glu-C. Proteolytic peptides were fractionated by reverse phase high performance liquid chromatography on a C18 column. Individual TIF1 protein fragments were sequenced using automated Edman degradation on a Hewlett-Packard G1005A automated protein sequencer. TIF1 cDNA and Genomic DNA Cloning.—The first part of the TIF1 gene was obtained by reverse transcriptase-PCR. mRNA was prepared with the Fast Track 2.0 mRNA isolation kit (Invitrogen) and subjected to cDNA synthesis with a peptide 4 (DPAEKRD) reverse complement

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2 M. Mohammad and G. M. Kapler, unpublished results.
primer (5'-TCYTTTTCGCGAARTC) and Superscript reverse transcriptase (Life Technologies, Inc.). 40 cycles of PCR amplification were subsequently performed with Taq polymerase (PerkinElmer Life Sciences), using the degenerate peptide 4 reverse complement primer in combination with a peptide 1 (GETVFSAAT) primer (5'-GGIGARAC-IIGITTTYTCGCGAGACCC). To compete with the primers, annealing and extension steps were performed for 1 min at 40 °C and 4 min at 50 °C, respectively. 3'-RACE and 5'-RACE were carried out with non-degenerate primers. For 3'-RACE, cDNA synthesis and PCR amplification were performed with a 3'-anchor primer containing the sequence 5'-GAGGATCCGGGTTACA(T/I). The gene-specific primer for 5'-RACE contained the sequence 5'-GGATCCGACGTTTTTATATTTTAATGTCG-ACCC. NESTED PCR amplification was performed using the gene-specific primer 5'-TACTGTTATGCTTTTAAA with the nested specific primers 5'-CATGGATAAACATCTGACGATGCATGCTC. Nested PCR amplification was performed using the gene-specific primer 5'-TACTGTTATGCTTTTAACTCC in combination with the 5'-RACE Abridged Anchor primer provided by the manufacturer. All PCR products were cloned into the Stratagene site of pUC118 and sequenced.

**DNA Transformation with TIF1 Antisense Ribosome Plasmids and PSE Deletion/Reinsertion Plasmids**—For antisense inhibition studies of the TIF1 gene, two selected TIF1 gene fragments were cloned in both orientations into the variable loop of the 28 S ribosomal RNA gene of the sea urchin vector p5318DN. Dyck's syngamy oligonucleotides containing the relevant region of the gene, NotI adapters, and a diagnostic internal Sall site were ligated into p5318DN. Insert orientation was verified by sequencing, the 5' -coding nonsense oligonucleotides are as follows: antisense 1 (As-1; S-1 corresponds to the reverse complement sequence), 5'-GGCGGCCCAATTATCTTAGCTAATTATGGAATTTTATATTATTGTATGTTTTTAACTCC in combination with the 5'-RACE Abridged Anchor primer, and a 5'-anchor primer containing the relevant region of the gene, NotI adapters, and a diagnostic internal Sall site were ligated into p5318DN. Insert orientation was verified by sequencing, the 5' -coding nonsense oligonucleotides are as follows: antisense 1 (As-1; S-1 corresponds to the reverse complement sequence), 5'-GGCGGCCCAATTATCTTAGCTAATTATGGAATTTTATATTATTGTATGTTTTTAACTCC in combination with the 5'-RACE Abridged Anchor primer provided by the manufacturer. All PCR products were cloned into the Stratagene site of pUC118 and sequenced.

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**Results**

**TIF1 Binds to Pause Sites Elements in Vitro**—Previous genetic studies demonstrated that type I elements regulate two replication-based processes, initiation and elongation of replication forks (4, 6, 22). Although the role of adjacent PSEs in either process had not been elucidated, their phylogenetic conservation (36), co-localization with the physical sites for replication fork arrest (22), and juxtaposition to nucleosome/non-nucleosome borders at replication origins (2) suggested similar functional roles. Furthermore, the physical proximity of type I and PSEs raised the possibility that proteins bound to these different elements might physically interact.

