Monoclonal antibodies to a phosphoprotein from chromatin of rat liver

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Three monoclonal antibody subclasses (IgG1, IgG2a, and IgM) were raised to the phosphoprotein B₂ (*M_r* 68 000, pI 6.5–8.2) which has been shown previously to be associated with the nucleosomes of rat liver nuclei. These antibodies do not show any significant cross reactivity with CM-cellulose ‘unbound’ non-histone chromosomal proteins, bovine serum albumin or histones. Further verification of the specificity of these antibodies to this phosphoprotein was carried out using both ‘dot’ blot and immunological transfer analysis (‘Western blot’). The monoclonal antibodies (IgG1 and IgG2a) could also be used to semi-quantify the phosphoprotein B₂ in rat liver nuclei. The high specificity and unlimited availability of this type of probe provides a means to study the role(s) of this phosphoprotein in the overall scheme of actively transcribed chromatin.

In recent years the highly heterogeneous non-histone chromosomal proteins, several of which are associated with the core particles of nucleosomes (Liew & Chan, 1976; Defer *et al.*, 1978; Zachau *et al.*, 1982; Klug, 1983; Klug *et al.*, 1983), have come under close scrutiny as potential regulators of gene expression (see reviews by Liew, 1979; Philips *et al.*, 1980; Cartwright *et al.*, 1982; Liew *et al.*, 1984b). For example, HMG 14 and 17 appear to be potential regulators due to their ability to confer sensitivity to deoxyribonuclease in certain selective transcribable regions of the genome (see reviews by Weisbrod & Weintraub, 1979; Johns, 1982). Attempts to find other such potential regulators, however, have been hindered due in large part to the inability to assay for any specific biological function (see reviews by Bustin *et al.*, 1982; Bekhor, 1984). This problem has been partially circumvented in recent years by the development of immunological probes (both polyclonal and monoclonal) (Bustin *et al.*, 1981; Elgin *et al.*, 1981; Turner, 1981; Vanderbilt & Anderson, 1983) that can serve as tools to aid in the isolation, quantification and precise localization of many of these components.

In a previous study we described the production of a polyclonal antibody to a rat liver nuclear phosphoprotein (B₂; *M_r* 68 000, pI 6.5–8.2) (Zhao & Liew, 1982). This non-histone chromosomal protein has been identified in the nucleosome

structure and subsequently demonstrated to be preferentially associated with actively transcribed nucleosomes (Liew & Chan, 1976; Chan & Liew, 1977, 1979; Liew *et al.*, 1984a). In the present report we carried this immunological approach one step further with the production of several monoclonal antibodies to this phosphoprotein. The characterization and the high degree of specificity of these antibodies has been verified and will be described.

Materials and methods

Preparation of nuclei and isolation of the phosphoprotein B₂

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/100 mM-phenylmethanesulphonyl fluoride, pH 8.0). Livers that were not used immediately were frozen in liquid N₂ and stored at –70°C. Isolation of nuclei was carried out at 4°C as described previously (Chan & Liew, 1977).

Nuclei were used to obtain non-histone chromosomal proteins by the phenol-buffer extraction procedure (Shelton & Allfrey, 1970). It has been found that this method resulted in an overall higher yield of this phosphoprotein along with ensuring the removal of both contaminating nucleic acids and phospholipids (Teng *et al.*, 1971; Suria & Liew, 1974a). Briefly, nuclear pellets were washed

Abbreviations used: HMG proteins, high mobility group proteins; SDS, sodium dodecyl sulphate.

with 140 mM-NaCl/0.1 mM-phenylmethane-sulphonyl fluoride. Acid-soluble proteins (i.e. histones and HMG) were then removed by extraction with 0.25 M-HCl/0.1 mM-phenylmethanesulphonyl fluoride. The pellet containing acid insoluble proteins was then dissolved in phenol/Tris buffer. The resulting phenol-soluble non-histone proteins were then fractionated by CM-23 cellulose column chromatography (Chan & Liew, 1979). The column was first eluted with equilibrating buffer (8 M-urea buffer, pH 9.2) and the eluate, consisting of 'unbound' non-histone proteins, was saved for later immunological studies. The 'bound' non-histone chromosomal protein fraction, which contained the phosphoprotein B₂ was then eluted with the same equilibrating buffer containing 300 mM-NaCl. This eluted 'bound' protein fraction was then purified by an additional CM-23 cellulose column passage using equilibrium buffer (pH 10). Isolation of the phosphoprotein B₂ was then carried out by two-dimensional polyacrylamide-gel electrophoresis (Suria & Liew, 1974b; Jackowski *et al.*, 1976). The phosphoprotein, which was subsequently identified by soaking the gel in 1 M-KCl for 3 min, was cut and eluted from the gel slice by one-dimensional SDS/polyacrylamide-gel electroelution (Chan & Liew, 1979).

