ISOLATION OF THE RIBOSOMAL RNA GENE FROM TETRAHYMENA IN THE STATE OF TRANSCRIPTIONALLY ACTIVE CHROMATIN

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Received July 13, 1976

SUMMARY:

The chromatin form of the gene coding for ribosomal 17S and 25S RNA can be isolated from exponentially growing Tetrahymena cells in a yield of 30-40%. The final purity with respect to DNA is more than 95%. The isolated ribosomal chromatin contains endogenously active RNA polymerase and sediments as a 50S particle on sucrose gradients.

Investigations of eukaryotic gene structure and function have been complicated by the extreme complexity of the genetic material and the problems associated with its fractionation. However, recent studies have established that bulk chromatin is organised upon a repeating DNA-histone subunit (1-12). Evidence has also been obtained that genes active in transcription are organised upon a similar repeating subunit (13), although the structure of transcriptionally active chromatin needs further clarification.

Very little is known about the factors which control the transcription of specific gene sequences in chromatin. Although cell free systems, using isolated nuclei or chromatin, generally show a low transcription efficiency compared to the in vivo rate, such systems have been successfully used to demonstrate that chromosomal nonhistone

proteins control the specific transcription of globin genes (14-18) and integrated viral genomes (19,20). The studies of specific gene transcription and the roles of chromosomal molecules would be considerably simplified if individual genes could be isolated in their chromatin state. Although present methods of chromatin fractionation do not allow this to be done for most eukaryotic genes it might be possible to purify amplified genes as chromatin, especially if such genes are extra-chromosomal.

The rRNA[x] gene of Tetrahymena pyriformis is both highly amplified (170-200 copies per haploid DNA amount (21)) and extra-chromosomal, being located in the nucleoli of the macronucleus (22,23). Each of the approximately 3000 rDNA molecules of a single cell is a giant palindrome containing two genes (24).

In this paper we describe a procedure for the isolation of the ribosomal chromatin of Tetrahymena. The r-chromatin is released from the nucleolar structure and rendered soluble by very mild trypsination. The gene isolated this way is transcriptionally active, possessing endogenous RNA polymerase activity. At the final stage of purification the r-chromatin is more than 95% pure with respect to DNA.

MATERIALS AND METHODS:

Agarose was obtained from Miles; ultrapure sucrose from Schwarz/Mann; ribonucleoside-5'-triphosphates from Boehringer; micrococcal nuclease, trypsin and soybean trypsin inhibitor from Worthington; [5,6-^{3}H]-uridine-5'-triphosphate, [^{32}P] orthophosphate and [methyl-^{3}H]-thymidine from The Radiochemical Centre, Amersham England; proteose peptone and

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x
Abbreviations:
rRNA = ribosomal 17 and 25S RNA
rDNA = the DNA coding for rRNA
r-chromatin = rDNA in its protein associated form.

yeast extract from Difco. Other chemicals were analytical grade.

Tetrahymena pyriformis strain GL (amicronucleate) was grown with good aeration at 27°C in a medium containing 1% proteose peptone, 1% glucose and 0.1% yeast extract? The cells were harvested in exponential phase and the nuclei were prepared as described by Mita et al. (25) except that 0.1 M sucrose was used instead of 0.25 M and 10 mM NaCl was added to the nuclei preparation buffer.

The endogenous RNA polymerase activity was measured by incubating 35 µl sample with 55 µl of an assay mixture containing: 10 mM $(NH_4)_2SO_4$; 15 mM $MgCl_2$; 10 mM 2-mercaptoethanol; 0.5 mM EDTA; 50 mM Tris-HCl pH 7.2; 5 mM KCl; 200 µM of each of ATP, GTP and CTP; 8 µM UTP (15 Ci/mmol). After 15 min at 27°C 75 µl was plated on Whatman 3MM discs and washed 5 times with icecold 5% TCA before counting. 1 U RNA-polymerase incorporates 1 pmole UTP into TCA-insoluble material in 15 minutes at 27°C.