Previous studies identified three in vitro type I element binding activities, TIF1–3 (27). As an initial step toward studying PSE-binding proteins, gel shift analysis was performed with crude S100 extracts and a radiolabeled single-stranded PSE3 oligonucleotide. Three stable gel shift complexes were detected, two of which co-migrated with type I element gel shift complexes that are mediated by the TIF1 protein (Fig. 2A) (25). To address whether the upper two PSE complexes were mediated by TIF1, TIF1 was directly assayed for PSE binding, following purification to homogeneity by conventional and sequence-specific oligonucleotide affinity chromatography (Fig. 2B). Experiments with a radiolabeled type IB element oligonucleotide and cold PSE competitor demonstrate that TIF1 binds to PSEs (Fig. 2C, left panel, crude S100 extract; right panel, purified TIF1 protein). TIF1 displayed a greater affinity for the PSE than for the type I element, both in studies with crude extracts and purified TIF1 protein. This result was confirmed using the radiolabeled PSE3 oligonucleotide (Fig. 2D, compare lanes 3 and 5). Thus, TIF1 binds to a known replication determinant, the type I element, and to an additional phylogenetically conserved sequence, the pause site element.

**Cloning and Sequencing of the TIF1 Gene**—Affinity-purified TIF1 (Fig. 2B) was obtained in amounts sufficient for peptide microsequencing. In addition to the amino terminus, peptide sequence information was obtained for four internal fragments following limited proteolysis with endo-Lys-C or endo-Glu-C (Fig. 3A, underlined segments). These sequenced segments were used to design primers for reverse transcriptase-PCR amplification of the TIF1 cDNA. Due to the high degree of codon degeneracy in the amino-terminal peptide and high A + T content of the corresponding degenerate primers, TIF1-specific PCR products were not obtained using amino-terminal primers in combination with reverse complement primers from any of the four internal peptide sequences. However, a single 490-bp PCR product was obtained with the peptide 1 forward and peptide 4 reverse complement primers (data not shown).

The predicted amino acid sequence of the cloned PCR product

16 h in 10 mTris-HCl (pH 7.5) and refreezing with 1% PFPY for 105 min. Harvested cells were washed with 10 mM Tris-HCl (pH 7.5), resuspended in 100 mM potassium phosphate (pH 7.0), and treated with 0.5 mM potassium permanganate at 20 °C for 20 min. Reactions were quenched by the addition of mercaptoethanol. Cells were subsequently washed with 10 mM Tris-HCl, and DNA was isolated as described previously (3). Permanganate-reactive sites were identified following 25 cycles of primer extension with Taq polymerase (35). 500,000–750,000 cpm of 5'-end-labeled primers were used per reaction. Reaction products were alcohol-precipitated and analyzed on 6% polyacrylamide, 8 M urea gels. The gels were exposed 48–72 h. Oligonucleotides flanking the relevant region of the gene, NotI adapters, and a diagnostic internal Sall site were ligated into p5318DN. Insert orientation was verified by sequencing, the 5' -coding nonsense oligonucleotides are as follows: antisense 1 (As-1; S-1 corresponds to the reverse complement sequence), 5'-GGCGGCCCAATTATCTTAGCTAATTATGGAATTTTATATTATTGTATGTTTTTAACTCC in combination with the 5'-RACE Abridged Anchor primer provided by the manufacturer. All PCR products were cloned into the Stratagene site of pUC118 and sequenced.
includes perfect matches to peptides 2 and 3, indicating that this product encompasses the majority of the TIF1 coding region. The remainder of the TIF1 cDNA was obtained by 5'- and 3'-RACE using non-degenerate primers (data not shown). The amino terminus of purified TIF1 protein lacks the first 23 amino acids of the TIF1 open reading frame, raising the possibility that TIF1 is post-translationally modified in vivo.

Sequence Conservation between TIF1 and a Sequence-specific Single-stranded DNA-binding Protein in Plants—The TIF1 gene predicts a protein mass of 21.5 kDa, which is in agreement with data obtained by mass spectrometry for purified TIF1 protein. The predicted isoelectric point of 8.8 is consistent with that of a DNA-binding protein. BLAST analysis revealed similarity between TIF1 and two plant proteins (Fig. 3B). Remarkably, one of these proteins, p24 from *Solanum tuberosum* (potato), is a sequence-specific single-stranded DNA binding factor. p24 is an integral part of a multiprotein complex, PBF-2, that induces transcription of the pathogen-resistance gene PR-10a, upon exposure to plant pathogens. The *Arabidopsis thaliana* cDNA ortholog is 80% identical to p24, with strong conservation at positions that are shared with TIF1.

