Structure of active chromatin: isolation and characterization of transcriptionally active chromatin from rat liver

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Rat liver nuclei were isolated in low-ionic-strength buffer in the absence of bi- and multi-valent cations. Digestion of these nuclei by endogenous nuclease, micrococcal nuclease and DNase I revealed that a minor chromatin fraction was preferentially digested into poly- and oligo-nucleosomes. Southern blot hybridization with various active gene probes confirmed that these chromatin fragments represent coding and 5' upstream regions of transcriptionally active chromatin. Active chromatin fragments were released selectively into the medium, with inactive chromatin remaining inside the nuclei, under the above ionic

conditions. The inclusion of bivalent cations during the digestion of nuclei reversed the solubility behaviour of active chromatin. Rearrangement and exchange of histone H1 between chromatin fragments was prevented by using low-salt conditions in all steps in the absence of bivalent cations. All histones, including H1, were present in stoichiometric amounts in this active chromatin fraction. Active nucleosomes showed a lower electrophoretic mobility than bulk nucleosomes in an acrylamide/agarose composite gel in the absence of Mg^{2+} , but were selectively bound to the gel in the presence of this ion.

INTRODUCTION

The study of the structure of transcriptionally active chromatin has been impeded by the difficulty in isolating it. Several techniques have been developed, including: (i) isolation of mono- and short oligo-nucleosome sequences after brief digestion of nuclei with micrococcal nuclease (MNase) [1], (ii) isolation of oligo- and poly-nucleosomes that are soluble in 0.15 M NaCl and are enriched in active genes after digestion of nuclear chromatin with MNase [2], (iii) isolation of active chromatin in the form of oligonucleosomes soluble in 2 mM $MgCl_2$ after digestion of nuclear chromatin with DNase II [3], and (iv) elution of active chromatin after its preferential binding to Hg–Sephacrose [4], HMG–Sephacrose [5] or cDNA–Sephacrose [6]. In most of these studies, the concentrations of uni- and bi-valent cations were such that they could cause rearrangement and exchange of the linker histone H1 among chromatin fibres. This would change the original native distribution of H1 on active chromatin fibres, and thus cause a change in chromatin folding behaviour. MNase and DNase II also do not show a reasonable level of preferential digestion of active chromatin. Recently it has been observed that MNase-digested active gene sequences are preferentially insoluble [6a].

In view of the above, we attempted to devise a method for the isolation of active chromatin that yields active chromatin fragments of longer size and with higher-order folding, in which the rearrangement and exchange of H1 histones is prevented. Thus, during the isolation of nuclei and active chromatin, bi- and multi-valent cations were excluded and the univalent cation concentration was maintained at between 15 and 25 mM. The digestion of nuclei by endogenous nucleases is found to occur in the absence of bivalent cations. Furthermore, a minor chromatin fraction was digested to oligo- and poly-nucleosomes in a preferential manner. On brief incubation of nuclei at 37 °C, the preferentially digested chromatin fraction was selectively released into the medium. The biochemical and structural characteristics of these active chromatin fragments are reported here.

MATERIALS AND METHODS

All operations were carried out between 0 and 4 °C, unless specified otherwise.

Isolation of nuclei

Adult male rats (150–200 g) of the Charles Foster strain (albino) were used. Rat livers were excised and washed with buffer containing 10 mM Tris/HCl, pH 7.4, and 150 mM NaCl. Minced tissue was homogenized in a Potter–Elvehjem homogenizer with 9 vol. of buffer containing 12 % (w/v) sucrose, 10 mM Tris/HCl, pH 7.8, 2.5 mM EDTA and 1 mM PMSF. EDTA was included in the homogenization buffer to chelate endogenous Ca^{2+} and Mg^{2+} .

The homogenate was filtered through two layers of cheese-cloth and centrifuged at 660 g for 5 min over a sucrose cushion [15 % (w/v) sucrose in buffer A (10 mM Tris/HCl, pH 7.8, 10 mM NaCl, 1 mM PMSF)] in a swing-out rotor (HB4) of a Sorvall RC-5C centrifuge. Crude nuclear pellets were washed with 12 % (w/v) sucrose in buffer A, and then twice with Triton X-100 (0.2 %) in buffer A, followed by pelleting over 15 % (w/v) sucrose in buffer A. Pellets were further washed with 12 % (w/v) sucrose in buffer A to remove traces of Triton X-100. Nuclei were suspended in buffer A (pH 7.0) at a concentration of 1–6 mg of DNA/ml.

Digestion of nuclei and DNA analysis

Nuclei suspended in buffer A (1–6 mg of DNA/ml) were incubated at 37 °C. Aliquots of equal volume were drawn after various time intervals and mixed in a solution containing 0.1 % SDS, 0.2 M NaCl and 10 mM EDTA (final concentrations). The resulting solutions were incubated overnight with Pronase (0.5 µg/µg of DNA) at 37 °C and the nucleic acids were extracted with phenol/chloroform (1:1, v/v). Ethanol-precipitated nucleic acids were further treated with RNase to degrade RNA. DNA samples were electrophoresed (50 µg per lane) in a 0.7 % agarose

Abbreviations used: MNase, micrococcal nuclease.

