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Major professor

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CHARACTERIZATION OF A NUCLEAR CARBOHYDRATE BINDING PROTEIN 35-RIBONUCLEOPROTEIN COMPLEX

BY

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ABSTRACT

In previous studies, (Carbohydrate Binding Protein 35, M_r ~35000) CBP35 had been identified in the nucleus and the cytoplasm of cultured mouse 3T3 fibroblasts. Treatment of Triton X-100 permeabilized 3T3 cells with ribonuclease A released CBP35 from the nuclei, while treatment with deoxyribonuclease I failed to do so. This was demonstrated by; a.) immunofluorescence analysis of the nuclear residue after detergent and enzymatic treatments, and b) immunoblotting analysis of the supernatant fraction derived from the different treatments. These results indicate that CBP35 may be associated with ribonucleoprotein elements of the 3T3 cell nucleus. In further experiments, fractionation of nucleoplasm derived from 3T3 cells on a cesium sulfate gradient, localized CBP35 in fractions with densities of 1.3-1.32 g/ml, corresponding to the range of densities reported for the heterogenous nuclear ribonucleoprotein complex (hnRNP). Conversely, fractionation of nucleoplasm on a Sepharose column derivatized with N-(ε-aminocaproyl)-D-galactosamine yielded a bound and eluted fraction that contained CBP35, RNA, as well as a set of proteins whose molecular weights match those reported for the core proteins of the hnRNP. These results suggest that CBP35 is associated with hnRNP.

Nucleoplasm derived from mouse 3T3 fibroblasts were

subjected to immunoprecipitation by antibodies directed against CBP35. This immunoprecipitate contained not only CBP35 but also the Sm antigen (B polypeptide), previously identified in small nuclear ribonucleoprotein particles (snRNP). Conversely, immunoprecipitation of nucleoplasm with autoimmune sera reactive Sm also co-precipitated CBP35. The B polypeptides of Sm antigen were not cross-reactive with their respective antibodies. However, coprecipitation was not observed if nucleoplasm was first treated with ribonuclease A. Thus it appears that CBP35 and the Sm antigen were associated via a ribonucleoprotein complex, a notion supported by their sedimentation positions on cesium sulfate gradients. These results implicating the association of CBP35 with snRNP, and the previous studies suggesting a similar association with the hnRNP provoke the possibility that the lectin is a component of the spliceosome that processes pre-mRNA. Saccharides and antibodies that bind to CBP35 inhibited splicing in an *in vitro* pre-mRNA splicing assay, while control reagents did not yield the same effect. These results support the conclusion that CBP35 may be associated with the spliceosome.

DEDICATION

To my parents

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LIST OF ABBREVIATIONS

ASF:	asialofetuin
BSA:	bovine serum albumin
CBP35:	carbohydrate binding protein 35
ConA:	Concanavalin A
CRD:	carbohydrate recognition domain
DME:	Dulbecco's modified Eagles medium
DNase:	deoxyribonuclease
ϵBP:	IgE binding protein
Gal-Sepharose:	Sepharose derivatized with N-(ϵ-aminocaproyl)-D-galactosamine
HMG:	high mobility group protein
hnRNP:	heterogenous nuclear ribonucleoprotein complex
LBP:	laminin binding protein
PAGE:	polyacrylamide gel electrophoresis
PBS:	phosphate buffered saline
PMSF:	phenolmethylsulfonyl fluoride
RCA:	*Ricinis communis* agglutinin
RNase:	ribonuclease
SBA:	soybean agglutinin
SDS:	sodium dodecyl sulfate
snRNP:	small nuclear ribonucleoprotein particle
SV-40:	simian virus 40
TKM buffer:	20 mM Tris buffer containing 5 mM KCl,

1 mM $MgCl_2$, and 1 mM PMSF

UEA: *Ulex europaeus* agglutinin

WGA: wheat germ agglutinin

CHAPTER I

LITERATURE REVIEW

INTRODUCTION

There is a general consensus that the potential to encode information in carbohydrate structures is enormous. Many of the possible roles of carbohydrate recognition in cellular function have been explored for extracellular molecules and events such as specific cell-cell recognition and cell adhesion to the extracellular matrix. Our interest in these possibilities, however, can now be extended to the intracellular compartment as well, mainly as a result of the demonstrations that carbohydrate structures such as glycoproteins and glycosaminoglycans can be found in the cytoplasm and the nucleus (1). These advances in nuclear and cytoplasmic glycosylation have, in turn, stimulated interest in the intracellular localization of lectins. Defined as non-enzymatic and non-immune proteins which can selectively

bind specific carbohydrate structures, lectins appear to be good candidates for recognizing variations in isomers, alternative linkages, sequence of saccharide units, and branched structures.

