The 5S RNA gene minichromosome of Euplotes

Arthur E.Roberson, Alan P.Wolffe[1], Loren J.Hauser, and Donald E.Olins

U.T.-Oak Ridge Graduate School of Biomedical Sciences, Biology Division, Oak Ridge National Laboratory, Box 2009, Oak Ridge, TN 37831-8077 and [1]Laboratory of Molecular Biology, NIDDK, National Institutes of Health, Bethesda, MD 20892, USA

Received December 30, 1988; Revised and Accepted May 18, 1989

ABSTRACT

The macronucleus of the ciliated protozoan *Euplotes eurystomus* contains about 10^6 copies of a single type of 5S ribosomal RNA gene. This 5S gene DNA is only 930 bp long, is flanked by telomeres, and contains a single coding region of 120 bp which serves as a template for transcription *in vivo* and *in vitro*. The 5S gene minichromatin possesses four positioned nucleosomes and hypersensitive cleavage sites in the telomeric regions.

INTRODUCTION

The macronuclear minichromosomes of hypotrichous ciliated protozoa (e.g., Euplotes, Oxytricha, Stylonychia, Onychodromus) represent unique systems for fundamental studies of eukaryotic gene and chromatin structure. The macronucleus consists of linear DNA ranging in size from 400 to 20,000 bp and is the site of most nuclear RNA synthesis; the micronucleus contains high molecular weight DNA and functions as the germ line (1). Native macronuclear chromatin is soluble in low ionic strength buffers without nuclease treatment (2), and specific genes can be enriched as native intact chromatin structures (3). Each macronuclear DNA molecule is bounded by telomeres (4) and is believed to represent a single coding function plus regulatory information required for transcription and replication (for review, see 1). Euplotes macronuclear chromatin is composed of nucleosomes containing the four core histones and H1 plus nonhistone chromosomal proteins (2,3). In most of its properties it is typical of eukaryotic chromatin, with the exceptions of its low molecular weight and high solubility.

The mechanism of transcriptional regulation of the 5S ribosomal RNA gene is probably better understood than for any other eukaryotic gene (5,6). The transcription factor, TFIIIA, was the first eukaryotic transcription factor to be purified (7,8). TFIIIA binds to a promotor within the 5S RNA coding region (9−11) and positively regulates its expression (12,13). The first 281 amino acids of TFIIIA contain nine repeats of a conserved 30 amino acid sequence which are basic and thought to fold into finger-like projections upon complex formation with Zn^{+2} (14,15). Detailed footprint analysis showed that these projections interact with the major groove of the internal promotor on one face of the double helix (16). In addition to TFIIIA, two other transcription factors, TFIIIC and TFIIIB, are also required for 5S RNA gene transcription. These three factors cooperatively bind to a 5S RNA gene with 1:1 stoichiometry resulting in a stable transcription complex, requiring only RNA polymerase III and rNTPs for activity (17−19). In eukaryotes, transcription complexes are believed to direct RNA polymerase to the relevant regulatory regions of the genome (20).

This report is the only detailed analysis of a hypotrich 5S RNA gene, and describes the first complete DNA and chromatin structure to be established in Euplotes.

MATERIALS AND METHODS

Euplotes Cell Culture

Stock cultures of *Euplotes eurystomus* were obtained from Carolina Biological Supply Company and maintained in 150×15 mm culture dishes containing 6–7 previously boiled wheat seeds in Carolina Spring-water. Large-scale cultures of Euplotes were grown in trays containing 3 liters of Pringsheim salt solution as described by Cadilla et al. (2). The cells were fed three times weekly with live algae, *Chlorogonium elongatum*, grown in a medium consisting of 2.67 g/L yeast extract and 1.33 g/L anhydrous sodium acetate. Algae were harvested and resuspended in Pringsheim solution before being presented to Euplotes. The Chlorogonium strain was kindly provided by David Prescott (University of Colorado, Boulder).

Macronuclear DNA Preparation

Euplotes macronuclear DNA was isolated by a modification of Swanton's procedure (21). Euplotes were starved for 2–3 days to reduce algae and were concentrated by filtration over a nylon screen. Cells were pelleted from the concentrated suspension by centrifugation at 250×g for 5 minutes at 4°C. The cells were lysed by resuspension in ice-cold 10 *mM* Tris HCl, 0.01% (w/v) spermidine phosphate, 0.5% (v/v) triton X-100 (pH 6.8). Macronuclei were isolated from the cell lysate by two cycles of sedimentation through 40% (w/v) sucrose in the above cell lysis buffer at 250×g for 5 minutes at 4°C. Macronuclei were lysed by resuspension in ice-cold 0.1 *M* EDTA, 0.25 *M* NaCl, 10 *mM* Tris HCl, 0.5% (w/v) sodium sarcosine (pH 8.0). The nuclear lysate was clarified by centrifugation and proteinase K (Boehringer Mannheim) was added to the supernatant at 1 mg/ml. The mixture was incubated at 50°C for 12 hours. Afterwards, the macronuclear DNA was purified by cesium chloride gradient centrifugation in a Ti60 rotor at 35,000 rpm for 48 hours at 20°C. The DNA was then dialyzed and precipitated with ethanol.

Cloning

A library of Euplotes macronuclear DNA was prepared in the plasmid vector pUC9 essentially as described by Swanton et al. (21). Briefly, the 3′ termini of macronuclear DNA were extended with poly(dC) using terminal deoxynucleotidyl transferase (BRL) and this DNA was annealed to pUC9 which had been linearized with *Pst I* and similarly tailed with poly(dG). These recombinant plasmids were introduced into competent *E. coli* DH5 cells (BRL) according to the manufacturer's instructions. Transformed cells were plated on LB-agar containing 100 μg/ml ampicillin. Colonies were replica plated onto nitrocellulose disks (Schleicher and Schuell) and the library was screened by hybridization with the nick translated 5S RNA gene of *Tetrahymena thermophila*, generously provided by D. S. Pederson (22).

