Identification of Carbohydrate Binding Protein 35 in Heterogeneous Nuclear Ribonucleoprotein Complex†

James G. Laing and John L. Wang*
Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824
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ABSTRACT: In previous studies, a lectin designated as carbohydrate binding protein 35 (CBP35) was identified in the nucleus and cytoplasm of cultured mouse 3T3 fibroblasts. In the present study, we observed that treatment of Triton X-100 permeabilized 3T3 cells with ribonuclease A released CBP35 from the nuclei, while parallel treatment with deoxyribonuclease I failed to do so. This conclusion was based on (a) immunofluorescence analysis of the nuclear residue after detergent and enzymatic treatments and (b) immunoblotting analysis of the supernatant fraction produced by these treatments. These results indicate that CBP35 may be associated with the ribonucleoprotein elements of the 3T3 cell nuclei. In corroboration with this conclusion, fractionation of the nucleoplasm derived from 3T3 cells on a cesium sulfate gradient (1.25–1.75 g/mL) localized CBP35 in fractions with densities of 1.30–1.32 g/mL, corresponding to the range of densities reported for heterogeneous nuclear ribonucleoprotein complex (hnRNP). Conversely, when nucleoplasm was fractionated on an affinity column of Sepharose derivatized with *N*-(ε-aminocaproyl)-D-galactosamine, the bound and eluted fraction contained RNA, as well as a set of polypeptides whose molecular weights matched those reported for the core particle of hnRNP. One of these polypeptides was identified as CBP35. These results suggest that CBP35 is a component of hnRNP.

Carbohydrate binding protein 35 (CBP35, M_r 35 000)[1] is a lectin that binds specifically to galactose and galactosyl-*N*-acetylglucosamine-containing glycoconjugates (Roff & Wang, 1983). This protein, initially identified and purified from cultures of mouse 3T3 fibroblasts, has now been found in a variety of adult and embryonic tissues (Crittenden et al., 1984). When a highly specific antibody directed against CBP35 was used, immunoblotting and immunofluorescence studies have shown that the lectin could be found in the nucleus of a cell (Moutsatsos et al., 1986) and that the level of expression and its nuclear localization may be dependent on the proliferation state of the cell (Moutsatsos et al., 1987). In the course of these studies, we observed that when serum-starved, quiescent cultures of 3T3 cells were stimulated by the addition of serum, the percentage of cells showing nuclear staining due to CBP35 increased prior to the onset of DNA synthesis in the synchronized cell population. Moreover, there was a distinct fluorescence pattern within the nucleus, characterized by prominent punctate intranuclear staining (Moutsatsos et al., 1987). This suggested the possibility that CBP35 might be associated with certain subnuclear structures.

In order to obtain some insight on the nature of the subnuclear structure with which CBP35 may be associated, we have sought conditions and treatments of permeabilized 3T3 cells that would release CBP35 from the nucleus. We report in the present paper the identification of CBP35 in the heterogeneous nuclear ribonucleoprotein complex (hnRNP). This conclusion is based on the differential effects of ribonuclease A (RNase A) and deoxyribonuclease I (DNase I) on release of CBP35 from the nucleus and on the immunochemical identification of CBP35 in hnRNP.

Experimental Procedures

Cell Culture and Antibody Reagents. Antiserum directed against CBP35 was generated in New Zealand White female rabbits (Roff & Wang, 1983). This antiserum had been characterized in terms of specificity in the following experiments: (a) immunoblotting of a single polypeptide (M_r 35 000) in sodium dodecyl sulfate (SDS) extracts (Moutsatsos et al., 1986) and in Triton X-100 extracts (Crittenden et al., 1984) of 3T3 cells; (b) specific isolation of CBP35 from Triton X-100 extracts of 3T3 cells by immunoaffinity chromatography (Moutsatsos et al., 1986); and (c) specific immunoprecipitation of CBP35 out of a partially purified preparation of endogenous lectins derived from 3T3 cells (Roff & Wang, 1983).

Swiss 3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DME) containing 10% calf serum (v/v) (Steck et al., 1982). The cells were synchronized by serum starvation (48 h in DME containing 0.2% serum), followed by the readdition of serum (10% v/v) (Moutsatsos et al., 1987). For labeling proteins with radioactive methionine, serum-starved 3T3 cells were restimulated by changing the medium to DME–10% serum containing [^{35}S]methionine (20 μCi/mL). The cells were incubated for 16 h and used for the isolation of nucleoplasm. For pulse labeling RNA with radioactive uridine, quiescent 3T3 cells were first stimulated in DME–10% serum for 16 h. The medium in the cultures was then changed to DME–10% serum containing [^{3}H]uridine (50 μCi/mL). In some experiments, the cells were treated with actinomycin D (0.04 μg/mL) for 15 min prior to being labeled with [^{3}H]-uridine. The cultures were then incubated for 30 min prior to being harvested.