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gel as described by Ali and Singh [7]. Gels were stained overnight with ethidium bromide (0.5 µg/ml) and photographed under UV light.

Isolation of active chromatin

Nuclei (1–6 mg of DNA/ml) in buffer A (pH 7.0) were incubated at 37 °C for 5–10 min. The reaction was stopped by placing in ice after addition of EDTA (2.5 mM final concentration). The suspension of nuclei was stirred gently for 15 min and then centrifuged at 12000 *g* for 10 min. The supernatant thus obtained is referred to as 'active chromatin'. Around 5–10% of total nuclear absorbance at 260 nm was extracted into the supernatant under these conditions. Supernatants showing higher than this level of extraction were found to be contaminated with bulk chromatin fragments, and were not used.

Preparation of ³²P-labelled DNA probes

Escherichia coli cells harbouring recombinant plasmid DNA were grown in L-broth containing an appropriate antibiotic. Plasmid DNAs were isolated by the method of Sambrook et al. [8]. Plasmid DNA was dialysed, digested with restriction nucleases and electrophoresed in agarose gels. DNA insert bands were eluted from the agarose gel by adsorption to DEAE-cellulose. DNA fragments were labelled with [α -³²P]dCTP using a random primer kit and protocol provided by New England Biolabs.

The following plasmids were used for the preparation of gene probes: a tryptophan oxygenase plasmid pUTO BP (+320 to –1930) (obtained from W. Schmid, [9]); an albumin plasmid pBR322, containing a 1.0 kbp cDNA insert, and cytochrome *P*-450 (obtained from Professor G. Padmanabhan, Indian Institute of Science, Bangalore, India); an α -fetoprotein plasmid pBR322 containing an 800 bp cDNA insert (from J. Bonner, [10]), a β -globin 300 bp cDNA probe (obtained from Dr. Veena K. Parnaik, CCMB, Hyderabad, India); and an α -globin gene probe, plasmid pBR322 containing a 2.1 kb insert (obtained from J. J. Lawrence, INSERM, Grenoble, France [11]).

Southern blot hybridization of DNA-containing gels with gene probes

DNA-containing gels were treated successively with 0.5 M NaOH and 1.5 M NaCl for 2 × 30 min and with 1 M Tris/HCl, pH 7.8, and 1.5 M NaCl for 90 min, and finally placed in 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate) for 30 min. DNA was transferred from the gel to a nitrocellulose filter by diffusion for 36 h in 10 × SSC. Filters were used for hybridization after baking at 80 °C for 2 h in a vacuum oven, and were prehybridized overnight at 37 °C in 25 mM potassium phosphate buffer, pH 7.4, 5 × SSC, 5 × Denhardt's solution, 50 µg/ml boiled salmon sperm DNA and 45% formamide. Blots were subsequently hybridized with denatured ³²P-labelled DNA probes [50 µg per filter; (0.25–1) × 10⁹ c.p.m./µg of DNA] in 25 mM potassium phosphate buffer, pH 7.4, 0.1% SDS, 5 × SSC, 5 × Denhardt's solution, 50 µg/ml denatured salmon sperm DNA, 45% formamide and 10% dextran sulphate, overnight at 37 °C. The filters were washed twice for 15 min each with 0.25 × SSC/0.5% SDS at room temperature. The blots were washed further with 0.1 × SSC/0.5% SDS at 65–80 °C for 0.75–1 h. Dried blots were exposed to X-ray film for 1–2 weeks at –45 °C.

Dot-blot hybridization

Active chromatin was extracted from nuclei after incubation for 5 min at 37 °C, as described above. The supernatant was collected

by centrifugation of the nuclear suspension after addition of 2.5 mM EDTA and shaking for 15 min on ice. Bulk chromatin was obtained by the same procedure, but after incubation of nuclei at 37 °C for 30 min. DNA was precipitated with 2 vol. of chilled ethanol, after treatment with RNase, and dissolved in 1 × electrophoresis buffer (40 mM Tris/HCl, pH 8.4/5 mM sodium acetate/1 mM EDTA). Salmon sperm DNA, utilized as control for non-specific hybridization, was digested with *Eco*RI. Thus the bulk chromatin and the salmon sperm DNA were brought to the same DNA size range as for the active chromatin. The DNA content of each sample was determined by the diphenylamine method [12].

DNA samples (4 µl) containing 1, 2, 4 or 8 µg of DNA were spotted on to nylon filters. The filters were dried and treated successively with denaturing solution (0.5 M NaOH/1.5 M NaCl) for 20 min and then with neutralization solution (1 M Tris/HCl, pH 7.8, 1.5 M NaCl) for 20 min. Filters were dried and baked at 80 °C for 90 min in a vacuum oven. The blots were hybridized as described for Southern blot hybridization, except that Denhardt's solution was omitted from the hybridization buffer.

