Purification of a Phosphoprotein from Chromatin of Rat Liver

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A simple and effective method to purify a phosphoprotein (B_2) (M_r 68000, pI 6.2–8) from phenol-soluble non-histone chromatin proteins of rat liver is described. The purification involved only two steps, CM-cellulose chromatography and preparative SDS/polyacrylamide (10%)–gel electrophoresis. The purified phosphoprotein B_2 was shown to be homogeneous by SDS/polyacrylamide-gel electrophoresis. The yield was 2% of total non-histone chromatin proteins. The acidic to basic amino acid ratio of phosphoprotein B_2 was less than 1, with high contents of glutamic acid, aspartic acid, arginine, lysine, glycine and alanine. The phosphate content of this protein is 0.3%.

Studies on chromatin reconstitution *in vitro* have indicated that non-histone chromatin proteins are associated with tissue-specific template activity as well as the specific control of gene expression (for reviews see: Stein *et al.*, 1974; MacGillivray & Rickwood, 1975; Chiu & Hnilica, 1977; Bekhor, 1978; Liew, 1979). Evidence also suggests that phosphorylation of non-histone chromatin proteins is involved in the regulation of gene expression (Stein *et al.*, 1974; Kleinsmith *et al.*, 1976; Jungmann & Russell, 1977). For example, phosphorylation and dephosphorylation of non-histone chromatin proteins were found to be associated with cellular activity (Kleinsmith *et al.*, 1966; Gornall & Liew, 1974; Ezrailson *et al.*, 1976; Thomson *et al.*, 1977) and the phosphorylation of nuclear proteins during hormone action (Jungmann & Schweppe, 1972; Liew *et al.*, 1973). It was also found that phosphoproteins bound selectively to homologous DNA (Teng *et al.*, 1971; Allfrey *et al.*, 1973) and enhanced the RNA synthesis in cell-free systems (Teng *et al.*, 1971; Kostraba *et al.*, 1975). Nevertheless, most of the work was done on a group of non-histone chromatin proteins which was enriched in phosphoprotein. In order to understand the precise role(s) of phosphoprotein in relation to gene regulation, specific phosphoprotein(s) should be isolated and characterized.

Kleinsmith (1973) isolated a group of nuclear phosphoproteins by DNA–cellulose chromatography. Kostraba *et al.* (1975) also succeeded in isolating a group of enriched non-histone nuclear phosphoproteins from Ehrlich ascites-tumour cells by salt and phenol extraction. MacGillivray *et al.* (1978) isolated a low-molecular-weight phosphorylated component of non-histone chromatin proteins by gel chromatography and electrophoresis from

Abbreviation used: SDS, sodium dodecyl sulphate.

mouse nuclei. Other low-molecular-weight non-histone chromatin proteins ($M_r \geq 30000$) were also isolated by several laboratories (Patel & Holoubek, 1974; Goldknopf *et al.*, 1975; Herrick & Alberts, 1976; Bluthmann, 1976). However, isolation of a specific non-histone nuclear phosphoprotein with relatively high molecular weight has yet to be achieved. In the present paper we describe a very simple method to isolate the specific phosphoprotein B_2 , which has previously been shown to be associated with the chromatin subunits of rat liver (Liew & Chan, 1976; Chan & Liew, 1977).

Materials

Chemicals

Acrylamide, NN' -methylenebisacrylamide and $NNN'N'$ -tetramethylethylenediamine were purchased from Eastman Organic Chemicals, Rochester, NY, U.S.A.; ultrapure urea and Tris base were obtained from Schwarz/Mann Co., Orangeburg, NY, U.S.A., and CM23 CM-cellulose was obtained from W. and R. Balston, Maidstone, Kent, U.K. All other chemicals were of reagent grade.

Animals

Male albino rats weighing about 200g were used. They had access to tap water and were fed on Purina Laboratory Chow. Food was removed the night before experiments.

Radioisotopes

[^{32}P]Phosphoric acid (carrier-free) was purchased from New England Nuclear Corp., Boston, MA, U.S.A.