Permeabilization, Digestion, and Extraction of 3T3 Cell Components. Cells for immunofluorescence analysis were seeded on coverslips and synchronized as described above. Sixteen hours after serum stimulation, the medium was removed, and the cells were incubated in 20 mM Tris, 5 mM

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* Address correspondence to this author.

[1] Abbreviations: CBP, carbohydrate binding protein; hnRNP, heterogeneous nuclear ribonucleoprotein complex; RNase, ribonuclease; DNase, deoxyribonuclease; SDS, sodium dodecyl sulfate; DME, Dulbecco's Modified Eagle's medium; TKM buffer, 20 mM Tris buffer containing 5 mM KCl, 1 mM $MgCl_2$, and 1 mM phenylmethanesulfonyl fluoride; PMSF, phenylmethanesulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline (20 mM Tris and 0.5 M NaCl, pH 7.5); Tris, tris(hydroxymethyl)aminomethane.

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KCl, 1 mM $MgCl_2$, and 1 mM phenylmethanesulfonyl fluoride (PMSF), pH 7.2 (TKM), buffer containing 1 mM vanadyl adenosine, 250 mM ammonium sulfate, and 0.5% Triton X-100 for 30 min at 4 °C (Herman et al., 1978; Fey et al., 1986). This incubation buffer was then removed, and the permeabilized cells were washed 3 times in TKM buffer. These permeabilized cells were subjected to the various treatments as detailed below.

Permeabilized 3T3 cells were incubated in TKM buffer containing 100 μg/mL DNase I (Sigma) and 1 mM vanadyl adenosine at 21 °C. After 30 min, ammonium sulfate was added to a final concentration of 250 mM and incubated for 5 min at room temperature (Fey et al., 1986). This medium was then removed, and the cells were washed 3 times in TKM buffer.

Permeabilized 3T3 cells were incubated in TKM buffer containing 25 μg/mL RNase A (Sigma) at 21 °C for 30 min (Fey et al., 1986). The medium was then removed, and the cells were washed 3 times in TKM buffer.

Coverslips that had been subjected to the various treatments or combinations of the extraction steps were then subjected to immunofluorescence analysis (see below).

For immunoblotting analysis of the supernatant fraction derived from the cells after various treatments, 3T3 cells were resuspended in permeabilization buffer for 30 min. The supernatant fraction of the Triton X-100 solubilized material was obtained after centrifugation at 1300*g* for 10 min and subjected to SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotting. Similarly, after each of the enzymatic treatments, the supernatant fraction was separated from the residue by centrifugation (1300*g*, 10 min).

Triton X-100 permeabilized cells were also suspended in TKM buffer containing 1 mM vanadyl adenosine and 100 mM lactose and incubated for 30 min at room temperature. The residue was removed by centrifugation (1300*g*, 10 min), and the extracted material was processed for immunoblotting.

Fraction of Nucleoplasm. Quiescent 3T3 cells were stimulated by the addition of serum. Sixteen hours after serum stimulation, the cells were harvested in 0.25% trypsin. The cells were pelleted by centrifugation (1300*g*, 3 min) and were resuspended in reticulocyte suspension buffer (10 mM Tris-HCl, 10 mM NaCl, 15 mM $MgCl_2$, 2 mM vanadyl adenosine, and 1.2 mM PMSF, pH 7.4). All subsequent steps were performed at 4 °C. The cells were lysed through repeated homogenization in a Dounce homogenizer, and the nuclei were collected by centrifugation (1300*g*, 10 min) (Fey et al., 1986). These nuclei were ruptured by sonication (4 × 15 s), and the nucleoplasmic material was removed from chromatin and other material by layering them on 25 mL of 30% sucrose in the same reticulocyte suspension buffer and centrifuging them for 15 min at 4500*g* in a Beckman SW27 rotor (Calvet & Pederson, 1978; Mayrand & Pederson, 1983).

The nucleoplasmic material was collected from the 30% sucrose interface and layered onto a preformed cesium sulfate gradient (1.25–1.75 g/mL). These gradients were centrifuged for 64 h at 112000*g* in a Beckman SW50.1 rotor (Calvet & Pederson, 1978; Mayrand & Pederson, 1983). The resulting gradients were collected with a Beckman fraction recovery system (0.5 mL/fraction), and the individual fractions were analyzed as outlined below. The density of each fraction was determined from the weight of 10 μL of the fraction. An aliquot from each fraction was mixed with scintillation cocktail (1:9 v/v), which consisted of 7 g of 2,5-diphenyloxazole (Research Products International Corp.), 333 mL of Triton X-100, and 667 mL of toluene. Radioactivity was determined on a Packard Tricarb 300CD liquid scintillation counter and expressed as disintegrations per minute (dpm). The remainder of the material in each fraction was analyzed by SDS–PAGE and immunoblotting.

