Transcribing chromatin is not preferentially enriched with acetylated histones

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Chromatin fragments of the RNA polymerase II–transcriptional complex were purified from the micrococcal nuclease digest of rat liver nuclei in the presence of *n*-butyrate, a potent histone deacetylase inhibitor. Polyacrylamide gel electrophoretic analysis in Triton acid–urea revealed that the extent of histone acetylation of the complex did not differ markedly from that of the total chromatin.

Chromatin *RNA polymerase* *Histone acetylation* n-*Butyrate* *Nuclease digestion*

1. INTRODUCTION

The reversible acetylation of specific lysine residues in the core histones [1] leads to an enzymatic alteration of the basic nature of defined regions of chromatin containing these histones [2]. Since the original suggestion in [3], the modification of histones has been repeatedly inferred as the mechanism by which inert chromatin regions are activated for RNA transcription [4]. Thus, numerous correlations have been reported between the increase in histone acetylation and the increased RNA synthesis [5] and evidence for enrichment of acetylated histones in the transcriptionally active chromatin (see [6]). Although these studies demonstrated an association between the rates and/or levels of histone acetylation and increased RNA synthetic capacity in a number of systems, the evidence has been considered circumstantial [7]. Therefore, it might be urgent to investigate the state of histone acetylation in highly purified chromatin isolated from the region being transcribed in vivo.

We found that, when rat liver nuclei were digested with micrococcal nuclease, the transcribing RNA polymerase II molecules were liberated in the form of two different chromatin–enzyme complexes (peak 1 and peak 2) [8,9]. These two forms could be separated by sucrose density gradient centrifugation or gel filtration, and distinguished by nuclease sensitivity, the possession of stimulating factors for chromatin transcription, RNP particles containing SnRNA, and DNA sequences that they transcribe [8–11].

We have now isolated the chromatin–RNA polymerase II complex (peak 1) in the presence of *n*-butyrate which inhibits histone deacetylation [12–14], and attempted to determine whether or not the chromatin being transcribed is enriched with acetylated histones.

2. MATERIALS AND METHODS

2.1. *Isolation of nuclei*

Rat livers were placed in a loose glass–Teflon homogenizer in 5 vol. 0.25 M sucrose/5 mM

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; SnRNA, small nuclear RNA

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$MgCl_2$/5 mM sodium butyrate/10 mM Tris–HCl, pH 8.0 (isotonic sucrose). The homogenate was filtered through cheesecloth, centrifuged for 10 min at 1000 × g, and the pellet rehomogenized in the same buffer containing 0.1% (w/v) Triton X-100. The homogenate was layered over 6–7 vol. 2.2 M sucrose/5 mM $MgCl_2$/5 mM sodium butyrate/10 mM Tris–HCl (pH 8.0) and centrifuged for 60 min at 76000 × *g* in a Hitachi RP-42 rotor. The nuclear pellet was washed twice in 5 vol. of the isotonic sucrose buffer.

2.2. *Micrococcal nuclease digestion of nuclei*

Nuclei were resuspended at 100 A_{260} units/ml in 80 mM sodium butyrate/0.2 mM dithiothreitol (DTT)/0.2 mM phenylmethylsulfonyl fluoride (PMSF)/0.1 mM $CaCl_2$/8% (v/v) glycerol/10 mM Tris–HCl (pH 7.5), warmed at 30°C for 2 min and digested with 300 units/ml of micrococcal nuclease (P-L Biochemicals) for an additional 15 min. The digestion was terminated by adjusting to 1 mM EDTA, centrifuged at 10000 × *g* for 10 min, and the supernatant was used for the subsequent fractionation. Under these conditions, 10% of the total chromatin DNA became acid-soluble, and 30% was released into the supernatant.

2.3. *Fractionation of chromatin*

The solubilized chromatin was applied to a column (1.5 × 140 cm) of Bio-Gel A-15m equilibrated with 80 mM NaCl/5 mM sodium butyrate/0.5 mM EDTA/0.2 mM DTT/0.2 mM PMSF/12.5% (v/v) glycerol/10 mM Tris–HCl (pH 7.5) and eluted with the same buffer at a flowrate of 10 ml/h at 2°C.

Preparative electrophoresis on a column (2.5 × 60 cm) of Sephadex G-10 was carried out as in [15], except that 5 mM sodium acetate/5 mM sodium butyrate/0.5 mM EDTA/0.2 mM DTT/0.2 mM PMSF/12.5% (v/v) glycerol/10 mM Tris–acetate (pH 8.0) was used as a buffer.

