Structure of active chromatin: covalent modifications of histones in active and inactive genes of control and hypothyroid rat liver

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Covalent modifications of histones in active and bulk chromatin fractions were studied in liver tissue from control and hypothyroid rats. The levels of acetylation and ubiquitination of histones were similar in the active and bulk chromatin fractions, and were not influenced by hypothyroidism. Histone H2A only was phosphorylated in control active and bulk chromatin fractions. The extent of this phosphorylation did not differ between the two fractions, but hypothyroidism greatly suppressed

it, indicating an association with tissue growth. ADP-ribosylation of histones was found to be mainly associated with transcriptional inactivation of the chromatin, while histone methylation was correlated with growth inhibition of the tissue, as observed with hypothyroidism. The validity of these conclusions will, however, depend upon the similarity of the turnover rates of these covalent modifications between active and inactive chromatin and between control and hypothyroid states.

INTRODUCTION

Histones undergo various types of covalent modification in nuclei, such as acetylation, phosphorylation, methylation, ADP-ribosylation and ubiquitination [1]. A large number of previous studies have indicated that the hyperacetylation and ubiquitination of histones has some role in the transcriptional activation of chromatin [2,3]. Reports on the roles of other covalent modifications of histones in the chromatin fractions are controversial, and in many cases contradictory [1]. Enrichment of hyperacetylated histones has been observed in nucleosomes that are preferentially solubilized by DNase I or micrococcal nuclease, and chromatin fragments enriched in active genes were recognized by antibodies against hyperacetylated histones [4]. Two populations of hyperacetylated histones, i.e. a minor one with fast turnover and another with slower turnover, have been identified [5–7]. Rapidly modified histones have been proposed to be located in transcriptionally competent genes [6]. Hyperacetylated histone H4 is distributed not only in active gene regions but also with other gene regions [8]. Decreased levels of ubiquitinated H2A have been reported during erythropoiesis [9]. Similarly, higher levels of ubiquitinated H2A have been reported in transcribed copia heat-shock genes, the transcribed dihydrofolate reductase gene of *Drosophila* [10] and the transcriptionally active fraction from chicken erythrocytes [2]. Ubiquitination of H2B appears to be required for ongoing transcription [11]. Histone H2A is constitutively phosphorylated and is the major phosphorylated protein in interphase cells, with a rapid turnover rate [12]. Association of phosphorylation with transcriptional activity has been shown by several investigators [12–15]. However, in contradiction to this, a relationship between H2A phosphorylation and the maintenance of heterochromatin [16] and chromatin inactivation [17] has also been suggested.

There are reports of increased levels of ADP-ribosylation in transcriptionally active chromatin [18], while some workers could not find any differences in the levels of ADP-ribosylation between transcriptionally active and inactive chromatin [19,20]. A cor-

relation between ADP-ribosylation and transcriptional inactivation has also been reported [21,22].

Most of the available sites in histones H3 and H4 are methylated in the brains of 12-day-old rats, while methylation is complete on all sites in adult rat brain [23]. No ongoing methylation of histones was found in mature avian erythrocytes [24]. The completeness of methylation in most non-dividing cells indicates that the methylation of histones is unlikely to have any role in the transcriptional activation of chromatin. Hypothyroidism is known to cause the arrest of liver growth in young rats, besides lowering the basal metabolic rate. Transcription of 10 % of the active genes is suppressed by hypothyroidism [25–27]. The structural changes in transcriptionally active and inactive chromatin associated with the cessation of growth have not yet been identified. This is, however, important in the sense that the suppression and induction of tissue growth occurs during aging, the action of anabolic hormones, the action of mitogens, tissue regeneration and tumour induction. Study of the structural changes occurring in chromatin during the above processes will lead to an understanding of their molecular mechanism. Using our method of the isolation of transcriptionally active chromatin, we have investigated the effects of hypothyroidism on the levels of various covalent modifications of histones in transcriptionally active and inactive chromatin of rat liver. In addition, comparisons of the levels of these modifications between transcriptionally active and inactive chromatin can give a clue as to their role in transcriptional activation.

MATERIALS AND METHODS

All operations were carried out at 4 °C, unless otherwise indicated.

