Histone Composition of a Chromatin Fraction containing Ribosomal Deoxyribonucleic Acid Isolated from the Macronucleus of *Tetrahymena pyriformis*

By RICHARD W. JONES

Sir William Dunn School of Pathology, University of Oxford,
South Parks Road, Oxford OX1 3RE, U.K.

(Received 28 October 1977)

The histone compositions of a chromatin fraction containing ribosomal DNA and of the remaining macronuclear chromatin of *Tetrahymena pyriformis* were analysed by gel electrophoresis. These chromatin fractions were used as models for transcriptionally active and inactive chromatin respectively. The extent of histone modification, as indicated by the distribution of histone between differently charged subspecies in acid-urea gels, is not grossly different in the two chromatin fractions. However, histone H1 is present, but may be differently modified in the two chromatin fractions. The histone/DNA ratio in ribosomal chromatin, measured after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of samples of chromatin, was found to be the same whether chromatin was extracted from growing or stationary organisms, and to be approx. 40% of this ratio in the remaining macronuclear chromatin. The implications of these results for the possible structure of transcriptionally active chromatin are discussed.

Evidence so far available suggests that fractions of chromatin containing DNA sequences that are actively involved in transcription do contain histone (Gottesfeld *et al.*, 1975). It has also been shown that transcriptionally active DNA sequences in chromatin from a variety of sources possess the periodic structure that is generally typical of DNA packaged with histone to form nucleosomes. Such sequences include the avian globin (Lacy & Axel, 1975) and ovalbumin (Garel & Axel, 1976) genes, and sequences coding for rRNA in *Xenopus laevis* (Reeves & Jones, 1976) and *Tetrahymena pyriformis* (Mathis & Gorovsky, 1976; Piper *et al.*, 1976). In most of these examples, the ratio of inter- to intra-nucleosomal DNA does not appear to differ from that in transcriptionally inactive chromatin. This implies that the histone/DNA ratios in active and inactive chromatin are the same. However, in sequences coding for rRNA in transcriptionally active cells from *X. laevis*, less of this DNA was protected from micrococcal nuclease digestion than in transcriptionally quiescent cells (Reeves & Jones, 1976). This could be interpreted to mean that either the histone/DNA ratio is less in transcriptionally active chromatin, or that the relationship between these components is altered. The former possibility is supported by the observation that the histone/DNA ratio is less in a transcriptionally active fraction of chromatin than in an inactive fraction (Gottesfeld *et al.*, 1975).

Abbreviations used: rDNA, ribosomal DNA, the region of DNA coding for rRNA; r-chromatin, chromatin containing rDNA; SDS, sodium dodecyl sulphate.

There is also evidence that the relationship between histone and DNA in active chromatin is not the same as in inactive fractions. For example, intranucleosomal DNA in these fractions differs in its susceptibility to digestion by deoxyribonuclease I (Garel & Axel, 1976; Weintraub & Groudine, 1976). The possibility that the relationship between histone and DNA in actively transcribing chromatin is radically different is suggested by electron micrographs of amphibian r-chromatin (Scheer *et al.*, 1976). These show the rDNA in an extended conformation, with few, if any, nucleosomes present.

The r-chromatin of *T. pyriformis* provides a source of a single species of chromatin in sufficient quantity that its protein composition can be measured. Its extrachromosomal location in the macronucleus allows it to be extracted, as described in the preceding paper (Jones, 1978), with a minimum of intentional exposure to disruptive mechanical forces or enzyme action. I have taken this material to be representative of transcriptionally active chromatin, and have analysed its histone composition with a view to providing information about the histone/DNA ratio in active chromatin, and about any contribution that histone modification may make to its structure.

Experimental

Growth of organisms and preparation of chromatin fractions

Conditions used for the culture of *T. pyriformis* syngen 1 D/1 and the preparation of r-chromatin

and bulk chromatin fractions from the macronuclei of growing or starved organisms are described in the preceding paper (Jones, 1978).

Radioactive labelling of DNA and of histone H4

Radioactive labelling of DNA and the measurement of radioactivities are described in the preceding paper (Jones, 1978).

To obtain radioactively labelled histone, organisms were harvested from a 4-litre culture and resuspended in 250 ml of 5 mM-KH₂PO₄/0.5 mM-MgSO₄ adjusted to pH 7.5 with KOH (Jones, 1978). This suspension of organisms was incubated at 28°C, with aeration, for 30 min in the presence of 1 mCi of sodium [³H]acetate (sp. radioactivity 225 mCi/mmol) (The Radiochemical Centre, Amersham, Bucks., U.K.). The cells were then harvested and macronuclei prepared. Histone was extracted and fractionated by gel-exclusion chromatography as described below.

