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Changes in chromatin structure accompany modulation of the rate of transcription of 5S ribosomal genes in *Tetrahymena*

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ABSTRACT

The chromatin structure of a single cluster of six tandemly repeated 5S ribosomal RNA genes (5S genes) in Tetrahymena thermophila has been characterized. Indirect end labeling experiments indicate that the actively transcribed 5S genes in macronuclei are rapidly cut by DNAse I near the putative internal promotor and just 5' to the transcribed region. When cells are starved to reduce 5S gene transcription rates, the DNAse I sensitivity of the intragenic site is reduced relative to the 5' site. In the nontranscribed 5S genes in micronuclei, neither of these sites is hypersensitive to DNAse I. Thus structural alterations accompany both the activation of transcription during macronuclear development and physiological changes in the rate of transcription of the 5S genes. These DNAse I data together with studies using Staphylococcal nuclease suggest that rapidly transcribed 5S genes may not be associated with histones as nucleosomes. In contrast, the genes in starved cell macronuclei appear to be associated with one nucleosome per 280 base pair tandem repeat.

INTRODUCTION

Development requires selective activation and modulation of gene transcription, which presumably occurs by controlling access to promotor sequences and by modification of the transcription machinery. We have studied the changes in the 5S ribosomal RNA genes (5S genes) of Tetrahymena thermophila which are associated with differential transcription. Tetrahymena is a ciliated protozoan with a germinal, diploid, transcriptionally inactive micronucleus, and a somatic, endoreplicated, transcriptionally active macronucleus which is derived from the micronucleus during conjugation (reviewed by 1).

Previous studies on the chromosomally located 5S genes and their flanking sequences (2,3) indicate that there are 150 to 300 copies per haploid genome in both micro- and macronuclei. The genes are distributed among about 30 widely spaced clusters containing one to about 16 genes. In those clusters which contain more than one

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gene, the 120 base pair coding sequences are separated by A+T rich spacers of about 160 base pairs to form a 280 base pair repeating unit. The large scale DNA rearrangements and deletions which occur during macronuclear development (reviewed by 4; for recent references, see 3,5,6) do not alter this level of 5S gene organization. In addition, there is no regular pattern to the rearrangements that occur in the sequences which flank different 5S gene clusters (3). This suggests that transcriptional activation of 5S genes during macronuclear development is not through DNA rearrangements.

Comparison of Tetrahymena and Xenopus 5S gene sequences (3), and in vitro transcription experiments by R. Hallberg (personal communication), suggest that the 5S genes of Tetrahymena are transcribed using an internal promotor as are the Xenopus genes (7,8,9). However, since macro- and micronuclei presumably have identical promotor sequences, regulation of Tetrahymena 5S genes cannot occur by differential affinity for the promotor. This, together with the fact that macronuclear development is accompanied by large scale changes in chromatin structure (10), and in histone composition (11,12; reviewed by 13), makes it likely that transcriptional activation of 5S genes during development involves alterations in chromatin structure. We have compared the chromatin structure of macro- and micronuclear 5S genes, concentrating on a single cluster which contains six genes. Specific regions in these presumably actively transcribed macronuclear genes are hypersensitive to DNAse I while the corresponding regions in micronuclei are not.

In vitro transcription studies (described below) and in vivo measurements (S. Libby and R. Hallberg, personal communication) indicate that when cells are starved, 5S gene transcription rates decline. Changes in the pattern of DNAse I hypersensitivity accompany this physiological response although the appearance of DNAse I hypersensitive sites has been previously correlated only with major developmental switches in gene activity. Staphylococcal nuclease cutting patterns also differ between growing and starved cells, suggesting that nucleosomal associations are altered with changes in transcriptional activity.

MATERIALS AND METHODS

Cell Culture, Nuclei Isolations

Tetrahymena thermophila strain B7 were grown axenically in 1% proteose peptone, 0.2% dextrose, and 0.003% sequestrine as described (14) to densities of 2 to 3 x 10^5 cells/ml. Starved cell cultures were prepared by gently washing cells into 10 mM Tris HCl, pH 7.5 and maintaining them in Tris for 18 to 24 hours at a concentration of 2 to 3 x 10^5/ml.

Nuclei were isolated by a modification of the method of Gorovsky et al (14), using a lysis buffer consisting of 4% Gum Arabic (Sigma), 0.1 M sucrose, 5 mM $MgSO_4$, 5 mM butyrate (to inhibit histone deacetylation), 5 mM EGTA (to inhibit nuclease activity), 1 mM β-mercaptoethanol, 1 mM spermidine-HCl, and 2 mM Tris base, brought to pH 7.0 with KOH. The spermidine, which enhances nuclear stability and partly inhibits histone proteolysis (14), was kept at a low concentration to reduce the possibility of spermidine induced nucleosome movement.

