Tetrahymena ribosomal RNA gene chromatin is digested by micrococcal nuclease at sites which have the same regular spacing on the DNA as corresponding sites in the bulk nuclear chromatin.

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ABSTRACT

Synchronised cells of *Tetrahymena pyriformis* GL were labelled with ³H thymidine at a stage in the cell cycle when only the mitochondrial and extrachromosomal nucleolar ribosomal DNAs were replicating. In this way it was possible to prepare nuclei labelled selectively in the DNA of the ribosomal RNA genes. Since the ribosomal RNA cistrons of these cells are also very active in serving as a template for transcription, experiments were performed to test whether these genes are organised upon a nucleoprotein subunit structure of the kind that has been found in the total chromatin of a wide range of eukaryotic cell types. *Tetrahymena* macronuclei were prepared labelled uniformly in their DNA with ³²P and labelled only in their nucleolar ribosomal DNA with ³H. Both the ribosomal genes and the bulk chromatin were then degraded *in situ* using micrococcal nuclease. The DNA fragments resulting from mild digestion were analysed on gels to reveal an identical DNA degradation pattern within both the ribosomal and bulk chromatins. It is concluded that the nucleoprotein structure of nucleolar rRNA cistrons possesses a periodic repeat along the DNA which is identical to that found in the substructure of unfractionated chromatin.

INTRODUCTION

Chromatin structure is based upon a repeating nucleoprotein subunit. The repeating unit can be visualised in the electron microscope¹⁻⁴, and there is now strong evidence that it consists of a specific assembly of histone molecules in association with approximately 200 base pairs of DNA⁵. Owing to the protective effect of the chromatin proteins the linkages between subunits are the sites on the DNA most susceptible to cleavage by micrococcal nuclease, and mild digestion of chromatin yields DNA fragments which are multiples of 150-200

base pairs in length⁴⁻⁸.

Whereas most of the chromatin of eukaryotic cells has an organisation based upon this subunit, there is as yet very little indication as to whether that fraction of the genome functioning as a template for RNA synthesis is organised in a similar way. Only a relatively small percentage of the genome of a differentiated cell is ever being actively transcribed into RNA and it would be of considerable interest to establish how the chromatin corresponding to this fraction differs from the template-inactive chromatin with respect to the organisation of proteins on the DNA. A first step towards such an understanding would be to determine whether the structure of template-active chromatin is based upon a subunit identical or different from the subunit which has been found to comprise more than 85-90% of the total chromatin of many eukaryotic cell types.

Procedures have recently been developed which enable a partial purification of the template-active fraction of mammalian chromatin to be achieved^{9,10}. However it might be doubted whether the chromatin fractionation methods currently in use are able to give preparations which have not undergone protein rearrangements during isolation and which are able to give a faithful reproduction of in vivo transcription in in vitro systems. It is possible to perform some initial experiments on the structure of active chromatin without using fractionation procedures by studying the nucleolar ribosomal RNA gene chromatin of a unicellular protozoan. Tetrahymena pyriformis possesses extrachromosomal rRNA genes that replicate during a short period at the beginning of the macronuclear S period at a time when there is no replication of the bulk macronuclear DNA^{11,12,13}. By providing synchronised cells of this organism with radioactive thymidine at this stage it is possible to label selectively only the mitochondrial and ribosomal RNA gene DNAs¹². Experimentally this is conveniently achieved by shifting starved cells to nutrient medium, whereupon the initiation of RNA synthesis precedes the onset of rRNA gene DNA (rDNA) replication (Leick, V., unpublished). It is probable therefore that newly replicated rDNA in the nuclei of these cells very rapidly enters the pool of rRNA genes

active in transcription. In this report this DNA is shown to be digested by micrococcal nuclease within isolated nuclei so as to give fragments of DNA identical in size to those obtained by digestion of the bulk chromatin of the nuclei.

EXPERIMENTAL PROCEDURES

Tetrahymena pyriformis strain GL (amiconucleate) was grown without shaking at 27° in 100ml complex medium (1.0%(w/v) proteose peptone, 0.1%(w/v) yeast extract, 0.1%(w/v) glucose) cultures, contained in 1500ml conical flasks. ^{32}P orthophosphate was present in the cultures at a concentration of 5 μCi per ml. The cells were harvested in exponential phase by centrifugation (4min at 600 x g) and the cell pellet gently resuspended in 50 times its volume of 0.004M succinic acid, 0.01M magnesium acetate, 0.01M Tris-HCl pH 7.3. Starvation was for 15h at 27° in this buffer. Afterwards the cultures were refed by the addition of 4 volumes of complex medium containing 2.5 μCi per ml ^3H thymidine (17 Ci per mmole, from Amersham U.K.) and incubated for between 100 and 110 min at 27°. Nuclei were then prepared from the cells.