Electrophoretic analysis of nuclear proteins

Proteins from nuclei or chromatin were extracted with 0.25 M HCl and precipitated with 20% (w/v) trichloroacetic acid, and the pellet was dried after washing with acetone. SDS/PAGE (13% gels) of these proteins was carried out by the procedure of Laemmli [13], and gels were stained with Coomassie Blue R250.

Electrophoresis of nucleosomes

Active chromatin was isolated in the absence of EDTA, treated with RNase (0.5 µg/µg of DNA) and concentrated against dry sucrose. DNA was digested with MNase (7.5 units/mg of DNA) (Sigma; 1 unit corresponds to an *A*₂₆₀ of 0.12/min per ml of reaction mixture, pH 8.0) at 37 °C for 1 and 2 min to convert it into short oligonucleosomes. The reaction was stopped by cooling after the addition of EDTA (2 mM final concentration). To isolate short oligonucleosomes from bulk chromatin, nuclei in buffer A were digested with MNase (10 units/mg of DNA) and RNase (0.5 µg/µg of DNA) at 37 °C for 2 min, and then centrifuged (12000 *g*, 10 min) after shaking for 15 min on ice with EDTA. The supernatant was collected and used for electrophoresis. Electrophoresis of active and bulk nucleosomes was carried out at 4 °C in a composite gel [14] consisting of 2.5% acrylamide, 0.125% bisacrylamide and 0.5% agarose in 5 mM Tris/HCl, pH 8.0, 10 mM sodium acetate and 0.2 mM EDTA. Gels were stained with ethidium bromide (0.5 µg/ml) and visualized under UV light.

Estimation of DNA, RNA and protein

Nuclei or chromatin were precipitated with 5% trichloroacetic acid. The pellet obtained on centrifugation (12000 *g*, 10 min) was washed once with 10% trichloroacetic acid and then with ethanol. Subsequently the pellet was suspended in 5% trichloroacetic acid and heated at 95 °C (15 min) to solubilize nucleic acids. Precipitated proteins were removed by centrifugation (12000 *g*, 10 min), and the clear supernatant was used for nucleic acid estimation. DNA was estimated by the diphenylamine method [12], while RNA was estimated by the orcinol reaction [15]; protein was measured by the method of Lowry et al. [16].

RESULTS

Digestion of rat liver nuclei with endonucleases

Isolated rat liver nuclei have been reported by Hewish and Burgoyne [17] to contain a Ca^{2+} - and Mg^{2+} -dependent endonuclease. The kinetics of digestion of nuclei in buffer A by endogenous nucleases were followed in order to find out whether the endogenous nucleases are active in the absence of exogenously added Ca^{2+} and Mg^{2+} , and whether chromatin subfractions can exhibit differential susceptibility to nucleases in low-ionic-strength buffer lacking bi- and multi-valent cations. Under these ionic conditions, bulk chromatin is not expected to form higher-order structure (30 nm thick), and should remain in fibre form (10 nm thick).

Nuclei were incubated at 37 °C in buffer A, and aliquots of DNA taken at various times were electrophoresed in a 0.7% agarose gel. Figure 1(A) shows the ethidium bromide-stained gel. An excess of nuclear DNA (50 μg) was loaded in each lane so that the digestion of even a minor chromatin fraction could be visualized by ethidium bromide staining. Isolated nuclei have all their chromatin in a high-molecular-mass form. It is apparent from Figure 1(A) that the endogenous nuclease is able to digest nuclear chromatin in the absence of exogenously added Ca^{2+} and Mg^{2+} . A minor chromatin fraction was digested rapidly to give oligo- and poly-nucleosomes (ranging in size from one to 100 nucleosomal long filaments; sizes determined as described in the accompanying paper [17a]) in the early phase, followed by digestion of bulk chromatin in a later phase. Digestion of bulk chromatin to give a similar size range took a much longer time. Both chromatin fractions were digested into normal nucleosomal repeat forms, with a similar repeat length. Digestion of the two

chromatin fractions is not initiated simultaneously, but takes place in a sequential manner, with digestion initiated first in the minor fraction, followed by the bulk fraction. A clear-cut boundary is formed between the undigested bulk chromatin DNA and the DNA in the minor chromatin fraction. This boundary becomes diffuse once digestion of the bulk chromatin is initiated, with complete overlap in the later phases of digestion. Similar digestion patterns have been obtained with MNase and DNase I (results not shown).

Preferential digestion of chromatin representing active gene sequences has been reported previously [18], again as a minor fraction, so it seemed probable that the preferentially digested fraction might represent transcriptionally active genes. It has been shown that phenobarbitone administration enhances the rate of synthesis of cytochrome *P*-450 *in vivo* and *in vitro*, and that actinomycin D inhibits this synthesis [19]. Hence the kinetics of digestion of the cytochrome *P*-450 gene were followed before and after induction with phenobarbitone. For this, nuclei were isolated from control and phenobarbitone-injected rats. The kinetics of digestion by endogenous nucleases in these nuclei are shown in Figures 1(A) and 1(B). It is clear that the rate and pattern of digestion of the bulk active chromatin were not changed by injection of phenobarbitone into the rats. However, a big difference was observed in the digestion rate of the cytochrome *P*-450 gene, as observed on Southern blot hybridization of these DNA gels (Figures 1C and 1D). In the uninduced state (control rats), the cytochrome *P*-450 gene was not digested in the early phase of digestion, whereas it was efficiently digested into oligo- and poly-nucleosomes in the early phase after induction by phenobarbitone. This observation demonstrates that the preferential nuclease-sensitivity of a gene depends on its transcriptional activity under our conditions.

