The Isolation and Partial Characterization of Low-Molecular-Weight Phosphorylated Component of the Non-Histone Proteins of Mouse Nuclei

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After labelling of mouse liver nuclei with [γ - ^{32}P]ATP *in vitro*, 10–20% of the radioactivity incorporated into the saline-soluble nuclear and HAP2 chromatin fractions was located in a low-molecular-weight component (component 10) with pI near 4.5 in urea. By using combinations of ion-exchange chromatography, preparative thin-layer isoelectric focusing and gel filtration, this component was isolated from both nuclear fractions. Recovery from the saline-soluble fraction was poor under conditions that allow endogenous phosphatases to be active. Component 10 was shown to be a phosphoprotein on the basis of enzyme-digestion experiments and the detection of phosphoserine and phosphothreonine. The ^{32}P radioactivity did not appear to be associated with phosphorylated basic amino acids. Its molecular weight was determined by gel chromatography and electrophoresis in sodium dodecyl sulphate/polyacrylamide gels as approx. 10000, and tryptic digestion of the reduced carboxymethylated protein in urea yielded two ^{32}P -labelled peptides. It has not been possible as yet to assign a function to component 10, though its similarity to other low-molecular-weight acidic proteins is discussed.

During the past decade much interest has centred on the control of transcription in higher organisms. The resulting evidence has indicated that, as judged by reconstruction studies carried out *in vitro*, the non-histone proteins of chromatin are associated with both the tissue-specific restriction of chromatin template activity (for review, see MacGillivray & Rickwood, 1975a) and the control of the expression of specific genes (Barrett *et al.*, 1974; Chiu *et al.*, 1975; Gilmour & Paul, 1975; Tsai *et al.*, 1976; Stein *et al.*, 1976; Park *et al.*, 1976). In particular the phosphoprotein fraction of the non-histone proteins has been implicated as playing a major role in such control processes (Kleinsmith *et al.*, 1976; Thomson *et al.*, 1977).

In this laboratory we have been interested in the identification of the nuclear proteins that regulate the expression of the genes coding for globin chains in erythropoietic cells. Our evidence to date shows

that this activity is primarily contained in a fraction of non-histone proteins, accounting for approx. 12% of the total nuclear proteins, which we have obtained by chromatography of salt/urea-dissociated chromatin on hydroxyapatite (Gilmour & MacGillivray, 1976). This fraction, designated HAP2 (Rickwood & MacGillivray, 1975), contains most of the chromatin non-histone proteins, but, like other preparations of non-histone proteins (Elgin & Bonner, 1970; Wu *et al.*, 1973, 1975; Yeoman *et al.*, 1975), has shown only quantitative tissue differences in its protein components (MacGillivray & Rickwood, 1974). These results suggest that the major polypeptides detected on electrophoresis of non-histone proteins may be normal constituents of all chromatins, e.g. enzymes or structural proteins, and that gene-regulatory proteins may be present in the nucleus in low concentrations. In fact, estimates of the number of regulatory proteins for a eukaryotic gene suggest that they may be at a concentration of 10^4 copies per nucleus or less (Linn & Riggs, 1975), i.e. probably beyond detection by many of the electrophoretic systems currently in use (see MacGillivray, 1977a).

If gene-regulatory proteins are indeed in such low concentrations in the nucleus, then it is necessary to develop procedures not only to separate them from the major constituents, but also to isolate them in sufficient purity for biochemical and biological

Abbreviations used: QAE-Sephadex, diethyl-(2-hydroxypropyl)aminoethyl-Sephadex; SDS, sodium dodecyl sulphate.

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characterization. Up to the present time a number of individual non-histone proteins have been isolated from chromatin by a variety of techniques. These include preparative polyacrylamide-gel electrophoresis of non-histone protein fractions (Knecht & Busch, 1971; MacGillivray *et al.*, 1972; Goldknopf *et al.*, 1975), ion-exchange chromatography of either whole chromatin itself (Elgin & Bonner, 1972) or of chromatin protein fractions (Patel & Holoubek, 1974; Goodwin *et al.*, 1975; Kostraba & Wang, 1975) and affinity chromatography on DNA-agarose (Bluthmann, 1976). In the present paper we describe a combination of procedures that we have adopted to fractionate groups of non-histone proteins and show their application in the isolation of a minor, but highly phosphorylated, protein (component 10) from mouse liver nuclei.

Experimental

Materials

As far as possible all chemicals were of A.R. grade and were largely obtained from British Drug Houses (Poole, Dorset, U.K.). A.R.-grade urea was prepared as an 8M stock solution, filtered through Whatman no. 1 filter paper and deionized immediately before use through a column of AG 501-X8 (D) mixed-bed resin (Bio-Rad Laboratories, Bromley, Kent, U.K.). Acrylamide, *NN'*-methylenebisacrylamide and *NNN'N'*-tetramethylethylenediamine were also purchased from BDH. Ampholine carrier ampholytes (40%, pH range 3.5–10) were obtained from LKB Instruments (South Croydon, Surrey, U.K.). Dithiothreitol, guanidine hydrochloride (type 1), deoxyribonuclease I, ribonuclease A, trypsin (diphenylcarbamoyl chloride-treated), acid phosphatase, bovine serum albumin (fraction V), ovalbumin, myoglobin, lysozyme and cytochrome *c* were obtained from Sigma (London) Chemical Co. (Kingston upon Thames, Surrey, U.K.). Blue Dextran, QAE-Sephadex A-25, Sephadex G-25, Sephadex G-50 (superfine grade) and Sepharose 6B were obtained from Pharmacia (G.B.) (London W.5, U.K.). AG 50W (X8) and Dowex 1 (X8) ion-exchange resins were obtained from Bio-Rad Laboratories. Coomassie Brilliant Blue R and Carbowax (mol.wt. 15000–20000) were purchased from G. T. Gurr (High Wycombe, Bucks., U.K.). Precoated thin layers of silica gel were obtained as either 13179 silica gel (Eastman-Kodak, Kirkby, Liverpool, U.K.) or polygram Sil G (Machery-Nagel and Co., Düren, Germany). [γ - 32 P]ATP (sp. radioactivity 9–15 Ci/mmol) was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Phenylmethanesulphonyl fluoride was purchased from Calbiochem