Preparation of nuclei: The cells were recovered by centrifugation and washed once in buffer (0.1M sucrose, 0.01M Tris-HCl pH 7.2, 0.01M NaCl, 0.05M NaHSO_3 , 0.003M CaCl_2 , 0.001M MgCl_2). The cell pellet was then resuspended in 20 times its volume of this buffer containing 0.3% nonidet P-40 (B.D.H.). The suspension was mixed for 3min using a pasteur pipette and then centrifuged 6min at 1000 x g. The pellet of nuclei was washed several times by resuspension in the above nuclei preparation buffer (minus nonidet) followed by centrifugation, until the washes were devoid of cytoplasmic material.

Analysis of the labelling of rDNA and total nuclear DNA: An aliquot of each preparation of nuclei was extracted twice with chloroform : isoamylalcohol (24:1) and the DNA precipitated with 70% ethanol at -20°. The remainder of the nuclei were then digested with micrococcal nuclease, the DNA of the digests recovered and stored at -20° before analysis on gels. The DNA sample from the extracted nuclei was analysed on both CsCl equilibrium density gradients and agarose gels. Provided the results showed a reasonable selectivity in the ^3H thymidine

labelling of rDNA, the samples from the nuclease digests were then analysed on gels.

Density gradients were prepared by dissolving 7.40g CsCl in 6ml 150mM NaCl, 25mM EDTA, 25mM Tris-HCl pH7.2 containing the DNA sample. Centrifugation was for 40-44h at 39,000rpm in the Beckman 65 rotor. DNA analysis on 0.12cm x 12cm x 16cm 0.6% agarose gels was according to the procedure of Hayward¹⁴. DNA samples were dissolved in 50μl gel buffer containing 10% glycerol, and applied to slots in the agarose. The gels were run for 15h at 15mA and the ³²P DNA bands then located by autoradiography. Segments of the gel were then sliced into 0.25cm sections. Each section was transferred to a vial, incubated overnight at 60° in 1ml NCS, scintillant added, and the ³H and ³²P contents determined.

Digestion of nuclei with micrococcal nuclease was performed on suspensions which had been washed twice in 0.1M sucrose, 1.5mM Tris-HCl pH 7.2, 10mM NaHSO₃, 10mM NaCl, 10mM MgCl₂ and 1mM CaCl₂. Finally the nuclei were resuspended in the same buffer to a DNA concentration of 0.2mg perml. After the enzyme (Worthington Biochemicals Co.) had been added, the nuclei were incubated at 28° for 10min, chilled on ice and NaEDTA pH 6.9 added to a final concentration of 20mM. Resistant DNA was purified by chloroform extraction and ethanol precipitation as described by Noll¹⁵.

Analysis of DNA fragments on 3% polyacrylamide, 0.5% agarose gels: DNA protected from nuclease digestion was analysed on 0.12cm x 15cm x 16cm slab gels using 0.04M Tris-acetate pH 7.9, 5mM sodium acetate, 1mM EDTA as electrolyte. Samples were dissolved in gel buffer containing 20% sucrose and 0.005% bromophenol blue and applied to slots in the gel. The gels were run at 17mA for 10h and segments corresponding to each sample sliced and counted as described above.

RESULTS

Upon transferring a starved culture of Tetrahymena to nutrient medium the nucleolar rRNA genes start to replicate before the bulk macronuclear DNA^{11,12,13}. This property was exploited so as to obtain a specific labelling of the nucleolar rRNA cistrons, and since the experiments described here were

dependent on a high selectivity of rDNA labelling it was necessary to analyse the ^3H thymidine incorporation for each preparation of nuclei. The total nuclear DNA was banded on CsCl equilibrium density gradients to determine whether the ^3H -DNA component banded predominantly to the denser side of the main DNA peak (Fig. 1). *Tetrahymena* rDNA has a density greater than the bulk macronuclear DNA^{12,16,17}, whereas mitochondrial DNA, the other DNA labelled by the nutritional shift-up procedure, bands as a light satellite on the gradients¹². The nuclei preparations did not contain mitochondrial DNA since no ^3H -DNA component lighter than main band DNA was ever observed (Fig. 1).

To establish whether the ^3H -DNA component of the nuclei comprised extrachromosomal rRNA cistrons, the nuclear DNA was analysed on agarose gels. Owing to its discrete molecular weight (12.6×10^6) and linearity^{16,17,18} *Tetrahymena* rDNA forms a sharp homogeneous band on agarose gels. When total nuclear DNA is applied to the gel, the rDNA species migrates ahead of a more

Figure 1:

CsCl isopycnic banding of the DNA labelled with ^3H thymidine within 105 min of refeeding starved *Tetrahymena* cells. The cells had been previously labelled with ^{32}P phosphate to uniformly label bulk DNA. In this diagram, as well as in Figs. 2 and 4, the background counts have been subtracted and ^3H counts corrected for the spillover of ^{32}P into the ^3H channel.

