Fractionation of chromatin by differential solubility in dilute salt

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ABSTRACT

Chromatin prepared from the livers of rats was fractionated on the basis of solubility in dilute NaCl. Neither of the fractions obtained was enriched in newly synthesized DNA. The salt-soluble fraction had a higher protein content (usually up to 50%) relative to the DNA, and contained 72% or more of the rapidly synthesized RNA. This RNA was found to be complexed with the salt-soluble deoxyribonucleoprotein, not merely co-solubilized with it. Also, polylysine-binding studies showed that about 70% or more of the nucleic acid phosphates were accessible as compared to about 40% in the unfractionated chromatin. These properties suggested that the soluble fraction was enriched in actively transcribed chromatin. In contrast molecular hybridization studies showed that the complexity of the DNA and its homology with cDNA transcribed from rat-liver polysomal mRNA were the same as those of DNA from unfractionated chromatin, or from the salt-insoluble fraction. This suggests that the criteria commonly accepted as distinguishing between euchromatin and heterochromatin in vitro are not invariably valid.

INTRODUCTION

Recent measurements of the homology between total cell RNA or mRNA by molecular hybridization techniques have shown that only a small proportion, usually less than 10%, of the base-sequences in the DNA of eukaryotic cells are represented in the population of RNA molecules (for review, see 1). This indicates that data from physical and chemical studies of whole chromatin must refer in the main to the inactive part of the chromatin, that is, that part which is not transcribed in vivo. These findings have led to many attempts to fractionate chromatin into the transcriptionally active and inactive portions, and many studies have been made with this aim in mind (see 2-4). Most of these attempts have relied upon a comparison of the transcriptional activity of the chromatin fractions in vitro, or on their association with rapidly labelled RNA, to determine whether the fractionation had been successful in separating active from inactive chromatin. Unfortunately, these criteria are prone to artifacts which are not always

obvious even when the data are evaluated very critically (4). Since the DNA sequences transcribed in vivo are a subset of all of the sequences in whole chromatin, a more definitive procedure to identify a chromatin fraction which is enriched in euchromatin is to measure the base-sequence complexity of the DNA extracted from that fraction and to determine whether this DNA is enriched in those sequences which are transcribed in vivo by molecular hybridization techniques (5,6,7).

We have used several methods, including the latter, to determine whether a fraction isolated from rat-liver chromatin comprises actively transcribed segments. The fractionation procedure used in this work is very simple, namely, differential solubility in dilute NaCl (cf. 5,8). This procedure was chosen on the basis of (i), studies which showed that the regions of DNA in chromatin which are accessible to cetylpyridinium chloride and polylysine are heterogeneous (9,10) and (ii), studies of solubility of rat-thymus chromatin over a range of NaCl concentrations which indicated that chromatin consisted of two distinct components (11). It has been found that, although the NaCl-soluble component of chromatin differs from whole chromatin and from the insoluble component in its capacity to bind polylysine and in its content of rapidly labelled RNA, the DNA sequences in this fraction are indistinguishable from those in whole chromatin or in the salt-insoluble fraction (12).

MATERIALS AND METHODS

Chemicals and radiochemicals: Poly-L-lysine hydrobromide of molecular weight 6000 was obtained from Miles-Yeda, Kankakee, Ill. U.S.A. 5- ^3H orotic acid (20 Ci/mmol), and 6- ^3H thymidine (25 Ci/mmol) were obtained from the Radiochemical Centre, Amersham. Rats were injected with 0.5 mCi (in 0.5 ml) of the former $1\frac{1}{2}$ h before death or 0.2 mCi (in 0.2 ml) of the latter 1-2 h before death.

Measurement of radioactivity: A Triton X-100 colloid method of counting was used (13), mixing 2ml of sample with 9 ml phosphor. The efficiency was measured by addition of an internal standard of tritiated water. The radioactivity of fractions from hydroxylapatite columns was measured in Instagel (14).

Preparation and analyses of chromatin: Chromatin was prepared from rat thymus glands or from rat-liver nuclei as described previously (15). Total nucleic acids in chromatin were measured spectrophotometrically taking $E_{1\text{cm}}^{1\%}$ at 260nm as 210; RNA was measured by the Schmidt-Thannhauser procedure (16)

as modified by Fleck and Munro (17). Total protein and 0.2M HCl-soluble protein (crude histones) were measured by a microbiuret method (18), or by the Folin method (19).