DNA-electrophoresis was conducted in slabs of 0.6% agarose. The buffer was 40 mM Tris, 1 mM EDTA adjusted to pH 7.9 with H_3PO_4. Samples for electrophoresis were dissolved in this buffer supplemented with 1% SDS and 10 mM EDTA.

RESULTS AND DISCUSSION

In order to isolate the r-chromatin nuclei were prepared as described in methods and subsequently extracted with 10 volumes of buffer A (12 mM Na-Citrate pH 7.1, 120 mM NaCl, 10% glycerol, 1 mM 2-mercaptoethanol). Gentle homogenization in a tight fitting glass homogenizer was found to be essential for a good yield of r-chromatin. After this treatment the basic structure of the nuclei was seen to be intact when stained preparations were examined under the light microscope. The nuclei were removed from the preparation by centrifugation at 500 xg for 4 minutes. Centrifugation of the supernatant at 10,000 xg for 10 min was found to pellet an r-chromatin containing fraction. Electrophoresis of the DNA of this 10,000 xg pellet on a 0.6% agarose gel (Fig. 1) separates the rDNA (MW = 12,6 x 10^6 (23,26)) from a larger DNA component (MW 30 x 10^6). The fastest moving DNA-band is rDNA according to the following criteria:

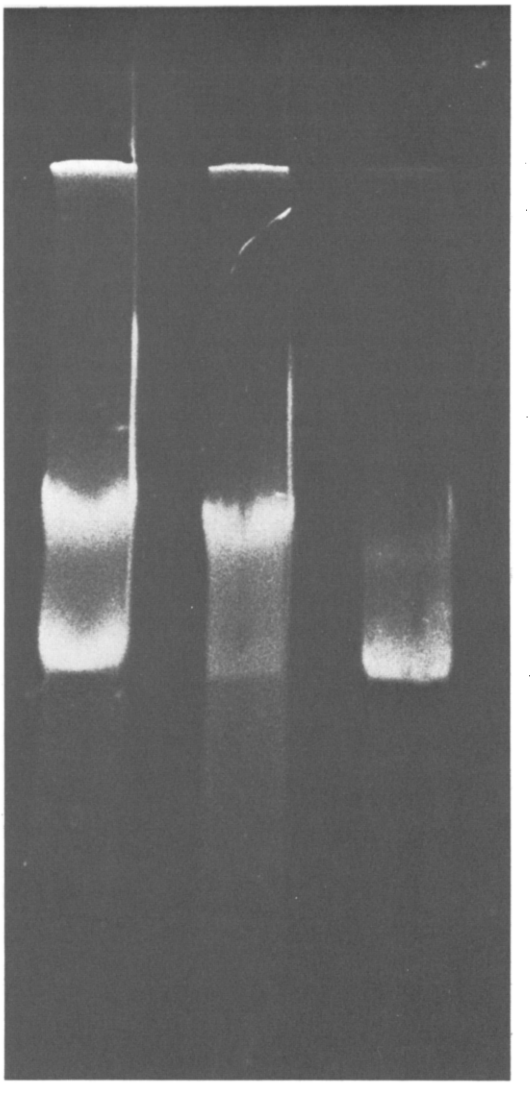

Figure 1

Agarose gel electrophoresis of 1)10,000 xg pellet obtained without trypsin treatment, 2)10,000 xg pellet and 3)150,000 xg pellet obtained after trypsin treatment. Samples numbered from left to right.

(a) It hybridizes to rRNA in the gel, using the *in situ* hybridization techniques described in ref.27;

(b) It gives DNA-fragments after cleavage by Eco R1 restrict-

ion endonuclease identical to those yielded by digestion of rDNA purified by other procedures (24); (c) It has the density on CsCl gradients and the size reported for rDNA (22,23,26). Gel electrophoretic analysis of the DNA of the 500 xg and 10,000 xg pellets indicated that 75-90% of the rDNA of the nucleus could be reproducibly obtained in the 10,000 xg pellet. In situ hybridization with rRNA confirmed this estimate of the yield.

