A chromosomal phosphoprotein is preferentially released by mild micrococcal-nuclease digestion

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[^{32}P]P_i was administered to rats (5 mCi/rat) 2 h before the isolation of liver nuclei. The isolated nuclei were subjected to mild micrococcal-nuclease digestion for 2.5, 5 and 10 min at 37°C, and the mononucleosomal fraction was subsequently isolated by sucrose-density-gradient centrifugation. The specific radioactivity of ^{32}P -labelled mononucleosomal fractions decreased with increased digestion times. A phosphorylated chromosomal protein, B₂ (M_r 68 000, pI 6.5–8.2), was demonstrated immunologically in the mononucleosomal fraction by using an antibody specific to this electrophoretically purified phosphoprotein. The incorporation of ^{32}P into this phosphoprotein, previously shown to be mainly through covalent linkage, was revealed by antibody precipitation followed by gel electrophoresis. The rate of release of acid-soluble nucleotides by micrococcal-nuclease digestion of liver nuclei from partially hepatectomized rats 16 h after operation was strikingly higher than that for sham-operated controls. After partial hepatectomy, an increase in ^{32}P incorporation into phosphoprotein in the monomer fractions specifically precipitated by this antibody was also found. This suggests that the phosphorylated non-histone chromatin protein B₂ is preferentially associated with the transcriptionally active chromatin.

The phosphorylation of both histone and non-histone chromosomal proteins has been studied for many years in order to elucidate their possible roles in cellular proliferation, growth and hormonal stimulation (Allfrey *et al.*, 1973; Liew *et al.*, 1973; Stein *et al.*, 1974; MacGillivray & Rickwood, 1974; Bradbury, 1975; Caplan *et al.*, 1978; Jungman *et al.*, 1981; Cooper & Spaulding, 1983; Cooper *et al.*, 1983; Cohen, 1983). The non-histone chromosomal proteins in particular are highly phosphorylated both *in vivo* and *in vitro* (Allfrey *et al.*, 1973; Offenbacher & Klein, 1975; Song & Adolph, 1983; Laskey, 1983). Two groups of non-histone chromosomal proteins, which co-sediment with the chromatin monomers generated by digestion with micrococcal nuclease and isolated by sucrose-density-gradient centrifugation, have been identified in our laboratory. One group of non-histone chromosomal proteins is highly phosphorylated, with M_r 68 000 and pI 6.5–8.2, and is associated with the monomers (Liew & Chan,

1976; Chan & Liew, 1977, 1979*a,b*; Zhao & Liew, 1982), and the second group of proteins is found associated with the heterogeneous ribonucleoprotein particles (Suria & Liew, 1979).

The present paper reports studies of the release of the phosphorylated chromosomal protein by micrococcal-nuclease digestion. This protein was identified immunologically in an attempt to locate it in the transcriptionally active nucleosomal fraction after partial hepatectomy (Mayfield & Bonner, 1972; Laks & Jungmann, 1980; Berkowitz & Riggs, 1981).

Materials and methods

All chemicals used were of reagent grade. Phenol was redistilled and stored at –20°C. Tris base and urea were purchased from Schwarz/Mann Co.; acrylamide, *NN'*-methylenebisacrylamide, *NNN'*-tetramethylethylenediamine and chloramine-T were from Eastman Organic Chemicals; ammonium persulphate, SDS, Coomassie Brilliant Blue R250, Bio-Rex 70, agarose and dithiothreitol were from Bio-Rad Laboratories; Ampholine carrier ampholytes (40%, pH range 3–10) were from LKB-Produkter; CM23 CM-cellu-

Abbreviation used: SDS, sodium dodecyl sulphate.

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lose was from Whatman; dimethyl sulphoxide was from Sigma Chemical Co.; Na¹²⁵I was from Amersham; micrococcal nuclease was from Boehringer Mannheim Corp.; sucrose was from Fisher Scientific Co.

Digestion of nuclei

Male Sprague-Dawley rats (180g body wt.), starved overnight, were used for all experiments. Two-thirds of the liver in the experimental group was removed by partial hepatectomy (Higgins & Anderson, 1931). Sham-operated rats were used as control. At 14h after operation, 5mCi of [³²P]P_i (carrier-free, New England Nuclear), was given to each rat intraperitoneally. Then 2h later, rat liver nuclei were isolated by centrifugation through 2.2M-sucrose in buffer A (10mM-Tris/HCl, pH 7.8, 3mM-MgCl₂ and 0.2mM-phenylmethanesulphonyl fluoride), as described previously (Liew & Chan, 1976). The purified nuclei were washed once with buffer A containing 0.25M-sucrose and then resuspended in digestion buffer, containing 10mM-Tris/HCl, pH 7.8, 0.1mM-CaCl₂ and 0.2mM-phenylmethanesulphonyl fluoride, at a final concentration of 120–130 A₂₆₀ units/ml. Nuclei were digested at 37°C with 1.4 units of micrococcal nuclease/A₂₆₀ unit (1 unit of enzyme/7.5 µg of DNA) for various times as designated in the experiments.