NaCl precipitation: 1.5 M NaCl was added to chromatin at a concentration of about 0.04% DNA, the mixture was shaken thoroughly, kept at 0°C for 15 min and then centrifuged at 8000g for 15 min.

Polylysine binding: Polylysine was added to chromatin to a final concentration of about 0.4 mM lysyl residues. Excess polylysine was estimated by addition of the anionic dye methyl orange, as described previously (9).

Isolation of DNA: Chromatin and chromatin fractions were homogenized in 0.1 M tris-HCl buffer, pH 8.0, containing 4% (w/v) sodium lauroyl sarcosinate, and incubated with Proteinase K (Boehringer) at 500 µg/ml for 18 h at 37°C. DNA was isolated by the hydroxylapatite procedure described by Hell *et al.* (20) and fragmented by sonication (14). The sizes of the DNA fragments were estimated by measuring sedimentation rates through 4-11% (w/w) sucrose gradients in 0.1 M NaOH, 0.9 M NaCl (21).

Synthesis of cDNA: Polysomes sedimenting faster than 100S were isolated from the livers of young adult male rats as described for mouse-liver polysomes by Young *et al.* (22). Polyadenylated RNA was isolated from the polysomes and transcribed with reverse transcriptase as described by Birnie *et al.* (23). The DNA was fractionated by sedimentation through an alkaline sucrose gradient and fractions containing cDNA sedimenting at 3-8S were pooled; the cDNA was precipitated with alcohol and de-salted by chromatography on Sephadex G-25 (23). Re-sedimentation of a portion of the cDNA showed that its mean molecular weight was 1.1×10^5 daltons; its specific activity was calculated to be 5000 counts/min/ng.

Molecular hybridization: Salt-free solutions of the DNA from chromatin, or chromatin fractions, or *E. coli*, were mixed with the cDNA, lyophilized, and redissolved in 0.5 M NaCl, 0.5 mM EDTA, 25 mM HEPES-NaOH, pH 6.8, 50% (v/v) formamide (23). The ratio of DNA to cDNA was 4.5×10^5 , and the concentration of DNA was 5 mg/ml. The mixtures were sealed in capillaries, heated at 70°C for 10 min, then incubated at 43°C. The capillaries were flushed out with 0.1 M NaCl, and the extent to which the DNA sequences had annealed was measured by chromatography on hydroxylapatite (14).

RESULTS

Solubility of chromatin in NaCl: Fig. 1 shows the variation of solubility of liver chromatin with NaCl concentration. The curve is similar to that

Fig. 1. Variation of solubility of chromatin with NaCl concentration. The graph is composite for 5 experiments using initial concentrations of chromatin ranging from 0.023% to 0.058% DNA. Chromatin-NaCl mixtures were kept in ice 15 min and then centrifuged at 3000g for 15 min. The amount of nucleic acid in the supernatant fluid was calculated from A_{260} values.

obtained previously with rat-thymus chromatin (11) in showing two components: one component, comprising about 70% of the nucleic acid, is precipitated between about 0.5 mM and 2 mM NaCl and the other between about 0.05 M and 0.1 M NaCl. In this study, the second component is referred to as the NaCl-soluble fraction, extraction being made either in 0.01 M or 0.05 M NaCl.

Chemical analyses of whole chromatin and of chromatin fractions: Whole thymus chromatin comprised about 36% DNA, 1% RNA, 47% histone and 16% non-histone protein. Whole liver chromatin comprised about 30% DNA, 3% RNA, 53% histone and 14% non-histone protein. The DNA and protein contents of the fraction soluble in 0.01 M NaCl or 0.05 M NaCl were rather variable, in the case of thymus ranging from 8 - 13% of the original DNA and 40 - 60% of the protein, the protein to DNA ratio being usually 30 - 50% higher than in the original chromatin. In the case of liver, the salt-soluble fraction usually contained 12 - 25% of the original DNA and 30 - 60% of the original protein, the protein to DNA ratio being 20 - 50% higher than in the original chromatin. Polyacrylamide gel electrophoresis of histones prepared from the salt-soluble fractions of rat thymus and rat liver showed bands similar to those of histones prepared from the original whole chromatin. As to the RNA content it has previously been found that there are two main components of chromatin RNA in liver. One is rapidly synthesized, as shown by its incorporation of injected [3 H] orotic acid, and the other stable and of lower molecular weight

(24); the NaCl-soluble fraction usually contained 72 - 88% of the former and 50 - 70% of the latter.

