Upon the observation of superbeads in chromatin

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ABSTRACT

There exist some indications that nucleases recognize "superbeads" in chromatin. We show that a chromatin extract of rat liver which contains so-called "superbead"-peaks can be separated in a Mg$$^{++}$$ soluble and a Mg$$^{++}$$ insoluble fraction. The Mg$$^{++}$$ insoluble fraction contains the full complement of histones and the expected DNA fragments, but has lost the characteristic peaks in sucrose gradient profiles. These discrete peaks are found in the Mg$$^{++}$$ soluble fraction of the chromatin extract. We give evidence that these peaks are RNP particles on the basis of their protein- and nucleic acid contents.

INTRODUCTION

The nucleosome core particle structure (110 X 57 Å) (1) has been studied extensively and its existence is well established (2),(3) There exists uncertainty however about the folding pattern of these nucleosomes into a higher order 250 Å fiber (4). A possible model deals with a helical arrangement of the nucleosomes into a solenoid (5),(6),(7). Another higher order structure of chromatin which on the basis of electron microscope data emphasizes the existence of discrete globular particles (=superbeads) of about 250 Å, has been presented (8),(9),(10). Further biochemical evidence in favour of the latter model came from the observation that nucleases were able to recognize and preferentially digest these irregularities (or superbeads) of the 250 Å fiber (11),(12). This was demonstrated by sucrose gradient centrifugation of chromatin extracts obtained after a brief nuclease digestion. We put forward evidences that the sucrose profile obtained by these authors should not be interpreted as a preferential digestion of superbeads by nucleases, but rather as a superposition of chromatin and RNP's.

MATERIALS AND METHODS

Rat livers were mixed in 6 mM MgCl$_2$, 0.2 M sucrose, filtered through 16
layers of cheese cloth, and nuclei were spun down by low speed centrifugation (1000g-10 min). Nuclei were purified by centrifugation through a 2.2 M sucrose cushion as described by Strätling et al. (12) Sometimes nuclei were further washed in 10 mM Tris-Cl pH 7.4, 10 mM MgCl₂, 0.5 % nonidet P40, .25 M sucrose and recuperated by low speed centrifugation.

Digestion conditions and chromatin extractions were identical to those of Strätling et al. (12) using 6 units of micrococcal nuclease (Worthington)/ml on a nuclei concentration of 2 × 10⁷/ml. Chromatin was usually obtained at an OD₂₆₀ of approx. 50.

All sucrose gradients were 9.8 ml 10-30 % (w/w) in 80 mM NaCl, 1mM phosphate pH 6.8, 1 mM EDTA. Centrifugation was carried out in a SW 41 rotor at 5°C, 38 Krpm for the time indicated under the figures. Gradients were analysed by pumping them from the bottom through a turbulence free flow cell mounted in a model 101 Hitachi spectrophotometer, whose transmission output is log converted by an Optilab multianalog 201. The Optilab output is then sampled by a 12-bit voltage monitor card located in a Hewlett Packard 6490 B multiprogrammer unit. This A/D card is under control of a program operating in a Real Time executive system. This program stores the sampled data (about 1000/gradient) on disc files. Other programs acces these data files, so it becomes easy to plot the OD profile, or to add up different profiles or to calculate different parameters as e.g. the OD₂₆₀ units surface under the profile.

Protein gel electrophoresis was performed on 17.5 % polyacrylamide SDS-gels (13). The nature of the nucleic acid contents of various fractions was determined by the orcinol method (14).