Methods

Preparation of phenol-soluble non-histone chromatin proteins

Rat liver nuclei were prepared as described previously (Chan & Liew, 1977). The phenol-soluble non-histone chromatin proteins were isolated by the method described by Teng *et al.* (1971). Briefly, the purified liver nuclei were suspended and stirred for 15 min in 0.14 M-NaCl (2 ml/g of liver tissue), followed by centrifugation at 3000g for 5 min. This extraction was then repeated. Histones were then removed by extracting twice with 0.25 M-HCl (2 ml/g of liver tissue). Lipids were removed by a chloroform/methanol mixture. The non-histone chromatin proteins were then suspended in TEM buffer (0.1 M-Tris/HCl, pH 8.4, 0.01 M-EDTA, 0.14 M- β -mercaptoethanol), and an equal volume of phenol saturated with TEM buffer was added. The mixture was stirred overnight in the cold-room, followed by centrifugation at 12000g for 10 min. The aqueous phase was collected and re-extracted with the buffered phenol as described previously. The two phenol extracts were combined and dialysed against 0.1 M-acetic acid containing 0.14 M- β -mercaptoethanol until the phenol phase was decreased to one-fifth of its original volume. The phenol extract was subsequently dialysed against 0.05 M-acetic acid/9.0 M-urea/0.14 M- β -mercaptoethanol for 24 h. Dialysis was continued in 0.1 M-Tris/HCl (pH 8.4)/8.6 M-urea/0.01 M-EDTA/0.14 M- β -mercaptoethanol for 2 h, followed by an equilibrating buffer consisting of 8 M-urea, 0.02 M-glycine, 0.02 M-Tris/HCl, pH 9.2, 0.05 % β -mercaptoethanol and 0.1 mM-phenylmethanesulphonyl fluoride.

CM-cellulose chromatography

Whatman CM 23 CM-cellulose (cat. no. 22232) was washed with 0.5 M-NaOH for 30 min and rinsed with water until pH 8 was reached. The CM-cellulose was finally packed into a glass column and equilibrated with equilibrating buffer. The protein samples (1–2 mg/ml) were dialysed in equilibrating buffer, pH 9.2, overnight. About 10 mg of protein was applied to the CM-cellulose column (1.5 cm $\times$ 6 cm). Chromatography was then performed at room temperature. The flow rate was 0.3 ml/min. After the unbound fraction was removed, the bound fraction was eluted with 0.3 M-NaCl in the equilibrating buffer, pH 9.2. The retarded fraction was dialysed against the equilibrating buffer, pH 10, to remove the NaCl. This fraction was then rechromatographed on the CM-cellulose column at pH 10. The unbound fraction was discarded and the protein that was retained in the column (enriched phosphoprotein fraction) was eluted by 0.3 M-NaCl in the equilibrating buffer, pH 10.

One-dimensional SDS/polyacrylamide-gel electrophoresis

Chromatin proteins (1–2 mg/ml) were solubilized by adding an equal volume of a mixture containing 5 % SDS, 1 % β -mercaptoethanol and 65 mM-Tris/HCl, pH 6.8. The SDS/polyacrylamide (10 %) gel electrophoresis was carried out as described by Laemmli (1970). The separating gels (0.6 cm $\times$ 6.5 cm) contained 10 % acrylamide, 0.27 % *NN'*-methylenebisacrylamide, 0.375 M-Tris/HCl, pH 8.8, 0.1 % SDS, 0.03 % ammonium persulphate and 0.05 % *NNN'N'*-tetramethylethylenediamine. The separating gel was overlaid with 0.1 ml of water and left overnight at room temperature before polymerization of the stacking gel. The stacking gels (0.6 cm $\times$ 0.5 cm) contained 3 % acrylamide, 0.08 % *NN'*-methylenebisacrylamide, 0.125 M-Tris/HCl, pH 6.8, 0.1 % SDS, 0.03 % ammonium persulphate and 0.05 % *NNN'N'*-tetramethylethylenediamine. The stacking gel was overlaid with water and allowed to polymerize for 1 h before electrophoresis. About 50 μ g of protein was layered on the stacking gel. Gel electrophoresis was performed at a constant current of 1 mA/tube until the tracing dye (Bromophenol Blue) had reached the bottom of the gel. The gels were fixed with a fixing solution consisting of 50 % (v/v) methanol and 10 % (v/v) acetic acid and stained with 0.1 % Coomassie Brilliant Blue in the fixing solution. The gels were destained with a solution of 5 % methanol and 10 % acetic acid.

Isolation of the phosphoprotein B₂ by SDS/polyacrylamide-gel electrophoresis

The enriched phosphoprotein fraction obtained from CM-cellulose column chromatography was dialysed overnight against two changes of equilibrating buffer, pH 9.2, to remove salt. The protein sample was then concentrated to a final protein concentration of about 1 mg/ml by Sephadex G-200 (concentration of the protein by ultrafiltration resulted in a low yield). To this fraction an equal volume of a mixture containing 5 % SDS, 1 % β -mercaptoethanol and 65 mM-Tris/HCl, pH 6.8, was added. The protein was fractionated by SDS/polyacrylamide (10 %) gel electrophoresis. After electrophoresis, the gels were immersed in 1 M-KCl for 3 min (Nelles & Bamberg, 1976). The protein bands in the gel were then made visible by precipitation of protein-SDS complexes after overnight storage in the refrigerator. Gel segments corresponding to the phosphoprotein B₂ were excised and ground into small pieces by a glass rod. The gel pieces were soaked in a mixture containing 5 % SDS, 1 % β -mercaptoethanol and 65 mM-Tris/HCl, pH 6.8. The gel mixture was then poured into a glass tube which contained a short polymerized SDS/polyacrylamide (10 %) gel (supporting gel, 1 cm $\times$ 1.5 cm) at the end of the tube. A dialysis-

tubing sac was tied at the anodic end under the supported gel. Electrophoresis was performed at 5 mA/tube for 48 h. The protein was collected in a dialysis-tubing sac.