Blotting and Hybridization

Colony transfers and nucleic acid blots were all performed using standard techniques. All hybridizations were in 5× SSC; 50 *mM* NaH_2PO_4; 0.25 mg/ml sheared, denatured salmon sperm DNA; 10 μg/ml polyadenylate; 1× Denhardt's solution; 50% (v/v) formamide (pH 6.8). The 5S RNA gene probe from Tetrahymena was used at a concentration of 10^7 cpm/ml and washed with 6× SSC, 0.1% (w/v) SDS at 42°C. The Euplotes 5S RNA gene probe was used at 10^6 cpm/ml and washed with 0.1×SSC, 0.1% (w/v) SDS at 65°C. Detection was with Kodak XAR-5 film.

DNA Sequence Analysis

For sequence analysis, a Euplotes 5S RNA gene insert was retrieved from the pUC vector by *Pst I* digestion and subcloned into the *Pst I* sites of M13mp18 and M13mp19. M13 subclones were propagated in *E. coli* JM107. Restriction enzymes which cleaved both the insert and the polylinker once were used to excise fragments of the insert, allowing sequencing to initiate at restriction enzyme sites within the insert. The DNA sequence was determined by the dideoxy technique essentially according to protocols in the BRL M13 Cloning/Dideoxy Sequencing Manual. DNA sequencing reaction products were analyzed by high resolution denaturing polyacrylamide gel electrophoresis using a Poker Face DNA Sequencer apparatus manufactured by Hoefer. Both DNA strands were sequenced twice with overlap between adjacent start sites.

Nucleosome Mapping

For the nucleosome mapping experiments, macronuclei were purified by metrizamide density gradient centrifugation as previously described (2). Nuclei were washed several times to remove metrizamide and were resuspended at a final concentration of $\sim 2 \times 10^6$ nuclei/ml in the buffer appropriate for each cleavage reagent. Micrococcal nuclease (Boehringer Mannheim) was used at 30 units/ml in 10 *mM* Pipes, 1 *mM* $CaCl_2$ (pH 7.0) at 37°C. At various times, reaction aliquots were removed and quenched with 2 *mM* EDTA. DNAse I (Sigma) was used at 0.5−10 units/ml in 60 *mM* KCl, 15 *mM* NaCl, 15 *mM* Tris HCl, 0.25 *M* sucrose, 0.5 *mM* DTT, 3 *mM* $MgCl_2$, 0.05 *mM* $CaCl_2$ (pH 7.4) at 25°C. The reaction was terminated after 3 minutes by addition of EDTA to 20 *mM* and SDS to 0.1% (w/v). Methidiumpropyl-EDTA·Fe(II), MPE·Fe(II), MPE kindly provided by Peter Dervan, was used at 5 μM in 15 *mM* Tris HCl, 15 *mM* NaCl, 60 *mM* KCl, 0.25 *M* sucrose, 5 μM $(NH_4)_2Fe(SO_4)_2$, 0.5 *mM* H_2O_2, 2 *mM* DTT (pH 7.4) at 25°C as described by Cartwright et al. (23,24). At various times reaction aliquots were quenched by addition of bathophenanthroline disulfonate to 6 *mM*. Immediately after a digestion was terminated, the DNA was precipitated with ethanol. DNAs were resuspended in 50 μl TE and purified by treatment with 20 μg/ml RNAse A at 65°C for 10 minutes followed by addition of 150 μl of a solution containing 0.5 mg/ml proteinase K in 0.1 *M* EDTA, 0.25 *M* NaCl, 10 *mM* Tris HCl, 0.5% SDS (pH 8.0) and incubation at 50°C for 1 hour. Afterwards, the DNA was extracted with phenol:chloroform:isoamyl alcohol at 25:24:1 and precipitated again with ethanol. As a control for the nucleosome mapping experiment, naked DNA was treated with 1 unit/ml micrococcal nuclease, 0.1−1.0 units/ml DNAse I, or 20 μM MPE·Fe(II) in the appropriate buffer and subsequently purified. The digestion products from macronuclei and naked DNA were electrophoresed through 1% agarose gels, transferred to nylon by the Southern technique, and analyzed by indirect end-labelling. Synthetic oligonucleotides 30 bases in length were prepared using an Applied Biosystems DNA Synthesizer and were 5′ end-labelled for use as hybridization probes.

In Vitro Transcription

To assay the transcription of the Euplotes 5S RNA gene *in vitro*, a soluble, cell-free extract of *Xenopus laevis* oocyte nuclei (GV supernatant) was prepared exactly as described by Birkenmeier et al. (25). The standard transcription reaction mixture was prepared by mixing 10 μl of GV supernatant with 2 μl J buffer [70 *mM* NH_4Cl, 7 *mM* $MgCl_2$, 0.1 *mM* EDTA, 2.5 *mM* DTT, 10% (v/v) glycerol, 10 *mM* Hepes (pH 7.4)] containing 30 units RNasin (Promega Biotec), 2 μl of a mix of nucleoside triphosphates [final concentration = 0.5 *mM* ATP, CTP, and UTP; 25 μM GTP and 10 μCi radioactive [α^{32P}]-GTP, specific activity = 2000 Ci/mmol (New England Nuclear)], DNA in 1 μl J buffer, and in some

```
-235          5'—CCAAAACCCCAAAACCCCAAAACCCCAAAACCCCAAAACCCCAAG
-190 TGAGAACTAAACACTTGAATTCAGATATAAGTATTATAATTCAGGAATCTGAGATTTACG
-130 GAATTATAAATCCAGATGGACTAGAGCATGCCCAAGCGTAAGTAAACGGTTTTTCAAAGA
-70  CTGGAGAGATGTTTTGGGTGAGTTCGATTTTAGGTGTTGAGTATATAAAGAGTGGCCTTA
-10  CTATAATTATCGCTATCGGCCATACTAAGCCAAATGCACCCGGATCCCTTCCGAACTCCGA
50  AGTTAAGCGGTTTAAGGCCTGTTAAGTACTGAGGTGGGGGACCACTCGGGAACTTCAGGT
110 GCTGATAGCTTTTTGCTCCTGAAGCTATCTTTTTGCACTCTTTCTTTTTATCTATCCACC
170 CTTCAGTACTTCTCACACTATCCAGGTTGGCGAGTTTTTGTTTCGTCGTGCTTGGCATAA
230 TGGAAAATCATCCTTCTTTGTGATAAAATAGAAAACAAAATGGCTAGTACAATCTTGAGA
290 CACCCGATGCTGGGTTTTGGGTACAAAGACTGCCAATACGTCCATCCCCTATCGAATTAC
350 CAAATATTGTTTTCTAATTTGGGTATTAAGTTCGAATCTCGCCAATATTTAAGTTTTTAT
410 TTCGCCATTAATCACAAATATGGCTAAATCTCAAATAAAAATCAGATATTAACTACCCAT
470 TTGGATTTAATATCCGATCCAAGACTCTATACCTGATGGTTGGAAGTTCTAAAACAATTT
530 ATTTTGTTGGGGAAGACAAAGAGTCGAATAAAACGCTAATTAATTTTATTGTACATCTTG
590 GCTTCAGTGCCATTATTAATTTTCAAGCTTTAAAGTTTTAGTTCAAGTCTGGAATCGTAG
650 CACGGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGGGGTTTTGG—3'
```