Extraction of histone

Two procedures were followed for the extraction of histone. (1) Bulk chromatin was sheared in water at concentrations between 200 and 500 µg of DNA/ml (estimated from the *A*₂₆₀ of the final solution), by using a Polytron homogenizer operated at half-speed for 15 s. Pelleted r-chromatin was suspended in 2 ml of water by using a vortex mixer. These solutions were made 0.2 M in H₂SO₄ and stirred on ice for 6 h or overnight, before being centrifuged at 15000 *g*_{av} for 1 h. The supernatants were removed and 0.2 vol. of 100% (w/v) trichloroacetic acid was added to them. After standing for 1 h at 0°C, precipitated protein was pelleted at 15000 *g*_{av} for 1 h. This pellet was washed three times with ice-cold ethanol, dried under vacuum, and finally dissolved in 8 M-urea/0.9 M-acetic acid/100 mM-2-mercaptoethanol for gel electrophoresis. (2) Alternatively, samples of sheared bulk chromatin or total r-chromatin fractions containing 5–20 µg of DNA were made up with water to a volume of 100 µl in 300 µl-capacity capped plastic centrifuge tubes. These samples were made 0.2 M in H₂SO₄ and the tubes rotated along their long axes at 5 rev./min overnight at 3–5°C. Insoluble material was removed by centrifuging at 15000 *g*_{av} for 1 h. H₂SO₄ was then removed by extracting the supernatants once with 2 ml of a solution of 2.5% (v/v) triethylamine in water-saturated diethyl ether (Lester-Smith & Page, 1948), and then twice more with 3 ml of water-saturated diethyl ether. Remaining ether was removed by a stream of air. The pH of the resulting solutions was not less than 3 at the end of this procedure. Urea and 2-mercaptoethanol were added to give final concentrations of 5 M (approx.) and 100 mM respectively.

Preparation of individual histones

Histones H1 and H2b were extracted from macronuclei by the method of Johns (1964). Histones HX (Johmann & Gorovsky, 1976), H2b, H3 and H4 were separated by gel-exclusion chromatography. Approx. 2 mg of histone, prepared by the first of the two methods described above, was dissolved in 200 µl of 8 M-urea/0.9 M-acetic acid/100 mM-2-mercaptoethanol and loaded on a 100 cm × 0.5 cm column of Bio-Gel P60 (50–150 mesh; Bio-Rad, Bromley, Kent, U.K.) (Johmann & Gorovsky, 1976). The column was eluted with 0.02 M-HCl/0.02% NaN₃/0.05 M-NaCl, pH 1.95, and fractions (2 ml) were collected at a flow rate of 10 ml/h. This method was also used to prepare radioactively labelled histone H4.

Polyacrylamide-gel electrophoresis

Acid-soluble proteins extracted from chromatin fractions were analysed by a modification of the method described by Panyim & Chalkley (1969). Solutions of 20% (w/v) acrylamide plus 0.133% (v/v) ethylene diacrylate, or 10% (w/v) acrylamide plus 0.067% (v/v) ethylene diacrylate, contained in addition 2.5 M-urea, 0.9 M-acetic acid, 0.25% (v/v) NNN'N'-tetramethylethylenediamine and 0.125% (w/v) ammonium persulphate. Exponential-acrylamide-gradient gels (20 cm long × 16 cm × 0.15 cm thick) were formed by pumping the 20% (w/v) acrylamide solution through 12 ml of 10% (w/v) acrylamide solution into the bottom of a slab-gel holder until the solution reached a point 2 cm from the top of the gel plates. Gels were left overnight before pre-electrophoresis at 10 mA for 20 h. Sample slots were then formed at the top of the gel with the 10% acrylamide solution described above.

It was necessary to make the slots after pre-electrophoresis, because slots formed before this closed up. The presence of a layer of acrylamide gel that had not been pre-electrophoresed did not affect adversely the running of the gel or the resolution of protein bands. The use of an acrylamide-gradient slab gel gave, in the present work, better resolution of protein components, and allowed the detection of smaller quantities of protein, than was possible with 20 cm-long tube gels.

Samples of acid-soluble proteins, prepared as described above, were loaded on these gels in volumes of 10–80 µl. In some gels, 20 µg of calf thymus histones (Sigma, Kingston upon Thames, Surrey, U.K.) were also run as standards.

SDS/polyacrylamide gels were made as described by Laemmli (1970), with the following modifications: 7.5–15% linear-acrylamide-gradient slab gels (20 cm long × 16 cm × 0.15 cm thick) were formed by pumping the gel solution into the bottom of a gel holder. No spacer gel was used. These gels were used for the

analysis of total chromatin protein. Samples of sheared bulk chromatin, or of r-chromatin, were taken up in a final volume of 100 μ l of sample buffer (Laemmli, 1970) and heated at 100°C for 3 min, then sonicated with a 3 mm-diam. probe at a frequency of 20 kHz and amplitude of 6 μ m for 1 min before being heated again at 100°C for 1 min. Samples, usually 50 μ l, were loaded on SDS/polyacrylamide gels, and the gels run at 30 mA until the Bromophenol Blue tracking dye reached the bottom of the gel. Portions from the samples were oxidized in a Packard Tri-Carb sample oxidizer for the measurement of radioactivity in DNA in the material for electrophoresis. In some gels molecular-weight markers (mol. wt. 14 300–71 500; BDH, Poole, Dorset, U.K.) were also run.

Staining of polyacrylamide gels

Acid/urea/ or SDS/polyacrylamide gels were stained overnight in a solution of either 0.1% Coomassie Brilliant Blue or 0.4% Amido Black in methanol/acetic acid/water (5:1:5, by vol.). Gels were destained in the same solvent. Gels, or photographic transparencies of gels, were scanned with a Joyce–Loebl double-beam recording microdensitometer. The amounts of material in protein peaks were measured by weighing the areas under the peaks.