Sepharose was derivatized with *N*-(ε-aminocaproyl)-D-galactosamine (Allen & Neuberger, 1975) and packed into affinity columns (0.8 × 3 cm) equilibrated with phosphate-buffered saline (10 mM sodium phosphate, 0.14 M NaCl, and 4 mM KCl, pH 7.4) containing 10 mM β-mercaptoethanol and 2 mM PMSF. Nucleoplasmic material (30% sucrose interface), derived from [^{35}S]methionine-labeled 3T3 cells, was fractionated over the affinity column. The column was washed and then eluted with the starting buffer containing 0.2 M mannose, followed by the same buffer containing 0.2 M glucose, and finally with the same buffer containing 0.2 M galactose. Aliquots of the effluent fractions were subjected to scintillation counting and SDS–PAGE, fluorography, and immunoblotting.

Indirect Immunofluorescence. After the permeabilized 3T3 cells were subjected to various treatments, they were fixed in 3.7% formaldehyde for 15 min and washed in Tris-buffered saline (TBS; 20 mM Tris and 0.5 M NaCl, pH 7.5) containing 0.5% bovine serum albumin. Each coverslip was incubated for 1 h in 100 μL of a 1:10 dilution of rabbit anti-CBP35 in TBS containing 3% normal goat serum. Cells were washed in TBS containing 3% normal goat serum and incubated in 200 μL of a 1:30 dilution of rhodamine-conjugated goat anti-rabbit IgG (Miles) in TBS for 1 h at room temperature (Moutsatsos et al., 1986). These cells were then washed 3 times with TBS and incubated for 15 min in the bis(benzimidazole) dye Hoechst 33258 (10 μg/mL; Sigma) (Cesarone et al., 1979; McKeon et al., 1984). These coverslips were washed 3 times in TBS and mounted in 70% glycerol–PBS containing 5% of the antibleaching agent *n*-propyl gallate (Sigma) (Moutsatsos et al., 1986). The slides were then viewed with a Leitz epiphase fluorescence microscope using a 40× objective lens. The Hoechst fluorescence was observed with a 430-nm barrier filter, and the rhodamine fluorescence was observed with a 580-nm barrier filter.

SDS–PAGE, Fluorography, and Immunoblotting. Samples were concentrated by centrifugation through a Centricon 10 filter. The concentrated material was then resuspended in 0.1% SDS containing 1 mM PMSF. Protein concentrations were assayed by the procedure of Bradford (1976). The proteins were separated on a 12.5% polyacrylamide gel in the presence of SDS (Laemmli, 1970). Fluorography was carried out as described by Bonner and Laskey (1974), using Kodak X-omat (XAR-5) film.

For immunoblotting analysis, the proteins separated on the SDS gels were electrophoretically transferred to nitrocellulose paper (400 mA at room temperature for 2 h) (Towbin et al., 1979). The nitrocellulose paper was then incubated overnight in saturating buffer (TBS containing 0.5% Tween-20). The paper was then incubated for 3 h in a 1:250 dilution of rabbit anti-CBP35 in saturating buffer (Moutsatsos et al., 1986). This paper was then washed 3 times with saturating buffer and incubated for an hour in 20 mL of a 1:1500 dilution of horseradish peroxidase conjugated goat anti-rabbit IgG (Bio-Rad). The blots were washed 2 times in saturating buffer and 2 times in TBS and then stained with 4-chloronaphthol as a substrate.

Results

Immunofluorescence Analysis of CBP35 in 3T3 Cells Treated with DNase I and RNase A. Serum-starved quiescent 3T3 cells were stimulated by the addition of serum; after 16