Figure 1. Nucleotide sequence of the cloned Euplotes macronuclear 5S RNA gene. The nucleotide sequence of the strand homologous to the RNA is shown. The coding region (positions +1 to +120), the putative replication origin (601−623), and the telomeric sequences are underlined.

experiments α-amanitin. Reactions occurred in capped conical tubes at 22°C for 1 hour. Reactions were stopped by the addition of 300 μl SETS [100 *mM* NaCl, 10 *mM* Tris HCl, 1 *mM* EDTA, 1% (w/v) SDS (pH 8.0)], phenol extracted, chloroform extracted, and nucleic acids precipitated by removing the aqueous phase and adding to it 1/10 volume of 3 *M* Na acetate and 3 volumes of ethanol. The labelled RNA was redissolved in 80% (v/v) formamide, 25 *mM* Tris borate, 0.5 *mM* EDTA (pH 8.3). Samples were analyzed by autoradiography following electrophoresis on a 10% (w/v) polyacrylamide gel containing 50 *mM* Tris borate, 1 *mM* EDTA, and 8 *M* urea. 5S RNA synthesis was quantified by excising a gel fragment containing the 5S RNA band and measuring Cerenkov radiation. Backgrounds were measured by excising and counting gel fragments on either side of the 5S RNA band.

RNA Preparation and Northern Analysis

Euplotes total cellular RNA was isolated by the guanidinium thiocyanate (GSCN) method essentially as described by Chirgwin et al. (26). Euplotes were starved for two days to reduce algae and were collected by filtration over a nylon screen. The screen was then held over a beaker and the Euplotes were washed from it into the beaker using a solution of 5 *M* GSCN, 0.1 *M* β-mercaptoethanol, 25 *mM* sodium citrate, 0.5% (w/v) sodium sarcosine, and 0.1% (v/v) antifoam A (Sigma) (pH 7.0). One gram of solid CsCl was added per 2.5 ml of lysate. Eight milliliter aliquots of lysate were layered over 2.5 ml cushions of 5.7 *M* CsCl in 0.1 *M* EDTA (pH 7.4). RNA was pelleted in an SW41 rotor at 35,000 rpm for 12 hours at 20°C. The RNA pellets were resuspended in 1 *mM* EDTA (pH 7.4) and precipitated twice from sodium acetate/ethanol. RNA was analyzed by electrophoresis in 1.2% (w/v) agarose gels containing 2.2 *M* formaldehyde in 10 *mM* NaH_2PO_4 buffer (pH 6.5). Northern blots were performed using standard techniques and hybridizations were carried out as previously described.

Table I. Properties of Euplotes Macronuclear 5S RNA Gene

Length:	930 bp
Coding region length:	120 bp
Copy number:	~10^6
GC% coding region:	53%
GC% flanking DNA:	37%
GC% telomeres:	52%
GC% overall:	39%
Homology of coding region to	
Euplotes woodruffi:	99%
Tetrahymena thermophila:	79%
Paramecium tetraurelia:	79%
Euglena gracilis:	72%
Xenopus laevis (somatic):	65%
Homology of Euplotes and	
Xenopus laevis (somatic) ICRs:	70%
Average nucleosome repeat length:	190 bp
Average nucleosome core length:	148 bp
Average nucleosome linker length:	42 bp

RESULTS

Cloning and Sequence Analysis

A library of *Euplotes eurystomus* macronuclear DNA was constructed in pUC9 and cloned into *E. coli* DH5. The library was screened by *in situ* colony hybridization using the nick-translated 5S RNA gene of *Tetrahymena thermophila* (22) as probe. Approximately 33,000 colonies were screened and plasmids were prepared from 19 positives. All of these plasmids contained inserts of equal size (930 bp) which co-migrated with the native Euplotes 5S RNA gene as judged by Southern analysis using the Tetrahymena 5S RNA gene as probe. These 19 isolates were further examined by restriction fragment length polymorphism (RFLP) analysis using six restriction enzymes, each cleaving the insert one or more times. No heterogeneity among the clones could be detected at this level of resolution. The cloned 930 bp macronuclear DNA insert was excised from the plasmid vector, gel-purified, and used as probe against native macronuclear genomic DNA on a Southern blot. A single species of 930 bp was observed, indicating that the cloned 5S RNA gene was a faithful representation of the native gene. These results taken together suggest that *Euplotes eurystomus* contains predominately one type of macronuclear 5S RNA gene.

