RELEASE OF EUCHROMATIC SEGMENTS BY Mg/Ca-DEPENDENT AUTODIGESTION OF CHROMATIN

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SUMMARY: Mouse TLT hepatoma chromatin was incubated in the presence of added $MgCl_2$ and $CaCl_2$ but in the absence of added exogenous nuclease. Fractionation of such autodigested chromatin by glycerol density gradient centrifugation resulted in a heterochromatin-enriched pellet and euchromatin-enriched non-pelletable fractions. This was determined by satellite DNA content analyses and analysis of nascent RNA distribution. Electron microscope examination of the chromatin revealed it to consist of a series of connected spherical particles (nucleosomes) having a diameter of 370 ± 70Å. This native structure of chromatin was not damaged by the autodigestion process.

It is well known that in most eukaryotic cells, chromatin is composed of heterochromatin, condensed regions devoid of transcriptional activity, and euchromatin, extended regions which include the transcriptionally active genes (1,2). In order to isolate these regions for study, most investigators have sheared their chromatin preparations by physical methods prior to fractionation. However, it has been recently demonstrated that physical shearing of chromatin damages its native structure (3,4). Several attempts have been made to shear chromatin enzymatically with added nuclease prior to fractionation (5,6).

We wish to report that the autodigestion of chromatin, presumably due to the action of the endogenous (Mg/Ca-dependent) endonuclease (7), yields chromatin segments which can be separated into eu- and heterochromatin-enriched fractions as determined by satellite DNA and nascent RNA distributions. Moreover, this method yields chromatin which retains its native (beaded) structure as determined by electron microscopy.

MATERIALS AND METHODS: Chromatin was isolated according to standard procedures as previously described (8) with several minor modifications from mouse TLT (Taper Liver Tumor) hepatoma cells which were grown in the ascites form in Swiss Webster mice. The final chromatin preparation was suspended in 10mM glycine buffer (pH 7.1) to a final concentration of 0.3 mg/ml or 0.75 mg/ml.

In cases where chromatin was autodigested in the presence of added Mg/Ca, a solution containing $MgCl_2$ and $CaCl_2$ was added to the chromatin prior to its incubation at 30°C for selected time periods. The final concentrations of Mg/Ca were varied since we have found that the optimal concentrations are dependent upon and proportional to the initial chromatin concentration (Paul and Duerksen, manuscript in preparation). Thus final concentrations of 0.1 mM $MgCl_2$ and 0.02 mM $CaCl_2$ are used with chromatin at a concentration of 0.3 mg/ml while final concentrations of 0.25 mM $MgCl_2$ and 0.05 mM $CaCl_2$ are used with chromatin at a concentration of 0.75 mg/ml. In cases where chromatin was autodigested in the absence of added Mg/Ca, the chromatin preparation was merely incubated at 30°C for selected time periods.

Chromatin was fractionated according to the method of Murphy *et al.* (9) by centrifugation through a 7.6%-76% glycerol gradient in a Beckman SW27 rotor at 20,000 rpm for 15-17 hr at 1°C. The absorbance profile of each gradient was recorded by pumping each gradient through a Beckman Acta III Spectrophotometer equipped with a flow-through cuvette.

For satellite DNA content analyses, selected adjacent fractions from each gradient were pooled and the DNA isolated by pelleting through 3M CsCl. The suspended DNA was analyzed by analytical CsCl equilibrium gradient centrifugation as previously described (10).

For analysis of nascent RNA distribution, mouse TLT hepatoma cells in ascites fluid were incubated at 37°C with [^{3}H]uridine (100 µCi/ml; specific activity 45 Ci/mmol) for 1 min prior to extraction, autodigestion (in the presence of added Mg/Ca) and fractionation as described above. The radioactivity in each 2 ml fraction was determined by precipitation of each sample with 10% (w/v) trichloroacetic acid. Precipitates, collected on Whatman GF/A filters, were counted in a liquid scintillation spectrometer.

Electron microscopy was performed with a Philips 300 electron microscope. Chromatin concentration was adjusted to 50 µg/ml and, after placing one drop onto a carbon-coated grid, negatively stained with 0.5% ammonium molybdate (pH 7.8) as described by Olins *et al.* (11).

