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The Distribution of Nuclear Proteins and Transcriptionally-Active Sequences in Rat Liver Chromatin Fractions

NICOLAI M. MIRONOV,* VICTOR V. LOBANENKOV* and GRAHAM H. GOODWIN**

Institute of Cancer Research, Royal Cancer Hospital, Chester Beatty Laboratories, London SW3 6JB, England

Chromatin fractions from rat liver nuclei digested by nucleases were separated by differential solubility into several fractions. Material solubilized during digestion (predominantly monomer nucleosomes and polynucleosomes) had the highest HMG14+17/DNA ratios but were not enriched in active gene sequences (albumin and c-Ha-*ras*1 genes). Material soluble in a low ionic strength buffer containing 0.2 mM $MgCl_2$ (monomer nucleosomes and polynucleosomes) contained in addition to the histones, HMG14 and 17 plus a 41K non-histone protein. This fraction was depleted in active gene sequences and enriched in inactive sequences. The insoluble material was highly enriched in active sequences and had the lowest HMG14+17/DNA ratio. This fraction could be further fractionated into a histone-containing 2 M NaCl-soluble fraction and a 2 M NaCl-insoluble matrix-bound fraction, both of which were enriched in active sequences. The results show that the HMG proteins do not partition with active sequences during fractionation of chromatin. The 41K protein may be associated with inactive chromatin fraction.

Regulation of gene activity is thought to be at least partly controlled by changes in the chromatin structure of genes and a number of differences between transcriptionally-active and inactive genes have been documented. Thus active genes are marked by DNase I 'hypersensitive' sites, have less 5-methylcytosine and are more susceptible to digestion by micrococcal nuclease or DNase I; the latter property may be due to the binding of HMG14 and 17 proteins to active nucleosomes (for references, see [1]). Evidence has been presented that genes are packed in looped domains attached to a residual framework called the nuclear matrix and it is thought that replication and transcription occurs at the matrix [2–5]. Evidence for an enrichment of active sequences on the nuclear matrix is rather controversial [6–9] and this may well be due to the different methods used to isolate nuclear matrix [23].

In this report a procedure is presented for fractionating nuclear chromatin following digestion by endogenous nuclease activity or by micrococcal nuclease. The distribution of chromosomal proteins and gene sequences in these fractions was investigated to see whether any nuclear proteins consistently partitioned with either transcriptionally active or inactive sequences.

* Address: All-Union Cancer Research Center, 24 Kashirskoye Shosse, 115478, Moscow, USSR.
** To whom offprint requests should be sent.

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METHODS

Rat-liver nuclei were isolated by homogenizing the tissue in 0.25 M sucrose in 30 mM Tris-HCl buffer, pH 7.6, containing 10 mM $MgCl_2$ and 0.03 mM mercaptoethanol followed by centrifugation at 800 g for 10 min. The crude nuclei were then centrifuged through 2.2 M sucrose in the same buffer at 85000 g for 1 h. Alternatively, rat-liver nuclei were prepared essentially as described [10] using a homogenization buffer of 0.34 M sucrose, 65 mM KCl, 15 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.5 mM spermidine, 0.15 mM spermine, 2 mM EDTA, 0.5 mM EGTA.

The nuclei were stored at −20°C in homogenization buffer containing 30% glycerol. All buffers contained 0.5 mM PMSF.

Rat-liver nuclei isolated in the presence of $MgCl_2$ were digested by endogenous nuclease activity during the isolation of nuclei. Rat-liver nuclei isolated in the presence of polyamines were digested by micrococcal nuclease in the same homogenizing buffer except EDTA and EGTA were 0.2 mM, $CaCl_2$ was added to 2.5 mM and mercaptoethanol to 0.5 mM. Concentration of nuclei was 30–40 A_{260}/ml and concentration of nuclease was 2 units per 1 A_{260} of nuclei. Nuclei were digested for 20–30 min at 37°C until 2±1% DNA was rendered acid-soluble.

Following digestion, the nuclear suspension was centrifuged at 800 g for 10 min. The pellet was washed with digestion buffer, centrifuged and the supernatants combined, giving a fraction of chromatin that is soluble in digestion buffer.