RESULTS

When nuclei preparation (without nonidet-P40 washing), chromatin digestion and extraction procedures and sucrose gradients analysis are performed as described in materials and methods, a sedimentation profile as in Fig. 1 was obtained. Similar profiles with characteristic peaks have been observed by others, under identical conditions. These discrete structures (designated 1,2,3, in the figures) have been shown to sediment at positions corresponding to multiples of 8 nucleosomes. These discrete particles were identified as "superbeads" resulting from the recognition and selective digestion by nucleases of irregularities along the 250 Å chromatin fiber (11, 12), in the same way as nucleases recognize nucleosomes. However, if the chromatin is condensed in a smooth helical structure, then these peaks could result from
Figure 1: Sucrose profile of a nuclear extract, obtained after a brief nuclease digestion. 2 O.D. 260 units were layered on a sucrose gradient as described in materials and methods and centrifugated for 2 hours. Numbers 1, 2, 3 refers to mono-, dimer- and trimer peak called superbeads by others (11,12).

a jump, of the acting enzyme, to the next turn of a helix. The fragments will therefore contain a preferred proportion corresponding to the size of a turn of the helix (about 8 nucleosomes per turn of helix (15)).

We felt however that it could not be excluded that these discrete structures might not be derived from chromatin. Indeed, it was observed during preparations that extensive washing of nuclei in buffers containing nonidet-P40 lowers the amount, and can even lead to a complete loss of "superbead" material (Fig. 2). Of course one cannot rule out the possibility that a nonionic detergent, such as nonidet-P40, alters the discrete higher order structures of chromatin and that as a consequence of this, nucleases no longer detect them. However, this cannot be the complete explanation, as the supernatants of sucrose purified nuclei, washed once in a very small volume of nonidet-P40 containing buffer, show the same discrete peaks in sucrose gradients (Fig. 3). This means that:

- these discrete structures are preserved even in the presence of the detergent.
- they can be release without adding Ca^{++} or nucleases.
- they are soluble in 3 mM MgCl_{2} (the washing buffer contains 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl_{2}, 0.2 % nonidet-P40, 0.25 M sucrose).

To explain the "spontaneous release" from nuclei of this material, one can of course state that in many studies, chromatin of globular appearance has been observed proximal to the nuclear membrane (16) and that such a material as a consequence of its localization could easily be liberated by endogeneous nucleases and subsequently eluted out of the nuclei, which had lost their membrane due to the action of the detergent (17). However, the above mentio-
Figure 2 : Sucrose profiles of extracts of the same amount of nuclei, digested to the same extent, after 2 1/2 hours of centrifugation. Profile a was derived from nuclei prepared without nonidet-P40 washing, while b is an extract of nonidet-P40 washed nuclei.

Figure 3 : Sucrose profile of the supernatant of nuclei washed in nonidet-P40 buffer, without adding Ca or nucleases, and without lysis of the nuclei. (Centrifugation was for 3 hours).

ned preparations were performed at 4°C, and identical results were obtained when EGTA, that selectively complexes Ca++, is included in all the preparation buffers (data not shown). These indications suggest that endogeneous nucleases were not activated during the isolation steps.

The solubility of these structures in 3 mM MgCl₂, did raise some doubt about the strict chromatin origin of the material. In order to positively identify its nature the following protocol was performed: rat liver nuclei are prepared without nonidet-P40 washing, and are very briefly digested by micrococcal nuclease. After lysis in 50 mM NaCl, 1 mM EDTA, 5 mM Tris-HCl, pH 7.5 and low speed centrifugation, chromatin is obtained (OD₂₆₀=58). After adding MgCl₂ to 1 ml of this total extract up to a final concentration of 3 mM, we centrifuged it and saved the supernatant (=Mg++ soluble fraction) (OD₂₆₀=31). The pellet (=Mg++ insoluble fraction) was solubilized in 1 ml lysis buffer (OD₂₆₀=26). The total extract, Mg++ soluble and Mg++ insoluble fractions were analysed on sucrose gradients (Fig. 4). The Mg++ insoluble material has completely lost the characteristic peaks. These appear in the Mg++ soluble fraction as might be expected from the above mentioned experiments. Mere addition of both profiles (Mg++ soluble and insoluble fractions)
yields a theoretical profile, perfectly coincident with the experimental profile of the total extract (Fig. 4). This proves that no fraction of the total extract was lost or irreversibly rearranged by the MgCl₂ precipitation step.