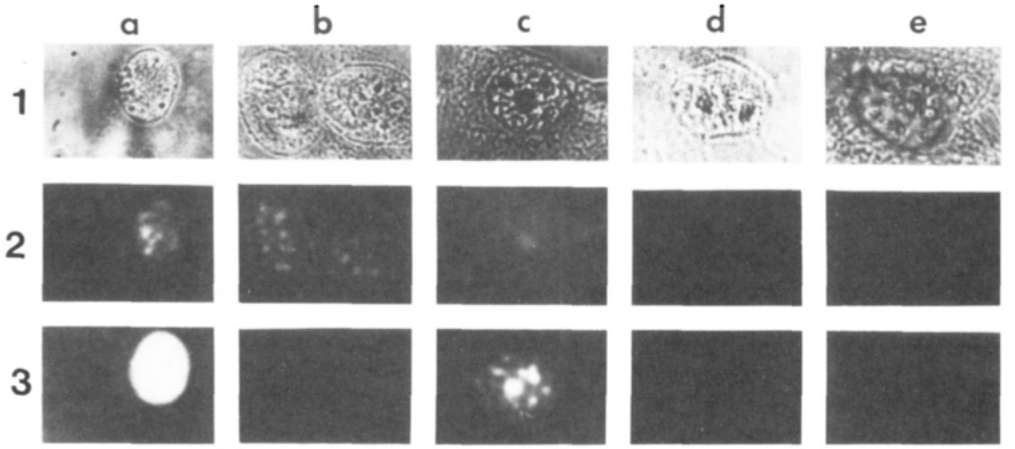

FIGURE 1: Effect of enzyme treatments on the localization of CBP35 in the nucleus of 3T3 cells. The cells were permeabilized in TKM buffer containing 1 mM vanadyl adenosine, 250 mM ammonium sulfate, and 0.5% Triton X-100 for 30 min at 4 °C. Parallel cultures of the cells were then subjected to enzyme treatment(s). After the treatment(s), the cells were fixed in 3.7% formaldehyde for 15 min prior to staining and light microscopy. Row 1, phase-contrast microscopy. Row 2, immunofluorescence staining with rabbit anti-CBP35 (1:10 dilution of antiserum) and rhodamine-conjugated goat anti-rabbit immunoglobulin (1:30 dilution) observed by using a 580-nm barrier filter. Row 3, fluorescence staining with the DNA-specific dye Hoechst 33258 (10 μg/mL) observed by using a 430-nm barrier filter. Column a, permeabilized 3T3 cells incubated in TKM buffer containing 1 mM vanadyl adenosine for 30 min at 21 °C as control. Column b, 3T3 cells, permeabilized as above, were incubated in TKM buffer containing DNase I (100 μg/mL) and 1 mM vanadyladenosine for 30 min at 21 °C. The cells were then extracted with 250 mM ammonium sulfate. Column c, permeabilized 3T3 cells were incubated in TKM buffer containing RNase A (25 μg/mL) for 30 min at 21 °C. Column d, permeabilized 3T3 cells were treated sequentially with DNase I, ammonium sulfate, and RNase A as described above. Column e, permeabilized 3T3 cells were treated sequentially with RNase A, DNase I, and ammonium sulfate.

h, the cells were subjected to chemical and enzymatic treatments. The cells were then fixed with formaldehyde and examined by phase-contrast microscopy (row 1, Figure 1), immunofluorescence detection of CBP35 (row 2, Figure 1), and staining with the DNA-specific dye Hoechst 33258 (row 3, Figure 1). The phase-contrast micrographs of Triton X-100 permeabilized 3T3 cells show that these cells have retained their nuclei (Figure 1, column a, row 1). These nuclei yielded the punctate staining pattern upon labeling with rabbit anti-CBP35 and rhodamine-conjugated goat anti-rabbit immunoglobulin (Figure 1, a-2). These nuclei also retained their DNA as seen by Hoechst dye staining (Figure 1, a-3).

It has been shown previously (Herman et al., 1978; Fey et al., 1986) that in vitro treatment of detergent-permeabilized cells with DNase I, followed by extraction with ammonium sulfate, effectively removes chromatin from the nuclear residue. In our experiments, cells treated by such a procedure retain nuclear structures, as seen in the phase-contrast micrograph (Figure 1, b-1). The nuclear residue is devoid of DNA (Figure 1, b-3) but retains the punctate intranuclear staining of CBP35 (Figure 1, b-2).

Detergent-permeabilized 3T3 cells were also treated with RNase A to see if this would affect CBP35 staining. RNase A digestion reduced the overall level of nuclear fluorescence due to CBP35 and removed the punctate intranuclear staining pattern (Figure 1, c-2). There was still staining of DNA by the Hoechst dye (Figure 1, c-3).

DNase I digestion and ammonium sulfate extraction followed by RNase treatment effectively depleted the nuclear residue of both chromatin and ribonucleoprotein elements (Fey et al., 1986). There was no staining for either CBP35 (Figure 1, d-2) or DNA (Figure 1, d-3). When the sequence of treatments was reversed, RNase A followed by DNase I and ammonium sulfate, the same results are obtained (Figure 1, e-2 and e-3). All of these results suggest that CBP35 is associated with the ribonucleoprotein fraction of the 3T3 cell nuclei.

Immunoblotting Analysis of CBP35 in the Material Released by DNase I and RNase A. In conjunction with these extraction procedures, we have monitored the proteins released

FIGURE 2: Immunoblotting analysis of the supernatant fractions derived from various treatment of 3T3 cells. Lane 1, supernatant fraction derived from intact 3T3 cells incubated in TKM, 1 mM vanadyladenosine, 250 mM ammonium sulfate, and 0.5% Triton X-100. Lane 2, supernatant fraction derived from treatment of permeabilized 3T3 cells with TKM buffer containing DNase I (100 μg/mL), 1 mM vanadyl adenosine, and 250 mM ammonium sulfate. Lane 3, supernatant fraction derived from treatment of permeabilized 3T3 cells with TKM buffer containing RNase A (25 μg/mL). Lane 4, supernatant fraction derived from treatment of permeabilized 3T3 cells with TKM buffer containing 1 mM vanadyl adenosine and 100 mM lactose. The samples were analyzed on SDS–PAGE (12.5% acrylamide) and immunoblotted with rabbit anti-CBP35 (1:250 dilution of antiserum) and horseradish peroxidase conjugated goat anti-rabbit immunoglobulin (1:1500 dilution). The arrows on the left indicate the positions of migration of molecular weight markers: ovalbumin (M_r 43 000); carbonic anhydrase (M_r 31 000); and soybean trypsin inhibitor (M_r 22 000). The arrow on the right indicates the position of migration of authentic CBP35.

