Physico-Chemical Studies of Isolated Chromatin Compared with in situ Chromatin after Partial Hepatectomy in the Rat

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Isolated Chromatin, Partial Hepatectomy, Molar Ellipticity, Thermal Stability, Flow Cytometry

Chromatin was isolated from rat liver cells at 0, 3, 5, 11, 18 and 24 h following partial hepatectomy. Consistent with findings in cultured cells stimulated to proliferate, there was an increase in chromatin molar ellipticity measured at 276 nm, and a decrease in thermal stability 3 to 8 h after surgery. These events occurred prior to the onset of DNA synthesis. These early changes between non-proliferating (G₀) and proliferating (G₁) cells, as well as later chromatin conformational changes observed at S and G₂ phases, mimic changes in template activity.

Results with sheared and unsheared chromatin (both with in vitro and in vivo systems) prove that structural and functional changes can be caused by even the slightest shearing during chromatin preparation, suggesting the loss of native chromatin organization. To eliminate this problem, experiments were also conducted using chromatin in situ. A flow cytometer (FCM) was used to study unfixed liver cell suspensions stained with ethidium bromide (EB). Fluorescence was measured in the green spectral range after addition of increasing amounts of EB. Experimental evidence is provided that the same alteration in chromatin conformation can be best detected using low molar ratios of EB per unit DNA due to greater fluorescence emission in G₁ respect to G₀ cells.

These correlated studies demonstrate that the same changes controlling chromatin organization in situ are detected also in the tertiary-quaternary structure of “isolated” chromatin. These changes in chromatin conformation are macromolecular events related to cell proliferation both at the G₀—G₂ and G₁—S transitions.

Introduction

The characteristics of the molecular and cellular events controlling the transition of cells from quiescence to proliferation are basic questions in cell biology (see review by Baserga and Nicolini [11]).

Several in vitro systems have been studied to date. The transition in vitro between confluent (G₀) and proliferating (G₁) cells, which have the same DNA content but differ in metabolic activity, has been studied at the molecular level [2] and the cellular level [3—5] with serum stimulated 3T3 mouse fibroblasts, WI-38 human diploid fibroblasts and phytohemagglutinin (PHA) stimulated lymphocytes. However, in addition to pitfalls inherent to an in vitro system, the nature of the cellular and molecular alterations observed in vitro may reflect a transient artifact induced by the serum or PHA stimulation, which may have little biological implication. Even though in vitro systems allow acquisition of useful information, and simplify analysis by dealing with homogeneous populations, an in vivo model for the study of the control of cell proliferation undoubtedly will take us a step further in characterizing these phenomena.

This investigation will use the partially hepatectomized rat as an in vivo model to study changes in liver cell chromatin conformation after stimulation. This model lends itself to study by many physicochemical methods. Higgins and Anderson [6] as morphologists used mitosis as an index of cellular activity.

Although they did not realize the full potential of this system to study the cell cycle in vivo, it became a model which has been exploited extensively. Until recently, objective identification of individual non-cycling cells was not possible. Now by using automated image analysis and flow cytometry (FCM) non-cycling (G₀) cells can be characterized and identified (Nicolini et al. [7], Kendall et al. [8], Nicolini et al. [9], Linden et al. [10]). Following up studies conducted with cultured WI-38 cells [2, 4, 11], in this communication conventionally prepared chromatin from non-cycling and cycling cells in vivo shall be quantitatively characterized by circular dichroism (CD) and thermal denaturation. The results are compared with FCM studies of chromatin

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in isolated intact nuclei, where it is in its natural site and micro-environment.

The parallel studies conducted both at the molecular and cellular levels reveal (with a striking symmetry of results among techniques) a change in the tertiary and quaternary structure of liver chromatin of cells stimulated to proliferate by partial hepatectomy. These changes are seen as an increase in the molar ellipticity at 276 nm and a decrease in the thermal stability of isolated chromatin. In the intact cell, increase in chromatin primary binding sites for ethidium bromide is observed by flow cytometry.