RESULTS AND DISCUSSION: When unincubated chromatin is centrifuged through a steep glycerol gradient, most of it pellets while only some of it (~10%) remains in the gradient, sedimenting at 58S (Fig. 1a). Similar analysis performed after incubation of the chromatin at 30°C for 2 hr results in the appearance of material sedimenting at 14S concommitant with a decrease in 58S material and pelletable chromatin (Fig. 1a). We interpret these results as indicating that autodigestion of chromatin has resulted in its degradation into 14S subunits. Incubation of this chromatin with micrococcal nuclease results, as has been demonstrated previously (12, 13, 14, 15), in its degradation into ~11S subunits (results not shown). At this time, we do not know the precise relationship between the 14S and 11S subunits. However, we feel that perhaps the 14S subunit is a dimer of the 11S subunit since the dimer has been reported to sediment at 15.9S (12) and 14.6S (13); alternatively, the 11S subunit might be a fragment of the 14S subunit aris-

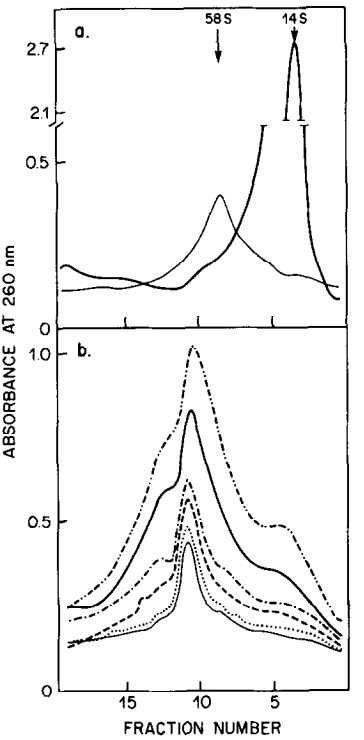

Figure 1. Glycerol density gradient analysis of chromatin autodigested under various conditions. Chromatin was isolated and suspended at a final concentration of 0.3 mg/ml in 0.01 M glycine, pH 7.1. *a*, Chromatin was autodigested at 30°C in the absence of added Mg/Ca for 0 hr (———) or 2 hr (▬▬); *b*, Chromatin was autodigested at 30°C in the presence of 0.1 mM $MgCl_2$/0.02 mM $CaCl_2$ for either 0 hr (———), 0.25 hr (····), 0.5 hr (---), 1 hr (-·-·-), 2 hr (▬▬) or 4 hr(--··--). Chromatin samples were subsequently layered onto 7.6% - 76% glycerol gradients and centrifuged in a Beckman SW27 rotor at 20,000 rpm for 16 hr at 1°C. Direction of sedimentation is from right to left. The pellets present in each gradient are not depicted. The indicated sedimentation coefficients were determined by sedimentation velocity studies performed with a Beckman Model E Analytical Ultracentrifuge.

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ing from overdigestion due to the action of both endogenous and added micrococcal nucleases (12).

Addition of $MgCl_2$ and $CaCl_2$ (hereafter termed "Mg/Ca") to glycine-chromatin results in a dramatic decrease in the viscosity of the chromatin solution, presumably due to Mg/Ca-induced aggregation of the chromatin fibers (16). Glycerol density gradient analysis of the Mg/Ca-chromatin also results in most of it pelleting while only some of it remains in the gradient; however, this chromatin now sediments at approximately 76S instead of 58S

(Fig. 1b). This increase in S value is probably related to the Mg/Ca-induced aggregation of chromatin.

Incubation of this chromatin for increasing time periods results in the digestion of initially pelletable material into chromatin segments whose absorbance profile distribution is extremely similar to that of undigested nonpelletable Mg/Ca-chromatin: the 76S material is still predominant although both "light" and "heavy" shoulders become prominent (Fig. 1b). Therefore, it appears that autodigestion of chromatin in the presence of added Mg/Ca results in its digestion into chromatin segments with sedimentation characteristics on glycerol gradients resembling those of undigested nonpelletable chromatin. We believe that the addition of Mg/Ca permits the digestion to proceed in a manner such that there is a release and maintenance of discrete-sized chromatin segments (from the pelletable chromatin) with properties similar to those of the initial nonpelletable chromatin segments (note that this is not the case for autodigestion in the absence of added Mg/Ca since the initial nonpelletable chromatin fraction is not maintained but, rather, is degraded into smaller segments). This tentative conclusion is supported by electron microscope studies (see results below) which indicate that the initial structural integrity of the chromatin is preserved throughout such autodigestion.