into the supernatant fraction of the various treatments by SDS–PAGE and immunoblotting with anti-CBP35. Triton X-100 permeabilization extracts CBP35 from the 3T3 cells (Figure 2, lane 1), consistent with the original isolation of CBP35 from Triton extracts of the same cells (Roff & Wang, 1983). The supernatant fraction, after DNase I digestion and ammonium sulfate extraction, yielded no CBP35 (Figure 2, lane 2). This is consistent with the observation that the intranuclear punctate staining remained with the nuclear residue after DNase I treatment (Figure 1, b-2). In contrast, RNase A treatment, either of permeabilized cells or of permeabilized and chromatin-depleted cells, removed the punctate intranuclear staining due to CBP35 (Figure 1, c-2 and d-2). Accordingly, the supernatant fraction after RNase digestion

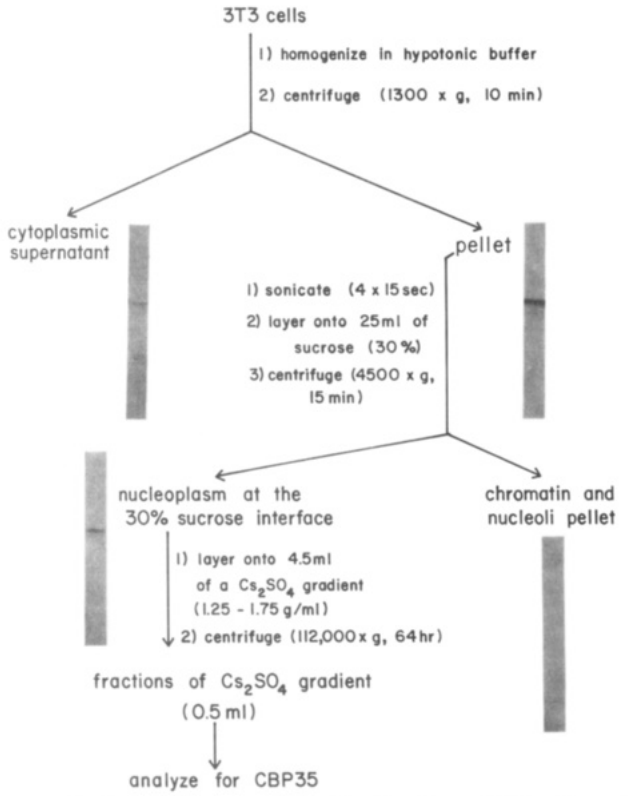

FIGURE 3: Schematic protocol for fractionation of 3T3 cells and tracking the presence of CBP35 in various fractions. Samples from each of the fractions were subjected to SDS–PAGE and immunoblotting analysis with anti-CBP35.

yielded CBP35 upon immunoblotting (Figure 2, lane 3).

We also tested whether the addition of lactose to the detergent-permeabilized 3T3 cells would release CBP35. Despite the fact that this lectin binds to galactose-containing glycoconjugates (Roff & Wang, 1983), the lactose treatment failed to release any CBP35 from the nuclear residue (Figure 2, lane 4).

Identification of CBP35 in Subnuclear Fractions. 3T3 cells were homogenized in hypotonic buffer and centrifuged to separate the cytoplasmic supernatant from the nuclei-containing pellet fraction. After sonication, the nuclei-containing pellet was centrifuged on a 30% (w/v) sucrose cushion to yield chromatin plus nucleoli material and nucleoplasm at the 30% sucrose interface (Figure 3). Throughout these fractionations, we tracked CBP35 by SDS–PAGE and immunoblotting. CBP35 was found in the nucleoplasm fraction, in the nuclei-containing pellet, and in the cytoplasmic supernatant (Figure 3). In contrast, no CBP35 was detected in the chromatin plus nucleoli material.

The nucleoplasm, containing CBP35 (Figure 3), was subjected to further fractionation by centrifugation on a Cs_2SO_4 gradient that ranged in density from 1.25 to 1.75 g/mL (Figure 4b). Immunoblotting of the individual fractions of the gradient showed that CBP35 could be found in fractions 2 and 3 (Figue 4b inset, lanes 2 and 3). In contrast, there was no CBP35 detectable in any of the other fractions of the gradient. Control experiments were carried out in which CBP35 was subjected to centrifugation in Cs_2SO_4 gradients. Immunoblotting analysis showed no CBP35 in fractions 2 and 3 under these circumstances. Therefore, the CBP35 protein alone failed to enter the gradient.