Measurement of radioactivity in polyacrylamide gels

Protein bands, identified by staining, were cut out from SDS/polyacrylamide gels, and dried overnight at 37°C. Then 0.15 ml of H₂O₂ (30%, w/v) was added, and incubated at 50°C for 6 h. Next 0.5 ml of NCS tissue solubilizer (Amersham/Searle Corp., High Wycombe, Bucks., U.K.) was added, and incubation continued for a further 4 h. Radioactivity was measured as described previously (Jones, 1978).

Protein determination

This was carried out by the method of Lowry *et al.* (1951), with bovine serum albumin (Armour Pharmaceuticals Co., Eastbourne, Sussex, U.K.) as standard.

Results

Analysis of acid-soluble chromatin proteins on acid/urea/polyacrylamide gels

In one of a number of analyses, acid-soluble protein was extracted, by the first of the two methods described above, from chromatin fractions pooled from three preparations. Fig. 1 shows this

protein separated on an acid/urea/polyacrylamide gel. Among the proteins extracted from bulk chromatin, histone H1 was identified by its solubility in 5% (w/v) trichloroacetic acid. When gels were stained with Amido Black, this histone gave a pale-blue colour, in contrast with the slate-blue of the other histones, as has been observed with histone H1 from other sources (Cohen & Gotchel, 1971). Histone H2b and its monoacetylated derivative (Johmann & Gorovsky, 1976) were identified by their solubilities in Johns's histone-extraction methods 1 and 2 (Johns, 1964). These and the other histones were identified in addition by their relative electrophoretic mobilities, which are very similar to those reported for other strains of *T. pyriformis* (Johmann & Gorovsky, 1976).

Fig. 1. Acetic acid/urea/polyacrylamide-gel electrophoresis of acid-soluble proteins extracted by the first method (see the Experimental section) from r-chromatin and bulk chromatin obtained from growing organisms

Samples of r-chromatin or bulk chromatin were pooled from three preparations. Sample (a) contained the acid-soluble protein extracted from r-chromatin containing 0.8% of the total radioactivity in DNA in both chromatin fractions. Samples (b), (c) and (d) contained 13.5, 27 and 41 μ g of protein, representing the acid-soluble protein extracted from bulk chromatin containing 0.25, 0.5 and 0.75% of the total radioactivity in DNA in chromatin. Protein bands were assigned to the different histones as described in the text. The positions of calf thymus histones (Th.H1, Th.H2a, Th.H2b, Th.H3 and Th.H4) are shown by arrows. Bands A, B and C are explained in the text.

Acid extracts of r-chromatin contained substantial quantities of non-histone protein. Nevertheless, I believe that most of the histones present were identifiable by comparing their electrophoretic mobilities with those of histones extracted from bulk chromatin. In addition, histone H1 could be identified by its colour when stained with Amido Black.

In some early preparations, there was evidence of proteolytic degradation of the histone extracted from both bulk and r-chromatin. Histones H1 and H3 were absent, and a prominent band was present running just ahead of histone H2b. In Fig. 1, histone H1, which is the most sensitive of the histones to proteolysis (Gorovsky *et al.*, 1974), is present in protein extracted from both bulk chromatin and r-chromatin, as is histone H3. This makes it unlikely that extensive proteolysis had occurred during the preparation of these chromatin fractions.

A number of protein bands in the extract from r-chromatin (Fig. 1) run close to the histones, but do not coincide with the position of any acid-soluble proteins present in the extract from bulk chromatin. Band A is always present in similar quantity relative to the histones, and is not likely to be a product of histone breakdown, since it does not appear in degraded preparations of bulk histone. Band B runs slightly behind any of the forms of histone H1, and is grey rather than blue when stained with Amido Black. It is therefore probably not a form of histone H1. Band C appears only in small amounts and its position corresponds to that of a prominent band seen in degraded preparations of bulk histone. Its presence may therefore indicate some proteolysis of histone. Unidentified bands also appear in the region of histone HX.

In comparing the histones from r-chromatin with those extracted from bulk chromatin, it is not possible to say whether additional bands appearing in the region of histones extracted from r-chromatin represent modified forms of histone or non-histone protein. The absence of bands that correspond to the position of histones from bulk chromatin can, however, be interpreted less equivocally.

Histone H1

Fig. 2 shows the densitometer trace of the histone-containing region of the gel shown in Fig. 1. Histone H1 from bulk chromatin appears as three distinct bands, the two more slowly migrating of which are probably due to phosphorylation of a faster-moving parent form (Gorovsky *et al.*, 1974). Histone H1 from r-chromatin, on the other hand, migrates predominantly as a single band with the same mobility as the fastest-moving form of histone H1 from bulk chromatin. It therefore seems probable that histone H1 is less extensively modified in r-chromatin than it is in bulk chromatin.

Fig. 2. Densitometer trace of the histone-containing region of the acetic acid/urea/polyacrylamide gel shown in Fig. 1. The densitometer traces are of the histone-containing regions of samples (a) (r-chromatin) and (b) (bulk chromatin) shown in Fig. 1.