(Hereford, U.K.). Hydroxyapatite was prepared by the procedure of Bernardi (1971).

Methods

All procedures were carried out at 4°C unless otherwise stated. Samples of column eluates were routinely monitored for absorbance at 280nm and for 32 P radioactivity by scintillation counting as previously described (Rickwood *et al.*, 1973; MacGillivray & Rickwood, 1974). Conductivity measurements were carried out with a type CDM2 conductivity meter (Radiometer A/S, Copenhagen, Denmark). Protein concentration was determined by the method of Schaffner & Weissman (1973).

Preparation of nuclear protein fractions

Mouse liver nuclei were prepared and incubated with [γ - 32 P]ATP as described by Rickwood *et al.* (1973). The saline-soluble fraction was obtained by extraction of the incubated nuclei with 140mM-NaCl/50mM-Tris/HCl (pH 7.4)/5mM-EDTA (Rickwood & MacGillivray, 1975), after which the resulting chromatin pellet was dissolved and dissociated in 2M-NaCl/5M-urea/1mM-sodium phosphate buffer, pH 6.8. The histones and non-histone proteins were then separated by chromatography on hydroxyapatite, as described by Rickwood & MacGillivray (1975). Thus after the unretained histones had been eluted from the column with the above solution (giving fraction HAP1), most of the non-histone proteins were obtained by increasing the concentration of the phosphate buffer in the salt/urea solution to 50mM (giving fraction HAP2).

Isolation of component 10 from chromatin fraction HAP2

Ion-exchange chromatography and gel filtration. The HAP2 fraction obtained as described above was made 0.1M with respect to phenylmethanesulphonyl fluoride and then concentrated some 5-fold to approximately 2mg/ml by dialysis against Carbowax at room temperature. The proteins in the concentrate were reduced by and maintained in urea/dithiothreitol as described previously (MacGillivray & Rickwood, 1974) before chromatography on QAE-Sephadex (MacGillivray & Rickwood, 1974). The major 32 P-labelled component, which was eluted at 300mM-NaCl of the salt gradient, was retained and concentrated to a volume of 1–3ml by treatment with Carbowax before dialysis for 24h at room temperature against 6M-guanidine hydrochloride/50mM-sodium acetate buffer (pH 6.4)/1mM-dithiothreitol. The sample was then applied to a column

(80cm×2.2cm) of Sepharose 6B equilibrated and run at room temperature in the same guanidine hydrochloride-containing solution (MacGillivray & Rickwood, 1974).

Recovery of ^{32}P -labelled material from the QAE-Sephadex and Sepharose 6B columns was over 90%, and the losses incurred during the preparation of the QAE-Sephadex fraction for gel filtration were not greater than 20%.

Thin-layer isoelectric focusing and gel filtration. The concentrated fraction HAP2 (2–4ml) containing up to 5mg of protein was reduced as described above and dialysed for 24h at room temperature against 8M-urea/2.5mM-dithiothreitol. The sample was then subjected to isoelectric focusing in a thin layer of Sephadex by the procedure of Radola (1973), as used in this laboratory (MacGillivray & Rickwood, 1975b; MacGillivray, 1976). Briefly, a layer (approximately 1mm thick) of Sephadex G-50 (superfine grade) previously swollen in 8M-urea/1mM-dithiothreitol containing 1% (w/v) Ampholines (pH 3.5–10) was prepared on a glass plate (20cm×20cm). A section (18cm×1cm) of the partially dried layer was removed and replaced by a slurry consisting of Sephadex G-50 (superfine grade) mixed with the HAP2 sample that had been made 1% (w/v) with respect to the pH 3.5–10 Ampholines. Focusing was accomplished at 10°C under conditions of constant voltage, i.e. 7.5V/cm for 16h followed by 15V/cm for 2h. After the layer had reached room temperature the pH gradient was determined at points along the gel by using a probe pH electrode (Russell pH, Auchtermuchty, Fife, Scotland, U.K.). A transfer print of the layer was then obtained by placing a 20cm×3cm strip of Whatman 3MM paper along the middle of the plate in the direction of the pH gradient, and after the strip had become moist it was dried and stained with Coomassie Blue by the procedure of Radola (1973). ^{32}P radioactivity on the strip was detected by using an Actigraph III strip Scanner (Nuclear-Chicago Corp., Des Plaines, IL, U.S.A.). Protein was recovered from sections of the gradient by scraping the Sephadex from appropriate areas of the plate and incubating it for at least 3h at 37°C in 10–20ml of 6M-guanidine hydrochloride/300mM-Tris/HCl (pH 8.3)/10mM-dithiothreitol. The Sephadex was then removed by centrifugation for 15min at 1000g (r_{av} , 18cm) or by filtration. Under these conditions recovery of ^{32}P -labelled material from the thin layer was always greater than 85%. The resulting extract was then concentrated to a volume of 1–3ml by treatment with Carbowax, dialysed for 24h at room temperature against 6M-guanidine hydrochloride/50mM-sodium acetate buffer (pH 6.4)/1mM-dithiothreitol and then applied to a gel-filtration column of Sepharose 6B as described above.

