Identification of a phosphoprotein in the nuclear matrix by monoclonal antibodies

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Previous work in this laboratory has established that a rat liver nuclear phosphoprotein (B_2 : M_r 68000, pI 6.5–8.2) is associated with actively transcribed nucleosomes, as demonstrated by its preferential release after mild treatment with micrococcal nuclease. In the present report we provide further immunological evidence ('Western Blot' analysis, solid-phase radioimmunoassay and indirect immunofluorescence) that in addition establishes the presence of this phosphoprotein in the nuclear-matrix protein fraction. This paradoxical localization suggests that this phosphoprotein may function in two separate and distinct roles within the realm of nuclear organization.

INTRODUCTION

It is becoming increasingly apparent that the chromosomal proteins [histones and non-histones (NHCP)] play a significant role in the diverse structural and functional properties of the eukaryotic genome (Scheidereit *et al.*, 1983; Bekhor, 1984; Liew *et al.*, 1984; Cote *et al.*, 1985). However, the continual search for specific regulatory proteins involved in gene expression has been centred on the NHCP class, owing to the tremendous heterogeneity of these proteins with respect to size, charge and solubility characteristics, which also rendered their investigation difficult (Teng *et al.*, 1971; Liew, 1979). Nevertheless, evidence has accumulated which suggests that those NHCP still tightly bound to DNA (approx. 5% of the total) are a group likely to be associated with specific regulatory control (Bekhor & Samal, 1977; Norman & Bekhor, 1981). In particular, those residual NHCP obtained by extracting nuclei with high salts (known alternatively as 'nuclear cage' or 'nuclear matrix') have come under close scrutiny, owing to their association with specific DNA single-copy sequences (Robinson *et al.*, 1982; O'Malley *et al.*, 1983). This nuclear protein matrix itself has been shown to be a general property of all eukaryotic cells, forming a structural matrix extending throughout the nucleus, to which chromatin appears to be attached at various points (Agutter & Richardson, 1980; Berezney, 1984). In addition, the nuclear matrix has been reported to contain high concentrations of heterogeneous nuclear RNA (Jackson *et al.*, 1981), small nuclear RNA (van Eekelen & van Venrooij, 1981) and specific steroid-binding sites (Barrack & Coffey, 1983). The nuclear matrix also appears to provide sites for DNA replication, post-translational RNA processing and intranuclear translocation (Agutter & Richardson, 1980; Berezney & Buchholtz, 1981; Ciejek *et al.*, 1982).

Previous work in our laboratory has been centred on characterization of a highly phosphorylated nuclear protein (B_2 : M_r 68000, pI 6.5–8.2) associated with mononucleosomal particles (Liew & Chan, 1976; Chan & Liew, 1977, 1979; Liew *et al.*, 1984). In the present paper we provide immunological evidence to demon-

strate that at least some form of this phosphoprotein also resides in the nuclear protein matrix. Its high degree of phosphorylation after partial hepatectomy in regenerating liver, coupled with its association with this nuclear matrix, suggest that this phosphoprotein may play a key role in some aspect of nuclear organization and/or function.

MATERIALS AND METHODS

Preparation of nuclei and nuclear-matrix protein fraction

Male albino rats (Wistar) weighing about 200 g were used in all experiments. After decapitation, livers were removed and placed in ice-cold medium A (250 mM-sucrose/3 mM-MgCl₂/10 mM-Tris/HCl/0.1 mM-phenylmethanesulphonyl fluoride, pH 8). Livers that were not used immediately were frozen in liquid N₂ and stored at –70 °C. Isolation of nuclei was then carried out as described previously (Chan & Liew, 1977).

Nuclear-matrix proteins were then isolated as previously described (Berezney & Buchholtz, 1981) with slight modification (Peter & Comings, 1980). The final nuclear pellet was resuspended at a concentration of 10⁸ nuclei/ml in TM buffer (250 mM-sucrose/5 mM-MgCl₂/50 mM-Tris/HCl/0.1 mM-phenylmethanesulphonyl fluoride, pH 7.4) and treated with DNAase I (2–5 µg/ml) and RNAase A (2–5 µg/ml) at 22 °C for 15 min. The nuclear suspension was then centrifuged at 770 g for 15 min and the resulting pellet was washed once in TM buffer. The nuclear pellet was then used in the subsequent preparation of the nuclear-matrix protein fraction. The nuclear-matrix fraction was then washed twice with TM buffer, dissolved in phenol/TEM buffer (0.1 M-Tris/HCl/0.01 M-EDTA/0.14 M-β-mercaptoethanol, pH 8.4) and solubilized as previously described (Suria & Liew, 1974).