A single 5S RNA coding region of 120 bases was found within the 930 bp macronuclear DNA molecule. A summary of selected properties of the Euplotes macronuclear 5S gene is presented in Table I. The *Euplotes eurystomus* coding region differs in only one nucleotide from that determined by sequencing 5S RNA from *Euplotes woodruffi* (27). The coding sequence is ~80% homologous to that of other protozoa such as Tetrahymena, Paramecium, and Euglena, and is ~65% homologous to the Xenopus sequence. The 3′ terminus of the coding region was observed to contain the canonical RNA polymerase III termination signal, $GCT_{4-5}GC$ (5). The RNA sequence readily conforms to the minimal model of eukaryotic 5S RNA secondary structure proposed by Erdmann and Wolters (28). This macronuclear gene was found to be flanked by telomeric sequences identical to those previously described in *Euplotes aediculatus* (4) including the conserved pentanucleotide TTGAA 17 bp inward from the C_4A_4 repeats. A DNA structure similar to those suggested to serve as replication origins in Oxytricha (29,30) and Stylonychia (31) was observed

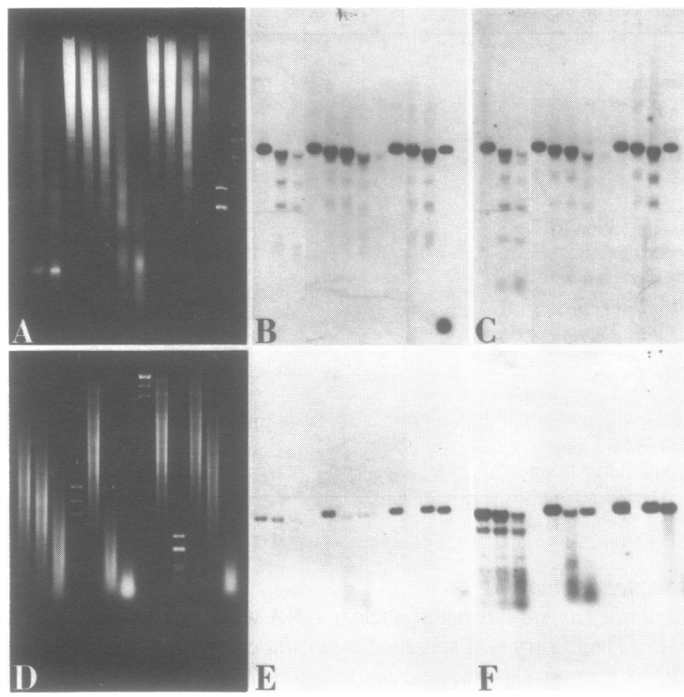

Figure 2. Digestion of Euplotes macronuclei and purified DNA.
Panel A: Ethidium bromide staining of an agarose gel loaded left to right (lanes 1−14) with macronuclear digestion products as follows: lanes 1−3, micrococcal nuclease digestion for 0, 1, 2 minutes; lanes 4−8, DNAse I digestion at 0, 0.5, 1, 3, 10 units/ml; lanes 9−11, MPE·Fe(II) digestion for 0, 5, 10 minutes; lane 12, native Euplotes macronuclear DNA (untreated); lanes 13 and 14, pUC9×*Pst I* ×*Hae III* and φX174RF×*Hae III* size markers.
Panel B: Autoradiogram of a Southern blot of the gel in panel A probed with the 5′ synthetic oligonucleotide probe.
Panel C: Autoradiogram of the blot in panel B after stripping and reprobing with the 3′ synthetic oligonucleotide probe.
Panel D: Ethidium bromide staining of an agarose gel loaded left to right (lanes 1−13) with naked DNA digestion products as follows: lanes 1−3, micrococcal nuclease digestion for 10, 40, 120 seconds; lane 4, φX174RF×*Hae III* size marker; lanes 5−7, DNAse I digestion at 0.1, 0.3, 1.0 units/ml; lane 8, λ×*Hind III* size marker; lane 9, native Euplotes macronuclear DNA (untreated); lane 10, pUC9×*Pst I*×*Hae III* size marker; lanes 11−13, MPE.Fe(II) digestion for 2, 7, 20 minutes.
Panel E: Autoradiogram of a Southern blot of the gel in panel D probed with the 5′ synthetic oligonucleotide probe.
Panel F: Autoradiogram of the blot in panel E after stripping and reprobing with the 3′ synthetic oligonucleotide probe.

approximately 50 bp inward from the 3′ telomere. An inverted repeat, ATTATTA, separated by 2 bp from the sequence TTTCAAGCTTTAAA, which is capable of forming a cruciform structure, is located between positions 601−623. These structural motifs are shared by the polyoma virus origin of replication (32) and ARS elements in yeast (33,34). Electron microscopic studies have indicated that putative replicating macronuclear DNA molecules generally show evidence of replication origins near one end of the linear molecules (35,36).

The GC% was calculated as a sliding local average along the macronuclear DNA molecule. Local GC% maxima were observed at the coding region and telomeres; while

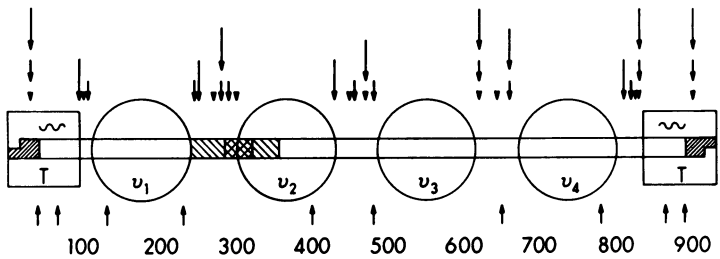

Figure 3. Chromatin structure of the Euplotes macronuclear 5S RNA gene. The central axis represents the 930 bp gene: the telomeres, the coding region, and the internal promotor are indicated by cross hatching. Cleavage sites of the DNA in chromatin are indicated above the axis, preferred cleavage sites in naked DNA for micrococcal nuclease are indicated below. Cleavage sites for micrococcal nuclease, DNAse I, and MPE.Fe(II) are represented by ↓, ↓, and ▾, respectively. The circles represent nucleosomes (ν_1, ν_2, ν_3, and ν_4) and the squares represent telomeric complexes. The wavy lines indicate the origins of the synthetic oligonucleotide probes used for indirect end-labelling.

a local GC% minimum was observed to coincide with the putative replication origin. Interestingly, within the coding sequence a depression in GC% was seen at the putative internal control region.