The digestion was terminated by placing the nuclear suspension on ice and adding EDTA (pH 7.0) to a final concentration of 2mM. The supernatant of the digested chromatin was centrifuged (Sorvall RC2B) at 12000g for 10min, and the supernatant from this spin was recovered. To ensure the maximal recovery of nucleosomes released from the digested chromatin, the pellet was suspended in 1mM-EDTA/1mM-Tris/HCl (pH 8.0)/0.1mM-phenylmethanesulphonyl fluoride and extracted at 4°C for 30min. The two supernatants were combined for chromatin fractionation.

Fractionation of digested chromatin

The combined supernatants (10–25 A₂₆₀ units/ml) were layered on linear sucrose-density gradients (5–30%, w/v) containing 0.2mM-EDTA/0.1mM-phenylmethanesulphonyl fluoride/10mM-Tris/HCl, pH 7.0. The gradients were centrifuged in a Beckman SW-27 rotors for 16h at 22000rev./min (113000g). The mono- di- and oligo-nucleosomal fractions were identified and collected separately. In some experiments the peaks of the mononucleosomal and oligonucleosomal regions (1ml/fraction) were specifically collected for identification of the phosphoprotein by radioimmunoassay. This procedure of obtaining monomer and oligomer peaks ensures that only minimal contamination resulting from neigh-

bouring regions occurs. Phosphoprotein in the mono-, di- and oligo-nucleosomal fractions was identified by precipitation with 8vol. of acetone, and the precipitate was subsequently dissolved in SDS-buffer [2.3% (w/v) SDS/5% β-mercaptoethanol/10% (v/v) glycerol/6.25mM-Tris/HCl, pH 6.8] for polyacrylamide-gel analysis as described previously (Chan & Liew, 1977).

Production of antibody to phosphoprotein

The phosphoprotein (*M_r* 68000, pI 6.5–8.2) identified in the monomer fraction was isolated by two-dimensional polyacrylamide-gel electrophoresis (Chan & Liew, 1979b). The extracted phosphoprotein was used to produce antibody by injection into a 2.5kg rabbit. The specificity and characterization of this antibody have been extensively investigated (Zhao & Liew, 1982). The concentration of phosphoprotein in both the mononucleosomal and oligonucleosomal fractions was determined by quantitative precipitation with the antibody and subsequent reaction with ¹²⁵I-labelled Protein A (Zhao & Liew, 1982).

In some experiments, ³²P-labelled mononucleosomal and oligonucleosomal fractions were made to react with antisera and subsequently precipitated with either Protein A (Zhao & Liew, 1982) or 25% poly(ethylene glycol) as described previously (Desbuquois & Aurbach, 1971). The precipitates were then dissolved in SDS-buffer, and the phosphoprotein fraction was resolved by one-dimensional SDS/polyacrylamide-gel electrophoresis.

Fractionation of non-histone chromosomal proteins and phosphoprotein B₂

Non-histone chromatin proteins of both sham-operated and partially hepatectomized liver were prepared by the phenol-buffer extraction method (Shelton & Allfrey, 1970; Suria & Liew, 1974) to ensure the complete removal of nucleic acids. Recoveries of non-histone chromosomal proteins from both regenerating liver and its control were similar. Phosphoprotein B₂ (*M_r* 68000) was identified after Coomassie Blue staining by comparison with standard protein *M_r* markers.

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Release of mononucleosomal fraction by mild micrococcal-nuclease digestion

³²P was given to rats 2h before the isolation of liver nuclei. Mononucleosomal fractions were then isolated by sucrose-density-gradient centrifugation as described in the Materials and methods section. As shown in Table 1, the specific radioactivity of

Table 1. *Specific radioactivity of ^{32}P in the nucleosomal fraction isolated by sucrose-density-gradient centrifugation* Nuclei labelled with ^{32}P *in vivo* were digested with micrococcal nuclease for 2.5, 5 or 10 min. The nuclei were lysed to obtain digested nucleosomes, followed by sucrose-density-gradient fractionation as described in the Materials and methods section. Mononucleosomes, dimers and oligonucleosomes were collected as shown in Fig. 3.