Glycerol or sucrose density gradient centrifugation has been used in the past in attempts to separate out euchromatic and heterochromatic fractions (9, 10, 17, 18). In order to ascertain whether the present method has achieved any such separation, it was decided to analyze the satellite DNA content of various chromatin fractions. Such analysis is based on the previously reported fact that satellite DNA is primarily restricted to heterochromatin (10, 19, 20). The results of this analysis appear in Table 1. It is clear that the chromatin which remains within the glycerol gradient is satellite DNA-deficient. Moreover, there appears to be a gradient of satellite DNA content with the more slowly sedimenting chromatin being more satellite DNA-

Table 1. Satellite DNA Content of Chromatin Samples Pooled from Various Glycerol Gradient Regions

Source of Chromatin	% Satellite DNA
1. Chromatin autodigested with added Mg/Ca	
a. Light chromatin (i.e., <76S chromatin)	5.0 (± 1.3)
b. Peak chromatin (i.e., ~76S chromatin)	6.3 (± 1.4)
c. Heavy chromatin (i.e., >76S chromatin)	9.2 (± 1.4)
d. Pelleted chromatin	11.6 (± 1.1)
2. Chromatin autodigested without added Mg/Ca	
a. 14S chromatin	8.6 (± 0.4)
b. Pelleted chromatin	10.5 (± 0.2)
3. Whole unfractionated chromatin	10.1*

* Standard deviation omitted since this value is extremely reproducible.

deficient than the more rapidly sedimenting chromatin (note that in the lightest chromatin region, there is a highly significant 50% reduction in satellite DNA content as compared to that found in whole chromatin). Furthermore, the pelleted chromatin is correspondingly satellite DNA-enriched. These results, indicating a gradient of chromatin satellite DNA content, are consistent with previous reports that heterochromatin is more condensed and therefore sediments more rapidly than euchromatin (1, 2, 9, 10, 18, 21). This data is interpreted as indicating that autodigestion of chromatin in the presence of added Mg/Ca results in the release of satellite DNA-deficient chromatin (i.e., euchromatin-enriched) from the initially pelletable chromatin. No significant differences were observed between corresponding fractions from chromatin autodigested for various periods up to 4 hr; however, preliminary results indicate that progressively longer digestion periods result in a relative decrease of the euchromatin content of the chromatin sedimenting within the glycerol gradient.

Similar analysis of the 14S segments obtained after autodigestion of

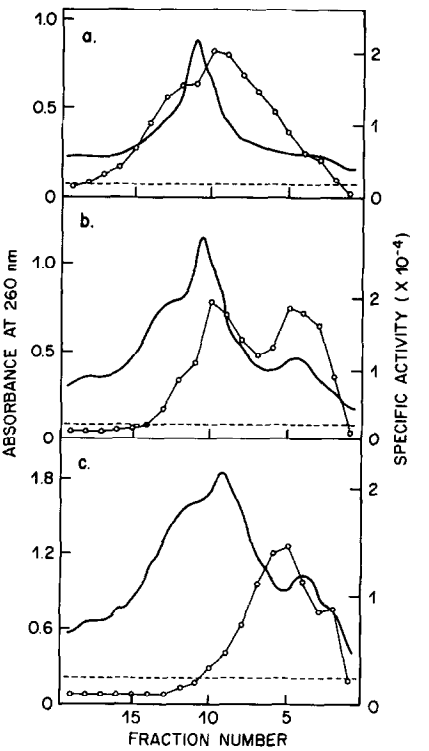

Figure 2. Distribution of nascent RNA on chromatin after various periods of autodigestion. TLT hepatoma cells in ascites fluid were incubated at 37°C with [^{3}H]uridine for 1 min prior to extraction of chromatin. Chromatin at a concentration of 0.75 mg/ml was autodigested at 30°C in the presence of 0.25 mM $MgCl_2$/0.05 mM $CaCl_2$ for either 0 hr (*a*), 1 hr (*b*), or 4 hr (*c*). After centrifugation and recording of the absorbance (——), each glycerol gradient was fractionated into 2 ml fractions; the specific activity in cpm/absorbance unit (o——o) was calculated after determining the TCA-precipitable radioactivity of each fraction. The specific activity of whole chromatin (i.e., unfractionated) is also indicated (---). The radioactivity in the pellets declined from (*a*) 2590 cpm to (*b*) 1022 cpm to (*c*) 687 cpm with the aforementioned digestion periods. These values all translate into specific activities which are estimated (estimated because the tight pellets are difficult to resuspend for accurate quantitation) to be below 50 cpm/absorbance unit.

chromatin in the absence of added Mg/Ca reveals that these segments are only slightly satellite DNA-deficient. Significantly, the corresponding gradient fractions from chromatin autodigested in the presence of added Mg/Ca have almost half the satellite DNA content of the 14S segments. Thus autodigestion in the presence of added Mg/Ca yields chromatin fragments which are significantly enriched for euchromatin.