There are excellent reviews on nuclear and cytoplasmic glycosylation (1) and on the organization and functional implications of carbohydrate recognition domains in animal lectins (2,3). In this literature review I have summarized updated information in these areas and discussed the available data pertaining to the subcellular localization of those carbohydrate-binding proteins that have been observed in the cell nucleus.

As will be detailed in this discussion, all of the lectins located in the nucleus are also found in the cytoplasm. The nucleus and the cytosol, which communicate through the nuclear pores, are topologically continuous although functionally distinct compartments. There are well-known examples of proteins (e.g., *Drosophila* heat shock protein hsp 70 (4)) and RNAs (e.g., snRNAs (5)) that can shuttle between the nucleus and the cytoplasm. What is more curious, however, is the considerable amount of evidence that indicates intracellular lectins also occur at the cell surface and, in some cases, in the extracellular medium. These latter sites belong to the extracellular compartment, which is continuous with the lumen of the endoplasmic reticulum, the Golgi stack, and the secretory vesicles, but

is topologically distinct from the intracellular compartment. These fascinating observations form the basis for much of the discussion to follow.

NUCLEAR BINDING OF NEOGLYCOPROTEINS IMPLICATES LECTINS

The existence of lectin molecules in the cell nucleus was first inferred from the binding of neoglycoproteins (6). When permeabilized baby hamster kidney (BHK) cells were incubated with fluorescein-labeled neoglycoprotein, fluorescence was observed in both the cytoplasm and the nucleus (7,8). Strongest labelling was observed for neoglycoproteins bearing α-L-rhamnose, whose structure resembles that of β-D-galactose (Gal) (9). Significant binding (> 3-fold over fluorescein-conjugated BSA) was also observed for BSA bearing glucose (Glc), N-acetylglucosamine (GlcNAc), lactose (Lac), fucose (Fuc),and mannose 6-phosphate residues. Similarly, cryostat sections of calf tissues incubated with fluorescein-conjugated Lac-BSA showed cytoplasmic and nuclear staining (10). This staining could be competed with non-fluorescent Lac-BSA, but not with Man-BSA. These results implicate the existence of specific carbohydrate-binding proteins in both the cytoplasm and the nucleus.

Karyoplasts obtained by enucleation of BHK cells also bound the same neoglycoproteins after permeabilization (8). Karyoplasts from exponentially growing cells bound a much

greater number of any given neoglycoprotein than did nuclei from density-inhibited cells. In the promyelocytic cell line HL60, *in vitro* differentiation into monocytes or granulocytes was accompanied by a decrease in the binding of neoglycoproteins in the nucleoplasm (11). These results suggest a correlation between the nuclear localization of the lectin(s) and the proliferation or differentiation state of the cells. Such a notion is consistent with the results of comparing the level of the nuclear lectin, Carbohydrate Binding Protein 35 (CBP35), in mouse 3T3 fibroblasts in quiescent versus proliferating cultures (12) (see below). Moreover, CBP35 has been found to be associated with ribonucleoprotein complexes (RNPs) of the nucleoplasm (13), consistent with the distribution of at least a portion of the neoglycoproteins bound to the nucleus. In all of the mammalian cells studied (BHK cells, HeLa cells, leukemic murine L1210 cells and rat hepatocytes), neoglycoproteins bearing rhamnose, Fuc, or Glc residues bound mostly to interchromatin spaces of the nucleoplasm and to nucleoli (7,8,14). Condensed chromatin, revealed by the DNA-specific dye Hoescht 33342, showed little or no binding of the neoglycoproteins.

The localization of neoglycoproteins in two non-mammalian cell types has been mapped to specific sub-structures within the nucleus. Nuclei of lizard ovarian follicle cells were highly labeled when ultra-thin sections

incubated in the presence of Man-ferritin were observed with electron microscopy (15). This binding can be specifically competed with Man-BSA. Labeling of nucleoli, chromatin, and the outer leaflet of the nuclear envelope was particularly strong. Due to the unique organization of the granular component of the nucleoli in the specialized granulosa cells, it was possible to conclude that the Man-binding sites were associated with the dense fibrillar component of nucleolus (i.e., the sites of rDNA gene transcription) and with the nucleolar granules (i.e. the site of ribosomal precursors assembly before export to the cytoplasm. In a study carried out on isolated macronuclei from the ciliated protozoan *Euplotes eurystomus*, neoglycoprotein labeling was found over nucleoli, interchromatin spaces, and most interestingly, the macronuclear replication bands (16). Inasmuch as these replication bands represent delimited areas of DNA synthesis, this suggests a co-localization of saccharide-binding components and sites of DNA synthesis.

The identity of the lectin(s) responsible for the binding of the various neoglycoproteins in the nucleus (and in the cytoplasm) has not been determined. Thus, it is not clear how many different lectins are present in the various regions of the nucleus or whether the various neoglycoproteins can bind to different subsites of one particular lectin. Extraction of nuclei or subnuclear components followed by affinity chromatography on specific

neoglycoprotein adsorbents will need to be carried out to purify the putative lectin(s), and the relationship of the saccharide-binding specificities of these isolated lectins with the labeling patterns of the neoglycoproteins will need to be established.