The overall 18% sequence identity between TIF1 and the two plant proteins is not particularly high. Sequence similarity, however, is ~40%, and there are only three small gaps in the sequence alignments, suggesting that these proteins are ancestrally related. Two conserved regions are evident in the aligned sequences, with the remaining positions of likeness being distributed more or less evenly throughout the proteins. The homologous regions encompass 24% of the TIF1 protein (region 1: 34% identity, 61% similarity to p24; region 2: 24% identity, 81% similarity to p24). TIF1 lacks the polyglutamine tract proposed to play a role in transcriptional activity by p24.

Antisense Inhibition of TIF1 Gene Expression, Transformation, and Phenotypic Analysis—To examine the role of TIF1 in vivo, we employed an antisense ribosome strategy to inhibit selectively translation of TIF1 mRNA (32). In this approach, C3

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3 L. Dangott, S. Saha, and G. M. Kapler, unpublished results.
Fig. 3. Predicted peptide sequence of the TIF1 protein and alignment with related eukaryotic proteins. A, predicted amino acid sequence of the TIF1 protein. Peptide sequences obtained by Edman degradation are underlined (amino terminus, peptides 1–4). Note that the predicted amino terminus of the TIF1 open reading frame (Met-1) differs from that of the sequenced TIF1 protein (Ser-24). B, sequence alignment of the T. thermophila TIF1 protein sequence with the PBF-2 protein subunit, p24, from S. tuberosum (potato) (GenBank™ accession number AF233432) and a cDNA of unknown function from A. thaliana (GenBank™ accession number AF332452). Positions of sequence similarity are indicated by + symbols. Numbers correspond to the predicted amino acid sequence for the respective proteins (with position 1 corresponding to the predicted initiator methionine for Tetrahymena TIF1). Black lines demarcate two blocks in the TIF1 protein that display the highest degree of sequence conservation with the two plant proteins.

rDNA plasmids carrying small insertions in the variable loop of the 26 S rRNA gene are introduced into the macronucleus of B rDNA recipients by conjugant electroporation (33). Expression of the plasmid-derived rRNA gene confers resistance to paromomycin, allowing for the selection of progeny that produces antisense ribosomes. Because C3 rDNA has a replication advantage over B rDNA (4), complete replacement of endogenous rDNA (encoding wild-type ribosomes) can be achieved if the antisense-targeted gene is not essential. Although the efficiency of antisense inhibition is construct-dependent and in most cases does not totally eliminate production of the targeted protein, dosage-dependent phenotypes can be uncovered.

Two overlapping fragments from the 5' end of the TIF1 gene were cloned into the 26 S D2 variable region of the C3 antisense rDNA vector, p5318DN (Fig. 4A), and the resulting plasmids were introduced into the developing macronucleus of mating T. thermophila cells by electroporation. Wild-type transformation frequencies were achieved for both antisense (As-1 and As-2) constructs and their respective sense (S-1 and S-2) controls. Both antisense and sense transformants grew more slowly than cells transformed with a vector lacking a TIF1 insert, suggesting that slow growth was not due to specific inhibition of TIF1 production. The ability of transformed C3 rDNA to replace endogenous B rDNA was evaluated following propagation of transformants for >80 fissions. Digestion with Hinfl generates unique fragments that are diagnostic for the endogenous B and insert-carrying C3 rDNA molecules. Southern blot analysis of clonal transformants with a TIF1 coding region probe revealed that the majority of the B rDNA had been replaced with C3 rDNA in all tested antisense transformants (Fig. 4B and data not shown). Because antisense replacement transformants are viable, either the targeted TIF1 gene is not essential or antisense inhibition was “leaky,” allowing for the production of sufficient levels of TIF1 protein to sustain vegetative growth.

To assess the abundance of TIF1 protein, extracts were prepared from wild-type, antisense, and sense transformants. Active TIF1 was detected by incubating extracts with a 5'-end-labeled oligonucleotide corresponding to the A-rich strand of the C3 type IB element, followed by UV cross-linking and SDS-polyacrylamide gel electrophoresis (27). Covalent cross-linking of the radiolabeled oligonucleotide to TIF1 generates a DNA-protein complex with a predicted mass of 36 kDa. As seen in Fig. 4C, TIF1 DNA binding activity was substantially reduced in clonal As-1 and As-2 transformants. Compared with empty vector and sense orientation controls, the greatest effect was detected in As-2 transformants, where an >20-fold reduction in the amount of TIF1 protein-DNA complex was observed.
Furthermore, the ratio of replicating to non-replicating rDNA was comparable in As-2 and control transformants that were synchronized by starvation and refeeding (data not shown), suggesting that there is no pronounced difference in replication efficiency. Although the sensitivity of the two-dimensional gel assays is not particularly high, these data suggest that TIF1 is (a) not the primary replication initiator protein, (b) functionally redundant with other initiator proteins, or (c) is not rate-limiting in antisense transformants (residual activity ~5%).