Figure 2:

Electrophoretic analysis on a 0.6% agarose gel of the DNA sample shown banded on CsCl in Fig. I. DNA migration was from left to right. Only a fraction of the bulk macronuclear DNA was of sufficiently low molecular weight to penetrate the gel (see text). (•): ^{32}P . (Δ): ^3H

diffuse DNA band (Fig.2). The slower moving peak consists of DNAs with molecular weights in the range $30\text{--}50 \times 10^6$. We have shown (unpublished results) that this band yields Eco R_I restriction endonuclease digestion products identical to those obtained from rDNA purified on CsCl gradients¹⁸, that it is the only DNA penetrating the gel which hybridises to the I7S and 25S rRNAs of *Tetrahymena*, and that no other *Tetrahymena* DNAs co-migrate with rDNA on this gel system. The DNAs of molecular weight $30\text{--}50 \times 10^6$ appear to arise as the result of the action of an endogenous nuclease on macronuclear chromatin¹⁹, since the higher molecular weight DNA of the nuclei (which does not enter the gel) slowly degrades to this size when nuclei (prepared as under methods) are stored at 0° . This degradation is evidently more selective than that catalysed by the endogenous Ca,Mg-dependent endonuclease of mammalian nuclei^{6,7}, and it has yet

to be determined whether it reflects a structural feature of macronuclear chromatin. However on agarose gels the relative labellings of the 12.6×10^6 and $30-50 \times 10^6$ MW bands provide a convenient assessment of thymidine incorporation into rDNA and bulk nuclear DNA. Fig. 2 illustrates the use of the technique to demonstrate the presence of at least 90% of the tritium label in rDNA in the total DNA from the nuclei of a preparation of starved-refed cells. From the knowledge that rDNA comprises about 1% of Tetrahymena macronuclear DNA^{18,20} it is possible to calculate, from the DNA content of the gel bands, the approximate proportion of bulk DNA that has degraded to the $30-50 \times 10^6$ MW size.

In order to study the digestion of rRNA gene chromatin by micrococcal nuclease it was necessary to establish whether these genes were as susceptible as the bulk chromatin to nuclease digestion within isolated nuclei. Starved cells uniformly labelled with ^{32}P were given a nutritional shift-up in the presence of ^3H thymidine, for a sufficient length of time to label the rDNA. Nuclei from these cells were then digested with micrococcal nuclease and the reaction monitored by measuring the relative rates at which ^3H and ^{32}P DNA labels became soluble in 5% trichloroacetic acid (Fig. 3). No difference could be discerned between the digestion rates for rDNA and total nuclear DNA. Cells labelled with ^{32}P before starvation were therefore used for the nuclease digestion experiments since their nuclei then yielded ^{32}P -DNA fragments which could serve as internal markers in gels of digestion products.

If nuclear DNA was isolated after a mild nuclease digestion and then examined on gels, it was resolved as a series of bands (Fig. 4). These DNA fragments from Tetrahymena macronuclei are known to have the same electrophoretic mobilities as the corresponding fragments obtained from calf thymus nuclei²¹. Furthermore the sizes of the larger fragments are integral multiples of that of the smallest fragment^{6-8,21}. The protected DNA analysed on gels in this study was from nuclei which were known to be labelled in bulk DNA with ^{32}P and in nucleolar rDNA with ^3H . Fig. 4 shows the gel patterns of two representative digests. The profiles shown represent intermediate stages of limited digestion since it is the intermediates of DNA break-

Figure 3:

Time course of the digestion of *Tetrahymena* nuclei by micrococcal nuclease (1.0 unit per ml, 28° incubation). The nuclei were labelled in rDNA with ^3H and in total DNA with ^{32}P . Aliquots of the suspension were taken at various times to test the ^3H (Δ) and alkali-stable ^{32}P ($\blacktriangle$) material rendered soluble in 5% trichloroacetic acid.

down that establish the periodicities of nuclease susceptible sites within chromatin. Also there is some controversy about the significance of the DNA fragments which remain after exhaustive chromatin digestion. The ^3H labelled rDNA fragments resulting from mild digestion can be clearly resolved on gels as a series of peaks (Fig. 4). Also these fragments co-migrate with the DNA digestion products of bulk chromatin run in the same gels. No appreciable or reproducible fluctuation in the $^3\text{H}/^{32}\text{P}$ ratio could be detected on the gel profiles, and this ratio was approximately the same both in the total DNA of a certain preparation of nuclei and in the gel bands corresponding to nuclease digestion fragments from the same nuclei. The quantitations indicated that the majority (at least 70-80%) of the newly-replicated rDNA had been degraded to the fragments represented by the ^3H -DNA bands on the gels.