This was explored further by studying the kinetics of digestion of various active and inactive genes in rat liver. Probes from the coding regions of the β -globin gene (inactive), the albumin gene (highly active) and the α -fetoprotein gene (weakly active), and from the 5' upstream region of the tryptophan oxygenase gene (moderately active) were used for this purpose. Autoradiograms obtained on Southern blot hybridization are shown in Figures 2(B)–2(E). The right-hand lane of Figure 2(B) represents the signal corresponding to digestion of the total tryptophan oxygenase gene 5' upstream sequences, as the whole of the bulk chromatin has been digested to give a mixture of oligo- and poly-nucleosomal fragments. The majority of the tryptophan oxygenase gene 5' upstream sequences were digested in the early phase, as the signal below the bulk DNA band obtained after 8 min of digestion was similar to the signal obtained after 30 min. Initially a fraction of the tryptophan oxygenase gene was digested to the level of poly- and oligo-nucleosomes, leaving the remaining material in an uncleaved form with long fibres (large bulk DNA band at the top of the lanes). Subsequent digestion of the other fraction of the tryptophan oxygenase gene also occurred, resulting in fragments with a similar size range. Digestion of this gene continued in this manner, with initiation of digestion of the bulk chromatin fraction in the later phase (Figures 2A and 2B). In contrast, the coding region of an inactive gene (β -globin) was digested into oligo- and poly-nucleosomes only in the late phase (last three lanes of Figure 2C). The coding regions of the albumin and α -fetoprotein genes were also digested in a preferential and batchwise manner (Figures 2D and 2E); the signal below the bulk DNA band after digestion for 8 min is the same as the signal obtained after 20 min of digestion. DNA fragments from one to 100 nucleosomes in length were obtained after digestion of these genes (see the accompanying paper [17a]). It is also clear from Figure 2 that the 5' upstream region of the tryptophan oxygenase

Figure 1 Effects of transcriptional induction of the cytochrome *P*-450 gene on its endogenous nuclease-sensitivity

Rat liver nuclei in buffer A (pH 7.0) were incubated at 37 °C for the times indicated. DNA (50 μg /lane) from these digests was analysed by electrophoresis. (A) Nuclei from control rat liver; (B) liver nuclei isolated 12 h after injection of phenobarbitone (8 mg/100 g body weight) into the rat. The stained gels of (A) and (B) were Southern-blotted on to a nitrocellulose membrane and hybridized to a α - ^{32}P -labelled cDNA probe from the rat cytochrome *P*-450 gene; autoradiograms in (C) and (D) correspond to (A) and (B) respectively.

Figure 2 Kinetics of nuclease digestion of active and inactive genes

Rat liver nuclei in buffer A (pH 7.0) were incubated at 37 °C for the times indicated. DNA (50 µg/lane) from these digests was analysed by electrophoresis (A). Stained gels identical to that shown in (A) were Southern-blotted on to a nitrocellulose membrane and hybridized to: (B) a ^{32}P -labelled probe for the 5' upstream region of the tryptophan oxygenase gene, (C) a ^{32}P -labelled cDNA probe for the β -globin gene, (D) a ^{32}P -labelled cDNA probe for the albumin gene and (E) a ^{32}P -labelled cDNA probe for the α -fetoprotein gene.

gene showed a greater nuclease-sensitivity than the coding regions of the active genes. Similar results were reported for the lysozyme gene in the chicken oviduct [20]. Although the α -fetoprotein gene is expressed at very low levels in the liver of young rats, its nuclease-sensitivity is similar to that of the albumin gene, which is expressed at a high level. The reason for this is not yet clear. In any case, active genes are preferentially digested in nuclei compared with uninduced and permanently inactive genes.