To obtain nuclei from growing cells, 10 liter cultures were concentrated at room temperature to 500 ml using a Millipore Pellicon Concentrator at a rate of about 500 ml/min and a pressure of no more than 0.5 kg/cm^2, all the while maintaining vigorous aeration of the culture. Cells were recovered from the concentrator filters using 500 ml of conditioned medium and the cell concentrate (one liter) was poured into a Waring blender containing 500 ml of cold 3 x lysis buffer and 0.3 ml of n-octanol/10^8 cells. Cells were ruptured by blending for 10 sec at moderate speed and then 0.1 M NaAcetate, pH 5.0, was added to a final concentration of 10 mM to reduce the pH to about 6. This step enhances the pelleting of nuclei while cell breakage occurs more readily at pH 7. The pH 6 mixture was blended for 10 sec at low speed and nuclei pelleted at 20,000g max for 15 min. The supernatant was reblended and recentrifuged once or twice to pellet most of the nuclei. Nuclear pellets were suspended in lysis buffer containing 0.1% NP40, combined, and micronuclei separated from macronuclei by filtration through 2 sets of 5 micron Nucleopore membrane filtures in a stirred ultrafiltration cell at 4°C. The use of NP40 concentrations higher than 0.1% during this step resulted in contamination of micronuclear preparations by nucleoli dislodged from macronuclei (data not shown). Filtered nuclei were pelleted at 10,000 g max for 5 min,

suspended in lysis buffer, pelleted at 2500g max for 5 min, and then washed twice in digestion buffer consisting of 85 mM KCl, 5 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM spermidine-HCl, 1 mM β-mercaptoethanol, and 5 mM $Na_{1.5}$Pipes (pH about 6.8). The elapsed time from cell disruption to nuclease digestion typically was 6 hours or less. Histones isolated at this point and analyzed by 2D electrophoresis showed no significant degradation (data not shown). Macronuclei isolated by this method support *in vitro* transcription reactions by all three RNA polymerases (Table 1 and unpublished observations).

To obtain starved cell nuclei, cells from a one liter culture were gently pelleted at room temperature, suspended in 60 ml of 10 mM NaCl and poured into a Waring blender containing 30 ml of cold 3 x lysis buffer and 0.2 ml of n-octanol/10^8 cells. All subsequent steps were identical to the isolation of nuclei from growing cells.

Nuclear Digestions, DNA Manipulations

Nuclei or DNA were suspended in digestion buffer at 20 A_{260} units/ml, brought to room temperature, and incubated for 2 to 20 minutes with DNAse I (Worthington) or Staphylococcal nuclease (Worthington or Sigma). In most nuclear preparations, no detectable endogenous digestion occurred during this time (data not shown). Preparations where endogenous digestion was evident were not used. For each nuclease and substrate, a 250 fold range of digestion was generated using 0.05 to 12.5 and 0.5 to 125 unit x min/ml DNAse I on DNA and nuclei, respectively; and 1 to 250 and 8 to 1000 Worthington unit x min/ml Staphylococcal nuclease on DNA and nuclei, respectively. Comparisons of different substrates were made using DNA samples which were similar in bulk average size. For the indirect end labeling experiments, digestions were brief enough to ensure that the restriction fragments analyzed had, on average, less than one cut per fragment. Digestions were stopped by the addition of one volume of 25 x NETS (25 x NETS is 400 mM NaAcetate, 25 mM H_4EDTA, 100 mM Tris base, and 25 mM SDS), and DNA purified as previously described (3).

DNA hybridization probes and methods for restriction enzyme digestion, nick translation, gel electrophoresis, blotting, and DNA:DNA hybridizations have been described (3).

In vitro Transcription and Analysis of RNA Products

RNA was synthesized using macronuclei isolated from growing or starved cells. Transcription reactions contained 5 x 10^7 nuclei in 0.25 ml of transcription buffer (50 mM Tris (pH 8.0), 50 mM $(NH_4)_2$

SO_4, 5.3 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM putrescine, 1 mM spermidine, 0.1 mM spermine, 2 mM β-mercapthoethanol, 0.63 mM each of ATP, CTP and GTP, and 0.3 mM aurintricarboxylic acid as a nuclease inhibitor (15). Each reaction contained 100-200 μCi of α-^{32}P-UTP or 5'-^{3}H-UTP (Amersham). To determine incorporation of labeled UTP into nucleic acids, aliquots of the reactions were precipitated with 10% TCA, 5 MM $Na_4P_2O_7$, and counted.

For hybridizations, RNA was isolated essentially by the method of Groudine et al. (16). Transcription reactions were terminated after 20 min by the addition of DNAse I (Sigma) to 20 μg/ml and incubated at 26°C for 5 min. The reaction mix was then made 0.1% SDS, 5 mM EDTA, 10 mM Tris-HCl (pH 7.4) and 100 μg proteinase K/ml, incubated at 37°C for 1 hr and phenol-chloroform extracted. Yeast tRNA was added to the aqueous phase to a final concentration of 100 μg/ml and precipitated for 1 hr at 4°C with an equal volume of cold 10% TCA. The precipitate was collected on a 25 mm (0.45 m pore size) nitrocellulose filter disk (Millipore HA) by suction and washed three times with 3% TCA, 30 mM $Na_4P_2O_7$. The disks were transferred to scintillation vials and 0.9 ml of a solution containing 20 mM HEPES [pH 7.5] 5 mM $MgCl_2$, 1 mM $CaCl_2$ and 25 μg of DNAse I were added and incubated for 10 min at 65°C. DNAse I digestions were terminated by the addition of EDTA and SDS to final concentrations of 125 mM and 1% respectively. The solution was transferred to a siliconized Corex tube and the disk incubated at 65°C in 1 ml of 1% SDS, 10 mM Tris-HCl (pH 7.5), 5 mM EDTA for an additional 10 min. The washes were combined, incubated at 37°C in the presence of 25 μg of proteinase K/ml for 1 hr, extracted with an equal volume of phenol-chloroform and ethanol precipitated.