Materials and Methods

Animals, partial hepatectomy and chromatin preparation

White, male, Sprague Dawley rats weighing 115 g to 125 g were obtained from Perfection Breeders (Douglassville, Pa.). All animals were allowed food and water ad libitum. Partial hepatectomies were performed according to the technique of Higgins and Anderson [6].

Chromatins were prepared by the method of Marushige and Bonner [12] with modification. All chromatin procedures were carried out at 4 °C unless otherwise indicated. Nuclei obtained after NaCl-EDTA (75 mM – 25 mM, pH 8) homogenization of cells, washing in NaCl-EDTA (75 mM – 25 mM, pH 8), followed by Tris pH 8 (50 mM) were swelled for 20 to 30 min in Tris pH 8 (25 mM). Next the nuclei were homogenized with 25 to 30 passes of a tight Dounce homogenizer (Kontes Glassware, Vineland, New Jersey) and were examined with a light microscope for intact nuclei. Following centrifugation through 1.7 M RNase in sucrose at 23,000 rpm for 3½ h in an SW27 rotor, the viscous chromatin pellet was not sheared but immediately suspended in 0.7 mM Na2HPO4·12 H2O, pH 6.8 to a final concentration between 0.5 and 1.0 A260 units, and analyzed by CD spectroscopy alone or during thermal denaturation.

Circular dichroism studies

CD measurements were performed on a Jasco model J-40 recording spectropolarimeter with CD. The instrument was standardized as described previously [13]. The experiments were conducted in N2 atmosphere using a fused quartz 1 cm cell. The time constant was 4 s, and when wavelength scanning mode was used, wavelengths were scanned at 25 nm/min. Dynode voltage did not exceed 500 V. The precision in band wavelength was 0.3 nm and the automatic slit program was used. The error of the measurement was less than or equal to 10% due to the signal to noise ratio at the sensitivity used.

The molar ellipticity \( \{ \Theta \} \) is expressed in degrees times centimeters squared per decimole of nucleotide residue, assuming a mean molecular weight of nucleotides of 330. In the range 270 – 280 nm, the molar ellipticity can be used as an indication of DNA conformation (Shih and Fasman [14]). CD measurements were corrected for light scattering artifacts by the method of Nicolini and Kendall [13].

Thermal denaturation studies

A specially constructed 1 cm fused quartz cell was used for thermal denaturation studies. This cuvette has a water jacket surrounding the sample cell for precise temperature regulation. Changes in \( \{ \Theta \} \) were monitored during the thermal denaturation studies on the modified J-40 spectropolarimeter equipped with a circulating bath and PG-UL thermostat. Temperature was increased at a rate of between 1 and 2 °C/min. The temperature was monitored at the cell jacket exit with a Bailey Amplifying Thermometer (Bailey Instruments BAT-4) using a thermocouple (Bailey Instruments IT-1) with a 0.8 s time constant. During measurements chromatins were suspended in 0.7 mM Na2HPO4·12 H2O, pH 6.8.

In studying DNA in chromatin, there is a transition prior to the helix to random coil transition of the double helix (B form). The \( \{ \Theta \} \) increases until it approximates a helical DNA (before the helix to random coil transition). This may be the result of the melting of superhelical elements [15 – 18, 13] and has been referred to (by operational definition) as the super helix to helix transition (where the major superhelical structural elements are presumably transformed to their double helical counterparts). The difference between \( \{ \Theta \} \) at room temperature and \( \{ \Theta \} \) at the end of this transition \( \{ \Theta \} \) at maximum, \( A \{ \Theta \} \) may be used as an indication of the shift of DNA from the superhelical conformation to that of relaxed DNA.