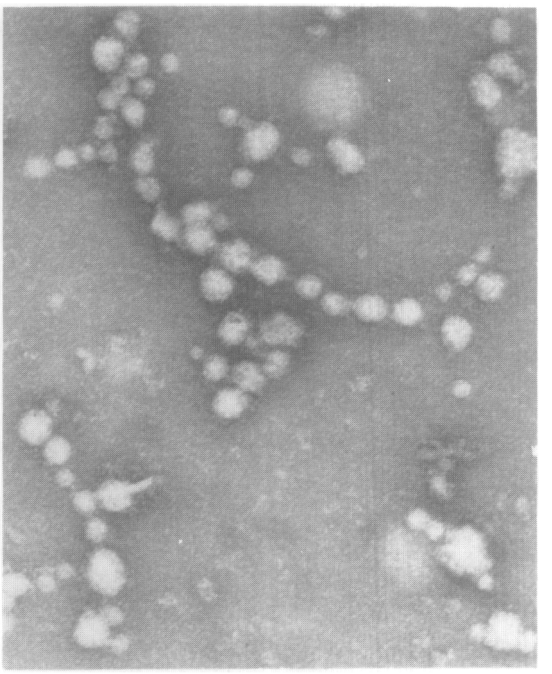

Figure 3. Electron micrograph of chromatin autodigested in the presence of added Mg/Ca after 1 hour of digestion. Chromatin concentration was adjusted to 50 μg/ml and, after placing one drop onto a carbon-coated grid, negatively stained with 0.5% ammonium molybdate (pH 7.8). Undigested chromatin has essentially the same appearance. Magnification: x 100,000.

In order to determine whether the euchromatin-enriched fractions described above correspond in any way to those which are transcribed *in vivo*, it was decided to examine the distribution of nascent RNA on the chromatin fractions. Cells were pulse-labelled with [^{3}H]uridine for 1 min prior to extraction and analysis of the chromatin. TLT hepatoma cells are particularly suitable for such experiments due to their extremely brief yet rapid rate of [^{3}H]uridine incorporation (22). As indicated in Fig. 2, the nascent RNA is predominantly associated with the chromatin which sediments within the glycerol gradients, i.e., the euchromatin-enriched fractions. As digestion proceeds, the distribution of nascent RNA shifts towards more slowly sedimenting, i.e., more euchromatic, chromatin (although the results are expressed in terms of specific activity, this shift is also observable when the actual cpm are

plotted). This data is interpreted as indicating that such autodigestion of chromatin yields progressively smaller fragments which, concomittant with being satellite DNA-deficient, are also enriched for nascent RNA.

Examination of both autodigested (in the presence of added Mg/Ca) and undigested chromatin by electron microscopy reveals it to consist of a series of connected spherical particles (Fig. 3). However, in marked contrast to the nucleosomes or "nu" bodies previously described, these particles are relatively large and variable in size. These particles have a mean diameter of 370 Å with a standard deviation of 70 Å as compared to previous reports of particle sizes ranging from 60 ± 16 Å (11, 23) to 137 ± 10 Å (24) to 150 Å (25). The variability among the reported sizes is at present inexplicable. However, as previously suggested (24), the method of extraction and preparation of the chromatin no doubt has a significant effect on its appearance under the electron microscope. Further studies are presently being pursued and preliminary results indicate that under conditions of autodigestion in the absence of added Mg/Ca, the aforementioned series of connected spherical particles are no longer maintained as intact structures.

We believe these results indicate that this method of isolating, shearing (i.e., autodigestion) and fractionating chromatin may be used for separating euchromatic from heterochromatic fractions and, moreover, maintenance of the initial structural features of the chromatin. In order to elaborate, further studies on DNA sequence complexity and histone and non-histone protein distributions are presently being conducted.

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