Histones H2b, H3 and H4

Protein bands corresponding to the positions of histones H3 and H4 and their mono- and diacetylated forms, and to the positions of histone H2b and its monoacetylated form, are seen in material from both bulk chromatin and r-chromatin. The monoacetylated form of histone H2b runs only slightly ahead of the unmodified form of histone H3, and these bands are not easily distinguished from each other in gel photographs or traces. Fig. 2 shows that the diacetylated forms of histones H3 and H4 (those forms closest to the anode) are present in smaller amounts relative to the corresponding parent forms than are the equivalent forms from bulk chromatin. The same may be true of the monoacetylated form of histone H4. These differences are not large. There is certainly no evidence from our results that modified forms of histones H2b, H3 and H4 are relatively more abundant among histones associated with r-chromatin than they are among those associated with bulk chromatin. However, we cannot exclude differences among more highly modified forms of these histones, and the complexity of protein bands in the region of histone HX among acid-soluble proteins from r-chromatin does not allow one to draw any conclusions about possible differences between r-chromatin and bulk chromatin in the composition of this histone.

The most striking observation was that substantially less histone was recovered from r-chromatin than would be predicted were the histone/DNA ratio the same as in bulk chromatin. Excluding histones H1 and HX, the histone/DNA ratio in the r-chromatin preparation analysed in Fig. 1 was 15% of that in bulk chromatin.

To overcome the possibility that acid-soluble

protein was lost during trichloroacetic acid precipitation and washing with ethanol, a second method was used to process small quantities of acid-soluble protein that eliminated the need for these steps (see the Experimental section). Use of this method allowed the extraction of histone from samples of bulk chromatin containing 10–20 μ g of DNA with the same efficiency as the first method achieved in extracting histone from chromatin containing between 1 and 2mg of DNA. Acid-soluble protein processed in this way after acid extraction from both r-chromatin and bulk chromatin was analysed on acid/urea/polyacrylamide gels (Fig. 3). The distribution of material between the different histone fractions is very similar to that observed previously (Fig. 2), though the histone (H2b+H3+H4)/DNA ratio in r-chromatin was greater (35% of that in bulk chromatin). Measurement of this ratio in eight preparations analysed by acetic acid/urea/polyacrylamide-gel electrophoresis gave a mean value of 30% of the ratio in bulk chromatin.

SDS/polyacrylamide-gel electrophoresis of total chromatin-associated protein

To obtain a more accurate measurement of the histone/DNA ratio in r-chromatin, a different

approach was adopted. Nucleic acid and protein in samples of chromatin were dissolved directly in sample buffer containing SDS (Laemmli, 1970), and samples were taken to measure the amount of radioactivity in DNA (see the Experimental section). Samples prepared in this way were analysed on SDS/polyacrylamide gradient gels (Fig. 4). Histones H4, H3, H2b and HX were the major protein components in samples of bulk chromatin (Fig. 4, sample *a*). These histones were identified by comparing their mobilities with histone fractionated by gel-exclusion

Fig. 3. Acetic acid/urea/polyacrylamide-gel electrophoresis of acid-soluble protein extracted from r-chromatin and bulk chromatin by the second method (see the Experimental section)

Acid-soluble proteins were extracted with 0.2M- H_2SO_4 from samples of r-chromatin (*a*) and bulk chromatin (*b*) containing 0.8% and 0.5% respectively of the radioactivity in DNA in the total chromatin obtained from a growing culture. The H_2SO_4 was removed from the samples, before electrophoresis, by extraction with trioctylamine, instead of precipitating the protein with 20% trichloroacetic acid and washing with ethanol (see the Experimental section). The traces are of the histone-containing region of the gel.

Fig. 4. SDS/polyacrylamide-gel electrophoresis of chromatin from growing organisms

Samples of r-chromatin (*b* and *c*) and bulk chromatin (*a* and *d*) from a growing culture, all containing equal amounts of radioactivity in DNA, were solubilized in sample buffer (Laemmli, 1970; also see the Experimental section), and analysed on a SDS/7.5–15% polyacrylamide gel. Also shown are the positions and molecular weights of markers run in the gel. Samples (*c*) and (*d*) contained in addition equal quantities of [3H]acetate-labelled histone H4, prepared as described in the Experimental section, and added to these samples immediately before electrophoresis. After electrophoresis, the protein bands containing histone H4 in samples (*c*) and (*d*) were cut out, and the radioactivity in them was measured (see the Experimental section). The radioactivities in adjacent portions of the gel were also measured to exclude the possibility that radioactivity from DNA contributed to the radioactivity in histone H4.

chromatography, and by their relative electrophoretic mobilities (Johmann & Gorovsky, 1976). Very little histone H1 was present in these samples, and amounts of this histone varied considerably in different preparations of both r-chromatin and bulk chromatin.

Among the proteins present in the r-chromatin fraction, the histones are minor components (Fig. 4, sample *b*). It is possible that non-histone protein migrated with the histones, and this would lead to an overestimate of the amount of histone associated with rDNA. Measurement of the amounts of histones H4, H3 and H2b+HX in r-chromatin and bulk chromatin (Fig. 4, samples *a* and *b*) showed that the ratio of each of these histones to DNA in r-chromatin was 50, 51 and 65% respectively of the equivalent ratios in bulk chromatin. It is therefore clear that less of these histones can be recovered from r-chromatin than from bulk chromatin, but that the ratio of these histones to each other is very similar in the two fractions. Analysis of eight preparations, excluding histone H1 from consideration, gave a mean value of 41% for the histone/DNA ratio in r-chromatin compared with that in bulk chromatin. This value is not greatly different from the values obtained when acid-soluble protein was extracted by the second of the two methods described above, and analysed on acetic acid/urea/polyacrylamide gels.