Immunization and preparation of monoclonal antibodies

Four BALB/c mice were immunized by subcutaneous injection with 100 µg of phosphoprotein B₂ mixed with an equal volume of complete Freund's adjuvant. Animals were then boosted over a 2 month period at 2 week intervals intraperitoneally as above (substituting incomplete adjuvant for complete). Three days after the fourth injection (an intravenous injection of 20 µg of B₂ protein in saline via the tail vein) spleen cells were harvested and fused with NS-1 myeloma cells using 50% poly(ethylene glycol) (Oi & Herzenberg, 1981) at a ratio of 8:1 (spleen:myeloma). The resulting hybrid cells were then seeded into 96-well microtitre plates containing a spleen feeder layer (10⁵ spleen cells/well) and maintained according to the method of Oi & Herzenberg (1981).

Solid phase radioimmunoassay

Radioimmunoassay was carried out as described by Goding (1983). The phosphoprotein B₂ was diluted to 50 µg/ml in phosphate-buffered saline and distributed into poly(vinyl chloride) microtitre wells (Dynatech) (100 µl/well). After overnight incubation at 4°C each well was emptied (supernatants could be saved for later use) and then washed twice with blocking buffer (0.1% bovine serum albumin in phosphate-buffered saline). Each well

was then treated with 100 µl of undiluted hybridoma culture fluid at room temperature for 30 min. Wells were then washed thoroughly with blocking buffer and then treated with 100 µl of a 1:500 dilution (in blocking buffer) of rabbit antiserum to mouse IgM and IgG (7S) (Bionetics). After 30 min at room temperature each well was again washed with blocking buffer and then treated with ¹²⁵I-Protein A (Pharmacia) [specific radioactivity 40 µCi/µg; (2-5) × 10⁴ c.p.m./well]. After a further 30 min incubation each well was washed thoroughly with phosphate-buffered saline and counted in a gamma counter (Beckman 4000). Further assays using plates coated with either histones, bovine serum albumin or 'unbound' proteins from the CM-23 column were also carried out in the same way. After determining which wells contained positive hybrids, cells were then expanded and further cloned at least two times by limiting dilution. Wells containing single-cell positive-producing hybrids were then expanded and either frozen down or injected into pristane-primed BALB/c mice [primed anywhere from 2 weeks to 2 months previously using (2-5) × 10⁶ cells/mouse] to produce higher titred ascites fluid.

Ouchterlony double immunodiffusion and immunoblots

Double immunodiffusion was carried out in 1% (w/v) agarose using barbital buffer (50 mM-sodium diethylbarbiturate/HCl, pH 8.6) (Ouchterlony, 1967). Immunoglobulins from positive culture fluids were concentrated by (NH₄)₂SO₄ precipitation (50% saturation) and placed in the centre well (2-3 µl). Goat antisera against purified mouse immunoglobulins (IgM, IgG₁, IgG_{2a}, IgG_{2b}, and IgG₃) (Miles) were placed in the surrounding wells. The plates were then developed for 24-48 h at room temperature, washed extensively for 5 days at 4°C and then stained with 0.2% Buffalo Black in 7% (v/v) acetic acid. The plates were then destained in 7% (v/v) acetic acid and photographed with Polaroid type 107 Land film.