Isolation of component 10 from saline-soluble nuclear extract

Component 10 was also isolated from the saline-soluble protein fraction by first passing a concentrated, reduced extract through Sepharose 6B as described above. The resulting low-molecular-weight peak containing component 10 was then applied either to thin-layer focusing or to a column of QAE-Sephadex by the procedures described above. In the case of the first combination of these techniques the recovery of ^{32}P -labelled component 10 was both variable and low (5–40%), a feature associated with marked losses of radioactivity during the concentration and analysis of the gel-filtration column fraction before the isoelectric-focusing step. In contrast, no large losses of ^{32}P -labelled material were observed when component 10 was prepared by gel filtration and ion-exchange chromatography.

Analysis of component 10

Enzyme digestions. Component 10 was prepared both from mouse liver chromatin HAP2 fraction by the ion-exchange-chromatography/gel-filtration procedures and from the saline-soluble nuclear extract by the combination of gel filtration and isoelectric focusing. Each preparation was dialysed extensively against water and freeze-dried in batches of volume 2–3ml each. For each enzyme digestion a freeze-dried sample was dissolved in a small volume of buffer and 0.5ml samples containing 3000–10000 c.p.m. were then mixed with 0.5ml of the same buffer containing 50μg of the enzyme. Controls consisted of a second 0.5ml portion diluted with an equal volume of buffer and incubated under the same conditions as the enzyme digests. After digestion, enzyme-treated and control samples were dialysed against the digestion buffer and the total radioactivity in each bag was determined.

In some experiments digestion was stopped by mixing each sample with an equal volume of 8M-urea/2% (w/v) SDS/2% (v/v) 2-mercaptoethanol. Each sample was then dialysed at room temperature against 8M-urea/1% (w/v) SDS/1% 2-mercaptoethanol/10mM-sodium phosphate buffer, pH 7, before electrophoresis in one-dimensional SDS/polyacrylamide gels by the procedure of Rickwood *et al.* (1973). The distribution of ^{32}P radioactivity was determined after sectioning each gel (Rickwood *et al.*, 1973).

Sensitivity to hydroxylamine and succinic acid (Sokol & Clark, 1973). The presence of acyl phosphates and phosphohistidine was examined in samples of component 10 prepared from chromatin HAP2 fraction by ion-exchange chromatography and gel filtration.

A freeze-dried batch of component 10 was dissolved in 100mM-sodium acetate buffer, pH 5.4, and a portion (0.5ml) was then mixed with an equal volume of 1.6M-hydroxylamine. As a control a second 0.5ml sample was mixed with 0.5ml of 1.28M-NaCl. After 10min at room temperature each sample was dialysed for 24h against the initial buffer before the radioactivity in each dialysis bag was determined.

For treatment with succinic acid a second freeze-dried batch was dissolved in water, a sample (0.5ml) mixed with an equal volume of 2M-succinic acid, pH 5.2, and a further 0.5ml was diluted to 1ml with water to act as a control. After incubation at 37°C for 30min each incubation was dialysed against water and the radioactivity determined.

Identification of phosphorylated amino acids. Preparations of component 10 obtained as described above were dialysed against three changes of 10mM-Tris/HCl, pH 8 for a total period of 48h, and finally against 1mM-Tris/HCl, pH 8, for 2h at room temperature. Samples were treated as follows.

(a) Phosphoserine and phosphothreonine (Schaffner, 1967). A portion (2ml) of the solution containing component 10 was mixed with an equal volume of 2M-HCl and hydrolysed in a sealed evacuated glass tube for 18h at 100°C. After concentration by rotary evaporation to approx. 1ml, 10 μ mol each of synthetic phosphoserine and phosphothreonine (Sigma) were added and the sample was applied to a column (40cm $\times$ 1cm) of AG 50W (X8) equilibrated with 50mM-HCl. Elution was then carried out at room temperature with the same solution, and samples of the fractions collected were assayed for ³²P radioactivity by scintillation counting and for amino acids by the ninhydrin reaction (Spackman *et al.*, 1958).

(b) Phosphorylated basic amino acids (Walinder, 1968). A second 2ml portion was mixed with an equal volume of 6M-KOH and hydrolysed in a sealed evacuated glass tube for 3h at 100°C. The hydrolysate was then diluted with 3vol. of water, followed by the addition of 10 μ mol each of phosphoarginine and phosphohistidine, synthesized by the procedures of Marcus & Morrison (1964) and Zetterqvist & Engstrom (1967) respectively. The solution was applied to a column (40cm $\times$ 1cm) of Dowex 1 (X 8; bicarbonate form), after which elution was carried out at room temperature with a linear gradient (total volume 500ml) of 200–800mM-KHCO₃. Samples of the fractions collected were used for the determination of ³²P radioactivity and amino acid content as described above.