The abundance of the 5S RNA gene in the Euplotes macronucleus was measured by dot blot hybridization (data not shown). Cloned 5S RNA gene was retrieved from the vector, gel-purified and used as a hybridization probe for a dot blot, containing a dilution series of this same DNA adjacent to a dilution series of native Euplotes macronuclear genomic DNA. Hybridization intensities were measured by densitometry and the amount of 5S DNA in macronuclear genomic DNA was calculated by comparison to the 5S DNA standard. About 1 in 200 macronuclear DNA molecules harbors a 5S RNA gene. Since there are $\sim 1.92 \times 10^8$ total DNA molecules per macronucleus (calculated from references 37–39), if 1 in 200 of these harbors a 5S RNA gene, this amounts to $\sim 9.6 \times 10^5$ copies per macronuclear genome. Since the average copy number of a Euplotes macronuclear gene is ~8200 (calculated from 37–39), the 5S RNA gene is amplified ~120-fold compared to a gene of typical abundance. This hyperamplification correlates well with the ~100-fold hyperamplification of the 7.5 KB gene coding for 19S and 25S ribosomal RNAs reported for Oxytricha (1,37). Presumably, as in other species, the macronuclear genes coding for these rRNAs are coordinately amplified so as to better synchronize the rates of ribosomal component production.

Chromatin Structure

To probe the native chromatin structure of the Euplotes 5S RNA gene, macronuclei from starved cells were treated with micrococcal nuclease, DNAse I, or MPE·Fe(II). Euplotes were starved for several days to reduce algae and to reduce macronuclear transcription to a minimal level. Purified macronuclei were resuspended in the appropriate buffers and treated with the cleavage reagents as described in **Materials and Methods**. DNA digestion products were purified and detected on Southern blots by indirect end-labelling using synthetic oligonucleotides as hybridization probes (Fig. 2). Thirty bases of DNA sequence immediately inward from the telomeres at each end of the coding strand were used as probes. The wavy lines in Figure 3 show the positions from which the probes were derived: the 5′ probe was derived from nucleotides −193 to −164 and the 3′ probe from +623 to +652. The positions of cleavage sites and protected regions are shown schematically

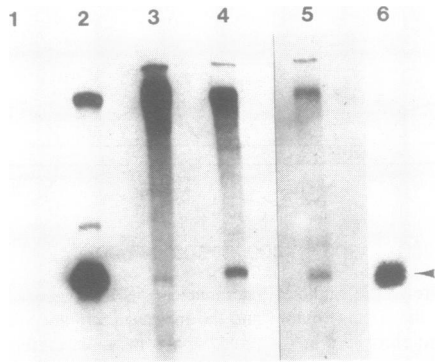

Figure 4. Transcription of the Euplotes macronuclear 5S RNA gene. Lanes 1–5 are an autoradiogram of a polyacrylamide gel containing *in vitro* transcription reaction products as follows: lane 1, negative control reaction with no added DNA; lane 2, positive control reaction with 200 ng of a circular plasmid containing a *Xenopus laevis* somatic 5S RNA gene; lane 3, reaction with 1 μg native Euplotes macronuclear DNA; lane 4, reaction with 500 ng of the circular pUC9 plasmid harboring the Euplotes macronuclear 5S gene in its *Pst I* site; lane 5, reaction with 100 ng of the gel-purified *Pst I* insert, the linear Euplotes 5S RNA gene. Lanes 1–4 were exposed 30 minutes, lane 5 was exposed 6 hours. Lane 6: Euplotes total cellular RNA was analyzed by northern hybridization using the nick-translated 930 bp Euplotes macronuclear 5S RNA gene as probe. The arrow in the margin indicates transcripts migrating at 120 nt.

in Figure 3. The measured linker lengths are: telomere-ν_1, 5 bp; ν_1-ν_2, 55 bp; ν_2-ν_3, 30 bp; ν_3-ν_4, 40 bp; ν_4-telomere, 10 bp. Many of the hybridization 'bands' are diffuse zones, indicating that accessible DNA stretches of some length ($\sim$ 45 bp) exist between the protected regions. The arrows in Figure 3 indicate the center of each cleavage zone and do not imply precise cutting sites. For all of the cleaved chromatin samples, a prominent band is observed at the top of the lane, about 60 bp shorter than the native gene. That this band is more intense than any of the others suggests that it results from cleavage of a hypersensitive site(s) (40). Since the telomeric sequences at each end of the DNA are identical, we contend that the prominent band results from cleavage of $\sim$30 bp from each end of the gene. The migration anomalies caused by single-strand DNA tails (41) suggest that the hypersensitive cleavage involves removal of the single-strand DNA tails. Preferential cleavage of the single-strand/double-strand boundary in chromatin was also reported by Gottschling and Cech (41). These initial hypersensitive cleavage events must be taken into account when mapping the positions of the other cleavage sites. The same cleavage sites were mapped when the blot was probed with oligonucleotides from opposite ends of the DNA molecule. All three cleavage reagents result in the same protection map for chromatin, although micrococcal nuclease demonstrated strong sequence preferences on naked DNA controls. The results are consistent with a model containing four positioned nucleosomes with an average repeat length of $\sim$190 bp (core length $\sim$145 bp) and telomeric complexes protecting $\sim$100 bp of DNA at each end (40).