The nuclear pellet was then resuspended at 20 A_{260} units per ml in a low ionic strength TM buffer (10 mM Tris-HCl, pH 7.6, 0.2 mM $MgCl_2$) which preserves the protein nuclear matrix [11]. Soluble and insoluble material was separated by centrifugation at 800 g for 10 min. The supernatant is the chromatin fraction that is soluble in the buffer of low ionic strength.

The pelleted insoluble material was further fractionated by resuspension in 2 M NaCl in TM buffer and centrifugation at 800 g for 20 min. The pellet was re-extracted with 2 M NaCl and the supernatants combined, giving a 2 M NaCl-soluble fraction and a 2 M NaCl-insoluble fraction.

DNA was isolated from the various fractions by proteinase K treatment and phenol extraction. RNA was removed by RNase treatment and alkaline hydrolysis (0.3 M NaOH, 65°C, 30 min).

Analysis of gene sequences was carried out by dot-blot hybridizations [12]. Before blot analysis, all samples were sonicated to an average length of 600 bp. Probes used were a plasmid containing the cDNA β-major mouse globin gene, the plasmid pRSA8 containing the rat albumin gene [13], and the plasmids contained c-Ha-*ras*1 gene insert [14]. After hybridization the DPT paper was washed as described [12] except that the temperature was 55°C instead of 65°C and 0.2 SSC was used instead of 0.1 SSC when the mouse globin probe was used for hybridization with rat-liver DNA.

To analyse the proteins in the chromatin fractions, the chromatin was dissociated with SDS and loaded onto SDS–polyacrylamide electrophoresis (SDS–PAGE) slab gels. Rat HMG14 and 17 were identified, migrating just behind the core histones by their co-migration with calf thymus HMG standards (rat HMG14 and 17 co-migrate with calf HMG17). Also, HMG proteins (and H1) were selectively extracted with perchloric acid (PCA) and identified with standards on SDS or acid-urea gels. The relative quantities of HMG14 plus HMG17 in the various fractions relative to the DNA in the fractions was determined by the scanning Coomassie Blue-stained gels. DNA in the fractions was determined by the method of Burton et al. [17].

RESULTS

Nuclei prepared from rat liver were fragmented by nucleases and the chromatin fractionated by differential solubility in buffers of differing ionic composition; rat liver nuclei isolated in the presence of Mg^{2+} were digested by the endogenous nuclease(s), nuclei isolated in the presence of chelating ions to inhibit endogenous nucleases were digested with micrococcal nuclease. After digestion, the nuclei were pelleted, washed with digestion buffer, and the combined supernatants retained. About 5% of the DNA was released into this first supernatant fraction during micrococcal nuclease digestion of rat liver nuclei. An agarose gel of the material shows the DNA to be mostly derived from mononucleosomes and poly-

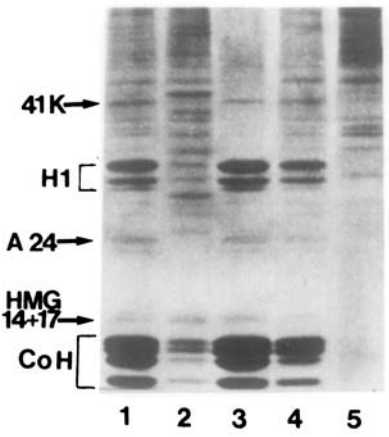

Fig. 1. SDS–PAGE of proteins from chromatin fractions obtained after digestion with micrococcal nuclease. Total proteins from: lane *1*, unfractionated nuclei; *2*, fraction released in digestion buffer; *3*, low ionic strength-soluble fraction; *4*, fraction soluble in 2 M NaCl; *5*, fraction insoluble in 2 M NaCl; *CoH*, core histones. Note: Rat HMG14 and 17 co-migrate on SDS gels.

nucleosomes (up to about hexanucleosomes, data not shown). The proteins in this fraction are shown in fig. 1 (lane *2*). All five histones are present, plus many non-histone chromosomal proteins. This fraction has the highest HMG14+17/DNA ratio (table 1).

The pelleted nuclei were then resuspended in a low ionic strength TM buffer, resulting in the solubilization of approx. 60% of the nuclear chromatin from rat liver nuclei. The DNA of this fraction was derived from a nucleosomal structure too, but the range of polynucleosomal sizes was higher than that of the previous fraction (mono- to decanucleosomes). Analysis of the protein of this low ionic strength soluble material reveals that it is composed of five major histones together with protein A24 (fig. 1,*3*). The HMG14+17/DNA ratio for this fraction is lower than the first fraction (table 1). The quantity of non-histone proteins migrating above H1 was very much lower than the other fractions; only two such proteins are seen on a two-dimensional (2D) electrophoretic gel (fig. 2), a protein of 41 (±1) kilodaltons (41K) and a protein termed P_X.