S-TYPE LECTINS ARE FOUND INSIDE AND OUTSIDE OF CELLS

Two Groups of S-type Lectins

Evidence for the presence of lectins in the nucleus has also been accumulated through immunofluorescence localization and immunoblotting of subcellular fractions using antibodies directed against polypeptides previously purified by saccharide-specific affinity chromatography. Such studies have identified Lac-/Gal-specific lectins belonging to the S-type family of animal lectins, as classified by Drickamer (2). Lectins are grouped into this S-type family on the basis of conserved amino acid residues within a characteristic carbohydrate recognition domain (CRD), which is clearly distinguishable from the corresponding CRD of the calcium-dependent, C-type lectins. Some S-type lectins have been reported to depend on the presence of reducing agents (dithiothreitol or β-mercaptoethanol) for maintaining carbohydrate-binding activity, but more recent studies suggest that, for certain of these lectins, the thiol dependence may be ascribed to an artifact of the extraction procedure rather than an intrinsic requirement of the protein itself (17).

For the purpose of the present discussion on nuclear lectins, it is convenient to subdivide the S-type lectins into two (arbitrary) groups: (a) the L-14 group and (b) the L-30 group. The L-14 group consists of all the S-type lectins from various species and tissues whose reported polypeptide molecular weight on reducing sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) ranges from 10,000 to 16,000 (Table 1). There is evidence that polypeptides of the L-14 group represent members of a multi-gene family (18,19,20,24). At least some of the L-14 proteins listed in Table 1 appear to be distinct. For example, the two chicken lectins, C-14 and C-16, have homologous, but not identical, sequences (19). Moreover, CBP13.5 and CBP16 isolated from the same source exhibit slightly different carbohydrate-binding affinities (21). Similar observations have been made for the rat lectins, RL-14.5 and RL-22, and the human lectins HL-14 and HL-22 (22,23). In general, the polypeptide of the L-14 group consists of a single domain, the CRD (Figure 1). In non-denaturing solvents, the native L-14 molecules are often comprised of dimers of the constituent polypeptides.

The L-30 group of S-type lectins is defined those whose polypeptide molecular weights have been reported to be in the range of 29,000 to 35,000 by SDS-PAGE (Table 2). These include proteins originally isolated from various sources and designated with different names, but all of

TABLE 1. L-14 Group of S-Type Lectins[a]

Name	Species	Tissue/Cell Type	M_r (in reducing SDS-PAGE)	References
CLL-II	chicken	intestine, liver	12,000	34,67
BHL	calf	heart, lung spleen, thymus	12,000	35,68
CHL	chicken	heart	13,000	39
Galaptin	rabbit	bone marrow	13,000	42,69
CBP13.5	mouse, human	lung, fibroblast	13,500	21,70
C-14	chicken	skin	14,000	19,71
H-14	human	placenta, hepatoma	14,000	40
HL-14	human	lung	14,000	18,20,23
M 3T3 L	mouse	fibroblast	14,000	72
RL-14.5	rat	lung, brain, intestine	14,500	22,37,38
L-14.5	mouse	fibrosarcoma, melanoma	14,500	43,46,47,74
mGBP	mouse	embryonic fibroblasts	14,735	48
L-15	marmoset	neonatal marmoset	15,000	77
CLL-I	chicken	muscle, liver	15,000	34,67
C-16	chicken	liver	16,000	19
CBP16	mouse, human	lung, fibroblast	16,000	21,70
Xenopus skin lectin	frog	skin	16,000	44,45
Electro-lectin	electric eel	electric organ	16,000	75
RL-22	rat	lung	22,000	22
HL-22	human	lung	22,000	23

[a] This table provides a comprehensive list of the L-14 group of S-type lectins. Sequence data indicate that some of these lectins are identical (see text).

Figure 1: Summary of the structural features of S-type lectins.

A. Domain content and features of sequences within domains in the L-14 and L-30 groups. Shown are the 13 invariant amino acid residues that occur in a 39-residue sequence in the carbohydrate recognition domains of all of the L-14 and L-30 proteins for which sequence information is available. Also shown is the 9-residue consensus sequence that is repeated in the proline-, glycine-rich domain of L-30. The letter n, designating the number of repeats, ranges from 5 in the human Mac-2 sequence to 10 in the rat εBP sequence. A single residue between invariant residues is denoted by a hyphen (-). Sequences of two or more residues are denoted by the symbol (~).

B. Hydropathy plot of murine CBP35, illustrating the distinctive patterns that characterize the two domains of an L-30 polypeptide. The deduced amino acid sequence of CBP35 was subjected to analysis by the program of Hopp and Woods (78). Positive values indicate hydrophilicity and negative values indicate hydrophobicity.