Investigation of factors possibly affecting the recovery of histone from r-chromatin

The estimate of the histone/DNA ratio in r-chromatin could be wrong if much of the radioactivity in this fraction were not in DNA, but I have shown in the preceding paper (Jones, 1978) that at least 90% of this radioactivity is in DNA. Since the radioactivities in DNA in r-chromatin and bulk chromatin were the result of several generations of growth of organisms in the presence of [^3H]thymidine, I have assumed that there were no differences in the specific radioactivities of their DNA that could lead to erroneous differences in their histone/DNA ratios.

The possibility that the difference between the histone contents of r-chromatin and bulk chromatin arises during the preparation of macronuclei cannot be excluded, though this seems unlikely. Fractionation of macronuclear chromatin on glycerol gradients (Jones, 1978) does lead to the two chromatin fractions being treated differently, and an attempt was therefore made to test whether alterations to this procedure altered the histone/DNA ratio in r-chromatin as a percentage of that in bulk chromatin.

Glycerol gradients loaded with macronuclei were left for either 10 min (the usual time) or 45 min before being centrifuged to separate the chromatin fractions. The histone/DNA ratios in r-chromatin or in bulk

chromatin did not depend on the time for which gradients stood before being centrifuged (Fig. 5). This result decreases the possibility that histone is preferentially lost from r-chromatin at this stage in its preparation, either as a result of proteolysis or for any other reason.

After centrifuging the glycerol gradients to separate r-chromatin from bulk chromatin, gradients were fractionated by pumping them through a gradient fractionator. This process exposed the r-chromatin-containing fractions to mild shearing forces, but the bulk chromatin fraction remained in the centrifuge tube. There is evidence that the interaction between histone and DNA may be different in transcriptionally active chromatin (Garel

Fig. 5. Effect of leaving loaded preparative gradients for different times before centrifugation on the amount of histone present in chromatin fractions

Glycerol gradients, loaded with suspensions of macronuclei, were left for 10 min (samples *a* and *c*) or 40 min (samples *b* and *d*) before centrifugation to separate chromatin fractions. The densitometer trace is of the histone-containing region of a SDS/7.5–15% polyacrylamide gel in which samples of r-chromatin (*c* and *d*) containing the same amounts of radioactivity in DNA, and bulk chromatin (*a* and *b*) also containing equal amounts of radioactivity, were electrophoresed.

& Axel, 1976; Weintraub & Groudine, 1976), and it therefore seemed possible that histone could be lost from r-chromatin during its collection from preparative gradients. Therefore the procedure used to collect r-chromatin from the preparative gradients was altered to test whether this would alter the amount of histone apparently associated with rDNA. I have shown (R. W. Jones, unpublished work) that the structure of r-chromatin is easily distorted by mild shearing forces, but that the addition of spermine and spermidine causes these structures to adopt a very compact conformation.

Chromatin was fractionated on two glycerol gradients, and r-chromatin was collected from one gradient in the usual way. The r-chromatin fractions in the second gradient were exposed by removing the top of the gradient. Spermine and spermidine were gently mixed with the appropriate portion of this gradient before it was removed with a wide-bore pipette and the r-chromatin pelleted by centrifugation. Fig. 6 shows that there was no significant difference between the amounts of histone extracted from r-chromatin collected in the presence or absence of these polyamines.

The protein compositions of r-chromatin and bulk chromatin differ greatly (Fig. 4). To test whether this influenced the recovery of histone from r-chromatin in SDS/polyacrylamide gels, equal quantities of radioactive histone H4, prepared by gel-exclusion chromatography of histone extracted from cells grown in the presence of [^3H]acetate, were added to samples of bulk chromatin (sample d, Fig. 4) and r-chromatin (sample c, Fig. 4) before elec-

trophoresis. The recoveries of radioactivities in the protein bands containing histone H4 from these samples were not significantly different.

Histone/DNA ratio in r-chromatin from starved cells

Fig. 7 shows the densitometer tracing of an SDS/polyacrylamide gel in which r-chromatin and bulk chromatin from starved organisms (Jones, 1978) were analysed. The histone H4/DNA, histone H3/DNA and histones H2b+HX/DNA ratios in r-chromatin were 42, 49 and 48% respectively of these ratios in bulk chromatin. In this and another preparation, the total amounts of these four small histones associated with rDNA were 46% and 41% respectively of the histone/DNA ratio in bulk chromatin. Therefore, as in chromatin fractions from growing cells, the ratio of these histones to each other is very similar in the two fractions, but the histone/DNA ratio in r-chromatin is less than in bulk chromatin. Assuming a similar histone/DNA ratio in bulk chromatin from growing and starved organisms, this ratio in r-chromatin is very similar in these two different metabolic states.