Table 1. *Comparison of HMG14+17/DNA ratios in different chromatin fractions*

Chromatin fraction	Endogenous nuclease digestion	Micrococcal nuclease digestion
Released during digestion		20.0
	–	11.0
Soluble in low ionic strength buffer	4.3	2.1
	2.4	
	3.4	
	4.4	2.5
Insoluble in low ionic strength buffer[a]	1.0	1.0

The data obtained in independent experiments are shown.
[a] Each HMG/DNA ratio obtained in separate experiments was normalized to the ratio in this fraction.

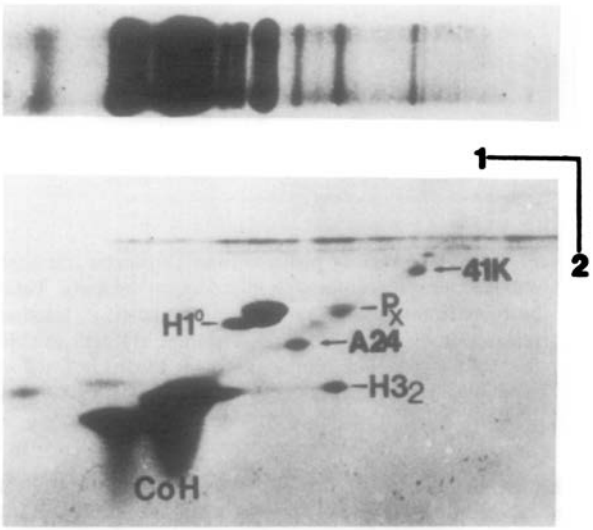

Fig. 2. 2D PAGE of HCl-soluble proteins from low ionic strength-soluble fraction of endogenous nuclease digested nuclei. First dimension (*1*), acid/urea; 2nd dimension (*2*), SDS; *CoH*, core histones.

The material insoluble in the low ionic strength buffer contained the bulk of nuclear non-histone proteins but had the lowest HMG14/17 to DNA ratio. Table 1 summarizes the quantitative data obtained using micrococcal and endogenous nuclease digestions.

The low ionic strength insoluble fraction can be further fractionated into a 2 M NaCl-soluble fraction and matrix fraction by dissociating the histones and other proteins. 2 M NaCl solubilizes most of the remaining DNA leaving behind 0.5–5% of the nuclear DNA as matrix-bound material. The DNA of these two fractions has the highest molecular weights (2–10 kb) with only a small amount of nucleosomal ladder visible in the 2 M NaCl-soluble fraction. The 2 M NaCl-soluble fraction has the five core histones plus HMG proteins and a reduced quantity of non-histones whilst the 2 M NaCl-insoluble fraction is comprised solely of high molecular weight non-histone proteins (fig. 1,*4*,*5*).

Table 2. *Ratio of concentration of gene sequences in DNA of chromatin fractions relative to that in total rat liver DNA*

Chromatin fraction	Albumin	c-Ha-*ras*1	β-globin
Released during micrococcal nuclease digestion	1.0 (±0.2)	1.2 (±0.2)	0.9 (±0.1)
Soluble in low ionic strength buffer	0.5 (±0.1)	0.5 (±0.1)	2.5 (±0.4)
2 M NaCl-soluble	1.8 (±0.3)	0.9 (±0.2)	0.8 (±0.2)
Matrix-bound	7.6 (±0.5)	4.5 (±0.3)	0.6 (±0.2)

Values were obtained by scanning dot-blots such as that shown in fig. 3 and averaging the results from five experiments using micrococcal nuclease.