Figure 1.

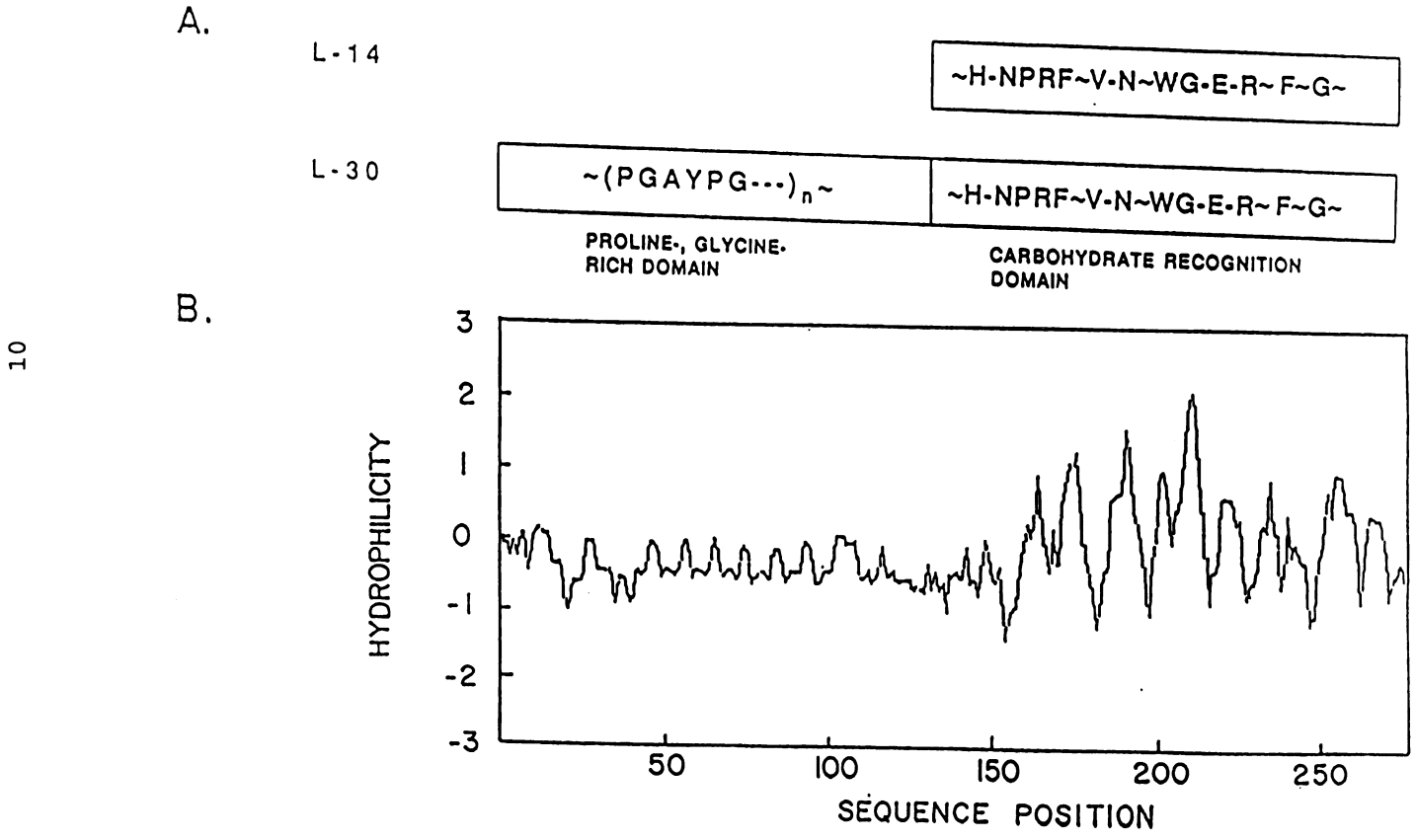
A.
L-14
~H-NPRF~V-N~WG-E-R~ F~G~
L-30
~(PGAYPG---)n~
~H-NPRF~V-N~WG-E-R~ F~G~
PROLINE-, GLYCINE-RICH DOMAIN
CARBOHYDRATE RECOGNITION DOMAIN
B.
HYDROPHILICITY
3
2
1
0
-1
-2
-3
50
100
150
200
250
SEQUENCE POSITION

Table 2. L-30 Group of S-Type Lectins[a]

Name	Species	Tissue/Cell Type	M_r (in reducing SDS-PAGE)	References
RL-29	rat	lung,brain	29,000	22,30,37,38
HL-29	human	lung	29,000	23,31
Hamster Lectin	hamster	BHK cells	30,000	33
εBP	rat, human	basophilic leukemia cells	31,000	27,50,51,76
Mac-2	mouse, human	macrophages	32,000	27,52,53,54
L-34	mouse	fibrosarcoma, melanoma	34,000	26,43,74
CBP35	mouse, human	lung, fibroblast	35,000	12,13,25, 50,51,70, 79,80,81,84
LBP	mouse	macrophage	35,000	28,33