Induction of hypothyroidism in rats

Rats of the Charles Foster strain were used for all studies, and were maintained on a control diet at room temperature. A number of rats from the same age group, with an average weight

of 40 g, were separated into two groups. One group was given normal drinking water and the other was given 0.05 % propyl thiouracil solution. Rats of each group were weighed daily until the group given propyl thiouracil solution became hypothyroid. The time period required was 3 weeks. All hypothyroid rats were compared with normal age-matched controls. Blood samples were obtained via cardiac puncture under light ether anaesthesia and centrifuged immediately after clotting (2000 g, 10 min). The serum was kept at -40°C until assayed for levels of triiodothyronine (T_3) and thyroxine (T_4). The quantitative estimation of T_3 and T_4 in rat serum was performed using RIAK-4 and RIAK-5 kits, following the instructions provided by Board of Radiation and Isotope Technology, Bombay, India.

Analysis of histone acetylation and ubiquitination

All buffers used for chromatin preparation contained 10 mM sodium butyrate to prevent deacetylation. Chromatin preparations were freed of non-histone chromosomal proteins by the following procedure. Chromatin preparations were adsorbed on hydroxyapatite in 0.35 M NaCl, and non-histone chromosomal proteins were removed as the supernatant after centrifugation (10000 g, 10 min). Histones were extracted from the pellet with 2 M NaCl and precipitated with 20 % (w/v) trichloroacetic acid. The precipitate was washed with acetone, dried and analysed by SDS/PAGE (13 % gels) as described by Laemmli [28], and by the acid/urea/Triton X-100 gel electrophoresis system of Zweidler et al. [29].

Analysis of the phosphorylation, ADP-ribosylation and methylation of histones

The chemical modifications of histones were studied *in vitro* by incubation of rat liver slices. Livers from control and hypothyroid rats were cut into 350 μm thick slices with the help of an automatic tissue chopper (Brinkman). Slices were transferred into conical flasks containing freshly prepared Krebs/Ringer phosphate buffer (1.2 mM MgSO_4 , 128 mM NaCl, 5 mM KCl, 2.8 mM CaCl_2 , 10 mM glucose, 10 mM sodium phosphate buffer, pH 7.4; 4 ml/g of tissue). When studying phosphorylation, sodium phosphate buffer was replaced with 25 mM Tris/HCl, pH 7.4. Cycloheximide (0.2 mM) was added to the incubation medium for the study of methylation. A mixture of 95 % oxygen and 5 % CO_2 was bubbled through the buffer and PMSF (0.1 mM) was added to inhibit proteolysis during incubation. Flasks were shaken at 37°C in a water bath. After 15 min, radioactive precursors were added, and shaking was continued for another 45 min. $\text{H}_3^{32}\text{PO}_4$ (25 $\mu\text{Ci}/\text{ml}$) was used for the study of phosphorylation, [*methyl*- ^{14}C]methionine (12.5 $\mu\text{Ci}/\text{ml}$; 31.0 mCi/mmol) for methylation and [^3H]adenosine (100 $\mu\text{Ci}/\text{ml}$; 14 Ci/mmol) for ADP-ribosylation. After incubation, the flasks were transferred on to ice. Liver slices were washed three times with Krebs/Ringer phosphate buffer to remove unincorporated material.

Nuclei and active chromatin were isolated from the liver slices by the method described in the preceding paper [29a]. Histones were extracted from the nuclei with 0.25 M HCl and electrophoresed in a 13 % polyacrylamide gel in the presence of SDS. For histones extracted from nuclei, protein was estimated by the method of Lowry et al. [30]. Protein estimation could not be done for histones extracted from active chromatin due to the low concentration. Therefore quantitative loading of histones could not be achieved with active chromatin. Variation in the RNA content of active chromatin and the low yield of histones on extraction made it difficult to calculate the amount of histones

extracted from A_{260} measurements of chromatin used in the extraction. Therefore, after staining with Coomassie Blue and destaining, each lane of the polyacrylamide gel was scanned by a Beckman DU 8-B spectrophotometer equipped with a slab gel scanning accessory. Peaks of the scans were cut and weighed. Amounts of protein ($\mu\text{g}/\text{unit mass}$) were calculated by dividing the amount of protein loaded for the nuclear histones by the total mass of the peaks in the lane. By multiplying the mass of individual histone peaks by this value, the amount of protein in each histone band from the active and bulk chromatin fractions could be estimated.