The resulting labeled RNA was analyzed for 5S specific RNA by dot hybridization (17) to a cloned 5S sequence (clone pDP5; 3). pBR322 DNA was used to assess nonspecific hybridization. Hybridizations were carried out in 5 X SSPE (0.9 M NaCl, 50 mM NaH_2PO_4, 40 mM NaOH, 5 mM Na_2 EDTA), 5X Denhardts (18), 10 mM SDS, and 100 μg/ml polyadenylic acid at 65°C for 36 hrs. After hybridization the filters were washed twice at room temperature in 2 X SSPE, 10 mM SDS, once at room temperature in 0.2 X SSPE, 1 mM SDS and once at 65°C in 0.2 X SSPE, 1 mM SDS. The filters were dried, dissolved in Triton-toulene-Spectrafluor liquid scintillant and

counted for 20 mins. To properly compare amounts of specific RNAs made, DNA on the filter must be in excess to input sequences. This was ensured by showing that filters containing twice the usual amount of DNA gave identical results, and that no additional hybrid was formed by subsequent incubation of the supernatant with a fresh filter containing the cloned DNA.

In some experiments, dual isotope labeling was utilized to eliminate the need for internal controls. Nuclei from each physiological state were labelled with ^{32}P-UTP or ^{3}H-UTP. Nuclei from growing and starved cells, labeled with different isotopes, were mixed prior to RNA isolation. The isolated RNA was then used to probe dot blots as previously described. No difference in hybridization was observed between the dual and single isotope experiments (Table 1).

RESULTS

Location of DNAse I cuts in 5S gene chromatin

In principle, cuts in 5S gene chromatin which occur at the same site in each 5S gene can be related to restriction sites within the 5S gene, using the gene itself as a probe. In practice, the fragments generated by this procedure were often too short to hybridize since the entire tandem repeat in Tetrahymena is only 280 base pairs and, because the coding region is itself DNAse I sensitive (10; see below), signal strength was rapidly lost with progressive DNAse I digestion (not shown). In addition, minor length heterogeneity among different tandem repeats (2; unpublished observations) diminished the accuracy with which cut sites could be mapped. Thus we chose to map nuclease cuts in 5S gene chromatin in relation to restriction sites in the single copy sequences which flank clusters of 5S genes in Tetrahymena. Nuclei and purified DNA were lightly digested with DNAse I. DNA was purified and cut with Hind III to generate a series of fragments bound on one end by a DNAse I cut, and on the other end by the Hind III cut. Such fragments can be visualized by hybridization with a short probe having one end at the same restriction site, and the position of the DNAse I cuts determined (19). The probes used in the work presented here were isolated from two clones which flank a single cluster of six 5S gene (3), as shown in Figure 1.

Figure 2 shows the pattern of DNAse I cuts in 5S genes in purified DNA, and on 5S genes in macronuclei from growing and starved

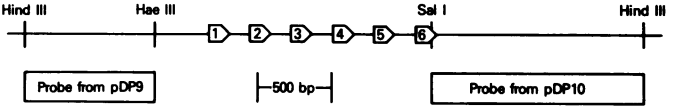

Figure 1. Map of the 5S ribosomal RNA gene cluster and hybridization probes used in indirect end label mapping.

The 5S gene cluster consists of six genes, indicated in the drawing by open arrowheads, pointing in the direction of transcription (5' to 3'). Cloning and characterization of clones pDP9 and pDP10 has been described (3).

cells, mapped from sites both up- and downstream of the cluster. Since Bam HI cuts 5S genes at position 30 in the coding sequence (3), DNA cut with Hind III and partially digested (e.g. Figure 6) or completely digested (Figure 2) with Bam HI serves to mark the position of 5S genes in the cluster. Comparison of partial Bam HI and and DNAse I cuts shows that DNAse I cuts each of the six genes similarly. By measuring the distance between cuts in the vicinity of each gene, and averaging these distances, a consensus 5S gene cutting pattern was derived. The locations of DNAse I and Staphylococcal nuclease cuts given in the text below, and the diagram in Figure 7, are based on this consensus pattern.

5S genes in macronuclei from growing cells are rapidly cut by DNAse I in a broad region centered at about position +70 in the coding sequence. Clearly, the great breadth of this DNAse I sensitive region is genuine and not due to any obvious technical artifact since the region is broad very early in digestion (Figure 2) and since analysis of Staphylococcal nuclease cuts (Figure 6) shows that the procedures used here are capable of fractionating what are presumably more homogeneously sized fragments as sharp bands. The 5S genes are also cut, somewhat less frequently, in a region centered about 34 base pairs 5' to the start of transcription. Purified DNA is also cut by DNAse I in these regions, at a lower frequency. The breadth of the cuts, even at very brief stages of digestion, makes it impossible to determine whether the positions cut by DNAse I are identical in free DNA and in chromatin. However, the greater intensity of the cuts in chromatin relative to those in free DNA, together with the fact that the pattern differs for starved and growing cell chromatin (discussed below), indicates that the macronuclear DNAse I pattern largely