Isoelectric focusing and two-dimensional polyacrylamide-gel electrophoresis

Nuclear-matrix proteins (350 µg) were subjected to isoelectric focusing in the first dimension (pH gradient

Abbreviations used: NHCP, non-histone chromosomal proteins; PBS, phosphate-buffered saline (0.01 M-sodium phosphate/0.14 M-NaCl, pH 7.2); DNAase, deoxyribonuclease; RNAase, ribonuclease.

3.5–10) and then subsequently fractionated by SDS/polyacrylamide-gel electrophoresis in the second dimension, as previously described (Suria & Liew, 1974; Jackowski *et al.*, 1976).

Preparation of monoclonal antibodies to B₂ phosphoprotein

Production of monoclonal antibodies (IgG₁, IgG_{2a} and IgM) to the phosphoprotein, along with verification of their specificity, was described previously (Halikowski & Liew, 1985).

Immunoblotting

Nitrocellulose transfer of nuclear-matrix proteins from two-dimensional gels and immunological detection of protein blots were carried out as described previously (Towbin *et al.*, 1979; Tsang *et al.*, 1983). Briefly, matrix proteins were first separated by the two-dimensional polyacrylamide-gel system and transferred to a Gene Screen (NEN) membrane by electrophoretic blotting. After transfer the membrane was allowed to dry in air for 30 min and was then incubated in blocking buffer (10% skim-milk powder in PBS, pH 7.2) for 1 h with gentle agitation. The membrane was then incubated with either monoclonal-antibody culture fluid (diluted 1:20) or ascites (diluted 1:500) in antibody incubation solution [10% skim-milk powder in PBS (pH 7.2)/0.3% Tween-20]. The membrane was then washed three times with PBS/0.3% Tween-20 (5 min each). The membrane was then incubated with ¹²⁵I-labelled goat anti-mouse IgG mixture (NEN) (1×10^7 c.p.m./sheet in antibody incubation solution) for 2 h. The membrane was then washed as above, dried, and exposed to Kodak X-ray film (XAR2) for 2–3 days before being developed.

Solid-phase radioimmunoassay

This was carried out as described by Goding (1983), with several modifications. The nuclear-matrix proteins were diluted to various concentrations (0.25–1 μ g) in PBS and distributed into poly(vinyl chloride) microtitre wells (Dynatech) (100 μ l/well). After overnight incubation at 4 °C, each well was emptied and then washed twice with PBS. Each well was then blocked with 5% (w/v) bovine serum albumin in PBS for at least 2 h. Each well was then treated with 100 μ l of IgG₁, IgG_{2a} or IgM, either as undiluted culture fluid or purified preparation (diluted 1:100 in PBS) for 1 h at room temperature. Wells were then washed thoroughly with 1% albumin in PBS and then treated with ¹²⁵I-labelled goat anti-mouse IgG mixture (NEN) (5×10^5 – 8×10^5 c.p.m./well). After a further 60 min incubation, each well was washed extensively with PBS and counted for radioactivity in a gamma counter (Beckman 4000).

Indirect immunofluorescence

Mouse BC3H1 muscle cells were cultured in Dulbecco's modified Eagle's medium with 10% (v/v) foetal-calf serum, 100 units of penicillin/ml, 100 μ g of streptomycin/ml and 0.25 μ g of fungizone/ml. Nuclear matrices were then prepared from cells growing on cover slips by transferring the cover slips individually through the various matrix extraction solutions (Chaly *et al.*, 1985). Final matrix preparations on cover slips were then fixed with 4% (w/v) paraformaldehyde in PBS for 30 min and rinsed in PBS (purified rat liver nuclei cytocentrifuged on to microscope slides were fixed and treated the same

Fig. 1. Solid-phase radioimmunoassay demonstrating the dependence of monoclonal-antibody binding on nuclear-matrix protein concentration

Microtitre wells were coated with a range (0.25–1 μ g) of nuclear-matrix protein diluted in PBS. Purified monoclonal IgG₁, IgG_{2a} and IgM (diluted 1:100) and ordinary culture fluid (C) were then added to the appropriate wells, followed by detection with ¹²⁵I-labelled goat anti-mouse IgGs (5×10^5 – 8×10^5 c.p.m./well).

way). The slips were then incubated with the battery of B₂ monoclonal antibodies in ascites fluid (diluted 1:100) for 30 min [normal mouse serum (diluted 1:100) was used as control] and then rinsed thoroughly in PBS. The secondary antibody fluorescein isothiocyanate-conjugated goat anti-mouse IgG+IgM (heavy and light) (Jackson ImmunoResearch; diluted 1:50) was then applied for 30 min, and again the slips were thoroughly washed with PBS. All cover slips were then mounted with 50% (v/v) glycerol/PBS and observed with a Zeiss Universal microscope (with epifluorescence illumination), and were recorded on Kodak CM-135 film.