Histone bands were sliced and digested with 30 % H_2O_2 overnight at 55°C , and the radioactivity of each band was counted using a Triton-, toluene- and dioxane-based scintillation fluid in a Beckman LS-100C liquid scintillation counter. To estimate background radiation, a piece of gel of same size without any protein contamination was taken from the gel and processed similarly. For measurement of phosphorylation, the gel was dried and exposed to X-ray film for 3 weeks.

All other methods were essentially as described in the preceding paper [29a].

RESULTS

Induction of hypothyroidism in rats

To induce hypothyroidism, water containing 0.05 % propyl thiouracil was given to the experimental group of rats; control rats received plain drinking water. The weight of the rats was recorded daily over a period of 3 weeks. The average weight was plotted against duration of treatment with propyl thiouracil (Figure 1). There was a linear increase in the body weight of control rats over more than a 2 week period; these rats grew by about 65 g in 2 weeks. Rats receiving propyl thiouracil showed a lower rate of growth than control rats from the beginning of drug treatment. This difference in the growth rate increased with increasing duration of treatment, and growth had almost ceased after 2 weeks. By this time, the body weights of propyl thiouracil-treated rats (weight gain ~ 30 g) were only about half those of control rats. The growth curve shows that propyl thiouracil has induced hypothyroidism in rats. In order to confirm this, levels of T_3 and T_4 were measured in the sera of control rats and

Figure 1 Effect of propyl thiouracil on the growth of rats

Rats were fed with potable water (△) or potable water containing 0.5% propyl thiouracil (○). Weights were taken daily and the average weights were recorded ($n = 25$).

Table 1 Levels of T_3 and T_4 in sera of control and hypothyroid rats

The levels of T_3 and T_4 in the sera of the control ($n = 25$) and hypothyroid ($n = 25$) groups of rats were measured as described in the Materials and methods section; means of these values are shown.

	Control	Hypothyroid
T_3 (ng/ml)	1.16	0.34
T_4 (μ g/dl)	8.8	< 1.0

Figure 2 Digestion of control and hypothyroid rat liver nuclei by endogenous nuclease

Nuclei from hypothyroid (A) and control (B) rat livers were incubated at 37 °C in buffer A (pH 7.0) for times indicated. DNA from these digests (50 μ g in each lane) was electrophoresed in a 0.7% agarose gel. Gels were stained with ethidium bromide.

Table 2 Enrichment of RNA and non-histone chromosomal proteins in active chromatin

DNA, RNA and protein were estimated in typical nuclear and active chromatin preparations from control and hypothyroid rat liver, as described in the Materials and methods section.

Material	RNA/DNA	Protein/DNA
Control nuclei	0.173	3.20
Hypothyroid nuclei	0.116	2.35
Control active chromatin	2.100	6.50
Hypothyroid active chromatin	0.855	2.35

hypothyroid rats after 3 weeks of propyl thiouracil treatment (Table 1). The levels of serum T_3 and T_4 in hypothyroid rats were 33 % and 13 % respectively of those in control rats. This confirms that the hypothyroid rats were actually depleted of thyroid hormones.

Protein and RNA content of nuclei and active chromatin from hypothyroid rat liver

The digestion of chromatin in nuclei by endogeneous nuclease in hypothyroid rat liver was measured as described in the preceding paper [29a] (Figure 2). Differential digestion of a minor chromatin fraction into oligo- and poly-nucleosomes also occurred with nuclei from hypothyroid rats. The rates of digestion of active and bulk chromatin fractions in hypothyroid nuclei (Figure 2B) were

Figure 3 Protein content of nuclei and active chromatin from control and hypothyroid rats

Proteins extracted with 0.25 M HCl from active chromatin (supernatant recovered after 5 min of incubation of nuclei at 37 °C) and nuclei were analysed by SDS/PAGE. The gel was stained with Coomassie Blue. Proteins were from 25 μ g of DNA containing active chromatin (lane a) or nuclei (lane b) from hypothyroid rats, and active chromatin (lane c) or nuclei (lane d) from control rats. Lane M contains marker proteins. Positions of histones are indicated.

similar to those in control nuclei (Figure 2A). The digestion pattern of the minor chromatin fraction in lanes corresponding to 2, 4 and 6 min was the same in control and hypothyroid rat liver nuclei. The digestion pattern of bulk chromatin in lanes corresponding to 18, 24 and 30 min was also similar in control and hypothyroid rat liver nuclei. By analogy with control nuclei, it can be inferred that the active chromatin in hypothyroid nuclei is also preferentially digested, at a similar rate. The nucleosomal repeat length of active and bulk chromatin is also the same as for the control group.