Two-dimensional polyacrylamide-gel electrophoresis

Chromatin proteins were subjected to isoelectrofocusing in the first dimension and subsequently fractionated by SDS/polyacrylamide-gel electrophoresis in the second dimension by the method described previously (Suria & Liew, 1974; Jackowski *et al.*, 1976; Chan & Liew, 1977). The Coomassie Blue-stained two-dimensional slab gels were dried under vacuum overnight. The ³²P-labelled non-histone chromatin proteins were detected by radioautography by using Kodak X-ray film (NS-54T). The gels were exposed to X-ray film for 2–3 days before being developed.

Protein determination and analysis

Protein determination followed the method described by Lowry *et al.* (1951). Bovine serum albumin was used as the standard.

The amino acid composition of the phosphoprotein was determined by an ion-exchange amino acid analyser. The protein was hydrolysed in 6M-HCl at 105°C for 21 h before analysis.

P_i determination

The phosphate content of phosphoprotein B₂ was determined by the method of Martin & Doty (1949). The phosphoprotein B₂ eluted from the SDS/polyacrylamide gel was precipitated with an equal volume of 25% (w/v) trichloroacetic acid and then with ether at 0°C. The phosphoprotein was hydrolysed with 1.0M-NaOH at 100°C for 15 min. A sample (10 µl) was taken for protein determination. The remainder was treated with ammonium molybdate and then extracted with 2-methylpropan-1-ol/benzene (1:1, v/v). The phosphomolybdate complex was measured at 660 nm in the presence of SnCl₂.

Results

An attempt was made to isolate a specific phosphoprotein B₂, which has previously been identified in chromatin subunits (Liew & Chan, 1976; Chan & Liew, 1977). However, to minimize the time required for the purification procedure, the preparation of chromatin subunits was omitted and phenol-soluble non-histone chromatin proteins were isolated directly from the nuclei. The non-histone chromatin proteins of rat liver were then fractionated by CM-cellulose column chromatography. The elution profile is shown in Fig. 1. Two fractions were obtained. In five separate experiments, about 60% (59.2 ± 3.8%) of

Fig. 1. Purification of the phosphoprotein (B₂) by CM-cellulose chromatography

The phenol-soluble non-histone chromatin protein (2 mg/ml) in equilibrating buffer (8M-urea, 0.02M-glycine, 0.02M-Tris/HCl, 0.05% β-mercaptoethanol, 0.1 mM-phenylmethanesulphonyl fluoride, pH 9.2) was applied to the CM-cellulose column. The 'unbound' fraction (60%) passed through. The 'bound' fraction (27%), which contained the phosphoprotein B₂, was eluted with 0.3M-NaCl in the equilibrating buffer.

Fig. 2. Fractionation of the phenol-soluble non-histone chromatin protein by SDS/polyacrylamide-gel electrophoresis

The phenol-soluble non-histone chromatin proteins and the purified fractions from CM-cellulose chromatography were mixed with an equal volume of 5% SDS in 1% β-mercaptoethanol/65 mM-Tris/HCl, pH 6.8. These proteins were subjected to SDS/polyacrylamide (10%) gel electrophoresis: (a) the phenol-soluble non-histone chromatin proteins; (b) the 'unbound' fraction from CM-cellulose chromatography; (c) the 'bound' fraction from CM-cellulose chromatography [the arrow (→) indicates the specific phosphoprotein B₂]; (d) the 'bound' fraction after rechromatography on the CM-cellulose column (enriched phosphoprotein fractions).

the proteins were not bound to the column. The 'bound' fraction ($27.5 \pm 6.0\%$) was then eluted from the column by 0.3 M-NaCl in the equilibrating buffer. This fraction was then rechromatographed under similar conditions, but with an increase in pH from 9.2 to 10.0. About 14% of the starting non-histone chromatin protein was eluted as the 'unbound' fraction and about 7% was finally eluted as the 'bound' fraction, which contained the phosphoproteins. About 86% of the non-histone chromatin proteins were accounted for after column chromatography.