Figure 4:

DNA fragments protected from nuclease digestion within nuclei labelled with ^3H in rDNA and with ^{32}P in bulk DNA. (A) and (B) are digests which contained respectively 0.8 and 1.6 units per ml micrococcal nuclease. Analysis was on 3% polyacrylamide, 0.25% agarose gels and the diagrams illustrate the region from the top of the gel (right) to the bromophenol blue marker (left).

DISCUSSION

The rRNA genes of exponentially growing Tetrahymena offer certain advantages for a biochemical analysis of a single transcriptionally active cistron from a eukaryotic cell type: (1) These genes are extrachromosomal linear DNA molecules of a discrete DNA size and are amplified about 200 times per haploid DNA amount in the macronucleus^{16-18,20}. Since all other nuclear DNAs are of higher molecular weight the small size of rDNA allows this species to be assayed on agarose gels (Fig. 2). (2) Each rDNA molecule probably encodes two molecules of 35S precursor rRNA¹⁸. The 35S precursor has a molecular weight of 2.5×10^6 and matures to one molecule each of 17S and 25S rRNAs^{12,13, 16-18}. Thus no more than 20% of the rDNA can consist of nontranscribed spacer regions¹⁸. (3) Replication of rDNA is initiated prior to the replication of the rest of the DNA after a nutritional shift-up¹¹⁻¹³. This allows the selective labelling of the ribosomal RNA genes in synchronously dividing cells.

Limited micrococcal nuclease digestion of mammalian nuclei results in a degradation of the DNA to fragments whose sizes are all multiples of a unit 150-200 base pairs in length⁴⁻⁸. The corresponding digests of Tetrahymena macronuclei contain DNA fragments which co-migrate on gels with digestion fragments from mammalian chromatin²¹ and it is probable therefore that the chromatin of Tetrahymena has a subunit structure similar to that of higher organisms. The newly-replicated rRNA genes are shown here to degrade to fragments of the same sizes and this result is probably due to an identical nucleoprotein particle periodicity along both the rDNA and the total nuclear DNA.

The cells used for these experiments were harvested whilst in a physiological state in which they are very actively synthesising rRNA. They were processed prior to the start of bulk DNA synthesis, and whereas the rRNA genes studied were probably active in transcription it might be argued that all rRNA synthesis could occur on nonreplicating rather than newly replicated nucleolar rDNA. We are currently attempting experiments in which the cells are given a cold thymidine chase at the point when they were normally harvested. They are then allowed to grow and divide further before nuclei are prepared.

Although the ^3H labelled rDNA probably very rapidly enters the pool of rRNA genes active in transcription there can be little doubt that it will enter this pool during a chase period. Since there is very little nontranscribed spacer region on Tetrahymena rDNA the nuclease digestion pattern must for the most part reflect the chromatin structure of the transcribed region.

Nucleolar ribosomal genes might be expected to have special structural features as compared to active nonribosomal genes in interphase eukaryotic cells. Transcription complexes have been studied by electron microscopy in several cell types²². These investigations revealed a wide spacing of growing RNA chains on nonribosomal genes and a very small spacing on ribosomal cistrons. The available evidence suggests that active ribosomal and nonribosomal transcription units both yield the DNA fragment pattern of whole nuclei when digested with micrococcal nuclease in their chromatin states. In addition to the experiments described here, Gottesfeld *et al*²³ have studied the digestion of the template-active chromatin fraction obtained when mammalian chromatin is fractionated according to its solubility in magnesium containing buffers¹⁰. They have found that the resulting DNA fragments have sizes identical to those of fragments from digests of unfractionated chromatin, even though they are to be found in nuclease resistant nucleoprotein particles containing nonhistone proteins in addition to histones. Also Lacy and Axel²⁴ have recently reported hybridisation studies on the DNA fragments protected by the nucleoprotein subunit structure. The reassociation kinetics reveal that most genomic sequences must be present in these fragments. Also the protected DNA from rat liver nuclei contains most of the sequences transcribed into mRNA in liver cells²⁴. From the point of view of a subunit structure, studies of DNA fragments obtained by mild nuclease digestion of chromatin are unlikely to reveal fundamental structural differences between the template-active and template-inactive portions of a genome. This is despite the demonstration that there are both protein composition and sedimentation differences between resistant nucleoprotein particles from transcribed and inactive regions²³.

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