Phosphopeptide 'maps'. Samples of component 10 in 6M-guanidine hydrochloride/300mM-Tris/HCl, pH 8.3, were reduced with dithiothreitol (Konigsberg, 1972) and carboxymethylated by the procedure

of Hirs (1967). The samples were then dialysed at room temperature for 24h against several changes of 8M-urea/0.1% (v/v) thiodiglycol/100mM-Tris/HCl, pH 8. Thereafter digestion with trypsin was carried out as described by Detoma & Westley (1970). Each sample was diluted with 3vol. of 100mM-Tris/HCl (pH 8)/0.1% (v/v) thiodiglycol, and 40 μ l of 10mM-HCl containing 40 μ g of trypsin was then added. After incubation at 37°C for 4h a further 40 μ g of trypsin was added and digestion continued for 18h. The trypsin digest was then applied to a column (58cm $\times$ 1.6cm) of Sephadex G-25 equilibrated at room temperature with 100mM-NH₄-HCO₃/10mM-2-mercaptoethanol. Elution with this solution led to the appearance of two ³²P-labelled peaks, the later eluted of which contained most of the radioactivity applied to the column. The fractions corresponding to the latter were combined, freeze-dried and extracted twice with 100mM-acetic acid by dissolution and freeze-drying. The final product was dissolved in 25–50 μ l of pyridine/acetic acid/water (25:1:225, by vol.), pH 6.5, and 10–15 μ l samples were applied near one corner of a silica gel thin-layer plate (20cm $\times$ 20cm), which had been previously sprayed with the pyridine/acetic acid/water mixture. Electrophoresis was then carried out at 10°C towards the anode at 500V (constant) for 4h, after which the plate was air dried and then subjected to ascending chromatography at room temperature at right angles to the direction of electrophoresis by using butanol/pyridine/acetic acid/water (15:10:3:12, by vol.). Chromatography was discontinued after the solvent front had migrated some 15cm, the plate air dried and ³²P radioactivity was located by radioautography by using KodirEx X-ray film.

Molecular weight. Preparations of component 10 were dialysed against water and freeze-dried before being dissolved in 8M-urea/1% (w/v) SDS/1% (v/v) 2-mercaptoethanol/10mM-sodium phosphate, pH 7, containing a mixture of proteins of known molecular weight (albumin, ovalbumin, trypsin, ribonuclease, myoglobin, lysozyme, cytochrome *c* and insulin). The samples were treated at 37°C, the SDS concentration was decreased to 0.1% (w/v) and 50–100 μ l containing 5000–10000 c.p.m. and 25 μ g of each of the marker proteins was applied to SDS/polyacrylamide gels and electrophoresed as described by Rickwood *et al.* (1973). The gels were stained with Naphthalene Black, scanned and the presence of ³²P radioactivity was determined in sections of each gel by scintillation counting (Rickwood *et al.*, 1973).

Alternatively, the freeze-dried preparation, containing 100000–300000 c.p.m. was dissolved in 1ml of 6M-guanidine hydrochloride/500mM-sodium acetate buffer (pH 6.4)/20mM-dithiothreitol containing 2mg of Blue Dextran, 5mg each of albumin, ovalbumin, trypsin, myoglobin, lysozyme and insulin,

and 1mg of tryptophan. This solution was then applied to a column of Sepharose 6B equilibrated and run in 6M-guanidine hydrochloride/1mM-dithiothreitol, pH 6.4, as described above. Each fraction was monitored for protein (A_{280}) and ^{32}P radioactivity (scintillation counting) (Rickwood *et al.*, 1973; MacGillivray & Rickwood, 1974).

Two-dimensional gels. Samples of component 10 in 8M-urea/2.5mM-dithiothreitol were applied to two-dimensional polyacrylamide gels (isoelectric focusing followed by electrophoresis in the presence of SDS) by the procedure of MacGillivray & Rickwood (1974).

Results

Isolation of component 10 from chromatin

We have previously shown that after labelling mouse nuclei with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and fractionation of the salt/urea-dissociated chromatin on hydroxyapatite, component 10 is eluted with most of the non-histone proteins in 50mM-sodium phosphate, pH 6.8 (Rickwood *et al.*, 1973; MacGillivray & Rickwood, 1974; Rickwood & MacGillivray, 1975). When this fraction, designated HAP2, is chromatographed on QAE-Sephadex at pH 8.3 (MacGillivray & Rickwood, 1974) a major ^{32}P -labelled fraction,

Fig. 1. Preparation of component 10 from the HAP2 chromatin fraction of mouse liver nuclei

(a) Chromatography of 14ml of a HAP2 protein preparation on a column (45cm $\times$ 1.6cm) of QAE-Sephadex A25 equilibrated in 5M-urea/0.5mM-MgCl₂/1mM-dithiothreitol/1mM-EDTA/10mM-Tris/HCl, pH 8.3. Before chromatography the proteins were concentrated and reduced as described in the Experimental section. The large arrow indicates the start of the salt gradient of total volume 200ml from 10mM-NaCl to 600mM-NaCl, both prepared in the above buffer solution. The column eluate was collected in 2ml fractions, each of which was monitored for A_{280} (—), ^{32}P radioactivity in 0.2ml samples (●) and salt concentration by conductivity (○). Fractions 110–120 eluted at 300mM-NaCl were combined as indicated by the double-headed arrow, concentrated and dialysed against 6M-guanidine hydrochloride/50mM-sodium acetate (pH 6.4)/1mM-dithiothreitol. (b) Isoelectric focusing of 3.8ml of a HAP2 protein on a thin layer of Sephadex G-50 (superfine grade). The proteins were concentrated and reduced as described in the Experimental section. Symbols: ○, pH gradient that formed after 18h; ●, distribution of ^{32}P radioactivity shown as a recorder trace of a scan of a transfer print of the layer taken along the line of the pH gradient. The area of the layer corresponding to pH 3.5–6 as indicated (double-headed arrow) was recovered from the plate and the proteins were extracted from the Sephadex by 6M-guanidine hydrochloride/300mM-Tris/HCl (pH 8.3)/10mM-dithiothreitol. (c) Gel filtration of 1.3ml of the concentrate obtained in (a) on a column (80cm $\times$ 2.2cm) of Sepharose 6B equilibrated in and eluted with 6M-guanidine hydrochloride/50mM-sodium acetate (pH 6.4)/1mM-dithiothreitol. Fractions (3ml) were collected and 0.2ml samples monitored for ^{32}P radioactivity (●). Component 10 was eluted in fractions 70–76, which were retained and combined as indicated (double-headed arrow). (d) Gel filtration of 3.4ml of a concentrate of the material prepared in (b). The conditions of chromatography were as described in (c). Component 10 was eluted in fractions 65–73, which were retained and combined as indicated (double-headed arrow).