As an index of the thermal stability of DNA superstructure in chromatin, one may calculate: \( A \{ \Theta \} / AT \), where \( AT = T \) (at maximum
A derivate (change in \( \Theta \)) with respect to temperature \( T \) is calculated to assign these transition points, based on a second order least square fit. The first instance the derivative increases is at the onset of the superhelix to the helix transition. The absolute maximum of the \( d\Theta/dT \) derivative is defined as \( T_{SH} \) (an operational definition of the temperature of superhelix to helix transition). The point where the derivative crosses the temperature axis \( (d\Theta/dT = 0) \) is the end of superhelix to helix transition and the onset of the helix to random coil transition \( (T_{SH})_{max} \). The absolute minimum is defined as \( T_m \) and is the inflection of the decrease in \( \Theta \) during the helix to random coil transition (50% of the DNA bases have melted). The \( T_m \) obtained in this method corresponds to the \( T_m \) computed from hyperchromicity studies. Derivative plots were constructed from profiles of \( \Theta \) versus temperature, using a second order least square fit, to allow precise determination of the transition points during denaturation of chromatin.

**Isolation of intact single liver cells, flow cytometry and \(^3\text{H}\)methyl-thymidine incorporation**

Single cell preparations were prepared from liver of animals sacrificed by ether anesthesia based on the methods of Jacob and Bhargava [19]. Dye binding studies using ethidium bromide (EB) (dissociation constant on the order of \( 10^{-6} \)) as the stain were carried out in a range of molar ratios from 0.1 to saturation \( (R \geq 2.5) \) using a cytofluorograf model 4800 A (Bio/Physics Systems, Mahopac, New York) with the techniques previously described [7, 10].

Animals were injected with \(^3\text{H}\)methyl-thymidine (spec. activity 6.7 Ci/mmol) in a concentration of 0.25 \( \mu \)Ci per g body weight. Forty-five min later they were sacrificed and DNA was extracted from the liver cells by the method of Scott *et al.* [20]. Samples were analyzed for \(^3\text{H}\) in an Intertechnique Refrigerated Scintillation Counter. The amount of DNA present in the sample analyzed was measured at 260 nm on a Gilford spectrophotometer. Profiles were constructed of counts per minute per mg DNA versus time after surgery (see Fig. 1).

**Results**

This investigation used the partially hepatectomized rat as an *in vivo* model to study changes in liver cell chromatin conformation after a stimulus to proliferate. Observations were performed using \(^3\text{H}\)methyl-thymidine incorporation, CD, thermal denaturation and FCM analysis. The results of the experiments follow:

\(^3\text{H}\)methyl-thymidine incorporation

\(^3\text{H}\)methyl-thymidine was not incorporated into DNA of cells isolated prior to and including 11 h post surgery (Fig. 1). By 18 h post surgery, there was a sharp increase in incorporation. The largest incorporation observed was noted 24 h post surgery. A similar kinetic was observed by Tidwell *et al.* [21].

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**Fig. 1.** Time course of change in mean molar ellipticity (-----) and \(^3\text{H}\)methyl-thymidine incorporation (--------) following partial hepatectomy in the young rat. \( \Theta \times 10^{-3} \text{deg cm}^2/\text{dmol} = \) molar ellipticity, degrees times centimeter squared per decimol. \( 10^4 \times \text{cpm/mg DNA} = \) Counts per minute per milligram of DNA. The standard deviations over 3 independent experiments are also shown (I).
Circular dichroism studies

As may be seen in Fig. 1 and Table I an increase in mean molar ellipticity (\(\langle \Theta \rangle_{276} \)) from control levels (2230 deg cm\(^2\)/dmol) was observed as early as 3 h post surgery (3100 deg cm\(^2\)/dmol). The mean \(\langle \Theta \rangle_{276} \) plateaued between 5 and 11 h (5 h, 3000 deg cm\(^2\)/dmol, and 11 h, 3100 deg cm\(^2\)/dmol). The peak mean value of \(\langle \Theta \rangle_{276} \) was observed with chromatin obtained 18 h after surgery (3700 deg cm\(^2\)/dmol) consistent with the wave of DNA synthesis. The CD spectrum of chromatin isolated 18 h after partial hepatectomy compared to the spectrum of control chromatin is presented in Fig. 2. Chromatin obtained 24 h post surgery showed a decrease from 18 h in mean \(\langle \Theta \rangle_{276} \) (3300 deg cm\(^2\)/dmol) but was still above control levels. In addition there was a blue shift in the spectrum compared to the control spectrum which was initially observed 3 h following surgery. At 24 h, the initial synchrony is lost. The liver cells which are proliferating exist as G\(_2\), M or S phase cells (Bucher and Swaffield [22]; our own results by FCM and pulse label with \([^{3}H]\)thymidine).