[a] This table provides a comprehensive list of the L-30 group of S-type lectins. Sequence data indicates that some of these lectins are identical (see text).

which exhibit saccharide-binding activity: (a) CBP35 (25) from mouse 3T3 fibroblasts; (b) L-34 (26) from mouse tumor cells; (c) Mac-2 (27) from mouse macrophages; (d) Laminin Binding Protein (LBP) (28), also from mouse macrophages; (e) IgE-Binding Protein (εBP) (29) from rat basophilic leukemia cells; (f) RL-29 (30) and HL-29 (31), from rat and human lung tissue, respectively; and (g) hamster lectin (32) from BHK cells. The results of Southern blot analyses of genomic DNA suggest that there is only one gene coding for the polypeptides of the L-30 group (26,27). For any given species, the few differences in the amino acid sequences reported for the different proteins (e.g. mouse CBP35, L-34, and Mac-2) are most probably ascribable to sequencing errors. Although alternative sites of transcription initiation and/or alternative splicing of the primary transcripts may lead to multiple mRNA species, the polypeptides of the L-30 group listed in Table 2 are most likely identical. CBP35 has been shown to form oligomers in the absence of reducing agents (33). The polypeptides of the lectins in the L-30 group contain two domains; the CRD is coupled to another domain, presumably responsible for mediating some as yet unidentified effector function (Figure 1). Unlike the L-14 lectins, the L-30 lectins appear to be monomeric in non-denaturing solvents (17). LBP has been reported to exist as a dimer in non-reducing SDS-PAGE (33).

Because lectins of both the L-14 group and the L-30

group belong to the S-type family, the CRDs of all these lectins are homologous, with conservation of 13 characteristic amino acid residues (Figure 1). Thus far, all of the lectins with S-type CRDs bind to lactosamine-based glycoconjugates (2,3). In addition to similar CRDs, the lectins of the S-type family share another key characteristic: they are found in both the intracellular as well as the extracellular compartment. I will review the data concerning the subcellular localization of the L-14 group and of the L-30 group separately.

Subcellular Localization of L-14

Comparisons of the immunofluorescence staining of live cells versus cells fixed with formaldehyde followed by permeabilization indicate that proteins of the L-14 group are found predominantly in the intracellular compartment. Within the cells, the staining is mostly cytoplasmic. Observations and interpretations pertaining to the nuclear localization of the L-14 group are somewhat more difficult to establish.

First, there are studies that specifically show the localization of L-14 in the nucleus as well as the cytoplasm. Cryostat sections of tissues subjected to immunofluorescence showed labeling of both the nucleus and cytoplasm in the following cases: Anti-CLL-I staining of adult chicken kidney (34) and anti-BHL staining of calf pancreas (10). A monoclonal antibody, designated 36/8, was

generated against BHL and stained the nucleus and cytoplasm of lymphoblastoid and leukemic cells (35). This monoclonal antibody immunoblotted polypeptides of apparent molecular mass 13, 36, 65, 80 and 130 kD in extracts of lymphoid cells. More recent studies have shown that the 36/8 antibody recognizes the tetrapeptide sequence Trp-Gly-Ala/Ser-Glu/Asp (36) and, therefore, it is not clear whether the nuclear staining component is a lectin or some other polypeptide carrying this epitope sequence.

Second, in a number of studies originally performed for other objectives (e.g., to show overlap in cells expressing lectins and lactoseries glycoconjugates), the investigators provide no specific conclusion concerning the intracellular distribution of L-14. Dorsal root ganglion neurons were subjected to immunofluorescence with anti-RL-14.5 and with a monoclonal antibody directed against a lactoseries glycoconjugate (37). Although the intracellular distribution of the lectin was not discussed in that particular report, the same investigators have recently concluded, on the basis of the previously published data, that RL-14.5 could be detected in both the nucleus and cytoplasm of the neurons (38). Similarly, reassessment of previously published immunocytochemical studies in non-neuronal cells has suggested the presence of RL-14.5 in the nucleus and cytoplasm.

Finally, immunofluorescence and ultrastructural studies

also have led to explicit statements that anti-L-14 antibodies failed to label the cell nucleus. In chicken embryo fibroblasts, anti-chicken heart lectin (CHL) staining was observed only in cells fixed and permeabilized, but not in unfixed cells, suggesting that most of the lectin was intracellular (39). The intracellular staining was ascribed only to the cytoplasm. When 17-day-old chicken embryonic keratinized epidermis was stained with gold-labeled anti-C-14 under electron microscopy, gold particles were found around desmosomes, tonafilament bundles, and the intercellular space, while the cell nucleus was free of the particles (40).