Preferentially digested active chromatin fragments are selectively released into the medium

The above experiments established that active chromatin is selectively fragmented by endonucleases on brief digestion of nuclei, and it appeared interesting to extract these fragments selectively. Nuclei were incubated briefly at 37 °C in buffer A (pH 7.0) and digestion was stopped by cooling after addition of EDTA to a final concentration 2.5 mM. After shaking for 15 min on ice, nuclei were centrifuged and the supernatant was collected. Figure 3(A) shows the agarose gel electrophoretic pattern of DNA from the nuclei, digested nuclei and supernatant. Only preferentially digested chromatin fragments were released from the nuclei, while bulk chromatin remained inside (lanes b and c). The enrichment of active gene sequences in the isolated active chromatin preparation was determined by dot blot hybridization. DNA from active chromatin and digested total chromatin, and digested salmon sperm DNA of a similar size range, were dot blotted on a nylon membrane. These blots were hybridized with the α -globin, albumin, tryptophan oxygenase and α -fetoprotein gene probes. Autoradiograms thus obtained were scanned and the fold enrichment of the active gene sequences in active chromatin was calculated. Even without quantification, the enrichment of active genes in early-phase-digested chromatin is

clearly visible on the autoradiograms (Figures 3B–3D). On hybridization with the albumin cDNA probe, salmon sperm DNA did not show any signal even with up to 8 µg of DNA spotted on to the nylon membrane (Figure 3B). This indicates the absence of non-specific hybridization. DNA from total chromatin showed a faint signal. The intensity of the signal increased proportionally with the amount of DNA spotted. In contrast, DNA from an active chromatin fraction gave a pronounced signal, the intensity of which also increased in proportion to the amount of DNA. On visual inspection, and as confirmed by scanning, approx. 4–5-fold enrichment of the coding region of the albumin gene was observed in active chromatin as compared with total chromatin. Specific hybridization took place in the case of hybridization with the probe from the 5' upstream region of the tryptophan oxygenase gene (Figure 3C). However, a slightly higher level of enrichment (5–6-fold) was found in active chromatin for this gene sequence. Enrichment of the weakly active α -fetoprotein gene coding sequence was much lower (2-fold) (Figure 3D) in active chromatin than for the highly and moderately active sequences. Figure 3(E) shows that the active chromatin does not contain a signal from an inactive gene (i.e. α -globin).

Preferential association of active gene regions with the nuclear matrix

Several groups have reported that actively transcribing gene sequences are selectively retained inside nuclei, in contrast with the efficient solubilization of inactive gene sequences on shaking of nuclei with EDTA or 0.4 mM Mg^{2+} after nuclease digestion [21]. However, in these studies, physiological concentrations of uni-, bi- and multi-valent cations were used during isolation and nuclease digestion, in contrast with the low-ionic-strength buffer

Figure 3 Enrichment of active genes in the active chromatin preparation

(A) Nuclei in buffer A (pH 7.0) were incubated at 37 °C for 5 min. Active chromatin was recovered in the supernatant and subjected to agarose-gel electrophoresis. Lane a, DNA from undigested nuclei (90 μg); lane b, DNA from digested nuclei (90 μg); lane c, DNA from the active chromatin fraction (10 μg). (B)–(E) Serial dilutions of DNA from *Eco*RI-digested salmon sperm DNA (a), bulk chromatin (b) and active chromatin (c) were dotted on to nylon membranes. Dot blots were hybridized to a ³²P-labelled albumin cDNA probe (B), a 5' upstream tryptophan oxygenase gene probe (C), an α-fetoprotein gene probe (D) and an α-globin gene probe (E), as described in the Materials and methods section.

lacking bi- and multi-valent cations used in these steps in our studies. Our aim was to find out whether the active chromatin fraction could also show differential insolubility inside nuclei on digestion and extraction in the presence of bivalent cations, as reported previously [20]. For this purpose, nuclei isolated in buffer A (pH 7.0) were incubated at 37 °C after the addition of Ca²⁺ and Mg²⁺ to final concentrations of 0.4 mM each. Digestion was stopped by cooling after addition of 0.4 mM EGTA (which chelates Ca²⁺), and the nuclei were centrifuged after shaking for 30 min on ice. DNA extracted from the supernatant was electrophoresed in an agarose gel (Figure 4). Differentially digested active chromatin was not released into the supernatant in the presence of 0.4 mM Mg²⁺ (Figure 4, lanes d and e), whereas it was efficiently extracted in the absence of bivalent cations (lanes b and c). This suggests that the selective retention of active gene fragments occurs by binding to the nuclear matrix in the presence of bivalent cations. In contrast, bivalent cations have very little

Figure 4 Selective association of active chromatin with the nuclear matrix

Rat liver nuclei were incubated in buffer A (pH 7.4) at 37 °C for 10 min to preferentially digest active chromatin, and with MNase (1.5 units/mg of DNA) at 37 °C for 10 min to digest bulk chromatin, with or without 0.4 mM CaCl₂ and 0.4 mM MgCl₂. Digestion was stopped by transferring the tubes to ice after the addition of 0.4 mM EGTA. Lane a, 100 μg of DNA equivalents from undigested nuclei; lane b, 100 μg of DNA equivalents from nuclei digested for 5 min in the absence of Ca²⁺ and Mg²⁺; lane c, supernatant of 200 μg of DNA equivalents from digested nuclei as for lane b; lane d, 100 μg of DNA equivalents from nuclei digested for 5 min in the presence of Ca²⁺ and Mg²⁺; lane e, supernatant of 200 μg of DNA equivalents from digested nuclei as for lane d; lane f, 20 μg of DNA equivalents from MNase-digested nuclei in the absence of Ca²⁺ and Mg²⁺; lane g, supernatant of 40 μg of DNA equivalents from digested nuclei as for lane f; lane h, 10 μg of DNA equivalents from MNase-digested nuclei in the presence of Ca²⁺ and Mg²⁺; lane i, supernatant of 20 μg of DNA equivalents from digested nuclei as for lane h.