Nitrocellulose transfer and immunological detection of the protein blots was carried out as described previously (Gershoni & Palade, 1983). Briefly, proteins were first separated in an SDS/10% polyacrylamide-slab gel and transferred to a nitrocellulose membrane by electrophoretic blotting. After the transfer the membrane was soaked for 1 h in blocking buffer [3% (w/v) bovine serum albumin in 10 mM-Tris/HCl (pH 7.4)/0.9% NaCl] at 40°C. The membrane was then incubated with either monoclonal antibody culture fluid (1:20) or ascites fluid (1:500) diluted in antibody incubation solution [50 mM-Tris/HCl (pH 7.4)/150 mM-NaCl/5 mM-EDTA/0.05% Nonidet P-40] for at least 2 h. The membrane was then washed

Fig. 1. Ouchterlony immunodiffusion patterns

Saturated $(\text{NH}_4)_2\text{SO}_4$ -precipitated culture fluid from each of the three hybridoma clones was placed respectively in the central well (2–3 μl). Peripheral wells contained: 1, goat anti-(mouse IgM); 2, anti-IgG₁; 3, anti-IgG_{2a}; 4, anti-IgG_{2b}; 5, anti-IgG₃. (a) Hybridoma IgM; (b) hybridoma IgG₁; (c) hybridoma IgG_{2a}.

with wash buffer [50mM-Tris/HCl (pH 7.4)/150mM-NaCl/5mM-EDTA/0.25% gelatin/0.1% SDS/0.5% Triton X-100] for at least 1 h with three changes of 20 min each. The membrane was then incubated with a 1:2000 dilution of peroxidase-conjugated goat anti-(mouse IgG) (Cappel) in antibody incubation buffer for at least 2 h. The membrane was then again washed for at least 1 h in wash buffer, then treated with substrate solution (25 μg of *o*-dianisidine/ml and 0.1% H_2O_2 in 10mM-Tris/HCl, pH 7.4). The colour was allowed to develop for 15–30 min and, following extensive washing with distilled water, it was photographed with Polaroid type 107 Land film.

Determination of phosphoprotein B₂ by radioimmunoassay

Liver nuclei (adult) were isolated as described earlier. Different amounts of nuclear protein (100 μg –1 mg) were then placed in 500 μl Eppendorf centrifuge tubes. After one wash in phosphate-buffered saline the nuclei were then incubated with either purified IgG₁ or IgG_{2a} (1:100) monoclonal antibodies (ordinary culture fluid was used as a control) at 37°C for 30 min. After three further washes in phosphate-buffered saline the nuclei were then incubated with ^{125}I -labelled goat anti-(mouse Igs) (New England Nuclear) (2.5×10^5 c.p.m./tube) at 37°C for 30 min. After three washes in phosphate-buffered saline the tubes were counted in a Beckman gamma 4000 scintillation counter. A standard curve using phosphoprotein B₂ (10–50 ng) was established concurrently as an aid to semi-quantification of B₂ levels in the nucleus.

Results and discussion

Previous work in our laboratory has been focused on the characterization of nuclear components, including non-histone chromosomal proteins

Fig. 2. Solid phase radioimmunoassay demonstrating the dependence of antibody binding on antigen concentration

Microtitre plates were coated with 100 μl of phosphoprotein B₂ (●), 'unbound' CM-23 proteins (□) and histones (○) at the indicated concentrations. Ascites fluid was diluted at 1:100 for detection followed by a mixture of rabbit anti-(mouse IgM), IgG (diluted 1:500) and finally by ^{125}I -Protein A (5×10^4 c.p.m./well). (a) Hybridoma IgM; (b) hybridoma IgG₁; (c) hybridoma IgG_{2a}.

in mononucleosomal particles isolated by micrococcal nuclease digestion and sucrose density centrifugation (Liew & Chan, 1976; Chan & Liew,

Fig. 3. 'Dot' blot analysis of the three monoclonal antibodies to the B_2 phosphoprotein

A range (0.01–2 μ g) of phosphoprotein B_2 , bovine serum albumin (B) or histone (H) respectively was applied directly to a nitrocellulose membrane and each was allowed to react with ascites fluid (diluted 1:1000) from each of the three clones followed by further incubation with horseradish peroxidase-conjugated goat anti-(mouse IgG + IgM) (diluted 1:2000) and colour development with *o*-dianisidine and H_2O_2 . (a) IgG₁; (b) IgG_{2a}; (c) IgM.