5S RNA Gene Transcription

The *in vivo* and *in vitro* transcriptional properties of this gene were investigated (Fig. 4). When Euplotes total cellular RNA was analyzed by northern hybridization using the entire 930 bp 5S RNA gene as a probe, only a single band of $\sim$120 nucleotides was detected (lane 6) even after a long exposure. This result suggests that this macronuclear gene codes for only a single size transcript *in vivo*. When a Xenopus oocyte nuclear extract was

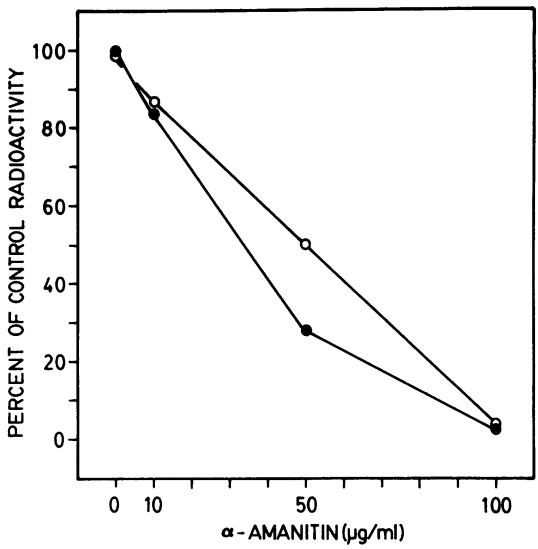

Figure 5. Sensitivity of *in vitro* transcription to α-amanitin. Radioactivity incorporated into 5S RNA is plotted as a function of α-amanitin concentration for the *Xenopus laevis* somatic 5S RNA gene (closed circles) and the Euplotes macronuclear 5S RNA gene (open circles). Both templates were presented in circular plasmids.

presented with the Euplotes 5S RNA gene, a specific transcript of ~120 nucleotides was generated. However, since 5′ end mapping experiments were not performed, precise initiation at position +1 of the coding region has not been established. The Euplotes 5S RNA gene was transcribed *in vitro* when present as closed circular form (contained in a plasmid), or as linear form (gel-purified from the plasmid, or as native macronuclear genomic DNA). Furthermore, under 'rate enhanced conditions' (42) the linear DNA template was transcribed as efficiently as the circular structure (data not shown). In transcriptional rate enhancement, transcriptional efficiency (defined as the number of transcripts generated per gene per hour) is seen to increase as the amount of 5S DNA is decreased while holding total DNA concentration constant (42). Presumably the carrier DNA sequesters repressor molecules, such as histones. Under rate enhanced conditions, *in vitro* transcriptional efficiency approaches that *in vivo*, and furthermore the *in vitro* transcriptional efficiency of linear templates approaches that of circular templates. The reason for lower transcription rates of linear templates at high concentration is the presence of free DNA ends, especially single-strand DNA at the ends, which bind transcription factors and RNA polymerase. These end effects are reduced at low linear template concentration. In an additional experiment, α-amanitin was used to demonstrate that the *in vitro* transcript was synthesized by RNA polymerase III (Fig. 5).

DISCUSSION

The DNA sequence, chromatin structure, and transcriptional properties of the Euplotes macronuclear 5S RNA gene have been characterized. The coding region of this gene is nearly identical to the 5S RNA sequence reported for *Euplotes woodruffi* (27) and is ~80% homologous to other known protozoan sequences. The coding region appears to be typical of all known 5S RNA sequences, being 120 nucleotides in length, conforming readily to

Table II. Nucleosome Positions on 5S RNA Genes from Various Organisms

Organism	Chromatin source	*in vivo* or *in vitro* reconstitution	Transcriptional state	Nucleosome positions[a]	Reference
Euplotes eurystomus	macronucleus	*in vivo*	presumably inactive	+63 to +213	this study
Drosophila melanogaster	embryos	*in vivo*	presumably active	not typical nucleosomes	24
Xenopus borealis (somatic type)	–	reconstitution	with and without TF111A	−85 to +78	48
Drosophila melanogaster	cultured cells and embryos	*in vivo*	presumably active	A phase: −69 to +76[b] and +97 to +241[b] B phase: +24 to +169[b]	51
Xenopus laevis (somatic type)	–	reconstitution	inactive	+20 to +200	52
Tetrahymena thermophila	macronucleus	*in vivo*	active inactive	nucleosome-free +98 to +244[b]	53
Xenopus laevis (oocyte type)	erythrocytes	*in vivo*	inactive	−30 to +110[b]	54
Xenopus laevis (oocyte type)	liver	*in vivo*	inactive	four	55
	cultured cells	*in vivo*	active	phases	
	–	reconstitution	inactive	observed	
Lytechinus variegatus	–	reconstitution	inactive	−71 to +74	56
Lytechinus variegatus	yeast	*in vivo*	unknown	−71 to +74	57

[a] Numbers are approximate and refer to nucleosome protected regions on or closest to the 5S gene coding region which begins at position +1.
[b] These numbers were estimated from figures.

the canonical 5S RNA secondary structure, and ending with the RNA polymerase III termination signal. The telomeric sequences flanking the *Euplotes eurystomus* gene are identical to those previously described in *Euplotes aediculatus* (4). The *Euplotes eurystomus* macronuclear 5S RNA gene also contains a structure similar to suggested (but untested) replication origins in Oxytricha (29,30) and Stylonychia (31). The high copy number of the Euplotes 5S RNA gene may correlate well with hyperamplification of the genes coding for 19S and 25S rRNAs (1,37). These protozoa are very large cells and probably require many copies of rRNA genes to produce enough ribosomes.

When the Euplotes 930 bp 5S RNA gene was used to probe a Southern blot of other hypotrich macronuclear DNAs, 5S RNA genes were identified as 680 and 600 bp in *Oxytricha nova*, 600 bp in *Stylonychia lemnae*, and 640 bp in *Onychodromus quadricornutus* (data not shown). The sizes of macronuclear DNA molecules coding for 5S RNA have been previously reported as 690 bp in *Oxytricha fallax* (43) and 200−300 bp in *Stylonchia mytilus* (44). The reason for length polymorphism of macronuclear 5S RNA genes among and within various hypotrich species is unknown, but possible explanations include differing lengths of nontranscribed spacer DNA flanking the 120 bp coding region and/or multiple copies of the coding region. Although the 5S genes of the other ciliates may contain multiple coding regions, the Euplotes macronuclear gene, which is the longest, does not. It would be interesting to compare the two versions of macronuclear 5S RNA genes in *Oxytricha nova* (680 and 600 bp) and see if these are differentially regulated. All of the macronuclear 5S RNA genes in *Euplotes eurystomus* reside on a single size DNA molecule (930 bp), and all of these are identical at the level of resolution afforded by RFLP analysis.