Polylysine binding: Fig. 2 shows typical binding curves for the NaCl-soluble fraction and for the original chromatin. In the latter case the amount of unbound polylysine decreased linearly with increasing amount of nucleic acid phosphate, the neutralising value being equivalent to 40% of the latter, i.e. to 42% of the DNA-phosphates, as the RNA was apparently inaccessible (see below). With the NaCl-soluble fraction, the decrease was much more rapid and was non-linear. Extrapolation of the initial part of the curve corresponded to 76% neutralisation of the nucleic acid-phosphates. In other preparations also this value was at least 70%. A rather similar non-linear curve had been found previously for the binding of polylysine by DNA-protamine (15) and by degraded DNA (10) but in both cases the amount of unbound polylysine remained constant and relatively high even at very high levels of DNA-phosphate, whereas here it dropped to zero. In the previous studies this was interpreted (10,15) as showing that the accessible zones were relatively small compared to those in chromatin or DNA and too small to bind the whole of each polylysine molecule. In the present case if the zones are small they must also be numerous to account for the high titration value. The non-linear curve could reflect the presence of such molecules plus others having a lower percentage of accessible phosphates but in longer stretches.

Salt and acid-solubility: To find if the NaCl-soluble fraction contained DNA-like molecules with loosely associated protein, the solubility in 0.15 M NaCl was measured since chromatin, but not DNA nor RNA, is insoluble in this medium. It was found that 16 - 30% of both nucleic acid and protein of the NaCl-soluble fraction was soluble in 0.15 M NaCl. Thus the protein to DNA ratio of the 70 - 80% of the fraction which is insoluble in 0.15 M NaCl, (presumably consisting of more highly associated nucleic acid and protein) was similar to that of the original salt-soluble fraction i.e. the bulk of the DNA, and possibly the whole, is protein-associated rather than free. Less than 10% of the nucleic acid of the NaCl-soluble fraction was soluble in 0.67 N perchloric acid, showing that this fraction was not highly degraded.

Association of RNA with DNA-protein: It was possible that the rapidly synthesized RNA was extracted independently in 0.01 M NaCl along with part of the chromatin rather than in true association with the latter. To check this, polylysine was added as a precipitating agent, at a range of concentra-

Fig. 2. Binding of polylysine to rat-liver chromatin (●) and to the fraction soluble in 0.05 M NaCl (Δ). Abscissa values are given as DNA plus RNA-phosphates, the RNA comprising 5% of the total nucleic acid in whole chromatin and 24% of the total in the NaCl-soluble fraction in this experiment.

tions, to find if co-precipitation of RNA and DNA-protein occurred. It had previously been shown that after mixing polylysine with chromatin in an amount sufficient to neutralise all accessible nucleic acid-phosphates, addition of RNAase caused slow degradation of virtually all the RNA (24). This suggested, by analogy with chromatin DNA, that the RNA is bound ionically to protein and so is inaccessible to polylysine. Thus, in the chromatin-polylysine complex, since only the DNA, not the RNA, is bound to polylysine, co-precipitation or otherwise of RNA with the DNA would show its extent of association with the DNA-protein. Fig. 3 shows that all the RNA is co-precipitated at the point of charge neutralisation suggesting that it is truly bound to the chromatin. The results indicate also that much of the RNA is associated with a relatively small proportion of the DNA, e.g. at a polylysine:DNA-P of 0.23, 76% of the radioactive RNA in the NaCl-soluble fraction is still in the supernatant fraction associated with 27% of the nucleic acid, i.e. 55% of the original radioactive RNA is associated with only 6% of the original DNA.