which contains component 10, is eluted at 300 mM NaCl in the salt gradient (Fig. 1a).

Alternatively isoelectric focusing of fraction HAP2 on a thin layer of Sephadex G-50 has been shown to give an initial separation of non-histone proteins (MacGillivray & Rickwood, 1975b). Fig. 1(b) illustrates the distribution of radioactivity in a pH gradient after focusing of ^{32}P -labelled HAP2 proteins. Under these conditions component 10 is the major ^{32}P -labelled component of the material focusing between pH 3.5 and 6 (MacGillivray & Rickwood, 1975b).

When the fractions containing component 10 obtained as described above were applied to a column of Sepharose 6B equilibrated with 6 M guanidine hydrochloride/1 mM-dithiothreitol, pH 6.5, most of the ^{32}P radioactivity was eluted as a well-defined peak of low molecular weight (Figs. 1c and 1d).

Figs. 2(a) and 2(b) show the result of two-dimensional gel electrophoresis of component 10 isolated by these procedures. The radioautogram shows that of the original HAP2 fraction (Fig. 2a) the low-molecular-weight component obtained from the gel-filtration step consists only of component 10 together with traces of more basic components with the same mobility in the presence of SDS. Similar results were obtained from preparations of component 10 isolated by using either ion-exchange chromatography or thin-layer isoelectric focusing as the first step in the fractionation of the HAP2 chromatin fraction.

Isolation of component 10 from the nuclear saline-soluble fraction

Analysis of the proteins extracted from mouse liver nuclei by 140 mM NaCl/50 mM-Tris/HCl (pH 7.4)/

1 mM-EDTA after incubation with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ has shown that component 10 is also a major phosphorylated constituent of this nuclear fraction (MacGillivray & Rickwood, 1974; Rickwood & MacGillivray, 1975). The techniques used to isolate component 10 from the saline-soluble fraction were similar to those described for its isolation from the HAP2 fraction proteins as described above. Thus the combination of gel filtration and thin-layer isoelectric focusing yielded two ill-defined peaks focusing between pH 5.5 and 7. Two-dimensional gel electrophoresis showed that the more acidic subfraction (subfraction 1) contained radioactivity corresponding to only component 10 of the original sample, whereas the more basic subfraction (subfraction 2) also contained traces of the minor components observed in preparations of component 10 obtained from HAP2 fraction proteins (Fig. 2b). By using gel filtration and ion-exchange chromatography, component 10 was eluted from QAE-Sephadex as a sharp band of radioactivity at 300 mM of the NaCl gradient. Two-dimensional gel electrophoresis showed that the radioactivity in this preparation corresponded only to component 10 of the original sample (results not shown).

Analysis of component 10

Enzymic and chemical analysis. Samples of component 10 were subjected to digestion by a number of enzymes and were also treated with hydroxylamine and succinic acid to detect acyl phosphates and phosphohistidine (Sokol & Clark, 1973). As judged by the effect on both total radioactivity (Table 1) and on that migrating in SDS/polyacrylamide gels (Fig.

Fig. 2. Two-dimensional gel electrophoresis

Reproductions of two-dimensional polyacrylamide gels of a preparation of component 10 and the HAP2 chromatin fraction from which it was obtained are shown. The upper horizontal axis represents isoelectric focusing in the first dimension, the pH gradient being indicated as shown in (a). The vertical axis shows separation in the second dimension by using gel electrophoresis in SDS, the position of marker proteins of known molecular weight ($\times 10^{-3}$) also being shown in (a). Proteins stained with Coomassie Blue are shown as lines or spots, and the position of ^{32}P -labelled polypeptides detected by radioautography is given by grains. (a) Total HAP2 chromatin fraction; (b) component 10 isolated from HAP2 fraction by ion-exchange chromatography/gel filtration (Figs. 1a and 1c).

Table 1. Sensitivity of component 10 to enzymes and chemical reagents

Component 10 was prepared from the saline-soluble nuclear fraction and dissolved in buffer as described in the Experimental section. Each sample was treated with enzymes or reagents as outlined below, after which each digest and its control (without enzyme or reagent) was dialysed against the original buffer. The radioactivity remaining in the dialysis bag is given as a percentage of that of the appropriate control.