Digestion time (min)	Type of nucleosomes	Specific radioactivity (c.p.m./ A_{260} unit)
2.5	Mononucleosomes	78 085
5.0	Mononucleosomes	41 854
10.0	Mononucleosomes	31 606
10.0	Dimers	36 160
10.0	Oligonucleosomes (> 28 nucleosomes)	26 140

^{32}P in the mononucleosomal fraction was increased more than 2-fold in the 2.5 min digestion as compared with the 10 min digestion. The rapid release of high-specific-radioactivity ^{32}P in the mononucleosomal fraction as compared with the dimers or oligomers indicated that phosphorylation of chromatin was preferentially directed towards nucleosomes more accessible to nuclease digestion. We have previously identified and characterized one of the major phosphorylated non-histone chromatin proteins, B_2 , as being associated with the monomers (Liew & Chan, 1976; Chan & Liew, 1977, 1979a,b).

Production of antibody to phosphoprotein B_2

In an attempt to resolve whether the mononucleosomal fraction contained the phosphoprotein B_2 , B_2 -specific antibody was produced by the method described previously (Zhao & Liew, 1982). As shown in Fig. 1(a), this antibody was specifically co-precipitated with the highly purified phosphoprotein B_2 (well 1) and with non-histone chromosomal proteins containing phosphoprotein B_2 (i.e. wells 2 and 5).

As shown in Fig. 1(b), antigen-antibody complexes could be detected by antiserum diluted 10-fold or less as revealed by counter-immunoelectrophoresis. Antigen was quantified by allowing the antiserum-antigen complex to react with ^{125}I -labelled Protein A (Fig. 1c).

Kinetic analysis of nuclei digested by micrococcal nuclease

Several investigators (Levy & Dixon, 1978; Bloom & Anderson, 1978; Berkowitz & Riggs, 1981; Guerrero & Alonso, 1983) have demonstrated that the transcriptionally active portion of the genome was more accessible to micrococcal-nuclease digestion than was the transcriptionally inactive portion. Berkowitz & Riggs (1981) reported that the oligomers, after a very mild digestion of rat liver nuclei by micrococcal nuclease, were enriched in transcriptionally active gene sequences. As shown in Fig. 2, the percentage

of acid-soluble nucleotides released from rat liver nuclei isolated from the 16h post-operative partially hepatectomized rats and the respective sham-operated control group is proportional to the incubation time. It was also demonstrated that the highly activated chromatin of the regenerating liver was more accessible to the enzyme digestion than was that of the sham-operated control.

Identification of the phosphorylated protein in the mononucleosomal fraction after the micrococcal-nuclease digestion

Nuclei isolated from both the partially hepatectomized and sham-operated control rat livers were digested with low enzyme concentrations (1.4 units/ A_{260} unit) at 37°C for either 5 or 25 min, followed by Tris/EDTA extraction of the lysed nuclei and centrifugation. The supernatant was fractionated on sucrose density gradients as shown in Fig. 3. The 11S region, which contained the mononucleosomal fractions from both regenerating liver and sham-operated controls, was isolated and mononucleosomal fractions were precipitated by the antisera of phosphoprotein B_2 . As shown in Table 2, the specific radioactivity of the immunoprecipitated mononucleosomal fraction of liver nuclei from both control and partially hepatectomized rats, derived from the 5 min digestion was higher than that from 15 min digestion as measured by co-precipitation of the B_2 -antibody complex with ^{125}I -labelled Protein A. It was also found that the amount of phosphoprotein B_2 in the mononucleosomal fraction had increased significantly at both 5 and 15 min after digestion. This increase in the amount of phosphoprotein B_2 in the mononucleosomal fraction was revealed by SDS/polyacrylamide-gel electrophoresis (Fig. 4). In three separate experiments, the phosphoprotein- B_2 fraction was isolated by SDS/polyacrylamide-gel electrophoresis and its specific radioactivity (c.p.m./ μg of protein) was determined. As shown in Table 3, a highly significant ($P < 0.005$) increase of 98% over the sham-operated control was found.