When all three fractions (total extract, Mg²⁺ soluble, and insoluble) were
checked on their protein constituents (Fig. 5), it was shown that the total extract contains many non-histone proteins. The Mg\textsuperscript{++} insoluble fraction reveals the full histone complement and less non-histones, whereas proteins from the dimer peak eluted out of a sucrose gradient were enriched in non-histone proteins, and no histones could be found. We believe that proteins which migrate in the proximity of H3 and H2B are also non-histones, because they migrate somewhat differently than these histones. The other histones (H4, H2B) are completely absent, and no histone degradation bands are observed (note that all protein samples were taken at the same time). On grounds of their mobility in SDS gels, we would rather favour a hn RNP and sn RNP origin for these proteins. The proteins of such particles have molecular weights in the range of 30,000-60,000 and 12,000-32,000 for hn RNP (8) and sn RNP (19, 20) respectively. Sometimes these particles appear to form complexes and are visualised in sucrose gradients as a sharp 30-40 S sedimenting peak. Finally we analysed the nucleic acid nature of our fractions: it could be deduced by orcinol colouring that about 50\% of the total extract consist of RNP. Whereas the Mg\textsuperscript{++} insoluble fraction did not contain any orcinol positive material, the Mg\textsuperscript{++} soluble fraction contains RNA.

**DISCUSSION**

As a result of the experiments and analysis described, we conclude that the nuclear extracts, isolated by techniques identical to those of others
(11, 12), are heterogeneous and that the discrete peaks are of a RNP rather than a DNP nature. Indeed, the total nuclear extract was separated in a Mg\(^{++}\) soluble and a Mg\(^{++}\) insoluble fraction. We denote the Mg\(^{++}\) insoluble fraction as chromatin, because of its sedimentation behaviour (sucrose profile), solubility behaviour (Mg\(^{++}\) precipitable), its presence of the full histone complement and its absorption maximum at 260 nm. Furthermore it did not contain any RNA. The Mg\(^{++}\) soluble fraction exhibiting the characteristic discrete peaks in sucrose gradient, does not contain any histones. By orcinol colouring we concluded that these structures contain RNA and that no DNA was present. These peaks were not part of the chromatin because they could be eluted out of the nuclei under conditions which we suspect have not activated the nucleases.

A further indirect argument in favour of the RNP nature of these peaks comes from a comparison between the nuclear extraction procedures used (11, 12) to obtain chromatin extracts and the extraction method of Samarina et al. (21) for hn RNP. Both use the same nuclear preparation and purification steps, and their extraction conditions differ only in the substitution of 1 mM MgCl\(_2\) for RNP extraction by 1 mM EDTA in the case of chromatin extraction. So it is obvious that RNP's which are solubilized in the presence of 1 mM MgCl\(_2\) will also be present in a 1 mM EDTA extract. This explains why we were able to separate a total nuclear extract (in 1 mM EDTA) into a Mg\(^{++}\) soluble RNP and a Mg\(^{++}\) insoluble chromatin fraction. The fact that the peaks can be digested with micrococcal nuclease does not exclude their RNP nature, because micrococcal nuclease is known to exhibit a RNase activity.

The results of Butt et al. (11) and Stratling et al. (12) cannot therefore be considered as arguments in favour of a superbead arrangement of chromatin. This does not mean that superbeads do not exist in chromatin in situ. Supposing superbeads do exist in chromatin as visualized in E.M. photographs of nuclei (16), then in sucrose gradient analysis. This may be explained in two ways:
- superbeads are not recognized by nucleases or are less resistant to nuclease digestion than nucleosomes, so they unfold after nuclease action.
- superbeads are not made up of a strict number of nucleosomes (10) but are rather heterogeneous in nucleosomal length.

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