Thermal denaturation

Table I shows that the control sample had the greatest value of mean \(\Delta \langle \Theta \rangle_{276} \) (4370 deg cm\(^2\)/dmol). Mean \(\Delta \langle \Theta \rangle_{276} \) remained relatively constant for chromatin isolated 3 h (3500 deg cm\(^2\)/dmol) 5 h (3600 deg cm\(^2\)/dmol) or 11 h (3500 deg cm\(^2\)/dmol) following surgery. The sample obtained 18 h following surgery showed the sharpest decrease in mean \(\langle \Theta \rangle_{276} \) (2900 deg cm\(^2\)/dmol) when compared with the control value. The mean \(\Delta \langle \Theta \rangle_{276} \) for chromatin isolated 24 h post surgery (3300 deg cm\(^2\)/dmol) increased toward the control.

Fig. 3 shows the mean \(\langle \Theta \rangle_{276} \) versus temperature for chromatin isolated from a control animal and that isolated 5 h following surgery. The temperature of helix to random coil transition \(T_m\) shows a decrease from 87 °C to 84 °C by 5 h following surgery, indicating a less stable structure (see Table II). The chromatin isolated 24 h following surgery had a \(T_m\) of 82 °C. Concurrently, the slight

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Table I. Mean chromatin molar ellipticity and \(\Delta \langle \Theta \rangle_{276} \) for rat liver chromatin at different time after partial hepatectomy.

<table>
<thead>
<tr>
<th>Time after surgery [h]</th>
<th>Mean (\langle \Theta \rangle_{276}) at room temperature [deg cm(^2)/dmol]</th>
<th>Mean (\Delta \langle \Theta \rangle_{276}) * [deg cm(^2)/dmol]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2230</td>
<td>4370</td>
</tr>
<tr>
<td>3</td>
<td>3100</td>
<td>3500</td>
</tr>
<tr>
<td>5</td>
<td>3000</td>
<td>3600</td>
</tr>
<tr>
<td>11</td>
<td>3100</td>
<td>3500</td>
</tr>
<tr>
<td>18</td>
<td>3700</td>
<td>2900</td>
</tr>
<tr>
<td>24</td>
<td>3300</td>
<td>3300</td>
</tr>
</tbody>
</table>

The error for each single measurement is due to the inherent resolution of the spectropolarimeter and photometric measurements and is less than or equal to 5% of the indicated values. The values are taken from a typical experiment. The overall error, taking into account also variability due to chromatin preparation and to the biological phenomena per se, is obviously larger (see standard deviations in Fig. 1). Both molar ellipticity and absorbance were corrected for light scattering artifacts [13].

* \(\Delta \langle \Theta \rangle_{276} \) is the difference between \(\langle \Theta \rangle_{276} \) measured at room temperature and \(\langle \Theta \rangle_{276} \) measured at the end of the superhelix to helix transformation.
Table II. Thermal denaturation and molar ellipticity values of rat liver chromatin isolated at different times after surgery.

<table>
<thead>
<tr>
<th>Time after surgery [h]</th>
<th>Melting temperature</th>
<th>Mean $\theta_{276}$ at room temperature</th>
<th>$\Delta \theta_{276}/\Delta T$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$T_{S0}$ [°C]</td>
<td>$T_m$ [°C]</td>
<td>[deg cm$^2$/dmol]</td>
</tr>
<tr>
<td>Control</td>
<td>73</td>
<td>87</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>71</td>
<td>84</td>
<td>19</td>
</tr>
<tr>
<td>24</td>
<td>71</td>
<td>82</td>
<td>—</td>
</tr>
</tbody>
</table>

The values are taken from a typical experiment.

change in the superhelix to helix transition was in the same direction, i.e., it decreased.