Analysis of TIF1 Antisense Transformants, TIF1 Binds to Type I and PSEs in Vivo—In vitro binding studies indicated that TIF1 is one of several proteins capable of binding to type I and PSEs (Fig. 2) (25). To assess whether TIF1 plays a regulatory role in type I- and PSE-mediated functions, in vivo footprinting was performed to determine the consequence of depleting TIF1 on PSE and type I element protein occupancy in native chromatin. Footprinting analyses were performed on As-2 and vector control transformants by treating intact, S phase synchronized cells with potassium permanganate (see “Experimental Procedures”). T and C residues within single-stranded regions are preferentially rendered susceptible to modification by this reagent. Thus, regions that are naturally prone to unwinding can be identified. In addition, protein-induced unwinding or stabilization of single strand DNA structures can be detected. The strength of a footprint depends largely on the in vivo “binding site occupancy.” Because at least three type I element binding activities interact with type I element oligonucleotides in vitro (27), in vivo type I element footprinting profiles may be a composite of several distinct DNA-protein complexes.

By using primer extension to map putative TIF1 protein-binding sites, attention was focused on the promoter- and domain 2-proximal type I and PSEs. Both the upper and lower strands of these regions were examined. In total, ~525 nucleotides were evaluated, and the relevant regions are shown in Fig. 6A. Examination of an upper strand segment spanning the promoter-proximal PSE3 and type IC element (and >50 nucleotides downstream) revealed no difference in the DNA footprint for wild-type and As-2 transformants (Fig. 6A, PSE + Type IC, upper strand, and data not shown, positions 1600–1820 examined in total). In marked contrast, a minimum of 13 sites of altered reactivity were evident in the corresponding segment of the lower strand (Fig. 6A, Type ID + Promoter lower strand, Type IC lower strand, and PSE3 lower strand, and data not shown; positions 1690–1870 examined in total). Two of these sites are located immediately downstream of the type IC element. The segment between the type IC and ID elements contains four positions of altered reactivity. These residues reside within a conserved sequence that is also found immediately downstream of the type IB element. However, none of these residues are affected in and around the type IB element. The remaining altered residues map within the conserved central domain of the tripartite PSE (Fig. 6A, PSE lower strand (22) and within the type ID element.

Although somewhat less pronounced, altered footprints were also detected in the Domain 2 origin region (Fig. 6A, Type IB upper strand and data not shown). Similar to the promoter-proximal segment, differentially modified residues are confined to one DNA strand. No differences were detected between wild-type and As-2 transformants for the lower strand of the C3 type IB element (Fig. 6A, Type IB, lower strand, positions 1110–1230 examined). In contrast, a minimum of six positions in the upper strand displayed reproducibly altered footprints (Fig. 6A, Type IB, upper strand, positions 1165–1240 examined; PSE2, positions 1050–1130 examined, data not shown). These same residues, although present downstream of the type IC...
element, are not modified there. Similar to the promoter region, altered footprints were detected at several sites as follows: within the PSE2 element, immediately upstream of the type IB element, and at several positions within the 42-bp segment downstream of the type IB element (the latter of which confers the C3 rDNA replicative advantage in C3/B heterozygotes (4) and induces replication fork arrest in C3 rDNA minichromosomes (22)). These data collectively indicate TIF1 in regulating the interaction of proteins at both origin- and promoter-proximal PSE and type I elements.