Discussion

The r-chromatin extracted from the macronucleus of *T. pyriformis* has been taken to be representative in its structure of transcriptionally active chromatin, and its histone composition has been compared with

Fig. 6. Effect of different methods of collecting r-chromatin from preparative gradients on the amount of histone present in r-chromatin fractions

The r-chromatin fractions were collected from preparative gradients, as described in the text, in the absence (a) or the presence (b) of spermidine (0.2 mM) and spermine (0.04 mM). Samples, containing equal amounts of radioactivity, were analysed on SDS/7.5–15% polyacrylamide gels. The densitometer traces are of the histone-containing region of the gel.

Fig. 7. SDS/polyacrylamide-gel electrophoresis of chromatin fractions extracted from starved organisms. Organisms were starved, and chromatin fractions prepared from them, as described in the preceding paper (Jones, 1978). Samples of bulk chromatin (b) and r-chromatin (a) contained equal amounts of radioactivity in DNA, and were analysed on SDS/7.5–15% polyacrylamide gels. The densitometer trace is of the histone-containing region of the gel.

that of the remaining macronuclear chromatin (bulk chromatin), which is likely to be relatively impoverished in transcribed sequences. The method used in the present paper to isolate r-chromatin depends on rDNA being the smallest species of DNA in the macronucleus (Gall, 1974), and as far as is known it involves no assumptions about the structure of transcriptionally active chromatin. I assume that when r-chromatin is isolated from growing cells, in which rRNA must be synthesized, then most of the rDNA molecules are involved in transcription. Approx. 80% of the length of double-stranded rDNA in the macronucleus codes for rRNA (Engberg *et al.*, 1976), and this makes it likely that any substantial alterations in histone composition directly related to the process of transcription would be detected.

The main conclusions of the present paper are, first, that with the possible exception of histone H1 there is little evidence of different modification of the histone associated with r-chromatin compared with bulk chromatin, and, secondly, that the major difference lies in there being substantially less histone associated with rDNA than with the same amount of DNA in bulk chromatin.

It has been suggested that histone modification, particularly histone acetylation, may play a role in making DNA available for transcription, presumably by altering the interaction between DNA and histone (Allfrey *et al.*, 1964; Vidali *et al.*, 1968; Pogo *et al.*, 1968; Ruiz-Carrillo *et al.*, 1975; Marushige, 1976). However, a comparison of the histones associated with transcriptionally active and inactive fractions of rat liver chromatin has shown no obvious difference in the extent of modification of these proteins in the two fractions (Gottesfeld *et al.*, 1974), and the present results appear to support this observation. The r-chromatin fraction used for the analyses described in the present paper is prepared in a very different way from the transcriptionally active chromatin fraction used by Gottesfeld *et al.* (1974). It therefore seems of added significance that, like them, in the present work no substantial differences in the modification of histones H2b, H3 and H4 could be detected when comparing transcriptionally active and inactive chromatin fractions. However, in this system, I have not excluded the possibility that histone HX, which is the nearest equivalent in *T. pyriformis* to histone H2a (Johmann & Gorovsky, 1976), is differently modified in the two chromatin fractions.

Histones H3 and H4 appear to have a central role in determining nucleosome structure in chromatin (Sollner-Webb *et al.*, 1976; Camerini-Otero *et al.*, 1976; Bosely *et al.*, 1976; Finch *et al.*, 1977), and it therefore seems significant that the extent of modification of these histones appears to be little different in the two chromatin fractions studied

in the present paper. Were acetylation of these histones to play a part in altering nucleosome structure in transcriptionally active chromatin, one would have expected to detect it in this system. The exception to these observations is the lysine-rich histone H1, which is less extensively modified in r-chromatin than in bulk chromatin. In the present work histone H1 in the chromatin fractions was not always recovered, but there seems little doubt that this histone is present in r-chromatin, and that its loss from both r-chromatin and bulk chromatin in some preparations is probably due to proteolysis. It is noteworthy that the presence or absence of histone H1 in any one preparation is paralleled in both chromatin fractions. There is little evidence that histone H1 is preferentially lost from r-chromatin. The presence of this histone in a transcriptionally active fraction of chromatin is at variance with the observations of others (Gottesfeld *et al.*, 1974).

However, the possibility cannot be completely excluded that the difference in modification of histone H1 in r-chromatin, when compared with histone H1 in bulk chromatin, may be due to their different susceptibilities to proteolysis.

Histone-H1 modification in *T. pyriformis* has been shown to be due to phosphorylation, and to be related to the growth rate of organisms, slowly growing organisms showing less extensive phosphorylation of histone H1 than do rapidly growing organisms (Gorovsky *et al.*, 1974). In a number of other eukaryotes, histone phosphorylation appears to be related to the stages of the cell cycle (Bradbury *et al.*, 1973; Hohmann *et al.*, 1976). In the light of this evidence, since both bulk chromatin and r-chromatin pass through the cell cycle, it is not easy to understand why histone H1 should be differently modified in these two fractions. It is possible either that r-chromatin does not participate in cell-cycle changes in chromatin structure involving histone-H1 phosphorylation, or that these changes are of short duration in r-chromatin, and so are not detected in an unsynchronized population of cells. Alternatively, the less extensive phosphorylation of histone H1 in r-chromatin than in bulk chromatin might be taken as support for the view that phosphorylation of this histone is related to transcriptional activity (Langan, 1969). However, in the system studied in the present paper, it appears that it is the absence of phosphorylation that is associated with transcriptional activity. This argues against the suggestion by Gorovsky *et al.* (1974) that the more extensive phosphorylation of histone H1 in rapidly growing organisms might be related to increased transcriptional activity.