Figure 5 Protein composition of active chromatin

(A) Proteins extracted with 0.25 M HCl from nuclei and chromatin were analysed by SDS/PAGE. Proteins were associated with 40 μg (lane a) or 20 μg (lane b) of DNA from active chromatin, or with 20 μg (lane c) or 40 μg (lane d) of DNA from nuclei. (B) Proteins present in supernatants recovered after incubation of nuclei at 37 °C for 5, 10 and 15 min were suspended in sample buffer, sonicated, heated and electrophoresed. Lane M, protein markers; lane a, proteins from 15 μg of DNA equivalents from nuclei. Lanes b–c contain proteins from the supernatant recovered after incubation of nuclei for 5 min (lane b; 20 μg of DNA equivalents), 10 min (lane c; 15 μg of DNA equivalents) and 15 min (lane d; 15 μg of DNA equivalents).

effect on the extent of extraction of bulk chromatin after digestion. Bulk chromatin is extracted efficiently in the presence or the absence of 0.4 mM Mg²⁺, after digestion.

Active chromatin fragments have a normal complement of histones and are enriched in RNA and non-histone proteins

Proteins were extracted from nuclei and active chromatin, and were analysed by SDS/PAGE (Figure 5A). All histones, including H1, were present in normal stoichiometric amounts in active

Table 1 Enrichment of RNA and non-histone chromosomal proteins in the active chromatin fraction

DNA, RNA and protein were estimated in typical nuclear and active chromatin preparations as described in the Materials and methods section.

Material	RNA/DNA	Protein/DNA
Nuclei	0.14	2.9
Active chromatin	0.90	10.6

Figure 6 Electrophoretic mobility of oligonucleosomes from active and bulk chromatin

Active and bulk chromatin fractions were digested with MNase in buffer A to give short oligonucleosomes. These were run in 0.5% agarose/2.5% acrylamide composite gels in the absence (A) and the presence (B) of 1 mM $MgCl_2$ as described in the Materials and methods section. (A) Lanes a and b contain oligonucleosomes from active chromatin, and lane c contains oligonucleosomes from bulk chromatin. (B) Lane a, active oligonucleosomes; lane b, bulk oligonucleosomes.

chromatin. Non-histone proteins were greatly enriched in the active chromatin fraction in comparison with nuclei (Table 1). In order to find out whether these non-histone proteins are bound to DNA or RNA, nuclei were incubated for 5, 10 and 15 min. Approx. 7.7%, 12% and 16% respectively of the total A_{260} was recovered in the supernatant after this treatment. Proteins after incubation were analysed on an SDS/polyacrylamide gel (Figure 5B). The supernatant of the 5 min incubation contained mostly non-histone proteins, and the histone content was very low. On further incubation this fraction maintained its histone content but became depleted of non-histone proteins and RNA. Active chromatin is also enriched in RNA (Table 1, and see the accompanying paper [17a]). It may be concluded that the non-histone proteins are components of ribonucleoprotein particles which are released in the early phase of incubation but become aggregated on further incubation.

Alteration of the nucleosomal structure of active chromatin

Alterations in the nucleosomal structure of active chromatin were assessed by comparison of the electrophoretic mobilities of

oligonucleosomes from the active and bulk chromatin fractions in a 0.5% agarose/2.5% acrylamide composite gel. Active chromatin and bulk chromatin were digested with MNase to convert them into short oligonucleosomes. When the short oligonucleosomal preparations from the two chromatin preparations were run in low-ionic-strength electrophoresis buffer, bands corresponding to mono-, di-, tri- and higher oligonucleosomes became visible (Figure 6A). Although the mobilities of the mononucleosomes from the two chromatin fractions were similar, the difference in mobility increased as the size of the oligomer increased. Oligonucleosomes from active chromatin had lower mobility as compared with their counterparts from bulk chromatin. On electrophoresis of these short oligonucleosomes in low-ionic-strength buffer containing 1 mM Mg^{2+} , a different picture was observed. Mg^{2+} was added to the buffer after the oligonucleosomes had entered the gel, to avoid aggregation of nucleosomes. In addition to decreases in the intensity of mono-, di- and higher nucleosomal bands on addition of 1 mM Mg^{2+} , a new band also appeared (Figures 6A and 6B, lane a). The mono-, di- and higher oligonucleosomes from active chromatin were totally retarded, while the mobility of the same bands from bulk chromatin was not affected (Figure 6B). In the case of bulk oligonucleosomes, a small fraction of mono- and di-nucleosomes remain fixed at a position identical to that of their counterparts in active chromatin.