The positions of altered footprints are schematically represented in Fig. 6B. With respect to type I elements, altered footprints were restricted to the upper strand (A-rich strand) at the Domain 2 origin region. In sharp contrast, promoter-proximal footprints were altered on the lower strand (T-rich strand). Because TIF1 can bind to either the A-rich or T-rich strand of type I elements in vitro (25), the in vivo foot printing results suggest that TIF1 can differentiate between promoter and origin-proximal elements in native rDNA minichromosomes. Thus, TIF1 has the potential to regulate one or more chromosomal functions by influencing the ability of a different subset of proteins to interact with origin versus promoter type I elements. Although the PSE footprints showed a similar in vivo strand bias, the orientations of the PSE2 and PSE3 elements are inverted with respect to one another. Thus, the A-rich strand is differentially modified at both sites. In conjunction with in vitro binding studies (Fig. 2) (25), the collective data argue that TIF1 binds directly to PSE and type I elements in vivo.

**Pause Site Elements Are Required for Macronuclear Transformation of Tetrahymena**—PSEs were initially identified by their co-localization with sites for replication fork pausing in T. thermophila and T. pyriformis rDNA minichromosomes (22, 36). They contain three blocks of sequence homology (26 bp in total) separated by two spacers of fixed length (18 and 8 bp). The origin-proximal PSEs in T. thermophila rDNA (Fig. 1, P1 and P2) map to the 5'-border of the nuclease-hypersensitive regions in domains 1 and 2, which encompass origins of replication. PSE3 is proximal to the rRNA promoter but does not reside within the minimal rRNA promoter (23). As a first
step toward testing the functional role of PSEs, the 52-bp tripartite PSE3 element was substituted with a DNA fragment of identical length and comparable A + T content in the rDNA transformation vector AN101. The resulting plasmid pPSE3 failed to transform mating Tetrahymena by conjugant electroporation (Table I). Reinsertion of the PSE3 sequence restored transformation to wild-type levels. Because the reinserted PSE reestablishes the sequence of the tripartite element, but alters the flanking DNA sequences by insertion of a PstI site and additional nucleotides, we conclude that the reinserted fragment encompasses an intact cis-acting regulatory determinant.

**DISCUSSION**

The *T. thermophila* DNA minichromosome has served as a useful paradigm for chromosomal DNA replication. The size of its regulatory region supercedes *S. cerevisiae* replicons, yet cis-acting regulatory determinants are less dispersed and more amenable to molecular analysis than in higher eukaryotes. Furthermore, long range interactions appear to play critical roles in the activation of *Tetrahymena* rDNA and higher eukaryotic replicons. A key cis-acting determinant, the multifunctional type I element, controls replication origin firing (4–6), mediates site-specific replication fork arrest (22), and activates transcription of the rRNA genes (23, 24). Promoter-proximal type I elements regulate distant replication origins (60). They modulate the binding of unknown proteins to additional sites in the initiator region through the presumed action of cognate type I element binding proteins (5). Thus, by identifying and understanding the regulation of type I element binding proteins, fundamental mechanisms for replication control should be illuminated.

Three distinct type I element binding activities (TIF1–3) were previously identified, based on the ability to bind to the A-rich strand of type I elements (27, 28). In addition, an ATP-dependent T strand binding activity, TIF4, has been recently identified. Developmental and cell cycle-regulated fluctuations in the abundance of TIF1–3 suggest that these proteins play distinct roles in rDNA metabolism. The most parsimonious model suggests that different proteins function as activators or repressors of DNA replication, in which temporally regulated expression of these activities provides the mechanism for controlling replication initiation. Although the in vitro DNA binding properties of TIF1–3 are consistent with a competition model, it remained to be determined whether these activities actually bind to type I elements in vivo and are able to modulate the binding of other proteins that recognize the same target site.

As a first step toward understanding regulation of the rDNA replicon, we report here the isolation of the gene encoding the type I element binding protein, TIF1. In vitro and in vivo characterizations demonstrate that, in addition to binding to type I elements, TIF1 interacts with phylogenetically conserved PSEs. We further demonstrate the essential nature of PSEs. Whereas TIF1 recognizes both type I and PSEs, additional, biochemically distinct proteins also bind to each determinant. Antisense inhibition studies demonstrate that TIF1 has the potential to regulate the binding of other proteins to type I and PSEs in vivo (presumably through its intrinsic ability to bind these targets). To our surprise, TIF1 displays a remarkable strand bias that may selectively modulate the access of different proteins to promoter- versus origin-proximal type I elements.