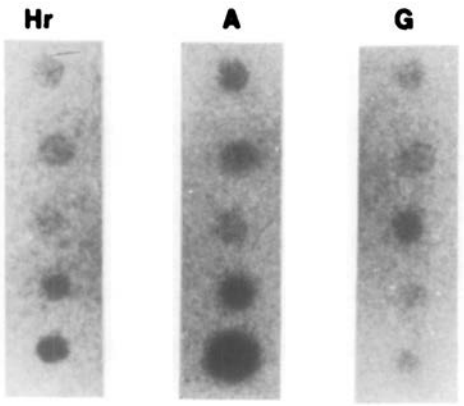

Fig. 3. Dot-blot hybridization of DNAs from chromatin fractions of micrococcal nuclease digested nuclei probed with *Hr*, c-Ha-*ras*1 gene probe; *A*, albumin and *G*, globin gene probe. DNA samples (5 μg) were from: *1*, unfractionated nuclei; *2*, fraction solubilized during digestion; *3*, the low ionic strength-soluble fraction; *4*, 2 M NaCl- soluble fraction; *5*, 2 M NaCl-insoluble matrix bound fraction.

Hybridization analysis of the DNA of the micrococcal nuclease fractions is shown in fig. 3. Two gene probes were used to analyse the distribution of transcriptionally active sequences, the tissue specific albumin gene which is highly transcribed in liver, and a 'housekeeping gene', the c-Ha-*ras*1 proto-oncogene. The β-globin gene was used to measure inactive sequences. Quantitative analysis of the dot blots (table 1) shows that the first fraction released into the digestion buffer (i.e. the fraction with the highest HMG/DNA ratio) is not markedly enriched in active sequences as compared with total liver DNA. The chromatin fraction soluble in the low-ionic strength buffer is depleted in active sequences and enriched in inactive sequences. The 2 M NaCl-soluble chromatin and the matrix-bound DNA which have the least quantity of HMG proteins are

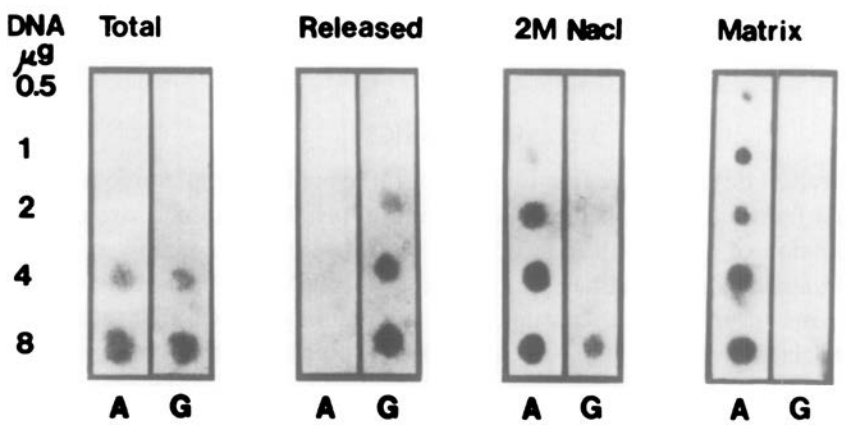

Fig. 4. Dot-blot hybridization of DNAs from chromatin fractions of endogenous nuclease-digested nuclei probed with *A*, albumin gene probe; *G*, globin gene probe. DNA samples loaded in increasing amounts onto DTP paper were from: Unfractionated nuclei (total); the fraction released in the low ionic strength buffer (released); the 2 M NaCl-soluble fraction (2 M NaCl); and the 2 M NaCl-insoluble fraction (matrix).

Table 3. *Ratio of albumin to globin genes in DNA isolated from rat liver chromatin fractions*

DNA	Average ratio	Range	No. of hybridizations
Total	1.0	–	6
Soluble in low ionic strength buffer	0.3	0–0.7	6[a]
Soluble in 2 M NaCl	6.6	3.0–17.0	5
Matrix-bound	25.0	25–∞	3[b]

[a] No hybridization to the albumin gene probe was found in two experiments.
[b] No hybridization to the globin gene probe was found in one experiment (shown in fig. 4).

enriched in albumin sequences. The matrix-bound DNA is highly enriched in both albumin and *ras* sequences.

Similar data were obtained for the three chromatin fractions obtained from nuclei digested by the endogenous nuclease (in this case the first fraction is not obtained). The albumin and globin probes were used (fig. 4) and table 3 summarizes the quantitative data obtained by scanning dot-blots of several experiments. It can be seen that again the highest enrichment for active sequences is in the low ionic strength-insoluble material; the albumin/globin ratio increases by a factor of 6 in the 2 M NaCl-soluble DNA and 25-fold or more in the matrix-bound DNA when compared with total unfractionated liver DNA. Again, the low ionic strength-soluble fraction is depleted in albumin sequences, the average albumin/globin ratio being about threefold lower than that of unfractionated DNA. Similar data to that obtained in fig. 4 on the distribution of HMG proteins and inactive globin gene and active N-*ras* gene sequences [36] were revealed for chromatin fractions obtained from human HT1080 cell nuclei digested with micrococcal nuclei or the restriction enzyme Hae III (data not shown).