DISCUSSION

In the preferentially digested chromatin fraction, the differential enrichment of genes of different transcriptional activities was observed on scanning the autoradiograms of dot blot hybridizations. Enrichments of 4–5-fold for the coding region of the highly active albumin gene, 2-fold for the low-activity α -fetoprotein gene and 5–6 fold for the 5' upstream regulatory region of the moderately active tryptophan oxygenase gene were observed. The induced cytochrome *P*-450 gene was also enriched. However, the inactive globin and uninduced cytochrome *P*-450 genes were depleted. Coding and 5' upstream regulatory regions were digested preferentially into poly- and oligo-nucleosomes (Figures 1 and 2). The degrees of enrichment of active genes in the active chromatin fraction were similar to previously reported values [22]. For example, the insoluble nuclear pellet from rat liver after digestion with MNase contained 15% DNA and was enriched in active genes without contamination by inactive genes. Some active chromatin fragments were released into the supernatant as well, suggesting that rat liver nuclei may contain more than 15% of the DNA as active genes [22]. It has also been reported that around 10% of DNA in mammalian cells is transcribed into heterogeneous RNA [23,24]. Active genes consisting of 5' upstream regulatory regions along with the coding region are comparable in size to those reported. Active chromatin isolated after nuclease digestion will contain both regions, due to their similar nuclease-sensitivities, thus bringing the active DNA content in this fraction to around 20%. Hence an enrichment of 5-fold in rat liver active chromatin seems reasonable.

Selective digestion of active genes into polynucleosomes in the early phase, as observed during the course of the present investigation, has not been reported previously. The technique for the fractionation of active chromatin in rat liver tissue is highly reproducible, and hence could be used in other tissues. A limitation in earlier nuclear digestions performed to isolate active chromatin was the inclusion of uni- and bi-valent cations at concentrations known to cause rearrangement of histone H1 [20,25–28]. In fact, poly- and short oligonucleosomes enriched in active genes isolated by these methods were depleted of between

60 and 100% of their expected H1 content [26,29,30]. It is important to note that the active chromatin fragments were easily extracted from nuclei in our studies, whereas these fragments remained selectively associated with the nuclear matrix on MNase digestion and extraction by other workers [21]. Indirect evidence for the association of active chromatin with the nuclear matrix is shown in Figure 4. Furthermore, the association of active nucleosomes in the presence of Mg^{2+} suggests that the attachment of active gene regions to the matrix may be mediated by Mg^{2+} , as seen for the polyacrylamide/agarose composite gel (Figure 6B).

All histones, including H1, were present in normal stoichiometric amounts in the active chromatin isolated in the present study (Figure 5). Some studies, using immunoprecipitation and hybridization of cross-linked chromatin fragments, have suggested that H1 is present in active chromatin, although in lower than stoichiometric amounts [30–34]. This partial depletion was attributed not to lower stoichiometric amounts but to the changed mode of interaction of H1 with active nucleosomes [34]. Thus our results also lend support to this school of thought.

The cause of the preferential nuclease-sensitivity of active genes was also investigated. Differences in higher-order folding cannot be the reason, since both active and bulk chromatin fractions are expected to lack higher-order folding at a univalent cation concentration of 15 mM. Both chromatin fractions have a polynucleosomal structure (Figures 1 and 2). A role for covalent modifications is also ruled out, since isolated nuclei have similar levels of acetylation and ubiquitination in the active and bulk chromatin fractions (see accompanying paper [34a]). Only ADP-ribosylation has been found to be very low in active chromatin in comparison with bulk chromatin. However, it is difficult to explain the selective nuclease digestion of active chromatin by a lack of ADP-ribosylation. Another likely explanation for this is the presence of non-histone proteins and supercoiling in active genes. The non-histone high mobility group proteins HMG14 and HMG17 have been implicated in the sensitivity to DNase I of active genes [35], although this could not be reproduced by others [36]. Our results also rule out the possibility of a role for non-histone proteins in the nuclease-sensitivity of active genes. (results not shown).

It has been proposed that the negative supercoiling of chromatin may be involved in the transcription of some eukaryotic genes inserted in circular vectors [37–40]. Ethidium bromide is known to cause release of negative supercoiling, and has been used to determine negative supercoiling in prokaryotic and eukaryotic chromosomes [41]. Ethidium bromide treatment of rat liver nuclei in our studies did not have any effect on the preferential sensitivity to MNase of active genes (results not shown), suggesting that negative supercoiling, if present, in liver nuclei has no role in this differential nuclease-sensitivity. Unfortunately, there is no direct way of finding out whether active genes in nuclei display negative supercoiling. Positive supercoiling has been implicated in the creation of DNase I-sensitive sites in active chromatin regions [42]. Further investigations in this regard are in progress.