BLAST sequence analysis of the TIF1 protein failed to identify similarity with known eukaryotic initiation proteins (ORC1–6, mcm2–7, and mcm10). However, homology to sequences in *S. tuberosum* (potato) and *Arabidopsis* was uncovered. The *Solomonum* p24 protein and predicted translation product of the *Arabidopsis* cDNA share 80% identity, suggesting that they are closely related in function. p24 is an integral component of a multiprotein complex that activates transcription by binding to cis-acting determinants in the PR-10a promoter (30). Two clustered sites of sequence conservation were observed between TIF1 and the two plant proteins, corresponding to 21- and 23-amino acid blocks that span 24% of the mature TIF1 protein. The internal TIF1 block contains 29% sequence identity and 81% sequence similarity to both the *Solomonum* p24 and *Arabidopsis* proteins. The 23-amino acid carboxyl-terminal block shows higher sequence identity to p24 (39%), with an overall similarity of 61%. Although the length of conserved sequence between the TIF1 carboxyl terminus and *Arabidopsis* protein is shorter (14 amino acids), the percent identity (64%) and similarity (78%) are higher. The regions of similarity between TIF1 and p24 do not encompass the proposed p24 transcription activation domain.

In addition to their sequence similarity, TIF1 and p24 share several distinctive biochemical properties. First, both proteins bind single-stranded DNA in a sequence-specific manner. Because both proteins can bind independently to either strand of their target sequence in vitro, they have the potential to interact with both strands in the melted duplex. Whereas p24 recognizes a palindromic sequence present on both strands, TIF1 binds to unique sequences in the complementary A-rich and T-rich strands (25). However, both proteins appear to bind to a single DNA substrate molecule in vitro, suggesting that they do not form a bridge within melted duplex DNA (25, 30). This was unexpected for TIF1, as the native protein is a homotetramer with at least four potential type I element binding sites. This biochemical property of TIF1 is reflected in the in vivo footprinting studies reported here, which demonstrate that TIF1 preferentially modulates the occupancy of just one of the two DNA strands at origin- and promoter-proximal type I elements. Remarkably, the affected strand differs at the origin and promoter. We speculate that this strand bias has the potential to regulate the access of different proteins to the respective type I elements.

To date, only a small number of sequence-specific single-stranded DNA binding proteins have been identified in eukaryotes, the majority of which modulate transcription by competing with other factors for DNA binding (30, 38–44). Although very little is known about how this class of proteins selectively recognizes their target (45, 46), sequence-specificity may be conferred in part by non-conventional DNA structure (47). For example, the protein recognition site in the chick *a2(I)* collagen gene promoter (which resembles the T-rich strand of the rDNA type I element) contains a pyrimidine tract that is predicted to exist in a non-B DNA form (41). Similarly, the *Solomonum* p24 DNA target is predicted to have an unconventional, non-B DNA structure. By having cloned the gene for TIF1, the DNA binding domain(s) can now be elucidated. Because TIF1 preferentially binds to opposite strands at the origin and promoter type I elements in vivo, it will be of particular interest to determine whether the same protein domain mediates both interactions.

| Plasmid                | Transformation frequency<sup>a</sup> |
|------------------------|--------------------------------------|
| Wild type              | 100%                                 |
| Pause site 3 deletion  | 0% (n = 5)                           |
| Pause site 3 reinsertion| 100% (± 5%) (n = 2)                  |

<sup>a</sup> Relative to wild-type controls for each transformation experiment (n).
Unraveling the complexities of how different PSE and type I element binding proteins regulate replication initiation, elongation and transcription will in all likelihood require genetic strategies for manipulating the expression of more than one gene. The emerging picture suggests that several proteins compete for binding to the same cis-acting regulatory determinant in vivo. The in vivo type I element footprints that we detected differ considerably from in vitro footprints obtained with purified TIF1 protein (25). This could reflect a lower binding site occupancy in vivo, structural differences between the two DNA binding substrates (single-stranded oligonucleotides versus breathing duplex DNA), or in vivo competition between TIF1 and other proteins. Experiments with antisense transfectants suggest that TIF1 affects the occupancy of a significant percentage of target sites. If this were not the case, the in vivo footprints of wild-type and As-2 transformants would have been indistinguishable. Because TIF1 binds to both PSE and type I elements in vitro and affects the protein-DNA composition at these sites in vivo, the regions that encompass these two elements may be largely single-stranded in native chromosomes. Both determinants reside within the three nucleosome-free regions in the 5′-NTS (3), are accessible to single-stranded DNA binding proteins (this paper), and are predicted to reside in a large DNA unwinding element (48).