Although there is little question that the L-14 proteins are found within cells, there is also considerable evidence that many of these lectins can be found outside as well (41-48). Moreover, the situation need not be static; there can be a shift from an intracellular to an extra-cellular location with differentiation or upon application of specific stimuli. Several striking observations by Barondes and colleagues are worth citing to illustrate this point. The skin of *Xenopus laevis* contains a lectin of the L-14 group that is found in the cytoplasm of granular and mucous gland cells (44). Upon injection of epinephrine, the lectin is externalized directly from the cytoplasm without the involvement of secretory vesicles. In another example, CLL-I, which is concentrated intra-cellularly in developing

chick muscle (19-day embryo), is exported from polynucleated myotubes and appeared extracellularly with maturation (2-day-old chick) (45). The process of externalization was studied in a mouse L2 myogenic cell line (46,47). Immunohistochemical localization during intermediate stages of externalization suggests that the lectin becomes concentrated in evaginations of the plasma membrane, which in turn pinch off to form labile lectin-rich extracellular vesicles. Thus, a novel mechanism for lectin export from the intracellular to the extracellular compartment has been suggested.

A growth inhibitory substance was purified from mouse embryonic fibroblast condition media. The amino acid sequence of this peptide fraction was identical to L-14. This protein was called galactose binding protein or mGBP. Curiously the inhibitory effects of mGBP on mouse embryonic fibroblast growth were not reversed by the presence of lactose. These results suggest that mGBP may play a regulatory role in the extracellular matrix independent of its carbohydrate binding activity (48). All of these results are consistent with the notion that L-14 found extracellularly may play a regulatory role in cell growth.

Subcellular Localization of L-30

The initial identification of L-30 in the nucleus of a cell was CBP35 (12). CBP35 was initially isolated from

extracts of mouse 3T3 fibroblasts on the basis of its Gal-/Lac-specific carbohydrate-binding activity. Although rabbit anti-CBP35 detected a small amount of the lectin on the surface of the 3T3 cells (by immunofluorescent staining, by agglutination of live cells, and by immunoisolation of ^{125}I-lectin after surface iodination), the predominant portion of the lectin was found to be intracellular by immunofluorescent staining of fixed and permeabilized cells. There was prominent labeling of the nucleus and variable staining of the cytoplasm. Cytoplasmic areas devoid of phase-dense intracellular vesicles stained diffusely, whereas areas rich in vesicular bodies stained in a highly reticular manner. When nucleoplasm or cytoplasm was subjected to sedimentation on cesium sulfate or sucrose density gradients, the position of migration of CBP35 was consistent with that of an RNP. In contrast, sedimentation of nuclease treated nucleoplasm or cytoplasm shifted CBP35 to the position of a free polypeptide (13,49).

The protein εBP was originally identified in rat basophilic leukemia cells as an IgE-binding protein (29). From a comparison of the amino acid sequence and binding properties of εBP with those of CBP35, it was established that the two proteins are rat and murine homologues, respectively, and that the εBP exhibited carbohydrate-binding activity (50). Subcellular fractionation studies on rat basophilic leukemia cells showed that the majority of

the εBP was intracellular, including in the nucleus (51). A small proportion of εBP was found on the cell surface. There is sufficient sequence data on RL-29 (30) to indicate a close relationship, if not identity, with rat εBP. Immunocytochemical studies both in non-neuronal cells and dorsal root ganglion neurons have shown that RL-29 can be detected in both the cytoplasm and the nucleus (37,38).

Two lectins designated L-14.5 and L-34 were purified on the basis of their carbohydrate-binding activity, from tumor cells such as murine melanoma cell lines B16-F1 and K-1735, fibrosarcoma UV-2237-IP3, and carcinoma HeLa-S3 (43). These proteins were located by immunofluorescence staining at the cell surface and, after fixation and permeabilization, in intracellular pools. The presence of L-34 (and of L-14.5) at the cell surface was established by lactoperoxidase-catalyzed ^{125}I-labeling, followed by immunoprecipitation. It has been reported that normal rat embryonal fibroblasts express only L-14.5 but, upon neoplastic transformation, also express L-34. Among related tumor cell variants of K-1735 melanoma, the UV-2237 fibrosarcoma and the A31 angiosarcoma, expression is highest in those cells that exhibit the greatest metastatic potential. It was thus suggested that the presence of L-34 at the cell surface may be related to neoplastic transformation and progression towards metastasis.