The r-chromatin extracted from the nuclei appears to be bound in a complex structural assembly originating from the nucleoli. We endeavoured to solubilize the r-chromatin and thereby bring it into such a form that further purification was possible. Among a large number of different procedures limited trypsination has so far proved most successful. The 500 xg supernatant containing about 50 µg/ml of protein was incubated with 0.1 µg/ml trypsin for 5 min at 25°C followed by 30 min at 0°C, the reaction being terminated by the addition of 1 µg/ml soybean trypsin inhibitor. After a centrifugation at 10,000 xg for 10 min the r-chromatin was found in the supernatant, whereas the higher molecular weight contaminating DNA was pelleted. The r-chromatin itself could be pelleted by centrifuging the 10,000 xg supernatant at 150,000 xg for 75 min. At this stage the purity with respect to DNA was more than 90% (Fig.1).

The 150,000 xg pellet was resuspended in buffer A and sedimented in a 10-30% sucrose gradient in buffer A minus glycerol, as shown in Fig 2. The DNA profile across the gradient shows a certain very reproducible heterogeneity. Since the DNA in all DNA-containing fractions across the gradient has the size and density of rDNA (results not shown)

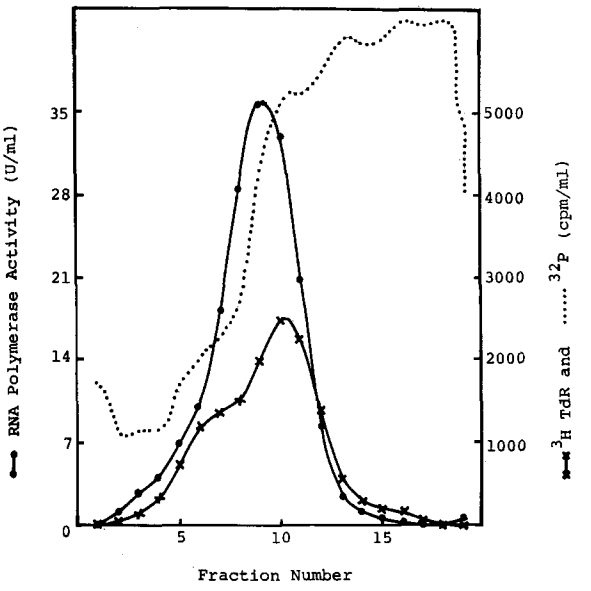

Figure 2

The 50 k pellet from a 100 ml culture labelled with 100 µCi ^{3}H-Thymidine and 50 µCi ^{32}p-orthophosphate was resuspended and sedimented in the SW41 rotor of the Beckman ultracentrifuge at 25 k rpm for 13 hrs. The direction of sedimentation was from right to left. 600 µl fractions were collected and 100 µl plated on Whatman 3 MM discs, washed in 5% TCA and counted for radioactivity. The specific activity of DNA was 21,000 cpm/µg of ^{3}H-counts. Endogenous RNA-polymerase was assayed as described under Methods. The RNA polymerase peak sediments at 50S, as judged by its cosedimentation with the Tetrahymena 50S ribosomal subunit. rDNA has a sedimentation coefficient of 26S (26).

the heterogeneity is not caused by a bulk chromatin contamination and could be explained in terms of two populations of r-chromatin, possibly a mixture of gene monomer and dimer. The purification procedure is summarized in Fig. 3.