The DNA, RNA and protein content were estimated in typical preparations of nuclei from control and hypothyroid rat livers (Table 2). The nuclei from hypothyroid rats had a lower content of protein and RNA. This was also reflected in the active chromatin preparation, which was obtained as 'supernatant' after brief incubation of these nuclei at 37 °C. Active chromatin from hypothyroid rats had a lower content of RNA and protein in comparison with the active chromatin from control rats. Proteins from these preparations were analysed further by SDS/PAGE (Figure 3). It is evident that the histone/DNA ratio was the same in the active chromatin and nuclei of control and hypothyroid rats. All the histones were present in stoichiometric amounts. However, the non-histone protein content of hypothyroid nuclei and active chromatin was lower than in the controls. As discussed in the preceding paper [29a], this may be due to a lower content of ribonucleoprotein particles in hypothyroid nuclei than in control nuclei, as a result of a lower rate of transcription [31].

Acetylation and ubiquitination

Before measuring acetylation and ubiquitination, non-histone proteins were removed from active and bulk chromatin by hydroxyapatite adsorption. Figure 4(A) shows the SDS/PAGE patterns of the proteins in these chromatin fractions. It is clear that the non-histone proteins have been completely removed. Histones were present in similar stoichiometric amounts in the active and bulk chromatin fractions from both control and hypothyroid rat livers. A band corresponding to ubiquitinated H2A, as observed by Strätling [32], is also visible in all the lanes.

Figure 4 Acetylation and ubiquitination of histones in the active and bulk chromatin fractions from nuclei of control and hypothyroid rats

Nuclei and active chromatin were isolated from control and hypothyroid rat liver. Histones were extracted with 0.25 M HCl after removal of non-histone proteins as described in the Materials and methods section, and were analysed by electrophoresis in (A) a 13% polyacrylamide gel in the presence of SDS and (B) an acid/urea/Triton X-100 polyacrylamide gel. The numbers near the histone labels (H2A, H2, H2B, H3 and H4) indicate the different extents of acetylation. The prefix 'u' indicates a ubiquitinated protein. Proteins were from: lane a, hypothyroid bulk chromatin; lane b, hypothyroid active chromatin; lane c, control bulk chromatin; lane d, control active chromatin. Lane M contains marker proteins.

The relative amount of ubiquitinated H2A was the same in all chromatin fractions. This indicates that the ubiquitination of H2A is not associated with transcriptional activation or with the growth of the liver tissue, as hypothyroidism is known to influence both transcription and liver growth.

Various acetylated forms of histones were resolved by electrophoresis in an acid/urea/Triton X-100-containing polyacrylamide gel. The Coomassie Blue-stained gel is shown in Figure 4(B). It is evident that the level of acetylation of histones is lower for rat liver histones than that reported for HeLa cells

[1]. Only three bands, corresponding to non-acetylated, mono-acetylated and diacetylated forms of H3, H4 and H2B, were visible in the gel. No appreciable differences were observed in the acetylation levels between active and bulk chromatin, or between control and hypothyroid rat liver chromatin.

Phosphorylation

To study the phosphorylation of histones, the active and bulk chromatin fractions were isolated from control and hypothyroid rat liver slices preincubated in $H^{32}PO_4$ -containing medium. Histones from these chromatins were extracted with 0.25 M HCl and analysed by SDS/PAGE. Figures 5(A) and 5(B) show the Coomassie Blue staining and autoradiographic patterns respectively of the gel. It is clear that the majority of the ^{32}P radioactivity appeared in the H2A band in both active and bulk chromatin from control rat liver. Other histones were labelled to a very small extent. There was no quantitative difference in the stoichiometry of H2A phosphorylation between active and inactive chromatin from control liver, as shown by comparison of scans of the Coomassie Blue-stained gel and its autoradiogram (Figures 5C and 5D). Interestingly, ^{32}P labelling of histones in chromatin fractions from hypothyroid rat liver was decreased to a very low level. This was true for both the active and bulk chromatin fractions, and suggests that the phosphorylation of histone H2A is associated with the growth of liver tissue.