1977, 1979; Liew *et al.*, 1984). In particular, our attention has been centred upon a highly phosphorylated protein (B_2 ; M_r 68000, pI 6.5–8.2) associated with these particles. A polyclonal antibody produced in rabbits to this protein has allowed us to verify further its nucleosomal location and demonstrate that B_2 nucleosomes are preferentially released following limited nuclease digestion (Zhao & Liew, 1982; Liew *et al.*, 1984a). However, this polyclonal antibody approach carried several major limitations [e.g. production of antibody to minor contaminants present in any preparation of this protein and an unpredictable and limited availability of this probe (see review by Goding, 1983)]. It was essentially for these reasons that efforts were aimed at the establishment of a monoclonal antibody probe that would aid in our approach to elucidating the structure and function of this protein.

The inherent nature of phosphoproteins as poor immunogens was encountered during the production of polyclonal antibodies in rabbit. We therefore decided to carry out the production of a monoclonal antibody to this protein by using a relatively purified phosphoprotein from rat liver chromatin in order to eliminate lengthy and laborious manipulation of the screening procedures. Our initial screening of hybridomas that produced monoclonal antibodies to the phosphoprotein B_2 was carried out by solid-phase radioimmunoassay. Following extensive cloning

by limited dilution, three stable clones were identified, as shown in Fig. 1. The hybridoma IgM, IgG₁, and IgG_{2a} were recognized specifically by goat anti-(mouse IgM), (mouse-IgG₁) and -(mouse IgG_{2a}), respectively. These three classes of hybridoma, which produced the monoclonal antibodies to phosphoprotein B_2 , the ascites fluid (diluted 1:100) contained the monoclonal antibody which reacted significantly with the same concentration of phosphoprotein (i.e. antigen). Culture fluid of each specific clone gave similar results in all cases; however, the IgG_{2a} clone appeared to be of lower titre (Fig. 2c). These three antibodies appear to be highly specific in their interaction with determinants (i.e. epitopes) found on the phosphoprotein B_2 as opposed to their low cross reactivity with both 'unbound' CM-23 proteins, and histones. This low cross-reactivity with 'unbound' proteins is particularly interesting, since this fraction comprises nearly 60% of the total starting phenol-soluble proteins (Chan & Liew, 1977) and it would seem that because of the extreme heterogeneity of the non-histone chromosomal proteins in general that perhaps at least some of these proteins might share a few of the same antigenic determinants (epitopes) found on the phosphoprotein B_2 .

Fig. 4. Specificity of the IgG₁ monoclonal antibody using immunoelectrophoretic transfer ('Western blot')

Phosphoprotein B₂ CM-23 'bound' peak proteins and bovine serum albumin were fractionated by a SDS/10% polyacrylamide-gel electrophoresis followed by electrophoretic transfer to a nitrocellulose membrane as described in the Materials and methods section. The nitrocellulose membrane was then subsequently blocked with 3% albumin in Tris buffer, incubated with the B₂ IgG₁ ascites fluid (diluted 1:1000) and, following washing to remove non-specifically bound antibody, incubated with horse-radish peroxidase-conjugated goat anti-(mouse IgG) (1:2000). After thorough washing colour development was carried out with *o*-dianisidine and H₂O₂. (a) Bovine serum albumin, 2 µg; (b) phosphoprotein B₂ 2 µg; (c) CM-23 'bound' protein, 5 µg.

The specificity of the monoclonal antibodies to the phosphoprotein was further verified by the use of two different immunological tests. Using the 'dot' blot technique, in which a range of B₂ concentrations was applied directly to a nitrocellulose membrane and then probed with the various monoclonal antibodies, a distinct colour reaction was detectable (Fig. 3). When histones (H) and bovine serum albumin (B) were used at the same concentration, no cross-reactivity was seen to

Fig. 5. Solid-phase radioimmunoassay demonstrating the dependence of purified IgG monoclonal antibody binding on antigen concentration

Microtitre plates were coated with 100 µl of B₂ phosphoprotein (●), 'unbound' CM-23 proteins (□), histones (○) and bovine serum albumin (△) at the indicated concentrations. Purified DEAE Affi-Gel Blue IgG₁ (a) and Protein A-Sepharose IgG_{2a} (b) were diluted at 1:100 for detection followed by a mixture of rabbit anti-(mouse IgG) (diluted 1:500) and finally by ¹²⁵I-Protein A.

occur with any of the three antibodies. This test was then carried out using electrophoretic blotting of the proteins from a 10% polyacrylamide gel to the nitrocellulose membrane followed by probing with the IgG₁ antibody. As shown in Fig. 4, this antibody did not show any cross-reactivity with bovine serum albumin and was able to react with B₂ directly or with the CM-23 'bound' proteins which contained approx. 23% of the phosphoprotein B₂.