The chromatin purified on a sucrose gradient contained at least a 10 fold excess of RNA over DNA in the peak fraction. At present we do not know what fraction of this RNA is associated with the r-chromatin. At the final sucrose gradient step the r-chromatin is not totally pure, but

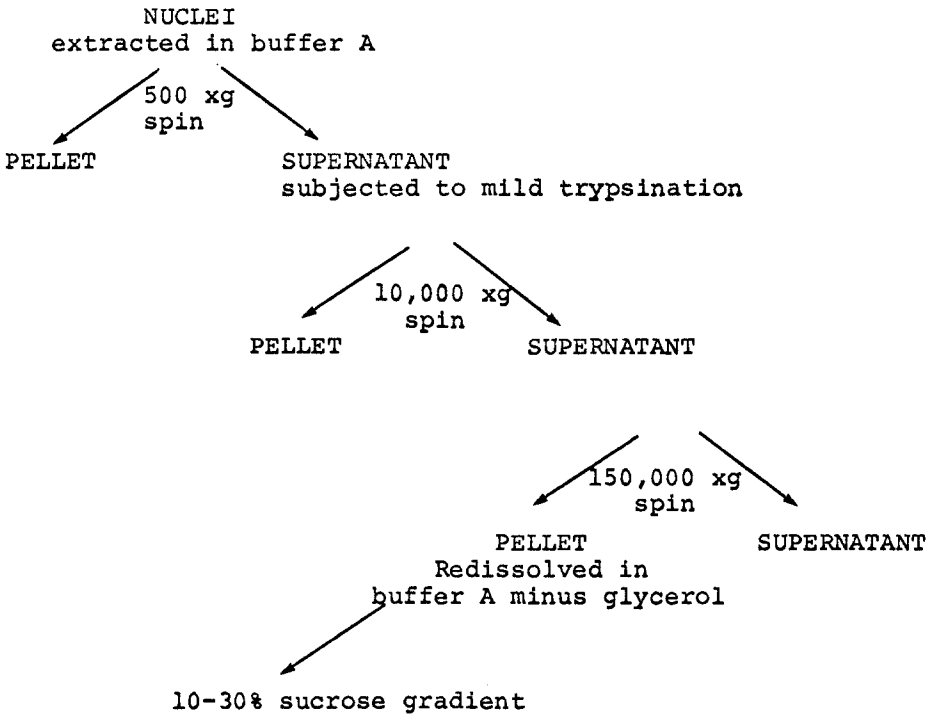

Figure 3

Flow diagram for the isolation of r-chromatin.

contains co-sedimenting RNA (Fig.2) and protein. If necessary, however, this material could probably be eliminated by banding the r-chromatin on metrizamide equilibrium density gradients. The gene-associated RNA is apparently responsible for the solubility of r-chromatin in buffer A since we were unable to isolate r-chromatin by the above procedure, if at any stage a ribonuclease-treatment was introduced.

Yields of rDNA, RNA polymerase activity and protein during the steps of the isolation procedure are given in Table 1. The sucrose gradient peak contained at least 95% pure rDNA and the final purification with respect to protein was about 4000-fold over total cellular protein.

TABLE 1

Purification scheme for a typical 1-liter culture (8×10^7 cells)

	mg protein	µg rDNA	Endogenous RNA polymerase activity, Units
Cells	260	-	-
Nuclei	23	-	-
500 xg supernatant	4.5	9	2150
150,000 xg pellet	0.35	8	750
Pooled sucrose gradient peak	0.025	3.5	650

15-20% of the RNA polymerase activity was lost during the solubilization of the gene and another 10-20% was lost after the 10,000 xg spin. Finally about 35% was lost after the 150,000 xg spin. Together this amounts to a recovery in the 150,000 xg pellet of about 35% of the RNA polymerase activity found in 500 xg supernatant. (Table I). There is a high recovery of RNA polymerase activity compared to rDNA on the sucrose gradients (90% as against 45%). This could be explained by the rDNA-associated RNA polymerase becoming more active as a result of the manipulations involved. Alternatively only a fraction of the r-chromatin in the 150,000 xg pellet might be in a transcriptionally active state and only this fraction can be redissolved in buffer A. The latter suggestion is supported by the finding that a low speed centrifugation (10,000 xg for 5 min) of the resuspended 150,000 xg pellet removed an insoluble fraction of r-chromatin but did not cause any loss of endogenous RNA polymerase activity in the supernatant.