Methylation

Methylation was examined in the active and bulk chromatin fractions isolated from control and hypothyroid rat liver tissue preincubated with $[^{14}C]$ methionine in the presence of cycloheximide. Histones were extracted from the chromatin and analysed by SDS/PAGE (Figure 6A). Each lane of the Coomassie Blue-stained gel was scanned (Figure 6B) to measure the exact amount of histone present. Bands corresponding to histones were sliced out and dissolved in H_2O_2 , and radioactivity was counted in a liquid scintillation counter. Figures 6(C) and 6(D) show the radioactivity present in histones from both control and

Figure 5 Phosphorylation of histones in active and bulk chromatin fractions from nuclei of control and hypothyroid rats

Nuclei and active chromatin were isolated from ^{32}P -labelled control and hypothyroid rat liver slices. Proteins extracted with 0.25 M HCl were analysed by SDS/PAGE and stained with Coomassie Blue (A). The stained gel was autoradiographed (B). Protein was from: lane a, control active chromatin; lane b, control nuclei; lane c, hypothyroid active chromatin; lane d, hypothyroid nuclei. The exact protein content of each band was compared by densitometer scanning of the Coomassie Blue-stained gel (C) and the autoradiogram (D). Labels (a)–(d) indicate the lane scanned.

Figure 6 Methylation of histones in the active and bulk chromatin fractions from nuclei of control and hypothyroid rats

Nuclei and active chromatin were isolated from control and hypothyroid rat liver slices incubated with L-[methyl- ^{14}C]methionine in the presence of cycloheximide. Acid-extracted proteins were subjected to SDS/PAGE (A). Proteins were from control active chromatin (lane a) and nuclei (lane b), and hypothyroid active chromatin (lane c) and nuclei (lane d). Lane M contains marker proteins. Samples of 45 μg of histones were loaded for nuclei, whereas the exact amount of histones was not known for active chromatin. (B) Scan of Coomassie Blue-stained gel; labels (a)–(d) indicate the lane scanned. For (C) and (D), the stained histone bands were sliced and the radioactivity counted in a liquid scintillation counter. The specific radioactivity of the bands was calculated by dividing the c.p.m. of the band by the amount of protein. Specific radioactivity patterns of histones from (C) control rat liver and (D) hypothyroid rat liver are shown. Black bars show histones from active chromatin, and hatched bars show histones from nuclei.

Figure 7 ADP-ribosylation of histones in the active and bulk chromatin fractions from nuclei of control and hypothyroid rats

Nuclei and active chromatin were isolated from control and hypothyroid rat liver slices preincubated with [^3H]adenosine. (A) Proteins extracted with 0.25 M HCl were analysed by SDS/13%-PAGE and stained with Coomassie Blue. Proteins were from: lane a, hypothyroid nuclei; lane b, hypothyroid active chromatin; lane c, control nuclei; lane d, control active chromatin. Lane M contains marker proteins. Samples of 20 μg of histones were loaded for nuclei, whereas the amount was unknown in the case of active chromatin. The exact amount of protein in each histone band was calculated by scanning the gel (not shown) as described in the Materials and method section. For (B) and (C), the stained histone bands of (A) were sliced out and radioactivity counted in a liquid scintillation counter. The specific radioactivity of the bands was calculated by dividing the c.p.m. of the band by the amount of protein. The specific radioactivity patterns of histones from (B) control rat liver and (C) hypothyroid rat liver are shown. Black bars show histones from active chromatin, and hatched bars show histones from nuclei.

hypothyroid liver chromatin. The level of radioactive methylation of histones in control liver chromatin was quite low, but this was increased by hypothyroidism. In the case of the bulk chromatin,

these increases were mainly confined to H3 (8-fold), H2A (3-fold) and H1 (3-fold); methylation of the other histones remained at a low level. In the case of active chromatin, H3 and H1 again

showed increases in methylation. Only a small difference in methylation was observed between the active and bulk chromatin fractions in both control and hypothyroid rat liver. Therefore it can be concluded that the methylation of histones is associated with the arrest of the liver growth.