2.4. *Histone isolation and gel electrophoresis*

Histones were extracted by adjusting all samples to 0.4 N H_2SO_4, followed by incubation at 0°C for 1 h, and centrifugation at 20000 × *g* for 20 min. The acid-soluble proteins were dialyzed for 24 h against 0.1 N HCl and lyophilized.

Gels for electrophoresis contained 8 M urea and 0.37% (w/v) Triton X-100, as in [14].

2.5. *Assay for enzyme activities*

RNA polymerase II activity dependent on an exogenous DNA template was measured after recovery of the enzyme by precipitation with $(NH_4)_2SO_4$, as in [8]. Assay for the enzyme bound to the endogenous chromatin was carried out, without precipitation of the enzymes with $(NH_4)_2SO_4$, at 200 mM $(NH_4)_2SO_4$, in the absence of an exogenous template [14].

Histone deacetylase activity was assayed as in [16].

3. RESULTS AND DISCUSSION

Rat liver nuclei were prepared in the presence of 5 mM *n*-butyrate, a compound which potently inhibits histone deacetylase [12–14], and then digested with micrococcal nuclease to about 10% acid-solubility in a medium containing 80 mM *n*-butyrate.

Fig.1 shows the elution pattern of the solubilized chromatin from a column of Bio-Gel A-15m. In this particular experiment, the chromatography was carried out in the absence of 5 mM *n*-butyrate to determine histone deacetylase activity. RNA polymerases in each fraction, collected by precipitation with $(NH_4)_2SO_4$, were assayed using an exogenously added DNA template [8]. As shown in the figure, two peaks of RNA polymerase II activity (peak 1 and peak 2) appeared. Peak 2 was eluted just ahead of the monomeric nucleosomes, and peak 1 was at the region of larger chromatin fragments. Histone deacetylase activity was distributed just between peak 1 and peak 2.

The peak 1 complex, fractionated by gel filtration on a column of Bio-Gel A-15m, was subsequently purified by preparative electrophoresis on Sephadex G-10. As shown in fig.2, the chromatins were separated into the overlapping 'fast' and 'slow' migrating peaks. The peak 1 activity was electrophoresed in a well-defined region coinciding with the slow migrating peak (fractions 12–15). From these fractions, histones were extracted and analyzed on Triton acid–urea polyacrylamide slab gels which allow for resolution of both the individual histones and their variously acetylated species [14]. As shown in fig.3, histone acetylation in the slowly migrating peak (peak 1 complex) was not markedly different from that of the total

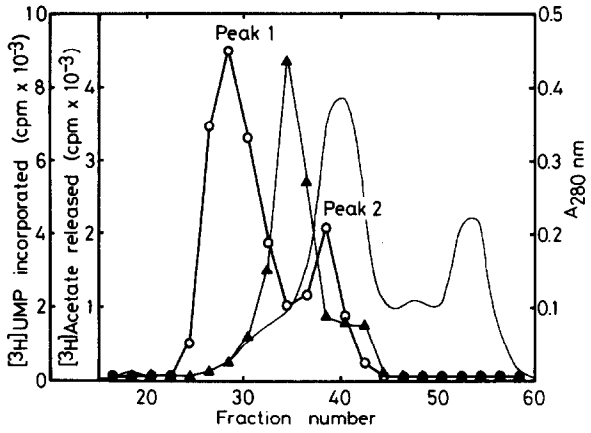

Fig.1. Fractionation of the solubilized rat liver chromatin on Bio-Gel A-15m: 20 ml of the rat liver nuclear suspension was digested with micrococcal nuclease to 10.2% acid-solubility; the solubilized chromatin, concentrated to 5 ml by ultrafiltration using an Amicon XM-300 membrane, was fractionated on a column of Bio-Gel A-15m, as described in section 2, except that 5 mM sodium butyrate was omitted from the chromatography. Samples (0.2 ml) of each 4-ml fraction were assayed for RNA polymerase II activity after recovering the enzyme by precipitation with $(NH_4)_2SO_4$ (○—○), and for histone deacetylase activity (▲—▲) (——) A_{280}.