As shown in Table II the temperature of superhelix to helix transition $T_{S0}$ did not vary significantly. The data were inhomogeneous in nature and did not lend themselves to statistical analysis.

The $\Delta T$ for the control liver sample was larger (30°C) than for liver chromatin isolated 5 h following surgery (19°C). The $\Delta \theta_{276}/\Delta T$ was less for the control sample (146) than for the 5 h sample (187). The lower $\Delta \theta_{276}/\Delta T$ indicates more stable superhelical structural elements in the more superpacked chromatin from the control sample.

**Flow cytometric studies**

In a previous communication [10], we have reported a detailed flow cytometric study on the rat liver cells following partial hepatectomy. Here we intend to present and summarize only the most relevant findings (Fig. 4).

The mean fluorescence per cell in the green emission range is plotted versus ethidium bromide concentration given as the molar ratio $R (\mu M \text{ EB}/\mu M \text{ DNA})$ for unstimulated $G_0$ (2C) and partial hepatectomy stimulated $G_1$ (2C; 5 h after partial hepatectomy) rat liver cells. As an independent FCM demonstration that the stimulated rat liver cells actually go into proliferation, we show also the concentration dependence of the mean fluorescence per cell for the new “G$_2$+M” cell population appearing 24 h after partial hepatectomy, corresponding to a third peak with higher fluorescence in the frequency distribution. The percentage of cells going into proliferation (wave of ‘S’ phase cells at 18 h, and “(G$_2$+M)” at 24 h) is about 40%. This corresponds to the previously reported [10] fraction of stimulated cells (G$_1$) with mean fluorescence intensity greater than the control (G$_0$) population in the rat liver 3 - 5 h following partial hepatectomy.

![Fig. 4. Mean fluorescence of rat liver cells as a function of ethidium bromide concentration given as molar ratio $R (\mu M \text{ EB}/\mu M \text{ DNA})$. Rat liver cells were isolated before $G_0$ (• • •) and 5 h after partial hepatectomy (G$_1$: ○ — ○). Liver cells were isolated also 24 h following partial hepatectomy and the mean fluorescence of “G$_2$+M” peak (X — • — •) obtained, as described previously [10].](image)

**Discussion**

This discussion will center on certain aspects of circular dichroism spectroscopy, thermal denaturation of isolated chromatin, flow cytometry of intact cells, and the manner in which these independent biophysical measurements relate in a coherent and comprehensive characterization of the $G_0$ – $G_1$ transition in vivo.

An obvious increase in molar ellipticity ($\{\theta\}_{276}$) of chromatin is observed with in vitro experiments
prior to DNA synthesis following stimulus of quiescent cells to proliferate (Nicolini and Baserga [23]). Excision of greater than 2/3 of liver tissue from the rat (a stimulus to the liver resulting in proliferation) causes up to 50% of the liver parenchyma to proliferate (Bucher and Swaffield [22]). Although not synchronized as well as in vitro systems, one would expect stimulated liver parenchyma to exhibit results similar to those obtained in vitro. Fig. 1 confirmed this expectation in vivo even though only 35–40% of the liver cells begin to proliferate as seen by autoradiography [22].

The initial increase in $\{\theta\}_{276}$ was followed by a leveling off between 3 and 11 h post surgery. The changes in chromatin conformation mimic the changes in chromatin-template activity (i.e. template activity levels off between 6 and 12 h post surgery, Bannai and Terayama [23]). It is noteworthy that the increase in $\{\theta\}_{276}$ following stimulus of quiescent WI-38 cells to proliferate which occurred within 2 h leveled off between 3 and 4 h [23]. Although $\{\theta\}_{276}$ did not increase further in chromatin isolated from liver as late as 11 h following partial hepatectomy, an increase of $\{\theta\}_{276}$ was observed in chromatin isolated 18 h post surgery (middle S phase), followed by a decrease 24 h post surgery (late S phase and G$_2$ + M phase). The time sequence described is consistent with chromatin conformational changes during the cell cycle of synchronized HeLa cells [25, 26].