Euplotes macronuclear DNA is organized in a typical nucleosomal structure containing the four core histones and an H1-like protein (2). The biophysical properties of bulk macronuclear chromatin resemble those of chicken erythrocyte chromatin (2). The nucleosome repeat length of bulk Euplotes macronuclear chromatin was found to be 186 bp (2). Approximately 100 bp of DNA is protected at each end of the 5S gene by telomeric complexes which contain internal hypersensitive sites, as was also observed in Oxytricha (40,41,45,46). In Oxytricha, two proteins (43 and 55 KDa) have been identified which specifically recognize and bind to the telomeric sequences (45,46). The telomeric complexes may function to protect the linear DNAs from exonucleolytic attack *in vivo*, may be involved in replication, may constitute anchors against which the linear DNAs could be torsionally strained, and may play a role in chromatin structure.

It has been suggested that nucleosome positioning is mediated inwardly along the macronuclear chromatin fragments by the telomeric complexes at the ends (41). Consistent with this model, we observe four nucleosomes occupying specific positions along the macronuclear DNA. However, if nucleosome positioning was purely statistical (47) and mediated inwardly by steric interactions with the telomeric complexes, one would predict that this molecule would have a symmetrical nucleosome structure with equal lengths of linker DNA between nucleosomes. Since this was not observed, other factors such as the DNA sequence itself and/or accessory proteins (e.g., transcription factors) must also influence nucleosome positioning. Relevant to this issue, we have observed (manuscript in preparation) that the Euplotes macronuclear polyubiquitin gene is about the same length as the 5S gene and is also composed of four nucleosomes. The positions of the nucleosomes, however, are quite different from those found on the 5S gene. Analysis of macronuclear chromatin in rapidly growing cells reveals that, at least, a significant fraction of the 5S genes possess the same nucleosome positioning (and no new hypersensitive sites) during

rapid growth as during starvation (data not shown). Presumably not enough 5S genes are actively transcribed to detect a difference in total chromatin. Chromatin fractionation (3) may allow us to resolve and characterize the active chromatin structure of this gene.

The positioning of nucleosomes on 5S RNA genes has been investigated in a number of organisms (Table II). Although specific positioning is observed in each case, no regular trend or pattern among organisms is readily apparent. The 5′ end, the 3′ end, or the entire coding region may be bound in a nucleosome. Evidently the 5S gene transcription machinery is able to recognize its target in a variety of chromatin mileux. Obviously packaging of this gene is a complex matter and may involve factors such as higher order chromatin structure. The results of Daniela Rhodes (48) suggest that TF111A can bind to the ICR even when the ICR is previously bound to histones, so perhaps nucleosome positioning is not crucial to the regulation of this gene. More important may be whether H1 is bound (49). In contrast to the results of Rhodes, Gottesfeld and Bloomer (50) were unable to generate transcriptionally active chromatin templates by *in vitro* reconstitution when histones were added to the DNA prior to TF111A. The positioning we present for the Euplotes gene resembles that reported for *Drosophila melanogaster in vivo* (51), the *Xenopus laevis* somatic gene reconstituted *in vitro* (52), and the *in vivo* positioning in starved *Tetrahymena thermophila* macronuclei (53) in that the 5′ end of the coding region is non-nucleosomal.

That a Xenopus oocyte nuclear extract (25) recognized and accurately transcribed the Euplotes 5S gene *in vitro* suggests that the transcriptional mechanism of this gene has been conserved over evolution as has its nucleotide sequence. Three forms of the Euplotes 5S gene were used as templates: the circular pUC9 plasmid harboring the Euplotes 5S gene in its *Pst I* site, the gel-purified *Pst I* insert (a linear structure), and native Euplotes macronuclear genomic DNA. Each template generated a specific RNA transcript of 120 nucleotides. Transcription of the Euplotes gene was about 5% as efficient as the Xenopus gene when both were presented in circular plasmids. In another experiment, rate enhanced conditions (42) were used to demonstrate that the linear DNA structure can be transcribed as efficiently as the circular structure. Current studies in our laboratory are being directed toward the development of a homologous system for *in vitro* transcription of Euplotes macronuclear 5S RNA genes.

ACKNOWLEDGEMENTS

The authors wish to thank Donald Brown, Lucia Cacheiro, Carmen Cadilla, James Finch, Elke Helftenbein, Adria Herrmann, Frank Larimer, Ada Olins, David Pederson, David Prescott, Bruce Suttle, and Marshal Swanton for their assistance and contribution of materials. Research sponsored by the Office of Health and Environmental Research, United States Department of Energy, under Contract DE-AC05−840R21400 with the Martin Marietta Energy Systems, by NIH Research Grant GM19334 and NSF Research Grant DCB870057 to D.E.O., and by NIH Genetics Training Grant GM07438 to U.T.-Oak Ridge Graduate School of Biomedical Sciences.

Research sponsored by the Office of Health and Environmental Research, United States Department of Energy, under Contract DE-AC05840R21400 with the Martin Marietta Energy Systems, by NIH Research Grant GM19334 and NSF Research Grant DCB870057 to D.E.O., and by NIH Genetics Training Grant GM07438 to U.T.-Oak Ridge Graduate School of Biomedical Sciences.