ADP-ribosylation

Liver slices from control and hypothyroid rats were incubated in medium containing [^3H]adenosine [33–35]. Active and bulk chromatin fractions were isolated and their histones were extracted with 0.25 M HCl. Histones were separated by SDS/PAGE (Figure 7A). The specific radioactivity of each histone band was calculated by the method described in the methylation section. Figures 7(B) and 7(C) show the patterns of specific radioactivity of the histones. ADP-ribosylation of all histones in bulk chromatin was much greater (2–10-fold) than for their counterparts in active chromatin. This was true for both the control and the hypothyroid rat liver. With hypothyroidism, increases in the ADP-ribosylation of H1 in inactive chromatin (2.5-fold) and of H4 in active chromatin (4-fold) were observed. This indicates that ADP-ribosylation is associated with transcriptional inactivation in a major way and with growth retardation in a minor way.

DISCUSSION

We did not observe any hyperacetylation of histones in the active chromatin preparation, or any difference caused by hypothyroidism (Figure 4). This is against the current belief that hyperacetylation is one of the factors involved in the transcriptional activation of genes [4]. Most convincing evidence in this regard has come from studies in chicken erythrocyte nuclei [2,6,36]. Hyperacetylated H4 in active chromatin has a very high turnover rate [6]. Failure to observe hyperacetylation in the active chromatin of rat liver may be due to inefficient inhibition of deacetylation by sodium butyrate during the isolation of nuclei and chromatin. This is supported by the observation that the nuclei isolated from rat liver by us and from hepatoma tissue culture cells [5] incubated with sodium butyrate have lower levels of hyperacetylation of H4 than nuclei from HeLa cells [37]. Therefore, in order to draw a definite conclusion, complete inhibition of deacetylation during chromatin isolation from rat liver has to be ensured.

Similarly, in contrast with results in the literature, we did not find any correlation between the ubiquitination of H2A and transcriptional activation in rat liver (Figure 4). This modification was also not affected by hypothyroidism. Strätling [32] was also unable to observe any differences in the levels of ubiquitinated H2A and H2B between transcriptionally active and inactive chromatin fraction from rat liver. The association of ubiquitination of histones H2A and H2B with transcriptionally active chromatin has been clearly demonstrated in chicken erythrocytes and *Drosophila* cells. The cell-cycle-dependence of ubiquitination and deubiquitination in *Physarum polycephalum* and mouse cells has also been demonstrated [38,39]. Following the replication of chromosomes in S-phase, the ubiquitination of a subset of H2A and H2B is an essential step for progression through the S/G2 boundary. From prophase to metaphase, these histones are deubiquitinated to allow full folding of the chromosomes, and they are then reubiquitinated [38,39]. A rapid turnover of bound ubiquitin has been demonstrated in both dividing and non-dividing cells [20]. Therefore it is possible that hyperubiquitinated groups in active chromatin are hydrolysed during the isolation of active chromatin from rat liver.

Rat liver slices incubated in $\text{H}_3^{32}\text{PO}_4$ -containing media incorporated radioactivity mainly into histone H2A. The phosphorylation of other histones occurred at a very low level. This is in accordance with the observation that the phosphorylation of H1 and H3 is cell-cycle-dependent, whereas phosphorylation of H2A occurs at a high level throughout the cell cycle, with a high turnover rate [14]. The level of phosphorylation of H2A was similar in both transcriptionally active and bulk chromatin, indicating that it is not linked to transcriptional activation. However, H2A phosphorylation was dramatically decreased in the active and inactive chromatin fractions from hypothyroid rat liver. This suggests strongly that H2A phosphorylation is associated with the growth of liver tissue. Our observation goes against reports suggesting a role for H2A phosphorylation in the transcriptional activation of chromatin [13–15,40], and also does not support the suggested role of H2A phosphorylation in the maintenance of heterochromatin and the transcriptional inactivation of genes [16,17]. H2A phosphorylation after G1 arrest in isoleucine-deprived cells also indicates that such phosphorylation does not have any role in cell growth [41]. No explanation is possible at present for these contradictory results. Phorbol esters have been reported to induce cell proliferation by activating protein kinase C, which is known to phosphorylate nuclear histones. Thus the phosphorylation of histones should be associated with the growth of the tissue. In the histone variant of H2A from *Xenopus laevis* oocytes, phosphorylation has been reported to be involved in nucleosomal spacing [42], which occurs during DNA replication associated with growth of the cell. The assembly of spaced nucleosomal arrays is likely to be a property of the core histones, being dependent upon their post-transcriptional modifications [43]. Our results are in accordance with this, showing that H2A phosphorylation is observed in growing control liver tissue, but is decreased on arrest of growth of the liver tissue as a result of hypothyroidism. Furthermore, we have observed incorporation of the [^{14}C]methyl group into histones H3 and H4 on incubation of slices from hypothyroid rat liver but not from control young rat liver. This level of incorporation is the same in both transcriptionally active and inactive chromatin. Thus the methylation of H3 and H4 appears to be associated with growth retardation. This is in accordance with the complete methylation of H3 and H4 in non-dividing cells [23]. The specific radioactivity incorporated into histone H3 is 6-fold greater than for H4, which agrees with the reported four sites of methylation in H3 as compared with only one site in H4 [1]. This is consistent with the reasonable turnover rate of methyl groups in H3 and H4 reported in cat kidney tissue [42]. However, we found very low levels of histone methylation in growing liver tissue, in contrast with reports on the methylation of H3 and H4 throughout the cell cycle [44].