In a parallel experiment, 3T3 cells were cultured in the presence of [^{3}H]uridine for 30 min to pulse-label the RNA species. The nucleoplasmic material derived from these labeled

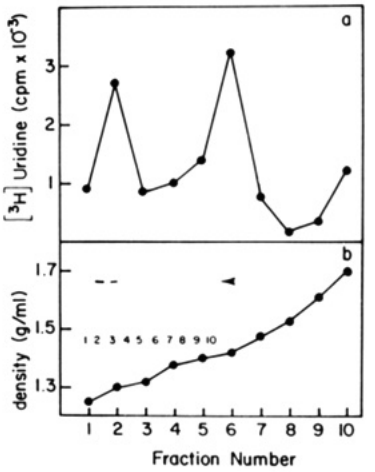

FIGURE 4: Representative profiles of RNA, CBP35, and density on Cs_2SO_4 gradient. (a) Serum-starved, quiescent 3T3 cells were stimulated by the addition of calf serum (10% v/v) for 16 h. The cells were then pulse-labeled for 30 min in the presence of [^{3}H]uridine (50 μCi/mL), and the nucleoplasm derived from these labeled cells was fractionated on the Cs_2SO_4 gradient. (b) The density of the individual fractions of the gradient was determined by weighing a 10-μL aliquot of each fraction. Inset: Immunoblotting analysis of individual fractions from the Cs_2SO_4 gradient. The samples were analyzed on SDS–PAGE (12.5% acrylamide) and immunoblotted with rabbit anti-CBP35 (1:250 dilution of antiserum) and horseradish peroxidase conjugated goat anti-rabbit immunoglobulin (1:1500 dilution). The arrow on the right indicates the position of migration of authentic CBP35.

cells was fractionated on a Cs_2SO_4 gradient, and the distribution of radioactivity was analyzed (Figure 4a). Two peaks of ^{3}H radioactivity were observed. The major peak was found in the range of densities of 1.40–1.50 g/mL, and it was due to ribonucleoprotein components, including rRNA-associated material (Wilt et al., 1973; Kloetzel & Shuldt, 1986). A second peak of radioactivity was found in fraction 2 ($\sim$1.3 g/mL). Therefore, the region of the Cs_2SO_4 gradient that yielded CBP35 upon immunoblotting also contained RNA (Figure 4a and Figure 4b inset).

The densities of fractions 2 and 3 were 1.3 and 1.32 g/mL, respectively (Figure 4b). This range of densities corresponded to the density of hnRNP as measured in Cs_2SO_4 gradients (1.3–1.35 g/mL) (Calvet & Pederson, 1978; Mayrand & Pederson, 1983). Moreover, SDS–PAGE and fluorography of this material isolated from [^{35}S]methionine-labeled cells revealed radioactive polypeptides with the following approximate molecular weights: (a) 68 000; (b) 53 000; (c) 43 000; (d) 37 000; (e) 35 000; (f) 34 000; and (g) 32 000 (Figure 5, lane 1). This set of proteins represents the polypeptides of the core particle of hnRNP (Pederson, 1974; Beyer et al., 1977; Choi & Dreyfuss, 1984; Wilk et al., 1985; Celis et al., 1986). These results indicate that CBP35 could be found in a hnRNP fraction. This notion is further supported by immunoblotting analysis of the 40S hnRNP fraction, isolated on sucrose gradients according to the procedure of Beyer et al. (1977). Immunoblotting with anti-CBP35 yielded a single polypeptide corresponding to the lectin (M_r $\sim$35 000).

Isolation of hnRNP by Saccharide-Specific Affinity Chromatography. The above results on the localization of CBP35 raised the possibility that CBP35 is a component of hnRNP and that one might be able to isolate hnRNP on the basis of saccharide binding. When nucleoplasm derived from [^{35}S]methionine-labeled 3T3 cells was fractionated on a column of Sepharose derivatized with *N*-(ϵ-aminocaproyl)-D-galactosamine, approximately 10% of the radioactivity was bound by the column. The bound material could not be eluted by the monosaccharides mannose and glucose, but the addition

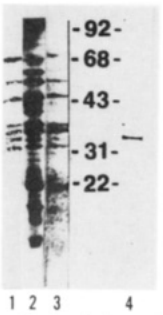

FIGURE 5: SDS–PAGE analysis of the polypeptide compositions of fractions derived from nucleoplasm subjected to Cs_2SO_4 gradient sedimentation and from nucleoplasm subjected to affinity chromatography on a Sepharose column derivatized with *N*-(ϵ-aminocaproyl)-D-galactosamine. Lane 1, material pooled from fractions 2 and 3 of the Cs_2SO_4 gradient (1.3–1.32 g/mL) shown in Figure 4. Lane 2, material that did not bind to the galactose affinity column (Figure 6). Lane 3, material that was bound to the affinity column (Figure 6) and specifically eluted with galactose. In lanes 1–3, the radioactive polypeptides were revealed by fluorography. Lane 4, immunoblotting analysis with anti-CBP35 of the material that bound to the affinity column (Figure 6) and specifically eluted with galactose. The numbers indicate the positions of migration of molecular weight markers.