Molecular hybridization analyses: The DNAs isolated from whole chromatin and the chromatin fractions were fragmented by sonication prior to their

Fig. 3. Precipitation by polylysine of rat-liver chromatin and of the fraction soluble in 0.01 M NaCl. Chromatin A₂₆₀ (x), dpm (O); NaCl-soluble fraction A₂₆₀ (Δ), dpm (●). Abscissa values are given as polylysine/DNA plus RNA-phosphates, the RNA comprising 11% of the total nucleic acid in the whole chromatin and 23% of the total in the NaCl-soluble fraction in this experiment.

use in molecular hybridization experiments. Fig. 4 shows that the fragmented DNA from whole chromatin, and that from the salt-insoluble fraction, migrated as a single broad band in alkaline sucrose gradients, with a peak molecular weight at about 2.8×10^5 daltons. In contrast, the DNA from the salt-soluble fraction, after fragmentation in the same way, showed a bimodal distribution in the alkaline gradients. Less than half of the DNA migrated at the same rate as that from whole chromatin; at least 60% was of significantly lower molecular weight, with a mean at about $6-10 \times 10^4$ daltons. Since the size of the fragments produced from the whole chromatin DNA was the limiting size obtainable by sonication under the conditions used in these experiments, this suggests that more than half of the DNA in the salt-soluble chromatin fraction had already been nicked quite extensively before it was extracted from the nucleoprotein complex soluble in 0.05 M NaCl. Whether this nicking was single or double-stranded was not determined.

If the DNA sequences in the salt-soluble fraction of chromatin had been a specific subset of sequences consisting of, say, 10% or 20% of those in whole chromatin-DNA, denatured DNA from this fraction would have

Fig. 4. Rate-zonal sedimentation of DNA fragments from whole chromatin (●), the salt-soluble fraction (△) and the salt-insoluble fraction (○) of chromatin through alkaline 4 - 11% (w/w) sucrose gradients; - - - shows the $S_{20,w}$ values at each point in the gradients.

reannealed 5-10 times more rapidly than that from whole chromatin. However, as shown by Fig. 5, while the fragmented DNAs from whole chromatin and the salt-insoluble fraction reannealed at the same rate, that from the salt-soluble reannealed more slowly. When the appropriate correction (25) was made to compensate for the difference in size between the DNA fragments from the salt-soluble and whole chromatin, the rates at which the DNAs reannealed became indistinguishable. Even if only a portion of the DNA in the salt-soluble fraction had consisted of a subset of the sequences in whole chromatin-DNA, that portion of the DNA would have reannealed faster. However, there is no suggestion from the curve in Fig. 5 that even as little as 20% of the DNA from the salt-soluble fraction annealed at a faster rate indicating that there was little, if any, enrichment of the latter in sequences which formed a specific subset of those in whole chromatin-DNA.

Fig. 5 shows also that cDNA transcribed *in vitro* from rat-liver polysomal polyadenylated RNA annealed at the same rate with DNA fragments from whole rat-liver chromatin as with fragments from the salt-soluble and salt-insoluble fractions of the chromatin. If the salt-soluble chromatin fraction had been enriched in the DNA sequences which were the *in vivo* templates for rat-liver polysomal polyadenylated RNA, the cDNA transcribed from the latter would have annealed more rapidly with the DNA from the

Fig. 5. Rates of reannealing of DNA, and of cDNA with DNA, from chromatin and chromatin fractions. Solid lines, reannealing of DNA fragments; dashed lines, annealing of cDNA transcribed from rat-liver polysomal polyadenylated RNA with DNA fragments from chromatin and chromatin fractions; ●, DNA from whole chromatin; △, DNA from the salt-soluble chromatin fractions; ○, DNA from the salt-insoluble fraction —■— shows the rate of reannealing of the cDNA in the presence of *E. coli* DNA (self-annealing).

chromatin fraction than with the DNA from whole chromatin. None of the cDNA-DNA reactions shown in Fig. 5 has gone to completion. This is probably due to a large proportion of the polyadenylated RNA from rat-liver polysomes consisting of a relatively few sequences, as had been shown to be the case for mouse-liver polysomal polyadenylated RNA (22). Thus, although the weight ratio to DNA to cDNA was relatively high (4.5×10^5), there was probably insufficient of some chromatin DNA sequences in the reaction mixture to hybridize completely all of the cDNA transcribed from the most abundant sequences in the polysomal polyadenylated RNA (see 7). However, this does not affect conclusions drawn from a comparison of the rates at which the cDNA annealed with DNA from the whole chromatin and the chromatin fractions. Thus, it must be concluded that there is no significant enrichment of either salt-soluble or salt-insoluble chromatin fractions with those DNA sequences which gave rise *in vivo* to the polyadenylated polysomal RNA in rat-liver.