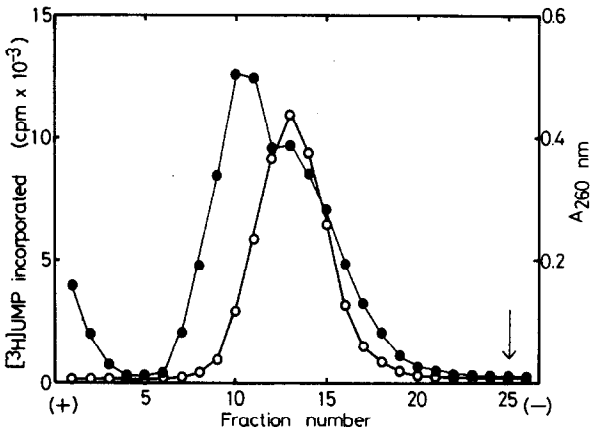

Fig.2. Sephadex G-10 column electrophoresis of peak 1 complex: 20 ml of rat liver nuclear suspension was digested with micrococcal nuclease to 10.4% acid-solubility, and the solubilized chromatin was chromatographed on a column of Bio-Gel A-15m, as described in section 2. The peak 1 fraction, concentrated by ultrafiltration using an Amicon XM-300 membrane, was subsequently electrophoresed on a column of Sephadex G-10. Portions (0.2 ml) of each 4-ml fraction were assayed for the endogenous chromatin-bound RNA polymerase activity (○—○). The arrow indicates the origin of electrophoresis. (●—●) A_{260}.

chromatin (fig.3, lane 7 *vs* lane 1), the most co spicuous being that of histone H_4. Although tl lagging fractions of the peak 1 complex (fractio 14–17) contained a band that co-migrated wi tetraacetylated H_4*, the distribution did not coi cide with that of the peak 1 activity, indicating th this species is not characteristic of peak 1.

It has been reported that each nucleus of the r liver contains about 2.9×10^9 basepairs of DN [17] and about 24000 molecules of RN polymerase II [18] per haploid genome. Takii these values into account and assuming that, in tl nucleus, 1/4 of RNA polymerase II molecules exi in the form of peak 1+ and each RNA polymera

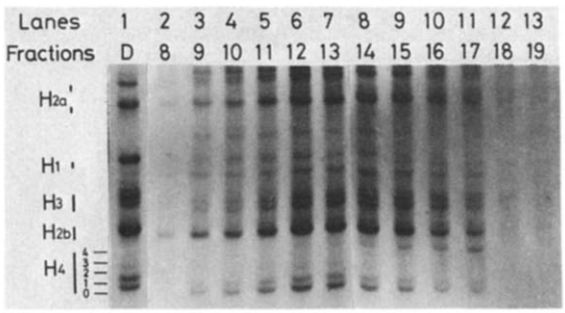

Fig.3. Triton acid–urea gel electrophoretic analysis of histones. Proteins were extracted with 0.4 N H_2SO_4 from the fractions of column electrophoresis (fig.2), and analyzed on 12% Triton acid–urea–polyacrylamide slab gels. Lanes 2–13, corresponding to the fractions 8–19, shown in fig.2. 'D' (lane 1) indicates the histones from the total nuclear digest kept at 2°C during the course of chromatin fractionation (~48 h). The gel was stained with Coomassie blue. Only the histone regions of the gel are shown.

* Preliminary experiments indicated that the band also co-migrated with histone H_4 in SDS–polyacrylamide gel electrophoresis

+ Peak 1 complex comprised about half of the transcribing RNA polymerase II molecules [11], which in turn represent half of the total enzymes contained in rat liver nuclei [19]

II molecule of peak 1 is complexed with dinucleosome [10,20], the peak 1 complex constitutes about 0.08% or 1/1250 parts of the total chromatin. Since our purification of the peak 1 complex was as high as 600-fold, on the basis of recovered chromatin-bound RNA polymerase II activity per A_{260}*, the purification was probably sufficient for an estimation of the level of histone acetylation in the complex. It should also be pointed out that deacetylation of the histones was not extensive during the isolation of peak 1, since the chromatin fractionation was carried out in the presence of a histone deacetylase inhibitor, and the peak 1 complex was not associated with histone deacetylase (fig.1).

These results led to the consideration that the chromatins being transcribed are not preferentially enriched with acetylated histones, and that histone acetylation probably does not play a direct role in facilitating chromatin transcription. Similar notions have been put forward in [21] and [22], studies using different approaches.

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* Peak 1 complex comprised approximately equal amounts of DNA and RNA

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