The Mac-2 antigen and LBP were both identified in mouse

macrophages on the basis of their cell surface localization. The former was originally described by a monoclonal antibody that stained the cell surface of macrophages and identified a M_r ~32,000 protein after surface iodination (52). More recent pulse-chase analysis and subcellular fractionation studies showed that although the majority of the Mac-2 was intracellular, a small proportion was found in the extracellular medium (27). In these experiments, actin could not be detected in the medium, suggesting that significant leakage of the cytosolic proteins did not occur. Since Mac-2 is expressed on thioglycollate-elicited peritoneal macrophages at a much higher level than resident macrophages, it may be induced during the process of inflammatory activation. Immunoprecipitation experiments of cytosolic extracts using monoclonal antibody directed against Mac-2, showed that the Mac-2 polypeptide precipitated with two associated glycoproteins, named M2BP-1 and M2BP-2. This interaction was shown to be carbohydrate mediated. One of these proteins M2BP-1 is secreted. The function of these proteins is not known (54).

The major non-integrin LBP of murine macrophages was identified as a M_r ~35,000 polypeptide on affinity columns of laminin-Sepharose (28). Amino acid sequencing of tryptic peptides of LBP revealed identities to sequences in CBP35. It was shown that LBP could be eluted from laminin-Sepharose by the addition of galactose or lactose. Cell surface

iodination followed by laminin-Sepharose affinity chromatography showed that LBP is the predominant macrophage surface protein that binds to laminin with high affinity, consistent with reports that implicate Gal residues on laminin as important determinants of cell adhesion (55). The intracellular localization of LBP was not reported, but since LBP and Mac-2 are presumably the same macrophage protein, the data on the large intracellular pool of Mac-2 also apply to LBP.

The hamster lectin was originally isolated from hamster kidney cells but is expressed in a variety different tissues. Triton X-100/Triton X-114 phase partition studies suggested that some of this lectin is associated with the plasma membrane. Immunolocalization studies of these tissues show that the lectin is found either in the cytoplasm space, or extacellularly (32). This dual localization mimics the behavior of the other L-30 lectins .

Issues Raised by Dual Intracellular and Extracellular Localization

In general, the fraction of L-14 or L-30 externally exposed at the cell surface represents only a small portion of that lectin in the cell. Inside the cell, the protein appears to be in the cytoplasm and/or nucleus rather than in the lumen of membrane-enclosed vesicles. This suggests that the lectin found in the extracellular compartment did not follow the classical endomembrane pathway of secretion.

Consistent with this notion, none of the cDNA clones identified for the L-14 or L-30 proteins has revealed an amino-terminal signal sequence for sequestration into the lumen of the endoplasmic reticulum. These observations on the S-type lectins parallel similar findings for a growing list of proteins with dual intracellular and extracellular localization: (a) proteins with nuclear functions such as probasin (56), simian virus 40 (SV40) large T-antigen (57), adenovirus E1A gene product (58), and La RNP identified by autoimmune anti-nuclear antibodies (59); (b) growth or differentiation factors capable of binding to cell surface receptors in autocrine or paracrine target systems such as the heparin-binding growth factors (including acidic and basic fibroblast growth factor) (60), platelet-derived endothelial cell growth factor (61), interleukins 1α and 1β (62), and the yeast mating hormone, a-factor (63); and (c) other extracellular proteins such as factor XIIIa (64) and ATL-derived factor (65). The data on probasin and on SV40 large T-antigen will be highlighted to contrast and compare the mechanisms of dual localization.

Probasin is a rat prostatic protein found both in secretions and in the nucleus of prostatic epithelial cells (56). Only one probasin mRNA could be detected by primer extension and S1 nuclease protection assays. *In vitro* translation of this mRNA demonstrated that a protein containing a signal sequence and a protein lacking a signal

sequence were synthesized by initiation at different AUG codons. In contrast, the SV40 large T antigen contains a nuclear localization signal, but no obvious signal sequence for secretion (57). Although the majority of T-antigen is found in the cell nucleus, a small fraction (< 5%) is localized at the cell surface. The protein is glycosylated and represents an example of a nuclear glycoconjugate (see below). It also undergoes post-translational palmitylation. Palmitylation is the only known structural difference between the plasma membrane and nuclear forms of T antigen (66), as none of the nuclear T antigen population is modified in this way.

In the course of this survey on nuclear lectins, it has also become apparent that the basis for designating a protein as intracellular or extracellular may need to be reconsidered. On the one hand, proteins with documented activities on DNA replication and RNA metabolism have now been found at the cell surface. In contrast the other, soluble factors with identified cell surface receptors have also been observed in the nucleus. Thus, it appears that we may be at the edge of discoveries with respect to novel mechanisms of protein externalization/internalization and new views of subcellular compartments in general.

CARBOHYDRATE BINDING PROTEIN 35

As is apparent from the above discussion, the S-type lectins have been studied from a number of perspectives.

Among these, murine CBP35 has been pursued most intensively as a nuclear lectin. On this basis, the properties of CBP35 will be highlighted. It should be kept in mind, however, that all of the L-30 proteins listed in Table 2 are either identical (within a given species) or are homologues (between species). Therefore, some of the information on CBP35 to be discussed below was originally obtained in a context other than as a nuclear protein.