Thymidine incorporation: There is evidence that newly synthesized DNA may differ from the bulk DNA in chromatin in its extent of association with protein (26,27). To find if the salt-soluble fraction contained newly synthesized DNA, the former was prepared using chromatin from rats injected

with [^3H] thymidine. Values of specific activity of the original chromatin, of purified DNA of the salt-soluble fraction and the salt-insoluble fraction were found to be respectively 137 dpm/ μg , 143 dpm/ μg and 131 dpm/ μg , showing that no separation of the newly synthesized DNA had occurred.

DISCUSSION

It has long been known that, in interphase nuclei, those parts of chromatin which are being transcribed can be distinguished from those parts which are not by several physico-chemical differences. In the main, the former consists of extended fibrils while the latter is composed of condensed, granular material. In addition, the cytological studies of Zirkin (28) indicate that active chromatin is associated with a larger proportion of non-histone protein than is inactive chromatin due, in part at least, to the accumulation of ribonucleoproteins. Also, autoradiographic studies of [^3H] uridine-labelled interphase nuclei have shown that newly synthesized RNA is confined to the fibrillar components of the chromatin (29). In many experiments the differences observed in vivo between active and inactive chromatin have been exploited, first, to fractionate isolated chromatin and, second, to identify the fractions which correspond to the regions of chromatin which are actively transcribed in vivo (see 4). However, it has not yet been proved that the biochemical and biophysical differences between active and inactive chromatin which can be predicted from the in vivo observations are all directly applicable to fractions obtained from isolated chromatin by in vitro procedures. The data obtained in this study clearly show that some, at least, of the commonly accepted criteria by which chromatin fractions enriched in active chromatin are identified are not unequivocal. The salt-soluble fraction of rat-liver chromatin has a number of properties expected of a euchromatin-enriched fraction: it contained 12-25% of the DNA, it had a higher ratio of protein to DNA than unfractionated chromatin, it had a higher capacity for polylysine than whole chromatin, and it was associated with about 80% of the rapidly synthesized RNA. However, when the base-sequence complexity of the DNA isolated from the salt-soluble fraction of chromatin was measured, it was found to be the same as the DNA from unfractionated chromatin. In a more critical experiment, the DNA from this fraction was found not to be enriched in the sequences which gave rise to rat-liver polysomal polyadenylated mRNA. Thus, the DNA sequences in the salt-soluble fraction are not a specific subset of the sequences in unfractionated chromatin and we must, therefore, conclude that this fraction of chromatin is not enriched

in euchromatin despite the other indications in favour of this conclusion.

Entirely similar conclusions have been drawn from studies of Drosophila chromatin fractionated by rate-zonal sedimentation (30) and of mouse Friend-cell chromatin fractionated by isopycnic sedimentation (6,7). In both of these studies, a putative euchromatin-enriched fraction was identified by its association with rapidly labelled RNA, higher ratio of protein to DNA, greater template activity with exogenous E. coli RNA polymerase, etc. However, in neither did molecular hybridization experiments show that the DNA of the putative euchromatin was enriched in those sequences which were being transcribed in the cells from which the chromatin was isolated. Taken together, these experiments and those described in this paper suggest that, although some methods do give rise to fractions enriched in active chromatin (see, for example, 5), it is essential to examine critically a wide variety of criteria before positively identifying a chromatin fraction as being composed of euchromatic fibrils.

Despite our failure to obtain a fraction of rat-liver chromatin which is enriched in the DNA sequences which are actively transcribed in vivo, it is interesting that the salt-soluble fraction obtained has so many properties in common with that isolated from Friend-cell chromatin by isopycnic sedimentation either after random shearing (6) or after shearing with DNAase II (7). The significance of the occurrence of a fraction of chromatin with just these properties is still completely obscure.

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