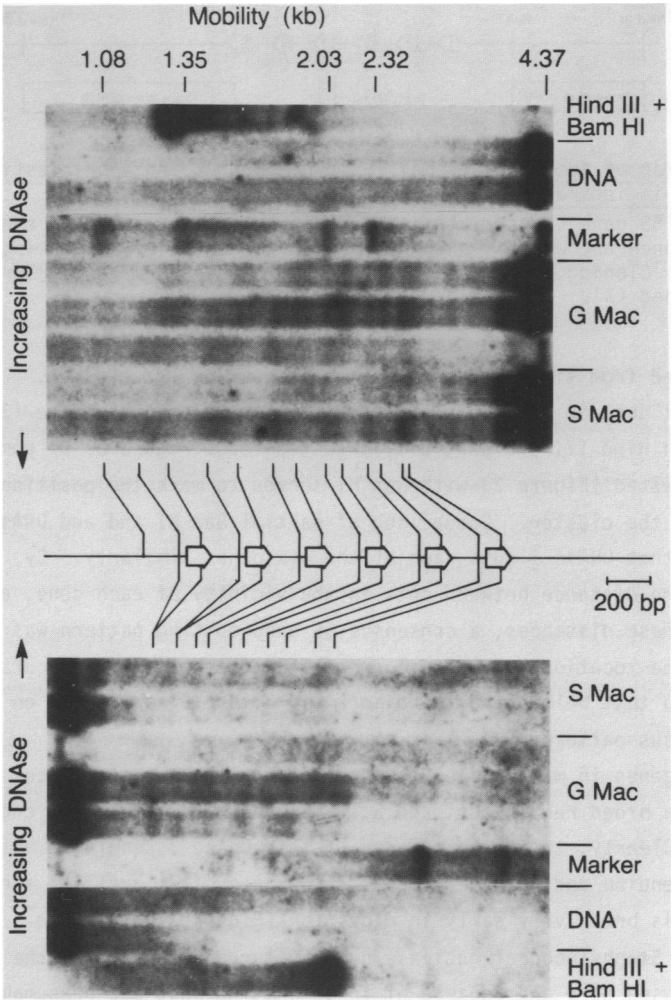

Figure 2. Indirect end label mapping of DNAse I cuts in the 5S genes.

Macronuclei from growing (G Mac) and starved (S Mac) cells, and purified DNA were digested with DNAse I to varying extents, as described in the Methods. DNA was purified, cut with Hind III, fractionated, blotted and probed with the probe from pDP9 (Figure 1). After autoradiography, the probe was eluted and the blot rehybridized with the probe from pDP10 (Figure 1). Fragment sizes were calibrated by hybridization to markers consisting of λDNA cut with Hind III, ϕX174 DNA cut with Hae III, and a 123 base pair oligomeric series purchased from BRL. As described in the text, a Hind III plus Bam HI double digest serves to mark the position of 5S genes in the cluster. Major DNAse I cuts made in macronuclei from growing cells have been mapped onto a drawing of the 5S gene cluster.

TABLE I. Summary of Transcription by Isolated Macronuclei

	Experiment #								
	1	2	3	4	5	6[1]	7[1]	8[1]	Mean ± s.d.
growing 5S[2]	.0480	.0539	.0333	.0478	.0798	.0301	.0700	.0260	.0486 ±.0178
starved 5S[2]	.0200	.0257	.0287	.0121	.0632	.00530	.0184	.00160	.0224 ±.0173
growing 5S[3] / starved 5S	3.9	5.91	2.96	7.94	1.83	7.10	7.23	5.63	5.32 ±2.05

[1] Dual isotope experiments (see Methods)

[2] Values are percent hybridization calculated as cpm bound to pDP5 filters less cpm bound to pBR322 filters divided by the total cpm added to the hybridization mix.

[3] Values are ratio of rates of transcription

$$= \frac{[\%\ \text{growing 5S}] \times [\text{total UMP incorporated-growing}]}{[\%\ \text{starved 5S}] \times [\text{total UMP incorporated-starved}]}$$

Note that on average, starved cell nuclei incorporate 0.61 as much UTP per nucleus as growing cell nuclei.

reflects features of chromatin rather than sequence preferences of the enzyme.

The frequency of cuts at the intragenic DNAse I hypersensitive site varies with transcriptional activity.

When cells are starved, the rate of accumulation of 5S RNA is reduced (S. Libby and R. Hallberg, personal communication). When the rates of transcription of sequences homologous to 5S genes are compared in nuclei isolated from growing and starved cells, 5S genes are transcribed about 5 times more rapidly in growing cell nuclei (Table 1).

When 5S gene transcription rates are reduced by starvation, the intragenic DNAse I hypersensitivity is largely lost, while the 5' DNAse I cut is unaltered or slightly enhanced (Figure 2). To show this phenomenon clearly, densitometric scans from digests of growing and starved cells have been aligned in Figure 3. Because these differences can be demonstrated by mapping from both up- and downstream directions, they cannot be due to any obvious artifact such as variable cutting at a position close to one of the reference Hind III sites.

Comparison of DNAse I cuts in macro- and micronuclei

Macro- and micronuclei isolated from growing cells and digested with comparable amounts of DNAse I are shown in Figure 4.

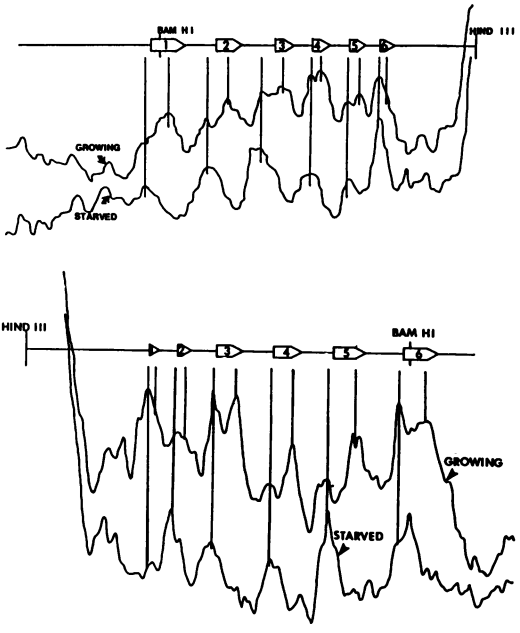

Figure 3. Reduced 5S gene DNAse I sensitivity with reduced transcriptional activity.