Protein determination

This was done as described by Lowry *et al.* (1951), with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Interest has been growing in recent years on the residual nuclear protein skeleton obtained after extraction of metaphase or interphase nuclei with nucleases and high salt (termed the 'nuclear matrix') (Berezney, 1984). This matrix has been found to represent 10–20% of the total nuclear protein mass and is composed of non-histone proteins (NHCP). Further detailed analysis of rat liver nuclear-matrix polypeptides by two-dimensional polyacrylamide-gel electrophoresis has demonstrated that these proteins are primarily in the M_r range of 55000–75000 (Peter & Comings, 1980). Of these, three major polypeptide fractions, of M_r 62000–70000, appear to be associated with the pore-

Table 1. Competition binding study demonstrating the epitope specificity for each of the monoclonal antibodies raised to the B₂ phosphoprotein

Microtitre wells were coated with 0.5 µg of purified B₂ protein and then probed with each of the monoclonal antibodies either alone or in the indicated combinations. After extensive washing, the wells were incubated with ¹²⁵I-labelled goat anti-mouse IgGs (5 × 10⁵–8 × 10⁵ c.p.m./well). The results (c.p.m.) represent averages of three separate experiments.

Monoclonal antibody		Combination	
IgG ₁	10940	+ IgG _{2a}	25836
		+ IgM	23861
IgG _{2a}	10067	+ IgM	9757
IgM	5187		

complex-lamina component (Gerace & Blobel, 1982). Until now, antibodies have been raised only against this lamina component of the nuclear matrix (Gerace *et al.*, 1978; Jost & Johnson, 1981). The following results present immunological evidence demonstrating that monoclonal antibodies raised to a rat liver chromatin protein (B₂: M_r 68000, pI 6.5–8.2) also react with a component of the nuclear matrix.

After the isolation of nuclear-matrix proteins, the presence of the phosphoprotein was verified by solid-phase radioimmunoassay. By coating of micro-titre plates with different concentrations of nuclear-matrix proteins (0.25–1 µg), it was clearly demonstrated that each of the monoclonal antibodies raised to the B₂ phosphoprotein (IgG₁, IgG_{2a} and IgM, 1:100 dilution) reacted with at least some component of the matrix (Fig. 1). Both IgG₁ and IgG_{2a} react with similar binding capacity to a component of the matrix preparation. It appears that the IgM monoclonal antibody reacts less strongly, possibly owing to its specific epitope being masked or altered by some aspect of the matrix isolation procedure. As shown in Table 1, it appears that the epitope of IgM is clearly distinct from that of IgG₁ and is either identical with or in close proximity to that of IgG_{2a}. The epitopes of IgG₁ and IgG_{2a} appear to be distinct and also possess non-competitive as well as additive binding properties. We have also investigated the distribution of the phosphoprotein in the supernatant fraction released by mild micrococcal-nuclease digestion, as compared with the residual pellet fraction. A semi-quantitative estimation by solid-phase radioimmunoassay with monoclonal IgG_{2a} has shown that 56% of the B₂ phosphoprotein is associated with the supernatant fraction and 44% with the residual pellet fraction.

Further demonstration of the presence of this phosphoprotein in the nuclear-matrix protein fraction was elucidated by using the two-dimensional polyacrylamide-gel system. As shown in Fig. 2(a), the relative complexity of these proteins is by far greater than can be demonstrated by one-dimensional gel analysis. The three major lamin protein bands (a, b, c), of M_r 60000–75000, are clearly present. In addition, a protein of M_r 68000–69000, which has been previously described as one of the major components of the intranuclear matrix

Fig. 2. 'Western Blot' verification of phosphoprotein B₂ association with nuclear protein matrix

(a) Two-dimensional gel-electrophoretic analysis of rat liver nuclear proteins (350 µg) (a, b, c indicate the positions of the major nuclear-pore-complex-lamina proteins; arrow indicates the position of the B₂ phosphoprotein). (b) Autoradiograph of Gene Screen membrane after exposure to phosphoprotein B₂ monoclonal antibody IgG_{2a} (diluted 1:500) and subsequent detection with ¹²⁵I-labelled goat anti-mouse IgGs (1 × 10⁷ c.p.m./Gene Screen membrane).

(Peter & Comings, 1980), is similar to the phosphoprotein B₂. On electrophoretic transfer and subsequent probing of the Gene Screen membrane with B₂-phosphoprotein monoclonal antibody (IgG_{2a}), this protein was demonstrated to react with this antibody (Fig. 2b). Identical results were obtained with the IgG₁ monoclonal antibody (results not shown).