DISCUSSION

This report describes the fractionation of rat liver chromatin into four fractions. The first fraction, which solubilized at about physiological ionic strength during the digestion of nuclei with micrococcal nuclease, consists mainly of monomer and polynucleosomes and has the highest level of HMG14/17 proteins relative to DNA. This agrees with other studies [12, 22] that have shown that these proteins are associated with the most nuclease-sensitive chromatin domains. Assuming the absence of protein rearrangements, it has lent support to the idea that HMG proteins are preferentially associated with nucleosomes of active genes, since transcriptionally active genes are considered to be more accessible to nuclease attack [18–20]. This study shows that in liver this supposition may not be valid, as the active albumin and *ras* genes are not noticeably enriched in this fraction from rat liver nuclei. Our inability to observe enrichment for active genes in the first

supernatant fraction, which is enriched in HMG14/17 proteins, is also in agreement with previous studies [20, 24, 35] that have shown the absence of specific binding of these proteins to nucleosomes containing active sequences. However, despite the mild digestion condition (~2% acid-soluble nucleotides), we have not excluded the possibility that the lack of enrichment of active sequences in the first supernatant fraction could be due to nuclease degradation of active sequences into non-hybridizing fragments once they are solubilized into the supernatant.

The second chromatin fraction solubilized with the low ionic strength buffer is mostly polynucleosomes and constitutes the major fraction of nuclear material. This soluble fraction has a 2–4-fold higher HMG/DNA ratio and it is enriched in inactive sequences and depleted in active sequences, compared with the insoluble fraction which is composed of a 2 M NaCl-soluble and matrix-bound fraction. A similar fraction solubilized in EDTA-containing low ionic strength buffer after micrococcal nuclease digestion of oviduct nuclei was found to be depleted in active sequences also [26, 27]. The properties of the low ionic strength-soluble fraction from endogenous nuclease-digested rat liver nuclei resemble that of inactive 'heterochromatin' since it is enriched in inactive sequences and strongly depleted in active sequences. It is also depleted of non-histone chromosomal proteins including the RNP proteins and proteins of high molecular weight. It is also depleted of RNA polymerase II activity and nascent RNA chains [28, 29]. 2D gel electrophoresis of the proteins in this fraction show in addition to the histones and the H3-dimer, two additional proteins; P_X, an unidentified protein, and protein P41 (MW 40000–42000), which is soluble in H_2SO_4, but insoluble in 5% $HClO_4$. This protein has been found to be more tightly bound to oligonucleosomes than H1 [30]. Protein P41 is similar to the protein Bu found associated with rat liver nucleosomes [33] and the 40K protein found in the nucleosome core particle [34].

The next fraction, the fraction insoluble in low ionic strength buffer, has the highest level of active gene sequences and the lowest level of HMG14/17 proteins. This fraction could be further fractionated into a histone-containing 2 M NaCl-soluble fraction and a 2 M NaCl-insoluble matrix-bound fraction, both of which were enriched in active sequences (table 2). The high enrichment of active sequences in the low ionic strength-insoluble fraction is principally due to the high level of active sequences in the matrix-bound DNA. This confirms previous findings that the high-salt-insoluble material which is in a non-nucleosome structure is enriched in active sequences [21, 27], though recent data has suggested that high ionic strengths can cause artificial aggregation of proteins onto transcribed sequences [23].

Thus, the analysis of the distribution of nuclear proteins and transcriptionally-active sequences in rat liver chromatin fractions shows that active sequences do not partition with HMG proteins during fractionation and are bound to matrix proteins, and that inactive chromatin may have an additional nucleosomal protein

of 41 000 MW. Our conclusions regarding distribution of HMG proteins, however, must be qualified by the consideration that these proteins are loosely bound within the nucleus and may therefore readily redistribute. HMG proteins may also be lost during nuclear isolation using aqueous buffers [37] and so the HMG content of the chromatin fractions may not reflect the in vivo distribution.

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