Lanes in Figure 2 where the patterns are most prominent were scanned, and densitometric profiles aligned below log scale drawings of the 5S gene cluster. Genes in the drawings are numbered one through six to emphasize that the orientation of the two drawings is the same.

While the patterns are similar, the intensity of the bands is greater in macronuclei than it is in micronuclei. The intensity of the micronuclear DNAse I pattern is similar to that of the free DNA pattern and could be due in part to DNAse I sequence preferences and/or to a low level of contamination of micronuclei by macronuclei.

Staphylococcal nuclease cuts in 5S gene chromatin

To further compare the 5S gene chromatin in different physiological and developmental states, and to learn about the involvement of nucleosomes in packaging 5S gene chromatin, nuclei were digested to varying extents with Staphylococcal nuclease, and DNA was purified, fractionated, and stained with ethidium bromide, as shown in Figure 5A. A series of broad bands with an oligomeric repeat of about 200 base pairs in the macronucleus and about 185 base pairs in the micronucleus can be seen, indicating that much of the Tetrahymena

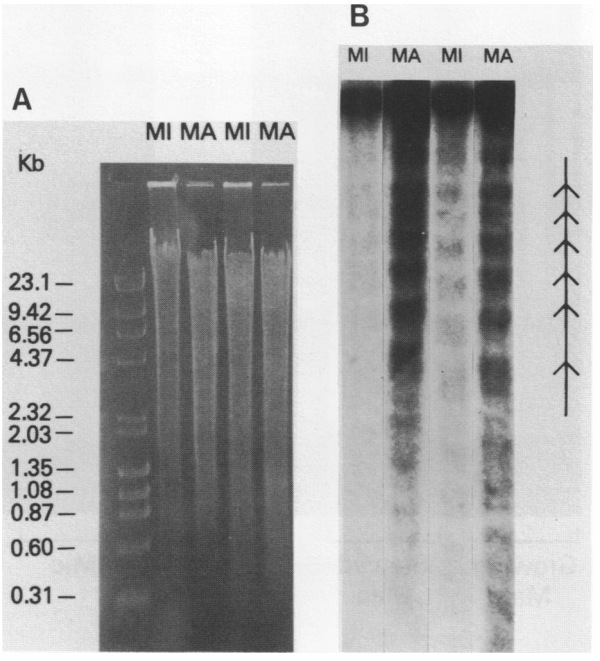

Figure 4. Micronuclear 5S genes lack DNAse I hypersensitive sites.

Macro- (Ma) and micronuclei (Mi) were digested to varying extents with DNAse I and DNA purified. The ethidium bromide stained gel in panel A shows that the extent of digestion of the samples used in comparing the two substrates is similar. Aliquots of DNA identical in amount to those appearing in panel A were digested with Hind III and analyzed as described in the legend to Figure 2. Hybridization with the probe from pDP9 is shown in panel B.

genome is associated with nucleosomes. The fuzziness and loss of clarity in the higher order oligomers indicates that the internucleosomal linker length heterogeneity is greater in Tetrahymena than in most other eukaryotes.

When the same gel is blotted and probed with the 5S gene, the pattern obtained is not a simple oligomeric series (Figure 5B). The region corresponding to dimers and higher order oligomers consists of a large number of poorly resolved bands. Several features of the 5S genes in Tetrahymena undoubtedly conspire to produce this pattern. First, as will be evident below, each 280 base pair tandem repeat is cut by Staphylococcal nuclease at two sequence preferred sites, located about 60 base pairs apart. Since these sites are cut at nearly