The IgG subclass immunoglobulins were then further purified by either DEAE Affi-Gel Blue column chromatography (Bio-Rad) or Protein A-Sepharose chromatography (Pharmacia). By use of solid phase radioimmunoassay, the specificity of antibody binding to phosphoprotein B₂ antigen concentration was clearly demonstrated as shown by a typical experiment in Figs. 5(a) and 5(b).

It is well known that non-histone chromosomal

Fig. 6. Semi-quantitative estimation of the phosphoprotein B_2 concentration in rat liver nuclei by using purified monoclonal IgG antibodies

Isolated liver nuclei (adult) were used to establish the content of phosphoprotein B_2 in the nuclear protein range (100 μ g–1 mg). Nuclei were then incubated with either purified IgG₁ (●) or IgG_{2a} (○), both at 1:100 dilution, as described in the Materials and methods section. The actual values were obtained by subtracting the control values (using ordinary culture fluid) from the IgG values. A standard curve using purified phosphoprotein B_2 (1–50 ng) is shown in the insert.

proteins are closely associated with nuclear activity (Allfrey, 1974; MacGillivray & Rickwood, 1974; Wang *et al.*, 1976; Cartwright *et al.*, 1982; Briggs *et al.*, 1983; Liew *et al.*, 1984a). For example, Hoefler & Lane (1980) recently demonstrated that a monoclonal antibody designated as DL 3C4 (IgG₁) raised against the large T protein of SV40 also recognized an antigenic determinant present on a host cell nuclear protein of M_r 68000. Using immunofluorescence it was shown that this antibody binds most effectively with cells undergoing a rapid growth rate, which may indicate that this protein has some vital role in initiating host DNA synthesis. The relationship between this protein and the phosphoprotein B_2 has yet to be determined. Our previous work using regenerating liver as a model system (Liew *et al.*, 1984a) showed that the phosphoprotein B_2 showed a maximum phosphorylation at 16 h post-hepatectomy, approximately the same time that DNA synthesis was initiated (Mayfield & Bonner, 1972; Liew & Gornall, 1975). The significant change in the phosphorylation of phosphoprotein B_2 following mild micrococcal nuclease digest (5–10% of DNA rendered soluble) (Liew *et al.*, 1984a) suggest that determination of its level in the nucleus may be pertinent in determining its role in actively transcribed chromatin. As shown in Fig. 6, a

typical experiment was carried out to determine the radioactivity in proportion to the concentration of nuclear protein. Subsequently the radioactivity was correlated with the actual amount of phosphoprotein B_2 in the radioimmunoassay. Thus a semi-quantitative determination of phosphoprotein B_2 in nuclear proteins using this battery of monoclonal IgG antibodies can be achieved. By the use of this assay both monoclonal antibodies can detect up to approx. 20 ng of phosphoprotein B_2 per mg of nuclear protein. This does not correlate well with the previous determinations (Chan & Liew, 1979). It is very likely that much of the phosphoprotein B_2 is highly complexed with the nuclear structure in such a way that many of its antigenic determinants (epitopes) are in fact masked so that these monoclonal antibodies are unable to recognize and bind to them. However we have already provided evidence that the phosphoprotein B_2 is preferentially released following mild micrococcal nuclease digestion (Liew *et al.*, 1984a) and it may be that when this phosphoprotein B_2 location in the more accessible regions of the genome is released by micrococcal nuclease digestion that a more accurate quantification can be achieved. In preliminary observations we found that an increase of phosphoprotein B_2 is evident in the regenerating liver nuclei as compared with its sham-operated control. These findings substantiate further that this protein is most likely involved in active nucleosomal structure and function.

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