Fig. 1. Identification of specific antibody produced against a phosphoprotein

An antibody was produced against a phosphoprotein as described previously (Zhao & Liew, 1982). Antiserum was made to react with several fractions of non-histone chromatin proteins, containing 20 μg each, as shown in (a): (1) represents phosphoprotein B₂, (2) the chromatin proteins that bound to the CM-cellulose column, (3) the chromatin proteins not bound to the CM-cellulose column, (4) a protein isolated from the heterogeneous-ribonucleoprotein particles, and (5) proteins isolated from the tightly bound chromatin complex (DNA-P) (Norman & Bekhor, 1981). (b) Counter-immunoelectrophoresis: control serum (C) and antiserum (Ab) which was

Fig. 2. Release of acid-soluble nucleotides from isolated nuclei after digestion with micrococcal nuclease for different times

Isolated nuclei (containing 200 A_{260} units) were suspended in 1.5 ml of 10 mM-Tris/HCl/0.1 mM- CaCl_2 /0.2 mM-phenylmethanesulphonyl fluoride, pH 8.0, and subsequently incubated with micrococcal nuclease (1.4 units/ A_{260} unit) at 37°C for 2.5–15 min. A sample of nuclear suspension was taken, and digestion arrested with 10% HClO_4 . After low-speed centrifugation, the A_{260} of the supernatant (acid-soluble nucleotides) was read. The total nucleic acids of the nuclear suspension were then obtained by acid hydrolysis at 75°C for 15 min, cooled, and the A_{260} was again read. The percentage of acid-soluble nucleotides released from the nuclei during the incubation was determined. R, regenerating rat liver nuclei; C, their respective control.

Fig. 3. Fractionation of nucleosomes on sucrose density gradients

Isolated nuclei were digested with micrococcal nuclease (1.4 units/ A_{260} unit) for 5 min (----) or 15 min (—) at 37°C. Mono-, di and oligo-nucleosome fractions were collected as indicated by bars.

diluted 1:1, 1:2, 1:4, 1:10 or 1:16, and made to react with 20 μg of phosphoprotein B₂ (Ag: antigen). (c) Excess of antiserum (■) or normal serum (□) was made to react with different amounts of phosphoprotein B₂ and subsequently with ^{125}I -labelled Protein A to determine antibody specificity (Zhao & Liew, 1982).

Table 2. Quantitative analysis of phosphoprotein B₂ in monomers as determined by immunoprecipitation

Equal amounts of monomer fractions derived from 5 and 15 min micrococcal-nuclease digestion and isolated by 5–30% sucrose-density-gradient centrifugation were precipitated by antisera to phosphoprotein B₂ and subsequently made to react with ¹²⁵I-labelled Protein A.

Digestion time (min) . . .	Radioactivity (c.p.m./A ₂₆₀ unit)	
	5	15
Sham-operated control	439	316
Partially hepatectomized	632	560
Change (%)	44	77

Fig. 4. Content of phosphoprotein in the mononucleosomal fraction

Mononucleosomal fractions were isolated from regenerating rat liver nuclei (R) and the sham-operated control (C) followed by a mild micrococcal-nuclease digestion for 5 min at 37°C. Equal amounts of mononucleosomal fractions were precipitated with 8 vol. of acetone and dissolved in SDS-buffer. The mononucleosomal proteins were fractionated by 10%-polyacrylamide-gel electrophoresis in the presence of SDS. The electrophoretic pattern was scanned at 570 nm to identify the phosphoprotein.

Table 3. Isolation of ³²P-labelled phosphoprotein by SDS/polyacrylamide-gel electrophoresis

Highly purified nuclei from three separate experiments were extracted with SDS-buffer. Equal amounts of nuclear proteins (200 µg) were analysed by SDS/polyacrylamide-gel electrophoresis. The phosphoprotein B₂ was identified after Coomassie Blue staining by comparison with its M_r standard. The radioactivity was determined by dissolving slices of gel containing phosphoprotein B₂ in 30% (v/v) H₂O₂, followed by liquid-scintillation counting. The results are means ± S.E.M. (n = 3).

	Radioactivity (c.p.m./µg)
Sham-operated control	297 ± 38
Partially hepatectomized	591 ± 56

The incorporation of ³²P into the mononucleosomal fraction was further analysed by using the antibody to precipitate the phosphoproteins. The mononucleosomal fraction was subsequently characterized by one-dimensional SDS/polyacrylamide-gel electrophoresis (Fig. 5). It was also found that the incorporation of ³²P into phosphoprotein B₂ in the regenerating liver was strikingly higher than in the sham-operated control [Fig. 5 (insert)]. It has been confirmed that the peak incorporation of ³²P into the monomer fraction was associated with phosphoprotein B₂, as analysed previously by acid hydrolysis that showed the presence primarily of phosphoserine (Chan & Liew, 1977).