Our results showed a much higher level of ADP-ribosylation of histones in transcriptionally inactive chromatin as compared with the active chromatin fraction. This is consistent with the suggestion of an involvement of ADP-ribosylation in transcriptional inactivation on the basis of decreased levels of ADP-ribosylation on germination of wheat embryos [21,22]. This does not rule out the possibility of an involvement of ADP-ribosylation in cell growth, as rapid cell growth also accompanies germination. We have found a significant decrease in the level of ADP-ribosylation on arrest of liver growth due to hypothyroidism. However, our results are in contrast with reports on the involvement of ADP-ribosylation [18], or the lack of its involvement [19,45], in transcriptional activation. The increased ADP-ribosylation in transcriptionally inactive chromatin may not be due to higher level of nicks in bulk chromatin as compared with active chromatin, since the inactive chromatin has lower nuclease-

sensitivity than active chromatin [46]. The poly(ADP-ribose) chain covalently links the N-terminal region of one H1 molecule to the C-terminal domain of other [47]. Thus ribosylated groups in the histones may lead to cross-linking of neighbouring nucleosomes, and in turn be involved in the stabilization of folded chromatin structures [1]. This can explain the enrichment of ADP-ribosylated groups in inactive chromatin, as inactive chromatin regions are thought to be more folded than active chromatin regions.

The specific radioactivity of the phosphorylated, methylated and ADP-ribosylated histones in nuclei will depend upon (i) the levels of these modifications in histones, (ii) their turnover rate and (iii) their rate of removal during isolation of nuclei and chromatin. Thus the above conclusions will be affected by the contributions of each of these factors in increasing or decreasing the level of radioactivity incorporated.