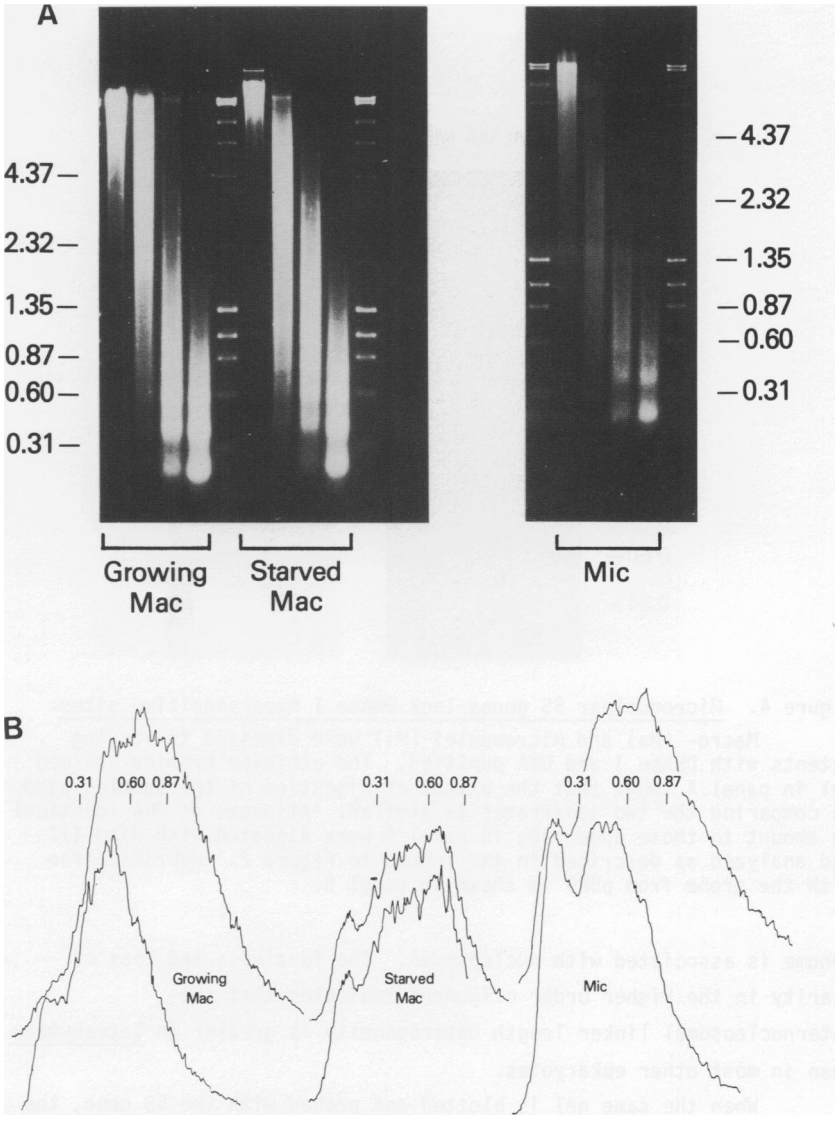

Figure 5. Pattern of Staphylococcal nuclease cuts in 5S genes.

Macronuclei from growing and starved cells, and micronuclei were digested to varying extents with Staphylococcal nuclease, and DNA purified, fractionated, and stained with ethidium bromide (panel A). The size of cofractionating marker fragments is indicated in kilobase pairs in both panels A and B. In panel A, the left most two lanes in each digestion series show the size range of material which was subsequently used in indirect end label mapping of cut sites (Figure 6). The DNA was blotted to nitrocellulose, hybridized to the 5S gene (3), and densitometric scans were made of the resulting

autoradiograms. Scans from the right most two lanes in each sample have been aligned and are shown in panel B. That the patterns for each sample consist of many closely spaced bands is evidenced by the fact that peaks visible in one scan are reproducibly visible in the second scan of the pair. In contrast to the complex pattern shown here (discussed further in the text), a more regular oligomeric repeat is seen when the same blot is probed with sequences from the large ribosomal RNA genes (not shown).

equal frequency (Figure 6), progressive digestion will produce DNA sizes of about 620, 560, 500, 340, 280, and 220 base pairs. Minor tandem repeat length heterogeneity among different 5S genes will add variability to these fragment sizes. Second, since the 5S gene clusters contain, on average, only 5 genes (3), a significant portion of 5S gene hybridization is to fragments which extend from the 5S genes into flanking regions. Cuts between nucleosomes spaced at, say, 200 base pair intervals in these flanking regions would produce a series of fragments of size (X + (200)n) base pairs, where X is the distance from a nuclease site in the 5S gene region to the nearest site adjacent to the 5S gene cluster, and where n=0, 1, 2... . Finally, any nucleosome directed cuts within the 5S gene regions may complicate the pattern further.

The locations of Staphylococcal nuclease cuts in the 5S genes were mapped as described for DNAse I. Inspection of Figure 6 shows that at a high extent of digestion, all substrates are cut identically. In each tandem repeat, two discrete cuts are made, one about 49 base pairs 5' to the start of transcription, close to the position where one of the two DNAse I cuts is made in macronuclei. The second site is about 60 base pairs further upstream. Clearly these cuts reflect a sequence preference of the enzyme.

At an earlier stage of digestion of macronuclei from starved cells, these sequence preferred sites are masked and a prominent cut is evident at about position 51 in the 5S gene coding region (indicated in the drawing in Figure 6 by upward pointing arrows). The initial occlusion of cuts at sequence preferred sites, together with cuts in a region which is not particularly sensitive in digests of free DNA suggests the presence of a specifically positioned nucleosome. Cutting at position 51 is also weakly evident in brief digests of micronuclei and in growing cell macronuclei.

Two interpretations of the rapid loss of the cut at position 51 and of the eventual dominance of the preferred sequence cuts can be