Enzyme or reagent	Buffer	Time	Temperature (°C)	Residual radioactivity (%)
Ribonuclease A	50mM-Tris/HCl, pH 7.5	2h	37	90
Deoxyribonuclease I	50mM-Tris/HCl, pH 7.5, containing 5mM-MgCl ₂	2h	37	98
Acid phosphatase	50mM-Sodium acetate, pH 5	24h	37	36
Trypsin	50mM-Tris/HCl, pH 8.1	24h	37	29
Hydroxylamine	50mM-sodium acetate, pH 5.4	10min	22	93
Succinic acid		30min	37	103

3), only trypsin and acid phosphatase caused a significant decrease in the amount of ³²P radioactivity associated with component 10. Similar results were obtained with component 10 isolated from both HAP2 and saline-soluble fractions. Confirmation that component 10 was a phosphoprotein was obtained by the detection of [³²P]phosphoserine and [³²P]phosphothreonine after partial acid hydrolysis of a purified preparation (Fig. 4). When component 10 was prepared at pH 6.4–8.3 by using ion-exchange chromatography on QAE-Sephadex in combination with gel filtration, no evidence was found for the presence of [³²P]phosphoarginine or [³²P]phosphohistidine (Fig. 5b).

Phosphopeptide 'maps'. Preliminary experiments showed that no phosphorylated products were obtained if freeze-dried preparations of component 10 were dissolved in 10mM-NH₄HCO₃ and then subjected to digestion by trypsin. Hence, to obtain reproducible results it was found necessary to reduce

Fig. 3. SDS/polyacrylamide-gel electrophoresis of component 10 after enzyme treatment

Component 10 was isolated from the chromatins HAP2 fraction (Figs. 1b and 1d) and digested with enzymes as described in Table 1. After digestion each sample was treated with 8M-urea/1% (w/v) SDS/1% (v/v) 2-mercaptoethanol/10mM-sodium phosphate, pH 7, and the samples were applied to SDS/polyacrylamide gels as described in the Experimental section. The distribution of ³²P radioactivity was determined after each gel had been cut into 2mm sections as described by Rickwood *et al.* (1973). (a) Control for phosphatase digest (24h in acetate buffer, pH 5, at 37°C), (b) acid phosphatase digest, (c) trypsin digest, (d) deoxyribonuclease digest.

and carboxymethylate the protein before digestion with trypsin in 2M-urea by the procedure of Detoma & Westley (1970). Subsequent gel filtration of the digest through Sephadex G-25 showed that in comparison with undigested material, most of the radioactivity was retarded by the column (Figs. 5a and 5b), indicating some degree of cleavage by the

Fig. 4. Analysis of component 10 for phosphorylated amino acids

Component 10 was isolated from the chromatin HAP2 or saline-soluble nuclear fraction by combinations of gel filtration and ion-exchange chromatography at pH 6.4–8.3 as described in Figs. 1(a), 1(c) and in the Experimental section. Samples were hydrolysed and phosphorylated hydroxy and basic amino acids were detected as described in the Experimental section. (a) Chromatography of an acid hydrolysate of component 10 on a column (40m×1cm) of AG50W (X8) equilibrated and eluted with 0.05M-HCl is shown. Synthetic phosphoserine and phosphothreonine were added to the hydrolysate before chromatography. Fractions (2ml) were collected and portions (0.5ml) were taken from each for the determination of amino acids by ninhydrin (Δ , A_{570}) and of ^{32}P radioactivity by scintillation counting ($\bullet$). (b) Chromatography of an alkaline hydrolysate of component 10 on a column (40cm×1cm) of Dowex 1 (X8; bicarbonate form). Synthetic phosphoarginine and phosphohistidine were added to the hydrolysate before chromatography. The arrow marks the start of a gradient of total volume 500ml between 200mM- and 800mM- KHCO_3 . Fractions (5ml) were collected and samples (1ml) were taken from each for the determination of amino acids by ninhydrin (Δ), ^{32}P radioactivity by scintillation counting ($\bullet$) and conductivity ($\circ$).

enzyme. Radioautography of the resulting peptide map showed the presence of two major ^{32}P -containing peptides (Fig. 6a). Similar phosphopeptide 'maps'

Fig. 5. Gel filtration of tryptic digest of component 10. Component 10 was isolated from the HAP2 chromatin fraction as described in Figs. 1(b) and 1(d) and was digested with trypsin as described in the Experimental section. Gel filtration was carried out on a column (58cm×1.6cm) of Sephadex G-25 equilibrated and eluted with 100mM- NH_4HCO_3 /10mM-2-mercaptoethanol. Fractions (2ml) were collected and the ^{32}P radioactivity of samples (0.2ml) was determined by scintillation counting ($\bullet$). (a) Undigested sample of component 10; (b) tryptic digest of component 10. Arrow (1) indicates the elution volume of Dextran Blue and arrow (2) shows the elution position of urea and buffer salts etc. as determined by refractive-index measurements. Fractions 34–42, corresponding to the ^{32}P radioactivity included by the gel, were combined and freeze-dried.

were given by digests of component 10 irrespective of whether it was obtained from the HAP2 chromatin fraction by the procedures described in Figs. 1(a) and 1(c) and Figs. 1(b) and 1(d) or from the saline-soluble extract as subfractions 1 and 2, as described in the Experimental section (see Figs. 6a, 6b and 6c).