Because antisense inhibition of TIF1 is not lethal, we anticipate that other proteins must bind to PSE and type I determinants in vivo to promote DNA replication and rDNA transcription. Although the precise role of TIF1 awaits future studies, the ability of this protein to influence the occupancy of type I and PSE in vivo argues that it must play a regulatory role in one or both processes. Whereas promoter-proximal type I elements regulate both DNA replication and transcription, origin-proximal (domain 2) mutations have been only shown to affect DNA replication. The observed loss or gain of hyper-reactivity to potassium permanganate at 14 rDNA positions in antisense transfectants is consistent with TIF1 modulating other protein-DNA interactions. The fact that TIF1 shows a strand bias at the origin and promoter-proximal type I elements in vivo, yet can bind to either strand in vitro, further suggests that TIF1 may regulate the access of different proteins to origin- and promoter-proximal regulatory determinants.

An early event in replication initiation is the localized unwinding of the DNA duplex. Although single-stranded DNA-binding proteins that recognize the human c-MYC origin region and yeast ARS elements have been identified (49–51), a role in DNA replication control has yet to be established. Furthermore, it is unclear whether these proteins interact with their target sites in vivo. We demonstrate that TIF1 has this capability. Whether TIF1 or any of the other known TIFs promote the initial unwinding event await future studies. Co-purification of TIF1 and an unrelated protein with intrinsic DNA helicase activity suggests a role in replication initiation and/or elongation. Replication initiation in yeast is concurrent with the conversion of the ARS-binding ORC complex from a pre-replicative complex to post-replicative complex state. This change is associated with the formation of an extended in vivo footprint at the origin that has been proposed to result from a conformational change in ORC (52). Although ORC binds duplex DNA in a sequence-specific manner, it binds nonspecifically to single-stranded DNA (53). Thus, unwinding of yeast origins may afford an opportunity for sequence-specific single-stranded DNA-binding proteins to compete with ORC for binding to ARS elements.

Chromatin structure has been proposed to play an important role in establishing functional replicons. In the case of Tetrahymena rDNA, this may operate at two levels: preventing interactions between cis-acting replication determinants and core histones, and facilitating unwinding for the recruitment of replication proteins. Nucleosome remodeling at the simian virus 40 replication origin facilitates the binding of the T antigen initiator protein with its DNA target in vitro (54). Chromatin reorganization may also play a role in the firing of the bovine papilloma virus replicon, through the association of cellular remodeling proteins with the viral E1 initiator protein (55). Physical interactions between human ORC1 and the histone acetyltransferase HBO1 suggest that chromatin structure may influence origin site selection or activation in higher eukaryotes (56). A functional relationship between nucleosome positioning and origin activation has been demonstrated recently (7) for yeast chromosomal replicons, in which positioned nucleosomes are proposed to facilitate the conversion of ORC from a pre- to post-replicative state.

The precise positioning of nucleosomes around Tetrahymena rDNA replication origins may play an important role in establishing an active replicon or regulating origin firing. We propose that the generally unwound structure of PSE/type I element regions is promoted in part by the absence of interactions with core histones in these nucleosome-free domains. The four type I elements reside well within the nucleosome-free regions (3), and PSE and type III elements map to the borders. The essential nature of PSEs may reflect a role in establishing the chromatin organization of the rDNA replicon. Their co-localization with replication fork pausing sites further suggests a role in the regulation of replication fork movement (22).

Future experiments for dissecting the role of TIF1 and PSEs in rDNA replication control will take advantage of powerful DNA transformation methodologies and genetic tools. By using germ line co-transformation to generate rDNA or TIF gene replacements, mutant strains can be created to analyze replication initiation, elongation, and transcription during early development and/or subsequent vegetative cell divisions. Heterokaryons that are homozygous for a given mutation in the germ line micronucleus, but contain a wild-type macronucleus, can be used to study the effect of null mutations in both non-essential and essential genes in subsequent generations (13, 57). Double and triple gene knockouts mutants can be created, for example, to identify functionally redundant or antagonistic roles for proteins that compete for binding to the biologically important type I and PSEs.

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Cloning and Biochemical Analysis of the Tetrahymena Origin Binding Protein TIF1: COMPETITIVE DNA BINDING IN VITRO AND IN VIVO TO CRITICAL rDNA REPLICATION DETERMINANTS
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