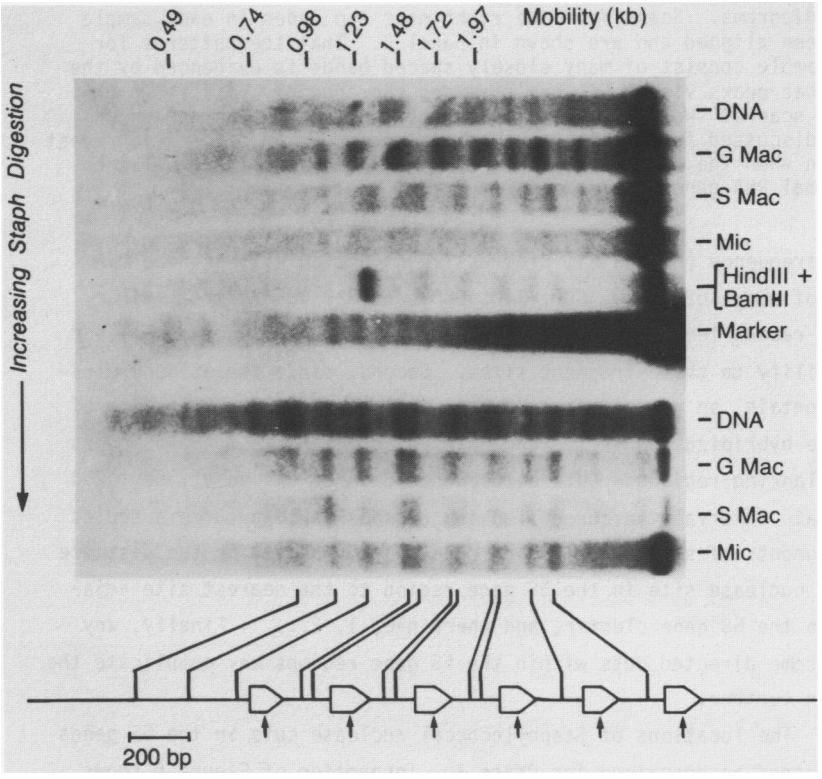

Figure 6. Indirect end label mapping of Staphylococcal nuclease cuts in the 5S genes.

Macronuclei from growing (G Mac) and starved (S Mac) cells, micronuclei (Mic), and purified DNA were digested to varying extents with Staphylococcal nuclease, and analyzed by the procedures described in the legend to Figure 2. Cuts most evident early in the digestion of macronuclei from starved cells are indicated by upward pointing arrows in a drawing of the 5S gene cluster. Cuts evident at later stages of digestion in all substrates, representing cleavages at preferred sequences, are indicated by lines extending from the photo to the same drawing. Only hybridization with the upstream probe from pDP9 is shown. Hybridization with the downstream probe gave comparable results (not shown), and was used in determining the position of nuclease cuts.

made. Those genes cut at position 51 may represent a subset which is rapidly degraded early in digestion. Alternatively, there may be a weakly bound nucleosome which directs cutting at position 51. Initial cuts could cause nucleosomes to move or dissociate so that position 51 is no longer uniquely accessible, and the preferred sequence cuts could quickly come to dominate the indirect end labeling pattern. The

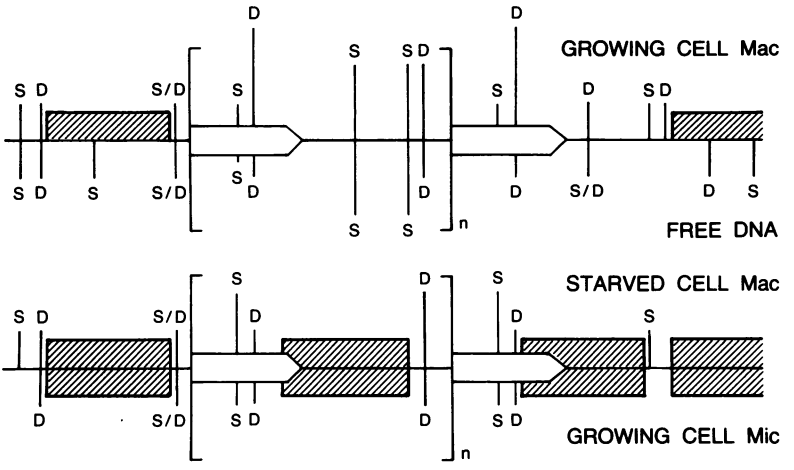

Figure 7. Consensus cutting pattern for 5S gene sequences.

Model 5S gene tandem repeats containing two genes and one spacer have been drawn to facilitate discussion of nuclease cuts and possible nucleosome positions (hatched boxes). The fact that all genes in a 5S gene cluster appear to be cut identically (Figures 2,3,4 and 6) is indicated by brackets on the drawing. Regions sensitive to DNAse I center around vertical lines headed with a "D", and cuts made by Staphylococcal nuclease are indicated by lines headed with an "S". The height of the lines is qualitatively related to the observed intensity of the cuts. As described in the text, the location of the cuts shown here reflects the average of data from all six genes, with one exception. Since we do not know the extent to which the intergenic spacer sequences extend into regions which flank 5S gene clusters, the cuts which map immediately up- and downstream of genes one and six, respectively, were not included in the averages leading to the consensus cutting pattern for the intergenic spacer. Because the standard deviation in measurements used to calculate the consensus pattern was generally about + 25 base pairs, and because the 5S gene DNAse I cuts are very broad, cuts on different substrates which map to nearly the same site have been drawn as occuring at the same site. Cuts evident in growing cell macronuclei and in free DNA are indicated, respectively, by lines above and below the upper drawing. Cuts evident in starved cell macronuclei and in micronuclei are indicated, respectively, by lines above and below the lower drawing, with one exception. Only the early, chromatin specific, Staphylococcal nuclease cuts are drawn for micronuclei and starved cell macronuclei, although sequence preferred cuts also appear on these substrates (Figure 6).

free DNA cuts appear to dominate the growing cell macronuclear pattern earlier in digestion than they do in the starved cell macronuclear pattern, suggesting that growing and starved cell 5S gene nucleosomes differ in binding affinity, or that growing cell 5S genes are more frequently nucleosome free.