Determination of molecular weight. Purified component 10 was electrophoresed in the presence of marker proteins on SDS/polyacrylamide gels. The mobility of the peak of ^{32}P radioactivity occurred just beyond the lower limit of accuracy of the calibration curve and marginally ahead of the position

Fig. 6. Phosphopeptide 'maps' of component 10

Component 10 was prepared from the HAP2 chromatin and saline-soluble nuclear fractions as described in Figs. 1(b) and 1(d) and in the Experimental section respectively. Samples were digested with trypsin and desalted by using Sephadex G-25 as described in Fig. 5(b). The ^{32}P -labelled fractions corresponding to the peptide material were freeze-dried and taken up in pyridine/acetic acid/water, pH 6.5. Samples were then subjected to electrophoresis and chromatography on silica-gel thin-layer plates before radioautography as described in the Experimental section. The application zone is shown in the bottom right-hand corner of each plate, the direction of electrophoresis being represented by the horizontal axis, and that of chromatography by the vertical axis. ^{32}P radioactivity is illustrated by grains. S.F. represents the position of the solvent front after chromatography. (a) Component 10 from HAP2 chromatin fraction; (b) component 10, subfraction 1 from saline-soluble nuclear fraction; (c) component 10, subfraction 2 from saline-soluble nuclear fraction.

of cytochrome *c* (Fig. 7a), indicating a mol.wt. of approx. 11000. When component 10 was rechromatographed on Sepharose 6B run in 6M-guanidine hydrochloride in the presence of marker proteins, the K_{av} value of the peak of ^{32}P radioactivity occurred on a non-linear portion of the calibration curve (see Bryce & Crichton, 1971), the data shown in Fig. 7(b) indicating a mol.wt. of approx. 10000.

Discussion

The study of nuclear non-histone proteins has often proved to be difficult unless carried out under highly denaturing conditions (see MacGillivray, 1976). In this investigation we have chosen component 10 as a suitable constituent to isolate, since it is a minor component and, moreover, being a phosphoprotein, belongs to a class of nuclear proteins that is considered to be implicated in the control of gene expression (Kleinsmith *et al.*, 1976; Thomson *et al.*, 1977). Thus the isolation and analysis of com-

ponent 10 may give an indication of the procedures required and the problems likely to be encountered during the study of gene-regulatory proteins.

The procedures used to isolate this component are based on those that have proved satisfactory in our hands and that are also compatible with current methods for the reconstitution of chromatin (see Gilmour & MacGillivray, 1976). For example, isoelectric focusing in thin layers of Sephadex was chosen, since focusing in sucrose-stabilized liquid gradients was marred by the formation of insoluble aggregates, and, apart from SDS, the most efficient solvent for gel filtration proved to be guanidine hydrochloride. Both the chromatographic and preparative isoelectric-focusing techniques themselves gave high recoveries of ^{32}P radioactivity. The losses that did occur were presumably associated with the concentration procedure used, and in addition the low-molecular-weight of component 10 might be expected to lead to losses on dialysis. In fact a marked loss of radioactivity was found at room

Fig. 7. Determination of molecular weight of component 10

Component 10 was isolated from a saline-soluble nuclear fraction as described in the Experimental section. Samples were mixed with proteins of known molecular weight before either electrophoresis in SDS/polyacrylamide gels or gel chromatography in Sepharose 6B run in 6M-guanidine hydrochloride as described in the Experimental section. (a) SDS/polyacrylamide-gel electrophoresis: plot of mobilities of component 10 and marker proteins against $\log$ (molecular weight). Mobilities were calculated by using the position of the peak values of absorbance of stained bands (marker proteins) and of ^{32}P radioactivity (component 10) relative to that of the Bromophenol Blue dye added to each sample. (b) Gel chromatography: plot of K_{av} of component 10 and marker proteins against $\log$ (molecular weight). The K_{av} of each component was calculated by using the elution volume of the peak of A_{280} (Blue Dextran and marker proteins) and the peak of ^{32}P radioactivity (component 10). Abbreviations used: A, albumin; OA, ovalbumin; T, trypsin; M, myoglobin; L, lysozyme; C, cytochrome c; I, insulin; 10, component 10.

temperature on dialysis against 8M-urea of samples containing component 10 that had been prepared from saline-soluble nuclear extracts. The wide range of recoveries (5–40%), and the fact that such losses were not evident when similar dialysis steps took place in the cold, suggest that they result from the action of phosphatases. In this respect Kleinsmith *et*

al. (1976) have isolated a non-histone protein phosphatase from the saline-soluble proteins of calf thymus nuclei that is active in the presence of 5M-urea.

The low molecular weight and acidic nature of component 10 aids in its isolation from nuclear protein fractions. Only two separation steps are required, i.e. thin-layer isoelectric focusing followed by gel filtration, or vice versa. However, the isolation of a polypeptide from a group of components with similar characteristics would require additional purification steps, such as further isoelectric focusing on a limited pH gradient or, as in our attempts to isolate basic proteins from the chromatin HAP2 fraction, a combination of ion-exchange chromatography, isoelectric focusing and gel filtration (MacGillivray, 1977b). Chromatography on QAE-Sephadex at pH 8.3 is a suitable alternative to isoelectric focusing, since not only has the protein composition of the fractions been determined (MacGillivray & Rickwood, 1973, 1974), but in addition the method can be used when acid-labile constituents are being studied.

The component 10 isolated in the present investigation belongs to a class of nuclear proteins that can be detected only because of an apparent high degree of phosphorylation. Previous work in our laboratory has shown that component 10 is not detectable by protein-staining methods, but in terms of radioactivity it accounts for 10–20% of the ^{32}P radioactivity incorporated in both saline-soluble nuclear and HAP2 chromatin fractions (Rickwood *et al.*, 1973; MacGillivray & Rickwood, 1974; Rickwood & MacGillivray, 1975). It is of low molecular weight, gel filtration and electrophoresis in SDS indicating a value of approx. 10000, and, as judged by its position in pH gradients in polyacrylamide gels (MacGillivray & Rickwood, 1974), has a pI near 4.5. However, the latter value can only be approximate, owing to the effect of high concentrations of urea on protein pI values (Ui, 1971) and on pH measurements (Bull *et al.*, 1964; Josephson *et al.*, 1971). Its elution from hydroxyapatite by 0.05M-phosphate belies its apparent degree of phosphorylation, and in fact it can be recovered along with the basic proteins of the HAP2 fraction by elution with 10mM-phosphate buffer. It is therefore possible that it is a highly modified basic protein. The component does not appear to be an artifact of the phosphorylation procedure *in vitro*, since it has been detected after culture of Landschutz ascites cells in the presence of [^{32}P]P_i (Rickwood *et al.*, 1973), nor does it appear to be a degradation product of a larger protein, since its presence is not diminished by the addition of NaHSO₃ to the media used for the isolation of nuclei and their incubation with [γ - ^{32}P]ATP (D. Rickwood & A. J. MacGillivray, unpublished work).