Fig. 2 shows the one-dimensional SDS/polyacrylamide-gel pattern of fractions obtained from CM-cellulose chromatography. As compared with the phenol-soluble non-histone chromatin proteins (Fig. 2, gel *a*), it was found that the unbound fraction (Fig. 2, gel *b*) contained most of the total non-histone chromatin proteins. However, the specific phosphoprotein B_2 (arrow) and some other proteins (Fig. 2, gel *c*) were bound to the column. Further

purification of the phosphoprotein B_2 was achieved by rechromatography of the bound fraction in the CM-cellulose column at a higher pH (pH 10). Under this condition most of the proteins in the unbound fraction were removed, leaving the phosphoprotein B_2 and two other major proteins (Fig. 2, gel *d*) bound to the CM-cellulose. These proteins (enriched phosphoprotein fractions) were again eluted by 0.3 M-NaCl in the equilibrating buffer.

The complexity of the non-histone chromatin proteins was revealed by the high resolving power of the two-dimensional polyacrylamide-gel electrophoresis. As shown in Fig. 3(*a*), the phenol-soluble non-histone chromatin proteins could be fractionated into more than 100 components with molecular weights ranging from 30000 to larger than 100000 and pI from 5 to 9.5. The isolation of specific phosphoprotein B_2 , which is shown in the rectangle, was carried out. Most of the non-histone chromatin proteins (Fig. 3*b*) did not bind to the CM-cellulose column under the experimental conditions, except

Fig. 3. Fractionation of phenol-soluble non-histone chromatin proteins and purified phosphoprotein by two-dimensional polyacrylamide-gel electrophoresis

For full details, see the Methods section. (a) Phenol-soluble non-histone chromatin proteins. (b) 'Unbound' fraction from the CM-cellulose chromatography, which contains the majority of non-histone chromatin proteins except the phosphoprotein B_2 and other basic proteins. (c) 'Bound' fraction from the CM-cellulose chromatography, which contains the phosphoprotein B_2 (rectangle) and other basic proteins of non-histone chromatin proteins. (d) Enriched phosphoprotein fraction, which was obtained from the rechromatography of the 'bound' fraction on the CM-cellulose column at pH 10. The phosphoprotein B_2 and two other basic proteins were observed.

for phosphoprotein B₂ and a few other proteins. Furthermore the non-histone chromatin proteins retained in the CM-cellulose column were relatively basic, with isoelectric points greater than 7 (Fig. 3c). Further purification of the phosphoprotein B₂ by rechromatography removed some of these basic proteins. The two-dimensional electrophoresis pattern of the enriched phosphoprotein fractions is shown in Fig. 3(d). Only three groups of non-histone chromatin proteins were observed. It should be noted that the phosphoprotein B₂ was resolved into five or six distinct fractions by this two-dimensional gel system.

Fig. 4 shows the radioautograph of the ³²P-labelled non-histone chromatin proteins on the two-dimensional gel. Fig. 4(a) shows that most of the phosphoproteins were not bound to the CM-cellulose column. In general there were three major groups of phosphorylated non-histone chromatin proteins, with mol.wts. around 20000, 38000 and

68000 in the phenol-soluble proteins (Fig. 4b). The phosphoprotein B₂ belongs to the high-molecular-weight group (*M*, 68000). However the phosphorylated protein B₂ and two other slightly phosphorylated proteins were retained in the CM-cellulose column (Fig. 4c) and could be removed by 0.3M-NaCl in the equilibrating buffer. As shown in Fig. 4(d), all these phosphoproteins were again retained in the CM-cellulose column during rechromatography at the higher pH.

Before the use of CM-cellulose chromatography, a DNA affinity column had been used to fractionate the phenol-soluble non-histone chromatin proteins. Rat liver DNA was incorporated into the CM-cellulose (2.5 mg of DNA/ml of DNA-CM-cellulose) and the affinity chromatography was performed by the method described by Potuzak & Wintersberger (1976). However, the protein recovery after passage through the column was very low (10–30%). In addition, the two-dimensional polyacrylamide-gel

Fig. 4. Radioautography of the purified ³²P-labelled phosphoproteins on two-dimensional polyacrylamide-gel electrophoresis. For full details, see the Methods section. (a) The 'unbound' fraction from the CM-cellulose chromatography. Most of the phosphorylated non-histone chromatin proteins were found in this fraction. (b) Phenol-soluble ³²P-labelled non-histone chromatin proteins. Three groups of highly phosphorylated non-histone chromatin proteins were identified, with mol.wts. of 20000, 38000 and 70000. (c) 'Bound' fraction from the CM-cellulose chromatography, which contains the phosphoprotein B₂ (rectangle) and a few other phosphorylated non-histone chromatin proteins. (d) 'Bound' fraction from the rechromatography on the CM-cellulose column (enriched phosphoprotein fraction). It was shown that phosphoprotein B₂ and two other phosphoproteins were again retained in the CM-cellulose column.