The changes in chromatin conformation are confirmed by the decrease in thermal stability for both superhelix to helix and helix to random coil transitions during the G$_0$ – G$_1$ transition. The variation in $\{\theta\}_{276}$ in terms of changes in the degree of supercoiling are confirmed by $\Delta\{\theta\}_{276}$. Table I reveals that control chromatin had the greatest value with mean $\Delta\{\theta\}_{276}$ equal to 4370 deg cm$^2$/dmol, decreasing to 3500 deg cm$^2$/dmol, 3600 deg cm$^2$/dmol, and 3500 deg cm$^2$/dmol for chromatin isolated 3, 5 and 11 h post surgery respectively. The chromatin isolated 18 h post surgery did exhibit the minimum mean $\Delta\{\theta\}_{276}$ in all samples (2900 deg cm$^2$/dmol). Concurrently, the melting temperatures of helix to coil transition ($T_m$) and the superhelix to helix transition ($T_{sh}$) decrease in the transition from G$_0$ to G$_1$ (Table II).

Questions remain open due to the artifacts inherent in the preparation of chromatin. One source of errors is the shearing of chromatin. It is well known that the high degree of DNA superstructure (arising from the supercoiling of the nucleosome filament into superhelices [13] or solenoids [27]) existing in native unsheared chromatin is disrupted by shearing. Furthermore, considering that the so-called “unsheared” (native) chromatin has an average molecular weight of 6 x 10$^6$ daltons, which is much smaller than in intact nuclei (≥ 10$^8$ daltons), the need to study chromatin in situ, without disruption that occurs with the most delicate isolation procedure, becomes obvious.

One method available to study chromatin in situ is with the flow cytometer. The second aspect of this discussion reviews FCM data collected on liver cells. It should be emphasized that all samples were obtained after sodium citrate perfusion of the liver and that, as observed with the light microscope, the leucocyte contamination was always below 5%.

By a plot of mean fluorescence per cell versus $R$ (added dye/DNA [7]) we obtained different fluorescence for G$_0$, G$_1$ and G$_2$ + M cells (Fig. 4). The quantum yield increased sharply until $R = 0.5$, when there was little further increase in quantum yield with increasing dye concentration. The chromatin conformational changes as detected by CD and thermal denaturation of chromatin may account for the increased uptake of EB in the higher (G$_1$) fluorescence peak (2C cells). A similar pattern for EB primary dye binding sites was observed in intact WI-38 and other cells stimulated to proliferate [4, 7], as well as in isolated chromatin and nuclei from cultured cells stimulated to proliferate (see review by Baserga and Nicolini [1]). Thus, our data show a remarkable agreement of results obtained with isolated chromatin and chromatin in situ. This agreement proves the value of data obtained in biophysical studies of isolated chromatin.

Recapitulating the experimental evidence obtained with FCM on the cellular level, and CD and thermal denaturation on the molecular level on rat liver cells at various time after partial hepatectomy the following conclusions may be drawn:

i) Differences in the chromatin structure during the various cell cycle phases (G$_0$, G$_1$, S, G$_2$ + M) may be characterized by the combined utilization of CD and thermal denaturation.

ii) All results obtained are consistent with the existence of a decreased degree of chromatin superpacking in G$_1$ cells indicated by: a) a higher amount of dye bound to the chromatin in the intact cell
(FCM data), and b) at the level of tertiary and quaternary structure by CD and thermal denaturation and FCM studies; the increase in mean fluorescence of G1 cells with respect to G0 cells being about 40 — 45%, similar to the relative increase in \( \theta_{276} \), i. e., 35 — 40%.

iii) The decreased thermal stability and increased \( \theta_{276} \) (chromatin DNA conformation) mimic the increase in chromatin template activity in rat liver cells following partial hepatectomy during the G0 to G1 transition, and during the G1 to S and S to G2 transitions. These results are similar to results reported for cultured cells.

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