Fig. 5. Analysis of immunologically precipitable proteins obtained from mononucleosomal fraction

The mononucleosomal fraction (1 A_{260} unit) derived from the micrococcal-nuclease-digested nuclei was precipitated by a phosphoprotein (B_2) antiserum. The antiserum-antigen complexes were precipitated by anti-rabbit immunoglobulin G. The precipitate was dissolved in 1% SDS buffer and analysed by SDS/10%-polyacrylamide-gel electrophoresis together with a standard protein marker gel (i.e. bovine serum albumin, M_r 67000) as described in the Materials and methods section. Insert: comparison of ^{32}P incorporation into phosphoprotein B_2 between the regenerating liver (○) and its control (□) as revealed by analysis of non-histone chromosomal protein (200 μ g) by one-dimensional SDS/polyacrylamide-gel electrophoresis. The fractions in the vicinity of the identified phosphoprotein B_2 (M_r 68000) were sliced into 1 mm-thick pieces and their radioactivity was determined. The major peak of ^{32}P incorporation (gel section 16) into nuclear proteins corresponds to the B_2 protein.

Discussion

Several investigators have reported that transcriptionally active portions of eukaryotic genomes are more accessible to micrococcal-nuclease digestion than are the inactive regions (e.g. see review by Weisbrod, 1982). It has been shown that moderate enzyme digestion preferentially released mononucleosomes enriched in transcriptionally active sequences (Levy & Dixon, 1978; Bloom & Anderson, 1978; Tsanev, 1983). Berkowitz & Riggs (1981) demonstrated that rat liver nuclei subjected to mild enzyme digestion could also yield mononucleosomes and oligonucleosomes enriched in transcriptionally active genes. Our experiments were performed by similar mild digestion of nuclei,

and we found that phosphoprotein B_2 was preferentially released with the mononucleosomal fraction. The specific radioactivity of ^{32}P in the mononucleosomal fraction was more than 2.5 times higher in the 2.5 min digestion than in the 10 min digestion. The rapid release of phosphoprotein B_2 with the mononucleosomal fraction implies that this phosphoprotein is associated with transcriptionally active genes.

Our finding was further affirmed by the following experiments, which used liver regeneration to stimulate cell proliferation and active gene transcription. We selected the 16 h post-operation period for optimum conditions to investigate the correlation between protein phosphorylation and active RNA synthesis, because two major events occur during liver regeneration: (i) maximal increases of RNA and protein synthesis at 12–18 h, and (ii) increases of DNA synthesis at 18–24 h (Mayfield & Bonner, 1972). It was found that ^{32}P incorporation into phosphoprotein B_2 in the nuclear fraction was significantly higher ($P < 0.005$) in the partially hepatectomized liver than in the sham-operated control (Table 3). Examination of the phosphorylated proteins by polyacrylamide-gel electrophoresis, both one-dimensional in the presence of SDS and two-dimensional, demonstrated a protein with M_r 68000 and pI 6.5–8.2 as the major highly phosphorylated polypeptide (Chan & Liew, 1979a,b; Liew *et al.*, 1984). It should be noted that ^{32}P incorporation also occurs in the nucleic acid and phospholipid nuclear components. To eliminate this possible contamination, we have used phenol and chloroform/methanol extraction to ensure their removal (Suria & Liew, 1974; Chan & Liew, 1977, 1979a). In addition, we used acid hydrolysis to show that most of the phosphoprotein- B_2 phosphorylation occurred on serine residues (Chan & Liew, 1977, 1979a). The specific radioactivity of this phosphoprotein in the mononucleosomal fraction obtained after nuclease digestion was increased by 44% and 77% under conditions of liver regeneration as compared with its control at 5 and 15 min respectively (Table 2). This finding suggests that monomers derived from less transcriptionally active nucleosomes were much less phosphorylated. This is again consistent with the observation that this protein is closely associated with transcriptionally active nucleosomes.

This phosphoprotein B_2 has been previously identified in the 11S fraction of chromatin from isolated nuclei (Chan & Liew, 1977), which was obtained by enzyme digestion and subsequently by sucrose-density-gradient centrifugation (Liew & Chan, 1976). Using an immunological approach, we have demonstrated that the antibody to the phosphoprotein specifically reacted with the mon-

omers, as revealed by polyacrylamide-gel electrophoresis (Zhao & Liew, 1982).

In conclusion, a phosphoprotein which is closely associated with nucleosomal structure was found to be highly phosphorylated during liver regeneration. It was found by immunoprecipitation that this phosphoprotein was a major phosphorylated non-histone chromosomal protein. The phosphoprotein is predominantly associated with transcriptionally active nucleosomes, as demonstrated by its release after limited micrococcal-nuclease digestion, thus suggesting a possible role for it in gene regulation.

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