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REFERENCES

- 1 Bellard, M., Gannon, F. and Chambon, P. (1977) *CSHSQB* **62**, 779–791
- 2 Ridsdale, J. A., Rattner, J. B. and Davie, J. R. (1988) *Nucleic Acids Res.* **16**, 5915–5926
- 3 Gottesfeld, J. M. (1977) *Methods Cell Biol.* **15**, 421–436
- 4 Boffa, L. C., Walker, J., Chen, J. A., Sterner, R., Mariani, M. R. and Allfrey, V. G. (1990) *Eur. J. Biochem.* **194**, 811–823
- 5 Weisbrod, S. and Weintraub, H. (1981) *Cell* **23**, 391–400
- 6 Vincez, C., Fronk, J., Tank, G. A. and Langmore, J. P. (1991) *Nucleic Acids Res.* **90**, 1325–1336
- 6a von Holde, K. E. (1988) in *Chromatin* (Rich, A., ed.), pp. 355–407, Springer-Verlag, New York
- 7 Ali, Z. and Singh, N. (1987) *J. Biol. Chem.* **262**, 12989–12993
- 8 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- 9 Becker, P., Renkawitz, R. and Schutz, G. (1984) *EMBO J.* **3**, 2015–2020
- 10 Jagodzinski, L. and Bonner, J. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3521–3525
- 11 Leder, A., Swan, D., Ruddle, F., Eustachio, P. D. and Leder, P. (1981) *Nature (London)* **293**, 196–200
- 12 Burton, K. (1959) *Biochem. J.* **62**, 315–323
- 13 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 14 Todd, R. D. and Garrard, W. T. (1977) *J. Biol. Chem.* **252**, 4729–4739
- 15 Schneider, W. C. (1957) *Methods Enzymology* **3**, 680
- 16 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- 17 Hewish, D. R. and Burgoyne, L. A. (1973) *Biochem. Biophys. Res. Commun.* **52**, 504–510
- 17a Tikoo, K., Hamid, Q. A. and Ali, Z. (1997) *Biochem. J.* **321**, 289–296
- 18 Reeves, R. (1984) *Biochim. Biophys. Acta* **782**, 343–393
- 19 Bhat, K. S. and Padmanaban, G. (1978) *FEBS Lett.* **89**, 331–343
- 20 Strätling, W. H. (1987) *Biochemistry* **26**, 7893–7899
- 21 Yaniv, M. and Cereghini, S. (1986) *CRC Crit. Rev. Biochem.* **21**, 1–26
- 22 Sikorska, M., Whitfield, J. F. and Walker, P. R. (1988) *J. Biol. Chem.* **263**, 3005–3011
- 23 Davidson, E. H. (1976) in *Gene Activity in Early Development*, Academic Press, New York
- 24 Holmes, D. S. and Bonner, J. (1974) *Biochemistry* **13**, 841–848
- 25 Caron, F. and Thomas, J. O. (1981) *J. Mol. Biol.* **146**, 513–537
- 26 Decluve, G. P. and Davie, J. R. (1989) *Biochem. J.* **263**, 179–186
- 27 Renz, M. and Day, L. A. (1976) *Biochemistry* **15**, 3220–3228
- 28 Ruiz-Carrillo, A., Puigdomenech, P., Eder, G. and Lurz, R. (1980) *Biochemistry* **19**, 2544–2554
- 29 Locklear, L., Ridsdale, J. A., Bazett-Jones, D. P. and Davie, J. R. (1990) *Nucleic Acids Res.* **18**, 7015–7024
- 30 Dedon, P. C., Saults, J. A., Allis, C. D. and Gorovsky, M. A. (1991) *Mol. Cell. Biol.* **11**, 1729–1732
- 31 Ericsson, C., Grossbach, U., Björkroth, B. and Daneholt, B. (1990) *Cell* **60**, 73–83
- 32 Hill, R. J., Watt, F., Wilson, C. M., Fifis, T., Underwood, P. A., Tribbick, G., Geysen, H. M. and Thomas, J. O. (1989) *Chromosoma* **89**, 411–421
- 33 Kamakaka, R. T. and Thomas, J. O. (1990) *EMBO J.* **9**, 3997–4006
- 34 Nacheva, G. A., Guschin, D. Y., Preobrazhenskaya, O. V., Karpov, V. L., Ebralidse, K. K. and Mirzabekov, A. D. (1989) *Cell* **58**, 27–36
- 34a Tikoo, K. and Ali, Z. (1997) *Biochem. J.* **321**, 281–287
- 35 Weisbrod, S., Groudine, M. and Weintraub, H. (1980) *Cell* **19**, 289–301
- 36 Reeves, R. and Chang, D. (1983) *J. Biol. Chem.* **258**, 679–687
- 37 Harland, R. M., Weintraub, H. and McKnight, S. L. (1983) *Nature (London)* **302**, 38–43
- 38 Luchnik, A. N., Bakayev, V. V., Zbarsky, I. B. and Georgiev, G. P. (1982) *EMBO J.* **1**, 1353–1358
- 39 Ryoji, M. and Worcel, A. (1984) *Cell* **37**, 21–32
- 40 Ryoji, M. and Worcel, A. (1985) *Cell* **40**, 923–932
- 41 Lilley, D. M. J. (1984) *Biochem. Soc. Trans.* **12**, 127–140
- 42 Freeman, L. A. and Garrard, W. T. (1992) *Crit. Rev. Eukaryotic Gene Expression* **2**, 165–209