electrophoresis pattern of the fractionated protein after column chromatography was not reproducible. It was later found that the addition of urea to the equilibrating buffer could increase the protein recovery from the column and provide a more reproducible result. Aggregation of the non-histone chromatin proteins either to the matrix of the cellulose or to themselves in the absence of urea was suspected to be the reason for the low recovery. Nevertheless, it was found that chromatography using CM-cellulose and DNA-CM-cellulose gave similar results under the high-urea condition. Therefore, the simple and effective CM-cellulose chromatography was used.

During the development of the chromatographic conditions for the CM-cellulose column, it was found

that less protein was retained in the column at a higher pH (cation exchanger). However, the specific phosphoprotein B₂ with pI 6.2–8 was still bound to the column. This property facilitated its purification by the CM-cellulose column. An attempt was also made to elute the phosphoprotein (B₂) from the column by using a salt gradient (0–0.3 M-NaCl). Two fractions overlapping each other were obtained. However, both fractions contained phosphoprotein B₂. In an alternative method, the proteins retained in the column were eluted by the pH 10 buffer followed by urea buffer containing 0.3 M-NaCl. Very little protein was eluted by the pH 10 buffer. Therefore rechromatography at a higher pH was required for better purification of the phosphoprotein B₂.

Based on the scanned gel area (Fig. 5a), it was

Fig. 5. Homogeneity of the isolated phosphoprotein (B₂)

The enriched phosphoprotein fraction obtained after CM-cellulose chromatography and the isolated phosphoprotein B₂ were fractionated by SDS/polyacrylamide (10%)-gel electrophoresis. After destaining, the gels were scanned at 550 nm. (a) Enriched phosphoprotein fraction. From the chart area, it was estimated that phosphoprotein B₂ constituted about 23% of the total protein on the gel. (b) Isolated phosphoprotein B₂. The scanned profile indicated that the B₂ preparation was homogeneous.

Table 1. *Protein recovery of the phosphoprotein B₂ from rat liver*

found that the percentage of phosphoprotein B₂ in the enriched phosphoprotein fraction was about 23%. Taking into account the protein recovery of the previous steps, it was estimated that the phosphoprotein B₂ constituted about 2% of the phenol-soluble non-histone chromatin proteins.

The phosphoprotein B₂ was finally isolated by excising the protein band from the SDS/polyacrylamide (10%) gel and eluted electrophoretically as described in the Methods section. The protein recovery of this final step was about 70%. Table 1 shows the yield of the phosphoprotein B₂ that could be obtained from 1 g of rat liver tissue. The isolated phosphoprotein B₂ was subjected to SDS/polyacrylamide (10%) gel electrophoresis. As shown in Fig. 5(b), only one band was observed, indicating that the isolated phosphoprotein B₂ was pure.

The amino acid composition of the phosphoprotein B₂ is shown in Table 2: the protein was enriched in glutamic acid, aspartic acid, arginine, lysine, glycine and alanine. The ratio of the sum of aspartic acid and glutamic acid to the sum of lysine, histidine and arginine was 0.93, indicating that the molecule was an unusual polypeptide of non-histone chromatin proteins. The *N*-terminal of the phosphoprotein B₂ was determined by the method described by Weiner *et al.* (1972). Preliminary results revealed that the amino group of the *N*-terminal amino acid was blocked.

The phosphate content of non-histone chromatin proteins and phosphoprotein B₂ was also determined, as shown in Table 3: the P_i content of phosphoprotein B₂ was 0.3%. We previously showed (Chan & Liew,

Table 2. *Total amino acid composition (mol %) of phosphoprotein B₂*

Values for proteins HMG1 and HMG2 are taken from Walker *et al.* (1976).

Amino acid	Phosphoprotein B ₂	HMG 1	HMG 2
Asp	8.9	11.1	10.1
Thr	3.3	2.9	2.4
Ser	6.7	5.6	7.6
Glu	13.0	19.4	18.4
Pro	7.0	7.1	7.8
Gly	11.5	5.7	6.5
Ala	9.3	9.1	7.5
Val	1.9	2.4	1.9
Cys	Not determined	Trace	Trace
Met	Not determined	1.4	1.4
Ile	2.5	1.8	1.8
Leu	6.9	2.0	2.4
Tyr	2.5	0.8	1.0
Phe	2.9	4.2	3.9
Lys	7.8	19.3	19.3
His	2.7	1.5	1.9
Arg	13.2	3.9	4.5
<i>N</i> -Terminal amino acid	Blocked (?)	Gly	Gly/Lys

Table 3. *Determination of P_i content of phosphoprotein B₂*

	Protein (μg)	Phosphate	
		(nmol)	(%, w/w)
Non-histone chromatin proteins	760	37.5	0.46
Phosphoprotein B ₂	320	10.0	0.30

1977) that most of the phosphate in phosphoprotein B₂ was covalently linked to serine residues, and a minor amount of phosphothreonine was also detectable.