The need to suppose that the modification of histone associated with transcribed DNA sequences is necessary to allow transcription to occur is perhaps

decreased by the observation that the histone/DNA ratio in r-chromatin is about 40% of that in bulk chromatin. If the relationship of histone and rDNA in r-chromatin is similar to that in bulk chromatin, then it is possible that a substantial proportion of the rDNA molecule is free of histone and that the DNA is available for transcription by virtue of this. A very similar result has been described by Gottesfeld *et al.* (1975), who found a histone/DNA ratio in their transcriptionally active chromatin fraction that was 53% of that in the inactive fraction. They suggested that, as a consequence of this, active chromatin had a more extended structure.

The histone/DNA ratio in r-chromatin may be the mean value for a heterogeneous population of rDNA molecules, possibly depending on whether they are involved in transcription or not, or depending on the stage of the cell cycle. However, preparations of r-chromatin from starved organisms give a very similar value for the histone/DNA ratio. It is likely that the r-chromatin in the macronuclei of starved cells is less actively involved in transcription, though this has not been demonstrated. Alternatively, since starved cells are arrested in the early G1 phase of the cell cycle (Cameron & Jeter, 1970), the histone/DNA ratio in r-chromatin may be typical of that phase of the cell cycle. The lower histone/DNA ratio in r-chromatin therefore appears to be a constant feature, independent of the metabolic state of the organism. This constancy could be taken as an argument against a model for chromatin structure in which transcriptional activity is associated with a decreased histone/DNA ratio, whereas decreased transcription of the same DNA sequences is associated with an increase in the amount of histone bound to them.

It is now well established that DNA in chromatin is wrapped round a core, composed of the four small histones, to give a compact beaded structure (Kornberg & Thomas, 1974; Kornberg, 1974). This structure has been made visible in electron micrographs prepared from chromatin (Olins & Olins, 1974), and the correspondence between electron-micrograph evidence and the model in which DNA and histone combine to form the compact bead or nucleosome has been demonstrated by Griffith (1975). It has also been shown that chromatin can be digested by nucleases to give multiples of a unit length of DNA (Hewish & Burgoyne, 1973; Sahasrabudde & Van Holde, 1974; Noll, 1974), and, as well as providing support for the nucleosome structure of chromatin, in which the DNA between nucleosomes is more exposed to enzyme action, this property has been used to suggest that particular chromatin fractions consist of DNA packaged with histone to form nucleosomes.

The results reported here show that, although all four of the small histones are present in similar

proportions to each other in both r-chromatin and bulk chromatin, there is only sufficient of these histones to package 40% of the rDNA in nucleosomes with the same structure as those in bulk chromatin. If the remaining DNA is unprotected from nuclease action, one would expect r-chromatin to be digested more rapidly or more extensively than bulk chromatin when exposed to micrococcal nuclease, but this is not the case. Mathis & Gorovsky (1976) have shown that both r-chromatin and bulk chromatin from the macronucleus of *T. pyriformis* possess a periodic structure, as evidenced by their susceptibility to micrococcal nuclease. Furthermore, the kinetics of digestion of these two fractions are indistinguishable, suggesting that the proportion of the DNA exposed between nucleosomes is the same in both cases. From this result one would expect the histone/DNA ratio to be the same in both chromatin fractions.

This same contradiction can be seen in studies on the structure of transcriptionally active chromatin from other eukaryotes. The transcriptionally active chromatin fraction prepared from rat liver nuclei by Gottesfeld *et al.* (1975) has a histone/DNA ratio that is 53% of that in the inactive fraction. However, it has also been shown that actively transcribing DNA sequences for globin, or ovalbumin, have a periodic structure that is apparently indistinguishable from that of the total nuclear chromatin (Lacy & Axel, 1975; Garel & Axel, 1976; Weintraub & Groudine, 1976), and Reeves & Jones (1976) have shown that the rRNA genes of cells from *Xenopus laevis* are largely protected from digestion by micrococcal nuclease.

This contradiction could be resolved if it were supposed that nucleosome-like structures can be formed in the absence of histone. Such a model would imply a structure for transcriptionally active chromatin in which a proportion of the DNA is packaged with non-histone protein, possibly including RNA polymerase, to give a periodic structure. The observations by Garel & Axel (1976) and by Weintraub & Groudine (1976) that nucleosomes in actively transcribing chromatin have properties different from those in inactive chromatin makes this a possibility worth considering. Furthermore, a precedent for such 'histone-free' nucleosomes already exists in the observation that the adenovirus chromosome is packaged with two non-histone proteins, to give a structure that can be digested with micrococcal nuclease to give a 200-base-pair repeat similar to the repeat observed when eukaryote chromatin is digested (Corden *et al.*, 1976).