DISCUSSION

Nuclease hypersensitive sites in 5S gene chromatin

Our nuclease mapping data are sumarized in Figure 7 where a model tandem repeat containing two 5S genes is diagrammed. The location of cuts within the tandem repeats represent averages based on all six genes. Sites mapping up- or downstream of the cluster are, of course, based only on cuts adjacent to genes one and six, respectively. It is now clear that DNAse I hypersensitivity is a feature of genes transcribed by all three Tetrahymena RNA polymerases (20,21; Pederson et al., submitted for publication). For a histone H4 gene (Pederson et al., submitted for publication), and for the 5S genes (this report) these DNAse I hypersensitive sites have been shown to be macronuclear specific. Because macro- and micronuclei are developmentally related and coexist in a single cell, these hypersensitive sites reflect an intranuclear differentiation, and not simply a response to a particular cytoplasmic state. It is also worth noting that while DNAse I hypersensitivity has been correlated in some cases with the selective modulation of methyl cytosine content (reviewed by 22,23), it clearly can depend on modulation of other components instead, since Tetrahymena lacks detectable levels of methyl cytosine (24).

To understand the basis for the 5S gene coding region hypersensitivity and its loss when cells are starved, it is helpful to calculate the approximate rate of transcription of these genes in growing cells. Growing Tetrahymena produce about 10^8 new ribosomes every two to three hour generation (25,26) requiring that each of the approximately 10^4 macronuclear 5S genes (150 to 300 per haploid amount X 45 to 90 haploid genomes per nucleus) produce about one ($10^8/(10^4$ x 120 to 180 min)) transcript per second. Assuming that eukaryotic RNA polymerase III elongation rates are between those of E. coli RNA polymerase (about 50 nucleotides per second) and T7 induced RNA polymerase (about 250 nucleotides per second) (reviewed by 28), the 120 base pair 5S genes in growing cells may be continually associated with at least one polymerase molecule. The measured decrease in 5S gene transcription rates brought about by starvation is based on the average activity of all 5S gene copies, and conceivably may vary from one cluster to the next. Nonetheless, the loss of coding region hypersensitivity when cells are starved suggests that the transcription complex itself confers DNAse I sensitivity. This

possibility is consistent with the breadth of the intragenic DNAse I sensitive region since a transcription complex could be located over any portion of the 120 base pair coding region. The sensitivity observed here may thus be formally analogous to the sensitivity that Wu observed over activated heat shock genes (19). There is no comparable basis for speculating on the structure which confers DNAse I hypersensitivity to the region just 5' to transcriptionally activated 5S genes, except to note that in Xenopus, and perhaps also in Tetrahymena, this region functions in accurate initiation (8) and in transcription efficiency (28). In Tetrahymena, this region is distinguished by its A+T richness, and by the presence of several short homopolymeric stretches, direct repeats, and palindromic sequences (3), and by sequences highly sensitive to Staphylococcal nuclease, even in purified DNA.

Placement of nucleosomes on 5S genes

Consideration of the DNAse I and Staphylococcal nuclease cutting data together places constraints on where nucleosomes are likely to lie with respect to the 5S genes (Figure 7). The coding region hypersensitivity in macronuclei from growing cells suggests that most of the transcribed region is nucleosome free. The early prominence of cuts at Staphylococcal nuclease preferred sequences in growing cell macronuclei suggests that this region and the intergenic DNAse I hypersensitive region are also nucleosome free. Assuming this, there then remains no stretch of DNA in the 280 base pair tandem repeat with sufficient length to form a nucleosome and suggests that in very rapidly transcribed genes, nucleosomes are (at least transiently) displaced by the transcription complex. A similar conclusion has recently been reached in a study of extremely active Drosophila heat shock genes (29). As proposed by Simpson et al. (30), transient displacement of nucleosome cores by polymerase molecules may require only a low amount of energy and, if it occurs, solves some of the topological problems inherent in transcribing DNA in chromatin.

In the less rapidly transcribed 5S genes of starved cells, the DNAse I hypersensitivity of the coding region is largely lost, the appearance of sequence preferred cuts by Staphylococcal nuclease is delayed, and a cut at position 51 is made by Staphylococcal nuclease early in digestion. This now leaves sufficient space such that the DNA between position 51 in the coding region and the DNAse I cut in

the following spacer region could associate with a nucleosome, as indicated in Figure 7. Progression from chromatin-specific cuts to cuts at sequence preferred sites with increasing digestion shown here, has also been seen by Thoma et al. (31) for the trp-1 gene chromatin of yeast, and may reflect a nucleosome modified so as to be readily displacable.

Aside from the cuts which occur in the preferred sequence region, nuclease cuts in micronuclei are indistinct and do not permit us to map nucleosome position(s). The absence of hypersensitive sites in micronuclei suggests that micronuclear nucleosomes could serve to inhibit a promotor specific factor from binding to the intragenic coding region, as has been suggested by the experiments of Bogenhagen et al. (32) and Gottesfeld and Bloomer (33). The timing of macro- and micronuclear S-phases in Tetrahymena suggests a way in which nucleosomes could serve this function. Since micronuclear S-phase preceeds macronuclear S-phase (34,35) a 5S gene promotor factor synthesized after micronuclear S-phase is complete would be present to compete with histones for binding to newly replicated macronuclear 5S gene chromatin. Such a model predicts that the Tetrahymena 5S gene promotor factor will be found in macronuclei but not in micronuclei, and that its message is only translated in cells which have completed micronuclear S-phase. Both of these predictions can eventually be tested.

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