Discussion

A simple method to isolate the specific phosphoprotein B₂ from the phenol-soluble non-histone chromatin proteins has been described. The procedure is easy to operate and the result is highly reproducible. It involves only two steps, CM-cellulose chromatography and SDS/polyacrylamide (10%)-gel electrophoresis. CM-cellulose chromatography at high pH provided an effective way to purify this protein. As revealed by two-dimensional gel electrophoresis, CM-cellulose chromatography removed over 90% of the non-histone chromatin proteins, which were devoid of the specific phosphoprotein B₂. Radioautography of the bound and unbound ³²P-labelled non-histone chromatin proteins on two-dimensional gels also confirmed that there was no cross-contamination (Fig. 4). About 2–4 µg of the purified phosphoprotein B₂ could be obtained from 1 g of rat liver tissue (Table 1). The purified phosphoprotein B₂ was shown to be homogeneous by one-dimensional SDS/polyacrylamide-gel electrophoresis (Fig. 5b). However, five distinct subfractions were revealed by two-dimensional polyacrylamide-gel electrophoresis, which may reflect varying degrees of phosphorylation. The heterogeneity and extent of phosphorylation of each phosphoprotein B₂ subfraction remains to be investigated. The heterogeneity of the purified chromatin fractions of the high-mobility-group (HMG) proteins was also observed by Johns and his co-workers (Goodwin *et al.*, 1976, 1977; Walker *et al.*, 1976). They found that protein HMG2 could be resolved into four fractions by isoelectrofocusing, whereas protein HMG1 exhibited a complex pattern. The multiple spots of protein that focused in the alkaline region of the isoelectrofocusing gel are probably not due to modifications *in vitro* arising from interactions between the Ampholines and the protein molecules (e.g. Gineitis *et al.*, 1978). As shown in Fig. 4, only certain proteins (e.g. B₂) exhibited multiple spots, whereas others did not; it follows that this electrophoretic heterogeneity was not a general artefact. Furthermore, we reported (Suria & Liew, 1979) that heterogeneous ribonucleoprotein particles solubilized directly in urea sample buffer without being subjected to the phenol-extraction procedure revealed an identical pattern of multiple spots. An alternative explanation might be that modification of proteins is due to phosphorylation (or ADP-ribosylation *in vivo*). It has been shown that phosphorylation of proteins modifies the charge of the protein molecules and that the various charged species are resolved as multiple spots on the iso-

electrofocusing gel. A phosphoprotein identified by MacGillivray *et al.* (1978), using salt extraction, hydroxyapatite chromatography and two-dimensional polyacrylamide-gel electrophoresis, was observed to be very similar to our findings in terms of molecular weight and isoelectric points. This finding lends further support to the fact that this phosphoprotein has isoelectrophoretic heterogeneity.

The specific phosphoprotein B₂ was shown to be associated with the chromatin subunits of the rat liver (Chan & Liew, 1977). A similar result was also observed by Bohm *et al.* (1977), who reported on the presence of a phosphoprotein with *M_r* 70000 in the chromatin subunits of ascites cells. There are only a few non-histone chromatin proteins that have been identified and characterized in chromatin subunits, e.g. HMG proteins by Goodwin *et al.* (1977), A-24 protein by Busch and his associates (Goldknopf *et al.*, 1977), a specific protein kinase by Simpson (1978) and DNA polymerase β by Schlaeger *et al.* (1978). It is also noteworthy that phosphoprotein B₂ is not likely to be one of the subunits of RNA polymerase (Seifart *et al.*, 1972; Keding *et al.*, 1974; Goldberg *et al.*, 1977), since none of the subunit species of RNA polymerase from rat liver have the same molecular-weight range as this phosphoprotein [e.g. molecular weights of rat liver RNA polymerase I subunits (Golberg *et al.*, 1977) are 205000, 125000, 51000, 44000, 26000 and 16000]. Busch and his co-workers isolated C-14 protein with *M_r* 70000 from Novikoff-hepatoma ascites. This protein, however, did not incorporate ³²P_i (Gordon *et al.*, 1977). It was also found that phosphoprotein B₂ is not one of the 40S-ribosomal-subunit proteins (Karn *et al.*, 1977; Beyer *et al.*, 1977). The amount of phosphoprotein B₂ in the chromatin subunits as compared with histone is very low. This suggests that phosphoprotein B₂ may not be an essential element of nucleosome structure, but may be one of the regulatory proteins in the chromatin of rat liver. In particular, the unusual amino acid composition of the B₂ protein, which has both high glutamic acid and aspartic acid contents and a high proportion of basic amino acids, arginine and lysine, suggests a polarization of charge which would confer both DNA-binding and histone-binding properties, as is the case for the HMG proteins (Goodwin *et al.*, 1976, 1977; Walker *et al.*, 1976).