The important reservation to be made is that the results reported here apply to ribosomal chromatin, and may not be generally applicable to transcriptionally active chromatin. Foe *et al.* (1976) have reported that r-chromatin can be distinguished in its

morphology from other transcriptionally active chromatin. The latter appears to contain DNA that is at least partially condensed, and contains beads that are identified as nucleosomes, whereas r-chromatin has a filamentous structure in which the DNA appears to be almost fully extended, and in which there is no evidence of nucleosomes. The latter observations are in agreement with those made by Scheer *et al.* (1976).

Foe *et al.* (1976) also observed that the structure of ribosomal chromatin appears to be independent of transcriptional activity, as judged by the number of transcripts associated with the chromatin strands. This observation may be related to that in the present paper that the histone content of r-chromatin does not differ between growing and stationary organisms.

I thank Ms. Jackie Lavis for her excellent technical assistance, and Dr. Michael Bramwell for many useful discussions.

References

- Allfrey, V. G., Faulkner, R. & Mirsky, A. E. (1964) *Proc. Natl. Acad. Sci. U.S.A.* **51**, 786–794
- Bosely, P. G., Bradbury, E. M., Butler-Browne, G. S., Carpenter, B. G. & Stephens, R. M. (1976) *Eur. J. Biochem.* **62**, 21–31
- Bradbury, E. M., Inglis, R. J., Mathews, H. R. & Sarner, N. (1973) *Eur. J. Biochem.* **33**, 131–139
- Camerini-Otero, R. D., Sollner-Webb, B. & Felsenfeld, G. (1976) *Cell* **9**, 179–193
- Cameron, I. L. & Jeter, J. R., Jr. (1970) *J. Protozool.* **17**, 429–431
- Cohen, L. H. & Gotchel, B. V. (1971) *J. Biol. Chem.* **246**, 1841–1848
- Corden, J., Engelking, M. & Pearson, G. D. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 401–404
- Engberg, J., Andersson, P. & Leick, V. (1976) *J. Mol. Biol.* **104**, 455–470
- Finch, J. T., Lutter, L. C., Rhodes, D., Brown, R. S., Rushton, B., Levitt, M. & Klug, A. (1977) *Nature (London)* **269**, 29–36
- Foe, V. E., Wilkinson, L. E. & Laird, C. D. (1976) *Cell* **9**, 131–146
- Gall, J. G. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3078–3081
- Garel, A. & Axel, R. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3966–3970
- Gorovsky, M. A., Keevert, J. B. & Pleger, G. L. (1974) *J. Cell Biol.* **61**, 134–145
- Gottesfeld, J. M., Garrard, W. T., Bagi, G., Wilson, R. F. & Bonner, J. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 2193–2197
- Gottesfeld, J. M., Murphy, R. F. & Bonner, J. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 4404–4408
- Griffith, J. D. (1975) *Science* **187**, 1202–1203
- Hewish, D. R. & Burgoyne, L. A. (1973) *Biochem. Biophys. Res. Commun.* **52**, 504–510
- Hohmann, P., Tobey, R. A. & Gurley, L. R. (1976) *J. Biol. Chem.* **251**, 3685–3692
- Johmann, C. A. & Gorovsky, M. A. (1976) *Biochemistry* **15**, 1249–1256
- Johns, E. W. (1964) *Biochem. J.* **92**, 55–59
- Jones, R. W. (1978) *Biochem. J.* **173**, 145–153
- Kornberg, R. (1974) *Science* **184**, 868–871
- Kornberg, R. & Thomas, J. (1974) *Science* **184**, 865–868
- Lacy, E. & Axel, R. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3978–3982
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Langan, T. A. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **64**, 1276–1283
- Lester-Smith, E. & Page, J. E. (1948) *J. Soc. Chem. Ind. London* **67**, 48–51
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Marushige, K. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3937–3941
- Mathis, D. J. & Gorovsky, M. A. (1976) *Biochemistry* **15**, 750–755
- Noll, M. (1974) *Nature (London)* **251**, 249–251
- Olins, A. & Olins, D. (1974) *Science* **183**, 330–332
- Panyim, S. & Chalkley, R. (1969) *Arch. Biochem. Biophys.* **130**, 337–346
- Piper, P. W., Celis, J., Kaltoft, K., Leer, J. C., Nielsen, O. F. & Westergaard, O. (1976) *Nucleic Acid Res.* **3**, 493–505
- Pogo, B. G. T., Pogo, A. O., Allfrey, V. G. & Mirsky, A. E. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **59**, 1337–1344
- Reeves, R. & Jones, A. (1976) *Nature (London)* **260**, 495–500
- Ruiz-Carrillo, A., Wangh, L. J. & Allfrey, V. G. (1975) *Science* **190**, 117–128
- Sahasrabudhe, C. G. & Van Holde, K. E. (1974) *J. Biol. Chem.* **249**, 152–156
- Scheer, U., Trendelenburg, M. F. & Franke, W. W. (1976) *J. Cell Biol.* **69**, 465–489
- Sollner-Webb, B., Camerini-Otero, R. D., Sollner-Webb, B. & Felsenfeld, G. (1976) *Cell* **9**, 179–193
- Vidali, G., Gershoy, E. L. & Allfrey, V. G. (1968) *J. Biol. Chem.* **243**, 6361–6366
- Weintraub, H. & Groudine, M. (1976) *Science* **193**, 848–856