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REFERENCES

- Van Holde, K. E. (1988) in *Chromatin* (Rich, A., ed.), pp. 111–148, Springer-Verlag, New York
- Decluve, G. P. and Davie, J. R. (1989) *Biochem. J.* **263**, 179–186
- Reeves, R. (1984) *Biochim. Biophys. Acta* **782**, 343–393
- Grunstein, M. (1990) *Trends Genet.* **6**, 395–400
- Covault, J. and Chalkley, R. (1980) *J. Biol. Chem.* **255**, 9110–9116
- Zhang, D. E. and Nelson, D. A. (1988) *Biochem. J.* **250**, 241–245
- Zhang, D. E. and Nelson, D. A. (1988) *Biochem. J.* **250**, 233–240
- Turner, B. M., Franchi, L. and Wallace, H. (1990) *J. Cell Sci.* **96**, 335–346
- Goldknopf, I. L., Wilson, G., Ballal, N. R. and Busch, H. (1980) *J. Biol. Chem.* **255**, 10555–10558
- Varshavsky, A., Levinger, L., Sundin, O., Barsoum, J., Ozkaynak, E., Swerdlow, P. and Finley, D. (1982) *Cold Spring Harbor Symp. Quant. Biol.* **47**, 511–528
- Davie, J. R. and Murphy, L. C. (1990) *Biochemistry* **29**, 4752–4757
- Prentice, D. A., Taylor, S. E., Newmark, M. Z. and Kito, P. A. (1978) *Biochem. Biophys. Res. Commun.* **85**, 541–550
- Allis, C. D. and Gorovsky, M. A. (1981) *Biochemistry* **20**, 3828–3833
- Prentice, D. A., Loechel, S. C. and Kito, P. A. (1982) *Biochemistry* **21**, 2412–2420
- Ruiz-Carrillo, A., Wang, L. J. and Allfrey, V. G. (1976) *Arch. Biochem. Biophys.* **174**, 273–290
- Gurley, L. R., D'Anna, J. R., Barham, S. S., Deavan, L. L. and Tokey, R. A. (1978) *Eur. J. Biochem.* **84**, 1–15
- Marian, B. and Wintersberger, V. (1982) *FEBS Lett.* **139**, 72–76
- Mullins, Jr., D. W., Giri, C. P. and Smulson, M. (1977) *Biochemistry* **16**, 506–513
- Malik, N. and Smulson, M. (1984) *Biochemistry* **23**, 3721–3725
- Wu, H. M., Dattagupta, N. and Crothers, D. M. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6808–6811
- Sasaki, K. and Sugita, M. (1982) *Plant Physiol.* **56**, 148–154
- Sasaki, K. and Sugita, M. (1982) *Plant Physiol.* **69**, 543–545
- Duerre, J. A., Quick, D. P., Trayno, M. D. and Onisk, D. V. (1982) *Biochim. Biophys. Acta* **719**, 18–23
- Sung, M. T., Harford, J., Bundman, M. and Vidalakis, G. (1977) *Biochemistry* **16**, 279–285
- Liaw, C., Seelig, S., Mariash, C. N., Oppenheimer, J. H. and Towle, H. C. (1983) *Biochemistry* **22**, 213–221
- Narayan, P., Liaw, C. W. and Towle, H. C. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4687–4691
- Seelig, S., Jump, D. B., Towle, H. C., Liaw, C., Mariash, C. N., Schwartz, H. L. and Oppenheimer, J. H. (1982) *Endocrinology* **110**, 671–673
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Zweidler, A., Urban, M. K. and Goldman, P. (1978) *Miami Winter Symp.* **15**, 531 (abstract)
- Tikoo, K., Gupta, S., Hamid, Q. A., Shah, V., Catterjee, B. and Ali, Z. (1997) *Biochem. J.* **321**, 273–279
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Leslie, J., DeGroot, L. J., Rue, P., Robertson, M., Bernal, J. and Scherberg, N. (1977) *Endocrinology* **101**, 1690–1700
- Strätling, W. H. (1987) *Biochemistry* **26**, 7893–7899
- Adamietz, P., Bredehorst, R. and Hiltz, H. (1978) *Eur. J. Biochem.* **91**, 317–326
- Ord, M. and Stocken, L. (1977) *Biochem. J.* **161**, 583
- Ueda, K., Omachi, H., Kawaichi, M. and Hayaishi, O. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 205–209
- Hebbes, T. R., Alan, W. T. and Crane-Robinson, C. (1988) *EMBO J.* **7**, 1395–1402
- D'Anna, J. A., Tobey, R. A. and Gurley, L. R. (1980) *Biochemistry* **19**, 2656–2671
- Finley, D., Ciechanover, A. and Varshavsky, A. (1984) *Cell* **37**, 43–55
- Mueller, R. D., Yasuda, H., Hatch, C. L., Bonner, W. M. and Bradbury, E. M. (1985) *J. Biol. Chem.* **260**, 5147–5153
- West, M. H. P., Pantazis, P. and Bonner, W. M. (1985) *J. Biol. Chem.* **260**, 4558–4560
- Gurley, L. R., Tobey, R. A., Walters, R. A., Hildebrand, C. E., Hohmann, P. G., D'Anna, J. A., Barham, S. S. and Deavan, L. L. (1978) in *Cell Cycle Regulation* (Jeter, J., Cameron, I. L., Padilla, G. M. and Zimmerman, A. M., eds.), pp. 37–60, Academic Press, New York
- Hempel, K., Thomas, G., Roos, G., Stocker, W. and Lange, H. W. (1979) *Hoppe-Seyler Z. Physiol. Chem.* **360**, 869–876
- Woleffe, A. (1993) *Chromatin Structure and Function*, pp. 55–57, Academic Press, New York
- Thomas, G., Lange, H. W. and Hempel, K. (1975) *Eur. J. Biochem.* **51**, 609–615
- Yukioka, M., Ykal, Y., Hasuma, T. and Inoue, A. (1978) *FEBS Lett.* **86**, 85–88
- Weintraub, H. and Groudine, M. (1976) *Science* **193**, 848–856
- Wong, M., Allan, J. and Smulson, M. (1984) *J. Biol. Chem.* **259**, 7963–7969