Nuclei from 1 g of rat liver tissue contained 1.5 mg of DNA, which corresponded to about 11.5 nmol of chromatin subunits (molecular weight of 200 base-pairs of DNA is 130000). About 0.2 mg of phenol-soluble non-histone chromatin proteins was obtained/g of liver tissue, from which about 4 µg (2% of 0.2 mg) or 0.059 nmol of phosphoprotein B₂ (*M_r* 68000) could be isolated. From this calculation, it was estimated that at least one molecule of phosphoprotein B₂ occurs per average of 200 chromatin subunits. Because phosphoprotein B₂ has been identified

in purified nucleosome monomers released by micrococcal-nuclease digestion (Chan & Liew, 1977; Liew & Chan, 1976), this finding also suggests that the phosphoprotein B₂ is associated with a subset of chromatin subunits. The size of the eukaryotic genome is about 3×10^{12} daltons, in which only 5–10% is expressed (Rabbitts, 1975; Matthews, 1975). If the phosphoprotein is involved in gene expression, it is expected that it should be localized in the active region of the genome. The proportion of phosphoprotein B₂ in chromatin (1 per every 200 chromatin subunits) is not inconsistent with a localization in transcriptionally active regions.

In conclusion, much evidence suggested that phosphoproteins are involved in regulation of gene expression (Kleinsmith *et al.*, 1966; Teng *et al.*, 1971; Jungmann & Schweppe, 1972; Johnson & Allfrey, 1972; Liew *et al.*, 1973; Shea & Kleinsmith, 1973; Johnson *et al.*, 1974; Stein *et al.*, 1974; Kostraba *et al.*, 1975; Ezrailson *et al.*, 1976; Thomson *et al.*, 1977). In particular, it was found that RNA polymerases are subject to phosphorylation (Jungmann *et al.*, 1974). Most of the earlier work was done on a complex group of non-histone chromatin proteins, enriched in phosphoprotein content. To gain a better understanding of the mechanism and the role of phosphoprotein in gene expression of the eukaryotic cell, isolation of a specific phosphoprotein is essential. We hope that future experiments on this phosphoprotein B₂ will provide a better insight into its localization and role in the mechanism of gene expression.