The RNA polymerase was found to sediment bound to the r-chromatin. When fractions from a sucrose gradient were assayed with either native or denatured calf thymus DNA added to the assay mixture, a minor stimulation (10-20%) with added template was only observed in the fractions having endogenous RNA polymerase activity. Under our assay conditions the amount of RNA synthesized endogenously in the peak fraction of the sucrose gradient was equivalent to about half the amount of DNA-template present.

The structure of r-chromatin from Tetrahymena was investigated recently by us and others (28,29). The experiments were conducted using a starvation-refeeding procedure that permits selective labelling of rDNA, since the rDNA is replicated preferentially during a nutritional shift-up (22). It was found that the newly replicated rDNA of starved-refed cells, active in rRNA-synthesis, is organised upon a repeating subunit structure. This was indicated by the finding that the sites accessible to micrococcal nuclease digestion on this rDNA have the same regular spacing as those on the bulk chromatin of isolated nuclei. Micrococcal nuclease has also been found to degrade a substantial fraction of the rRNA genes of Xenopus laevis cells, actively synthezising rRNA, to DNA-pieces which are monomer and oligomers of the 200 base-pair unit (30). Digestion of the isolated r-chromatin from exponentially growing Tetrahymena with micrococcal nuclease yielded DNA-pieces approximately 200 base-pairs long but no detectable oligomers of this fragment. This suggests that the r-chromatin may have undergone a structural change during

the purification, possibly a stretching (31). The DNA of r-chromatin is however protected against degration by micrococcal nuclease to the same extent as the DNA of bulk chromatin, represented by the 500 xg pellet material, Table II.

TABLE 2

Protection of r-chromatin against degradation by micrococcal nuclease. Samples of 500 xg pellet, 150,000 xg pellet and naked DNA (isolated from the 500 xg pellet) were suspended in a buffer containing 10 mm Tris-HCl pH 7.2, 10 mm NaCl, 3 mm $CaCl_2$, 1mm $MgCl_2$ and 0.1 m sucrose. Each sample contained 3 µg of DNA[x] and was digested with 0.75 units of micrococcal nuclease at 25° C. The time course of the reaction was followed by determinations of trichloroacetic acid - precipitable label in DNA at the indicated points of time.

Time	per cent acid-precipitable DNA		
(minutes)	500 xg pellet	150,000 xg pellet	DNA
0	100	100	100
2	100	87	46
5	96	83	9.0
10	88	81	2.2
15	80	76	<1

[x] (labelled with ^{3}H-thymidine to a specific activity of 28,000 cpm/µg).

We have described a procedure for the isolation of transcriptionally active r-chromatin from Tetrahymena, that involves a release from the nucleolar assembly by limited trypsination and subsequent purification of the gene by centrifugation techniques. The use of a proteolytic enzyme introduces the risk of degrading proteins associated with the gene. However, preliminary experiments using SDS-gel electrophoresis have indicated that no extensive proteolysis occurs in the procedure. Furthermore rDNA in the isolated r-chromatin is substantially protected against degradation

by micrococcal nuclease. Recent experiments (unpublished) have demonstrated that transcriptionally active r-chromatin can also be isolated using other proteolytic enzymes or very dilute ionic detergents as solubilizing agents. Characterization of the r-chromatin obtained by different procedures should enable us to detect any specific damage caused by trypsin.

Studies on the transcriptional properties of the isolated r-chromatin are now in progress.

ACKNOWLEDGEMENTS

This research was supported by grant No. 511-5670 from the Danish Natural Science Research Council, and contract No. 122-74-1 BioDK with Euratom, C.E.C., Brussels. P.W.P. acknowledges postdoctoral fellowships from Euratom and EMBO. We thank Professor K.A. Marcker and Dr. J.E. Celis for helpful advice and criticism.

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