REFERENCES

1. Kraut, H., Lipps, H. J., and Prescott, D. M. (1986) International Review of Cytology **99**, 1–28.
2. Cadilla, C. L., Harp, J., Flanagan, J. M., Olins, A. L., and Olins, D. E. (1986) Nucl. Acids Res. **14**, 823–841.
3. Cadilla, C. L., Roberson, A. E., Harp, J., Olins, A. L., and Olins, D. E. (1986) Nucl. Acids Res. **14**, 8501–8512.
4. Klobutcher, L. A., Swanton, M. T., Donini, P., and Prescott, D. M. (1981) Proc. Natl. Acad. Sci. USA **78**, 3015–3019.
5. Brown, D. (1982) The Harvey Lectures **76**, 27–44.
6. Brown, D. D. and Schlissel, M. S. (1985) Cell **42**, 759–767.
7. Engelke, D. R., Ng, S. Y., Shastry, B. S., and Roeder, R. G. (1980) Cell **19**, 717–728.
8. Sakonju, S., Brown, D. D., Engelke, D. R., Ng, S. Y., Shastry, B. S., and Roeder, R. G. (1981) Cell **23**, 665–669.
9. Pelham, H. R. B. and Brown, D. D. (1980) Proc. Natl. Acad. Sci. USA **77**, 4170–4174.
10. Sakonju, S., Bogenhagen, D. F., and Brown, D. D. (1980) Cell **19**, 13–25.
11. Bogenhagen, D. F., Sakonju, S., and Brown, D. D. (1980) Cell **19**, 27–35.
12. Wormington, W. M. and Brown, D. D. (1983) Dev. Biol. **99**, 248–257.
13. Bogenhagen, D. F., Wormington, W. M., and Brown, D. D. (1982) Cell **28**, 413–421.
14. Bohm, S. and Drescher, B. (1985) Studia Biophysica **107**, 237–247.
15. Vincent, A. (1986) Nucleic Acids Res. **14**, 4385–4391.
16. Fairall, L., Rhodes, D., and Klug, A. (1986) J. Mol. Biol. **192**, 577–591.
17. Setzer, D. R. and Brown, D. D. (1985) J. Biol. Chem. **260**, 2483–2492.
18. Segall, J. (1986) J. Biol. Chem. **261**, 11578–11584.
19. Bieker, J. J., Martin, P. L., and Roeder, R. G. (1985) Cell **40**, 119–127.
20. Brown, D. D. (1984) Cell **37**, 359–365.
21. Swanton, M. T., Greslin, A. F., and Prescott, D. M. (1980) Chromosoma **77**, 203–215.
22. Pederson, D. S., Yao, M.-C, Kimmel, A. R., and Gorovsky, M. A. (1984) Nucl. Acids Res. **12**, 3003–3021.
23. Cartwright, I. L., Hertzberg, R. P., Dervan, P. B., and Elgin, S. C. R. (1983) Proc. Natl. Acad. Sci. **80**, 3213–3217.
24. Cartwright, I. L. and Elgin, S. C. R. (1984) The EMBO Journal **3**, 3101–3108.
25. Birkenmeier, E. H., Brown, D. D., and Jordan, E. (1978) Cell **15**, 1077–1086.
26. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) Biochemistry **18**, 5294–5299.
27. Kumazaki, T., Hori, H., and Osawa, S. (1983) J. Mol. Evol. **19**, 411–419.
28. Erdmann, V. A. and Wolters, J. (1986) Nucl. Acids Res. **14** (Suppl.), r1–r59.
29. Kaine, B. P. and Spear, B. B. (1980) Proc. Natl. Acad. Sci. USA **77**, 5336–5340.
30. Kaine, B. P. and Spear, B. B. (1982) Nature **295**, 430–432.
31. Helftenbein, E. (1985) Nucl. Acids Res. **13**, 415–433.
32. Soeda, E., Miura, K., Nakaso, A., and Kimura, G. (1977) FEBS Lett. **79**, 383–389.
33. Stinchcomb, D. T., Thomas, M., Kelly, J., Selker, E., and Davis, R. W. (1980) Biochem. **77**, 4559–4563.
34. Montiel, J. F. et al. (1984) Nucl. Acids Res. **12**, 1049–1068.
35. Murti, K. G. and Prescott, D. M. (1983) Mol. Cell. Biol. **3**, 1562–1566.
36. Allen, R. L., Olins, A. L., Harp, J. M., and Olins, D. E. (1985) Eur. J. Cell Biol. **39**, 217–223.
37. Swanton, M. T., Heumann, J. M., and Prescott, D. M. (1980) Chromosoma **77**, 217–227.
38. Ammermann, D. and Muenz, A. (1982) Eur. J. Cell Biol. **27**, 22–24.
39. Steinbruck, G., Hass, I., Hellmer, K. H., Ammermann, D. (1981) Chromosoma **82**, 199–208.
40. Lipps, H. J. and Erhardt, P. (1981) FEBS Lett. **126**, 219–222.
41. Gottschling, D. E. and Cech, T. R. (1984) Cell **38**, 501–510.
42. Wolffe, A. P., Jordan, E., and Brown, D. D. (1986) Cell **44**, 381–389.
43. Rae, P. M. M. and Spear, B. B. (1978) Proc. Natl. Acad. Sci. USA **75**, 4992–4996
44. Lipps, H. J. and Steinbruck, G. (1978) Chromosoma **69**, 21–26.
45. Gottschling, D. E. and Zakian, V. A. (1986) Cell **47**, 195–205.

46. Price, C. M. and Cech, T. R. (1987) Genes and Development **1**, 783–793.
47. Kornberg, R. D. and Stryer, L. (1988) Nucl. Acids Res. **16**, 6677–6690.
48. Rhodes, D. (1985) The EMBO Journal **4**, 3473–3482.
49. Schlissel, M. S. and Brown, D. D. (1984) Cell **37**, 903–913.
50. Gottesfeld, J. M. and Bloomer, L. S. (1982) Cell **28**, 781–791.
51. Louis, C., Schedl, P., Samal, B., and Worcel, A. (1980) Cell **22**, 387–392.
52. Gottesfeld, J. M. (1987) Mol. and Cell. Biol. **7**, 1612–1622.
53. Pederson, D. S., Shupe, K., and Gorovsky, M. A. (1984) Nucl. Acids Res. **12**, 8489–8507.
54. Young, D. and Carroll, D. (1983) Mol. and Cell. Biol. **3**, 720–730.
55. Gottesfeld, J. M. and Bloomer, L. S. (1980) Cell **21**, 751–760.
56. Simpson, R. T. and Stafford, D. W. (1983) Proc. Natl. Acad. Sci. USA **80**, 51–55.
57. Thoma, F. and Simpson, R. T. (1985) Nature **315**, 250–252.

This article, submitted on disc, has been automatically converted into this typeset format by the publisher.