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References

- Allfrey, V. G., Inoue, A., Karn, J. & Johnson, E. M. (1973) *Cold Spring Harbor Symp. Quant. Biol.* **38**, 785–805
- Bekhor, I. (1978) in *The Cell Nucleus* (Busch, H., ed.), vol. 5, pp. 137–166, Academic Press, New York
- Beyer, A. L., Christensen, M. E., Walker, B. W. & LeStourgeon, W. M. (1977) *Cell* **11**, 127–138
- Bluthmann, H. (1976) *Eur. J. Biochem.* **70**, 233–240
- Bohm, J., Keil, G. & Knippers, R. (1977) *Eur. J. Biochem.* **78**, 251–266
- Chan, P. K. & Liew, C. C. (1977) *Can. J. Biochem.* **55**, 847–855
- Chiu, J. F. & Hnilica, L. A. (1977) in *Chemistry and Function in Chromatin and Chromosome Structure* (Li, H. J. & Eckhardt, R. A., eds.), pp. 193–253, Academic Press, New York
- Ezrailson, E. G., Olson, M. O. J., Guetzow, K. A. & Busch, H. (1976) *FEBS Lett.* **62**, 69–73
- Gineitis, A. A., Anachkova, B. & Ressev, G. (1978) *Anal. Biochem.* **87**, 157–161
- Goldberg, M. I., Perriard, J. C. & Rutter, W. J. (1977) *Biochemistry* **16**, 1655–1665
- Goldknopf, I. L., Taylor, C. W., Baum, R. M., Yeoman, L. C., Olson, M. O. J., Prestayko, A. W. & Bush, H. (1975) *J. Biol. Chem.* **250**, 7182–7187
- Goldknopf, I. L., French, M. F., Musso, R. & Busch, H. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5492–5495
- Goodwin, G. H., Nicolas, R. H. & Johns, E. W. (1976) *FEBS Lett.* **64**, 412–414
- Goodwin, G. H., Woodhead, L. & Johns, E. W. (1977) *FEBS Lett.* **73**, 85–88
- Gordon, T. J., Yeoman, L. C., Matsui, S. I., Goldberg, A. H. & Busch, H. (1977) *Biochemistry* **16**, 2384–2389
- Gornall, A. G. & Liew, C. C. (1974) *Adv. Enzyme Regul.* **12**, 267–285
- Herrick, G. & Alberts, B. (1976) *J. Biol. Chem.* **251**, 2124–2132
- Jackowski, G., Suria, D. & Liew, C. C. (1976) *Can. J. Biochem.* **54**, 9–14
- Johnson, E. M. & Allfrey, V. G. (1972) *Arch. Biochem. Biophys.* **152**, 786–794
- Johnson, E. M., Karn, J. & Allfrey, V. G. (1974) *J. Biol. Chem.* **249**, 4990–4999
- Jungmann, R. A. & Russell, D. H. (1977) *Life Sci.* **20**, 1787–1798
- Jungmann, R. A. & Schweppe, J. S. (1972) *J. Biol. Chem.* **247**, 5535–5542
- Jungmann, R. A., Hiestand, P. C. & Schweppe, J. S. (1974) *J. Biol. Chem.* **249**, 5444–5451
- Karn, J., Vidali, G., Boffa, V. C. & Allfrey, V. G. (1977) *J. Biol. Chem.* **252**, 7307–7322
- Kedinger, C., Gissinger, F. & Chambon, P. (1974) *Eur. J. Biochem.* **44**, 421–436
- Kleinsmith, L. J. (1973) *J. Biol. Chem.* **248**, 5648–5653
- Kleinsmith, L. J., Allfrey, V. C. & Mirsky, A. E. (1966) *Science* **154**, 780–781
- Kleinsmith, L. J., Stein, J. & Stein, G. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1174–1178
- Kostraba, N. C., Montagna, R. A. & Wang, T. Y. (1975) *J. Biol. Chem.* **250**, 1548–1555
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Liew, C. C. (1979) *Recent Adv. Genet. Res.* **2**, in the press
- Liew, C. C. & Chan, P. K. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3458–3462
- Liew, C. C., Suria, D. & Gornall, A. G. (1973) *Endocrinology* **93**, 1025–1034
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- MacGillivray, A. J. & Rickwood, D. (1975) in *Biochemistry of Differentiation and Development, Biochemistry Series* (Paul, J., ed.), vol. 9, pp. 301–361, Medical and Technical Publishing Co., Oxford
- MacGillivray, A. J., Johnston, C., MacFarlane, R. & Rickwood, D. (1978) *Biochem. J.* **175**, 35–46
- Martin, J. B. & Doty, D. M. (1949) *Anal. Chem.* **21**, 965–967
- Matthews, H. R. (1975) *Nature (London)* **256**, 167–168
- Nelles, L. P. & Bamberg, J. R. (1976) *Anal. Biochem.* **73**, 522–531
- Patel, N. T. & Holoubek, V. (1974) *FEBS Lett.* **46**, 154–157
- Potuzak, H. & Wintersberger, U. (1976) *FEBS Lett.* **63**, 167–170

- Rabbitts, T. H. (1975) *Nature (London)* **257**, 182–183
- Schlaeger, E. J., Van Telgen, H. J., Klempnauer, K. H. & Knippers, R. (1978) *Eur. J. Biochem.* **84**, 95–102
- Seifart, K. H., Benecke, B. J. & Juhasz, P. P. (1972) *Arch. Biochem. Biophys.* **151**, 519–532
- Shea, M. & Kleinsmith, L. J. (1973) *Biochem. Biophys. Res. Commun.* **50**, 473–477
- Simpson, R. T. (1978) *Nucleic Acid Res.* **4**, 1109–1119
- Stein, G. S., Spelsberg, T. C. & Kleinsmith, L. J. (1974) *Science* **183**, 817–824
- Suria, D. & Liew, C. C. (1974) *Can. J. Biochem.* **52**, 1143–1153
- Suria, D. & Liew, C. C. (1979) *Can. J. Biochem.* **57**, 32–42
- Teng, C. S., Teng, C. T. & Allfrey, V. G. (1971) *J. Biol. Chem.* **246**, 3597–3609
- Thomson, J., Stein, J. L., Kleinsmith, L. J. & Stein, G. S. (1977) *Science* **194**, 428–431
- Walker, J. M., Goodwin, G. H. & Johns, E. W. (1976) *Eur. J. Biochem.* **62**, 461–469
- Weiner, A. M., Platt, T. & Weber, K. (1972) *J. Biol. Chem.* **247**, 3242–3251