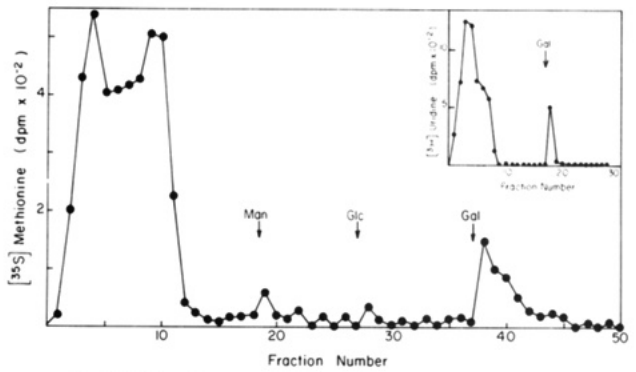

FIGURE 6: Affinity chromatography of nucleoplasm on a Sepharose column derivatized with *N*-(ϵ-aminocaproyl)-D-galactosamine. Serum-starved, quiescent 3T3 cells were stimulated by the addition of calf serum (10% v/v) and then cultured for 16 h in the presence of [^{35}S]methionine (20 μCi/mL). The nucleoplasm derived from these labeled cells was fractionated on the affinity column (0.8 × 3 cm) equilibrated with phosphate-buffered saline containing 10 mM β-mercaptoethanol and 2 mM PMSF. At the points indicated by the vertical arrows, the column was eluted with buffer containing 0.2 M mannose (Man), 0.2 M glucose (Glc), and 0.2 M galactose (Gal). Inset: Radioactivity profile of the fractionation of nucleoplasm, derived from cells labeled with [^{3}H]uridine, on the same affinity column. Serum-starved, quiescent 3T3 cells were stimulated by the addition of calf serum (10% v/v) for 16 h. Actinomycin D (0.04 μg/mL) was added 15 min prior to labeling with [^{3}H]uridine (50 μCi/mL) for 30 min. Nucleoplasm was isolated from these cells and fractionated on the affinity column. At the point indicated by the arrow, the column was eluted with buffer containing 0.2 M galactose. Fractions were precipitated with 10% trichloroacetic acid, and the precipitated radioactivity was determined by scintillation counting.

of galactose released the radioactive material (Figure 6). Immunoblotting analysis showed that this fraction contained CBP35 (Figure 5, lane 4).

More importantly, however, when this bound and galactose-eluted fraction was subjected to SDS–PAGE and fluorography to reveal the radioactivity polypeptides, eight bands were observed (Figure 5, lane 3): (a) M_r 68 000; (b) M_r 53 000; (c) M_r 44 000; (d) M_r 37 000; (e) M_r 35 500; (f) M_r 34 000; (g) M_r 32 000; and (h) M_r 22 000. The gel pattern corresponds closely to that of hnRNP isolated on Cs_2SO_4 gradients (Figure 5, lane 1) and to the polypeptides reported for the core particle of hnRNP (Pederson, 1974; Beyer et al., 1977; Choi & Dreyfuss, 1984; Wilk et al., 1985; Celis et al., 1986). The lone exception is the M_r 22 000 polypeptide observed in the affinity column bound material. Finally, the gel pattern of the bound and galactose-eluted fraction was considerably different from that of the unbound material (Figure 5, lane 2). At least eight other polypeptides, particularly those of low molecular weight (M_r 10 000–25 000), did not bind to the column.

When nucleoplasm derived from [^{3}H]uridine-labeled cells was subjected to the same affinity chromatography procedure, there was radioactivity bound to the column that could be specifically eluted with galactose (Figure 6, inset). In addition to the correspondence of the polypeptides to those of hnRNP, therefore, the galactose-bound fraction also contained RNA. All of these results suggest that galactose-specific affinity chromtography could be used to purify hnRNP from nucleoplasm. They provide, therefore, another line of evidence for the presence of lectin in hnRNP.

Discussion

The key conclusion derived from the experiments documented in the present study is that CBP35 is a component of hnRNP. This conclusion is based on three main lines of evidence. First, RNase A treatment of permeabilized 3T3 cells released CBP35 from the nuclear residue, whereas parallel treatment with DNase I failed to do so. This was ascertained by monitoring the presence of CBP35 remaining in the nuclear residue using immunofluorescence and by identifying the CBP35 released into the supernatant fraction after enzymatic treatment through immunoblotting. The release of CBP35 from the nuclear residue by RNase A parallels the behavior of a number of polypeptides of the hnRNP core particle. These include polypeptides designated A_1, group C proteins, and group D proteins of the core particle (Fey et al., 1986; Celis et al., 1986; Beyer et al., 1977).