Traditionally the nuclear matrix has been defined as that insoluble nuclear material remaining after a harsh fractionation procedure on cellular nuclei (i.e. detergent, nucleases, hyperosmotic buffers). In particular, questions have been raised as to whether the internal nuclear-matrix component itself represents some type of artifactual protein association induced by the isolation procedure. Indirect immunofluorescence (using all three monoclonal antibodies) was performed on cultured BC3H1 mouse muscle cells, which were extracted directly on cover slips to aid in localization *in situ* as described by Chaly *et al.*

Fig. 3. Indirect immunofluorescence of the nuclear matrix of BC3H1 muscle cells and isolated rat liver nuclei

Cultured BC3H1 muscle cells on cover slips were extracted sequentially with the various nuclear-matrix buffer solutions as described in the Materials and methods section. After fixation in 4% paraformaldehyde, the remaining nuclear material was probed with anti-(B₂ phosphoprotein) monoclonal antibody IgM (ascites diluted 1:100) or PBS (as control), and then detected with fluorescein isothiocyanate-conjugated goat anti-mouse IgG + IgM (Jackson ImmunoResearch; diluted 1:50). The cover slips were then mounted, observed by immunofluorescence microscopy and photomicrographed. (a) Final matrix preparation (magnification $\times 1250$). (b) Control (magnification $\times 1250$). (c) Normal rat liver nuclei (magnification $\times 600$).

(1985). This technique allows the cultured cells to remain attached to the cover slips and avoids mechanical disruption such as homogenization and centrifugation. It was found that, on treatment with very low concentrations of DNAase I, fluorescence became very prominent with IgM, with both nuclear peripheral and extensive meshwork-like staining (results similar to those in Fig. 3a). This pattern of fluorescence was maintained throughout the remainder of the extraction procedure (low-Mg²⁺ buffer, high-salt extraction, extensive DNAase-I and RNAase-A digestion), and the degree of final nuclear-matrix fluorescence is shown in Fig. 3(a). In addition, this sequential extraction procedure has been used successfully in the study of matrix antigens by other laboratories (Staufenbiel & Deppert, 1984; Lehner *et al.*,

1986). This observation strongly indicates that the B₂ phosphoprotein does indeed have an association with the nuclear matrix. The mild exposure to DNAase I may in fact result in a small degree of nuclear reorganization. However, it is more likely that this mild DNAase-I treatment in some way allows the bulky IgM more accessibility to its B₂ epitope, and moreover the pentameric IgM molecule will amplify the fluorescent signal. A small degree of cytoplasmic staining was also observed, which may be due to either synthesis of phosphoprotein B₂ *de novo* as the cells rapidly grow in culture or the leakage of the nuclear phosphoprotein into the cytoplasm as the result of the use of DNAase and RNAase during the extraction. The use of PBS as a negative control (Fig. 3b) demonstrates the lack of

non-specific background. Chaly *et al.* (1985) have also concluded, using 3T3 cells, that treatment with DNAase I and monoclonal IgM also gives a more pronounced staining pattern to the nuclear matrix. As shown in Fig. 3(c), isolated rat liver nuclei reacted with monoclonal IgM without the use of DNAase I. The fluorescent staining clearly demonstrates that this monoclonal IgM is also accessible to the B₂ protein, present in highly purified nuclei. We also carried out the same fluorescence studies with the IgG₁ and IgG_{2a} monoclonal antibodies, but these did not result in any marked fluorescence, suggesting either that their epitopes may be 'masked' or that the monoclonal antibodies themselves may not be useful for immunofluorescence.

Previous work in our laboratory has been centred on the immunological analysis of the B₂ phosphoprotein in hope of elucidating its functional significance. Interestingly, a polyclonal antibody raised to this phosphoprotein allowed us both to verify its nucleosomal location and to demonstrate its preferential release after micrococcal-nuclease digestion (Zhao & Liew, 1982; Liew *et al.*, 1984). This latter result, in particular, seems to contradict any presence of this phosphoprotein in 2 M-NaCl-treated matrix. In order to clarify this paradoxical situation, we hypothesize that this phosphoprotein may function in different roles, depending on its nuclear location. The presence of this phosphoprotein in the matrix, along with its binding to the nucleosome structure, may suggest that it serves to 'anchor' certain actively transcribed nucleosomes in the region of the pore-complex-lamina matrix subfraction, thus perhaps playing a role in some facet of RNA processing. Its presence on nucleosomes, such that it may be more accessible to nuclease digestion, may suggest a completely separate and distinct role more related to maintaining chromatin structure in a more 'active' conformation for DNA replication and/or RNA transcription. Interestingly, it has been reported that the *v-myc* and *c-myc* proteins may also have similar multiple associations with nuclei, the most prominent localization being a tight-binding fraction with the nuclear matrix (Eisenman *et al.*, 1985). It is currently unknown whether this B₂ phosphoprotein has any relation with the *v-myc/c-myc* protein family. In the future particular interest will centre on the degree of phosphorylation of this B₂ phosphoprotein in these two fractions, with emphasis on whether or not there is any significant difference and how these may be related to different functional states.

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