The conclusion that component 10 is a phosphoprotein is based on evidence derived from treatment with enzymes and other reagents together with the identification of [^{32}P]phosphoserine and [^{32}P]phosphothreonine in digests. Its protein nature is confirmed by the fact that on centrifugation of the ^{32}P -labelled HAP2 fraction in 30% CsCl/4M-urea component 10 moves with the proteins to the top of the resulting density gradient (MacGillivray, 1977a,b). In view of the fact that non-histone proteins from rat liver nuclei have been shown to contain ^{32}P -labelled basic amino acids after labelling both *in vivo* (Chen *et al.*, 1974) and *in vitro* (Schiltz & Sekeris, 1969), it is interesting that we did not detect such modified residues in component 10. In fact we could not detect significant amounts of these phosphorylated products in alkaline digests of more highly phosphorylated non-histone proteins (fraction HAP3) that bind tightly to hydroxyapatite (C. Johnston & A. J. MacGillivray, unpublished work) and, although a species difference may seem unlikely, it is possible that other classes of chromatin non-histone proteins possess phosphorylated basic amino acids. Only two major ^{32}P -labelled peptides were observed after tryptic digestion of the reduced and carboxymethylated component, indicating that perhaps the phosphoprotein was cleaved at relatively few sites. The pattern of phosphopeptides was not altered by further treatment of the trypsin digest with chymotrypsin (A. J. MacGillivray, unpublished work). The phosphopeptide 'maps' of component 10 isolated from HAP2 fraction or nuclear saline-soluble proteins were identical, as were those from the two subfractions obtained by thin-layer isoelectric focusing of the latter. The detection of these two subfractions appears to be associated with the shallow nature of the pH gradient in the thin-layer system and two-dimensional gel electrophoresis showed that the major difference between the two subfractions concerned the presence of minor components of pI near 4.7 and 6.5 in the more basic subfraction. These minor components were found in preparations of component 10 obtained from both HAP2 fraction and saline-soluble proteins but are probably not derived from the phosphoproteins that focus between pH 6 and 7 in HAP 2 proteins (Fig. 1b and Fig. 2a), since such phosphorylated species are virtually absent from the saline-soluble extract (MacGillivray & Rickwood, 1974; Rickwood & MacGillivray, 1975). In addition the minor components are not seen on two-dimensional gel electrophoresis of the original samples of HAP2 fraction (Fig. 2a) or saline-soluble proteins (MacGillivray & Rickwood, 1974; Rickwood & MacGillivray, 1975) and they may therefore represent degradation products of component 10 produced during its isolation.

Component 10 has been observed in all the tissues that we have examined (Rickwood *et al.*, 1973;

MacGillivray & Rickwood, 1974; Rickwood & MacGillivray, 1975). Other workers have reported the presence of a similar phosphorylated component in non-histone protein preparations from liver (Liew *et al.*, 1973) and fibroblasts (Pumo *et al.*, 1975) after labelling *in vivo*, whereas Grummt & Grummt (1974) have observed that it is located only in the nucleoplasm. A phosphorylated component of similar molecular weight has also been detected in vaccinia virus, but this would appear to be a modified basic protein (Rosemond & Moss, 1973), whereas the small phosphoprotein observed to be associated with the RNA-dependent DNA polymerase of avian myeloblastosis virus appears to be quite acidic (Tsiapalis, 1977). In addition to the properties described above, component 10 appears to belong to a class of nuclear proteins that is partly soluble and partly chromatin bound (see Comings & Harris, 1976). However, other experiments carried out in our laboratory show that the chromatin-associated fraction binds strongly, since it is not removed by treatment of chromatin with 0.35M-NaCl, conditions used to extract selectively many non-histone proteins (Goodwin *et al.*, 1975).

The function of component 10 remains obscure, but it should be noted that in addition to the nucleoprotein-associated components so far described proteins with similar characteristics, some of which are phosphoproteins, have a wide cellular distribution, e.g. in saliva (Bennick, 1974), in muscle (Childers & Siegel, 1976), in adenosine triphosphatase preparations (Dowd *et al.*, 1976), in cell membranes (Gaetjens, 1976) and in soluble extracts of whole cells (Lin *et al.*, 1974; Goldstein *et al.*, 1975; Huang *et al.*, 1975). It is noteworthy that many of these proteins bind Ca^{2+} and some have been implicated in Ca^{2+} transport (Huang *et al.*, 1975), whereas others have been identified as activators of cyclic nucleotide phosphodiesterases (Wolff & Brostrom, 1974; Lin *et al.*, 1974; Stevens *et al.*, 1976), and in the case of ubiquitin as an activator of adenylate cyclase itself (Schlesinger *et al.*, 1975). However, the presence of these proteins in the cell nucleus has yet to be confirmed.

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