Second, nucleoplasm of 3T3 cells, fractionated on a Cs_2SO_4 gradient, yielded CBP35 upon immunoblotting in fractions with densities of 1.30–1.35 g/mL. This range of densities corresponded to the density of hnRNP on Cs_2SO_4 gradients (Wilt et al., 1973; Calvet & Pederson, 1978; Mayrand & Pederson, 1983; Kloetzel & Schuldt, 1986).

Finally, when nucleoplasm was subjected to affinity chromatography on a Sepharose column containing covalently coupled galactose, the bound and eluted fraction yielded CBP35, along with polypeptides that correspond to the group A proteins (M_r ~32 000), group B proteins (M_r ~35 000), group C proteins (M_r ~37 000), group D proteins (M_r ~44 000), and the M_r 53 000 and 68 000 polypeptides of hnRNP (Beyer et al., 1977; Choi & Dreyfuss, 1984; Wilk et al., 1985; Celis et al., 1986). It appears, therefore, that the entire complement of polypeptides associated with hnRNP was bound to the column. When nucleoplasm derived from [^{3}H]-uridine-labeled cells was used in these affinity isolation procedures, the fraction bound and eluted with galactose also contained RNA.

The specificity of this recognition and binding was demonstrated by the fact that galactose, but not mannose and glucose, could elute the column-bound material. These results implicate that CBP35 in hnRNP can bind to galactose-containing glycoconjugates, which, in turn, co-isolates the other hnRNP components as a unitary structure. This is comparable to the immunoprecipitation of all the hnRNP polypeptides by a monoclonal antibody specifically directed against the C_1 and C_2 proteins of hnRNP (Choi & Dreyfuss, 1984).

The identification of CBP35 in hnRNP is also supported by results on the structure of the protein (Jia & Wang, 1988). We have obtained a cDNA clone for CBP35 from a λgt11

expression library derived from 3T3 cell mRNA. We have determined the complete nucleotide sequence of this cDNA clone. The deduced amino acid sequence indicates that the CBP35 polypeptide is divided into two domains: a carboxyl-terminal portion that is homologous to β-galactoside-specific lectins (Gitt & Barondes, 1986; Hirabayashi et al., 1987; Southan et al., 1987) and an amino-terminal portion that is homologous to certain hnRNP proteins, including human hnRNP protein C_1 (Swanson et al., 1987), brine shrimp hnRNP protein GRP33 (Cruz-Alvarez & Pellicer, 1987), and the deduced amino acid sequence of clone DL-4 (Lahiri & Thomas, 1986). This DL-4 clone was isolated from a human hepatoma cDNA library on the basis of its expression of a fusion protein reactive with chicken antibodies directed against bovine hnRNP proteins (Lahiri & Thomas, 1986). Therefore, the structure relatedness of CBP35 to hnRNP proteins, together with the present results, strongly suggests that the lectin is a component of hnRNP.

Our present identification of CBP35 as a component of the core particle of hnRNP is consistent with the report of Sève et al. (1986). Using quantitative microfluorometric analysis of neoglycoprotein binding as an assay, they have demonstrated the existence of carbohydrate binding sites in the ribonucleoprotein elements of the nuclei of BHK cells. The levels of carbohydrate binding sites were dependent on the proliferation state of the cell and parallel our analysis of the expression and nuclear localization of CBP35 (Moutsatsos et al., 1987). This suggests that CBP35 corresponds to the binding sites for the galactose-bearing neoglycoproteins of Sève et al. (1986).

It should be noted that the identification of CBP35 as a component of hnRNP does not preclude the presence of the lectin in other parts of the cell. In previous studies, we had shown that CBP35 is clearly found in the cytoplasm, as well as the nucleus of a cell, and that the quantitative distribution of nuclear versus cytoplasmic CBP35 may be dependent on the proliferative state of the cell (Moutsatsos et al., 1986, 1987). These observations, along with the results of the present study on the presence of CBP35 in subcellular and subnuclear fractions, raise the question of whether cytoplasmic CBP35 may also be associated with ribonucleoprotein complexes. In our original purification of CBP35 (Roff & Wang, 1983), 3T3 fibroblasts were extracted with Triton X-100, which removes the majority of the cytoplasmic lectin but leaves CBP35 in the nucleus (Moutsatsos et al., 1986). Such a Triton X-100 extract, upon a single cycle of affinity chromatography on a galactose-containing column, yielded CBP35 without co-isolation of the other polypeptides of the ribonucleoprotein complex. This is in direct contrast to our present results on the affinity chromatography of nucleoplasm, which results in the isolation of CBP35, as well as other polypeptides that show correspondence to the proteins of hnRNP. On the basis of such an analysis, we infer that cytoplasmic CBP35 is not complexed with ribonucleoprotein components. Obviously, the nuclear versus cytoplasmic forms of CBP35 need to be characterized in detail, their chemical difference(s) identified, the regulation of their interconversion analyzed, and their differential capability of complexing with hnRNP components studied.

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