Structure

Murine CBP35 is a monomer comprised of 264 amino acid residues. A hydropathy analysis of the deduced amino acid sequence (25) indicates that the protein consists of two structural domains (Figure 1), an arrangement that is also found in several of the C-type lectins (2). The carboxyl-terminal half of the CBP35 molecule contains both hydrophilic and hydrophobic regions, as is characteristic of many globular proteins. This region includes a 76-residue sequence that is about 35% identical with several members of the L-14 group of Gal-/Lac-specific lectins. Thirteen amino acid residues are invariant in every L-14 and L-30 for which sequence information is available (Figure 1). Consequently, the carboxyl-terminal half of CBP35 is assumed to harbor the CRD. In contrast, the amino-terminal half of the molecule contains neither a highly hydrophilic nor a hydrophobic region. This half includes a stretch of eight contiguous 9-residue repeat units having the sequence: Pro-Gly-Ala-Tyr-

Pro-Gly followed by three other residues. As a result, this stretch of the sequence is characterized by a high proportion of Pro and Gly. Certain of the core heterogenous nuclear ribonucleoprotein complex (hnRNP) proteins also exhibit two distinct domains with a largely unequal distribution of Gly and Pro residues in the two halves. Over a 107-residue region, the sequence of CBP35 is about 25% identical with some of the core proteins of hnRNP. This degree of identity is comparable to that found among the core hnRNP proteins themselves. Raz *et al.* (26) have pointed out that the amino-terminal half of murine L-34 also has 33.5% identity with the collagen α1 (II) chain of bovine cartilage.

The pI of murine CBP35 is 8.7, as determined both by calculation from the deduced amino acid sequence and experimentally by isoelectric focussing of recombinant CBP35 obtained by expression of a cDNA clone in *Escherichia coli* (79). When extracts of mouse 3T3 cells were subjected to two-dimensional gel electrophoresis and immunoblotting, two spots were observed, corresponding to pI values of 8.7 and 8.2. The pI 8.2 species represents a post-translational modification of the pI 8.7 polypeptide by the addition of a single phosphate group, probably O-linked. These two isoelectric variants have differential expression and localization in cells (see below).

Homologues of murine CBP35 have been identified in

other species, and those from rat (29,30) and human (31,53,76) have been sequenced. The rat and human lectins are comprised of 262 and 250 amino acids, respectively, in contrast to the 264 amino acids that make up the mouse lectin. However, the homology of the lectins among the three species is very high: for the carboxyl-terminal half, which represents the CRD, the identity is well over 80%; the amino-terminal half also has highly conserved sequences, including the 9-residue repeated sequence. Oda *et al.* (31) have suggested a third domain, that is located at the amino-terminal end (residues 10-39 of HL-29), is 46% identical to residues 18-47 of human serum response factor, a transcriptional activator.

Saccharide-binding Specificity

In the initial isolation (21), it was established that murine CBP35 could be eluted from asialofetuin-Sepharose columns by lactose or galactose, but not by mannose, sucrose, or N-acetylglucosamine. Lactose was more efficient than galactose, and concentrations as low as 250 μM caused 50% inhibition of binding of the lectin to erythrocytes (70). More extensive analysis of the saccharide-binding specificity has been reported for RL-29 and HL-29 (22,23). The binding activity was determined by measuring the concentration of various saccharides that gave 50% inhibition of binding of the lectin to asialofetuin-Sepharose columns. The affinity of RL-29 and HL-29 for lactose was found to be 57

to 100 times greater than that for galactose. The parts of the lactose molecule that were critical for binding include the hydroxyls at positions 4 and 6 of Gal and position 3 of Glc, since substitutions at any of these positions greatly reduce binding activity. Substitution at positions 2 of Gal or 1 or 6 of Glc have little effect on the binding activity. However, binding was enhanced 7 to 11 times by the incorporation of an acetamido group at position 2 of Glc (i.e., to yield N-acetyllactosamine). For RL-29 and HL-29, an even greater enhancement of binding affinity resulted from the addition of GalNAcα1- at position 3 and Fucα1- at position 2 of the Gal moiety of lactose derivatives(i.e. to yield blood group A tetrasaccharide), although the binding affinity of GalNac or Fuc themselves is low. These lectins also have high binding affinity for larger oligosaccharides (e.g., fetuin oligosaccharide) and glycopeptides (e.g., erythrocyte lactosaminoglycan glycopeptide) that have the lactosyl determinants discussed above.

The saccharide-binding specificity of the L-14 group of lectins is similar to that of the L-30 group, but there are sufficient differences in specificity between these groups to suggest the possibility of selective interactions with cellular glycoconjugates. For example, the L-30 lectins have a high affinity for blood group A tetrasaccharide, while L-14 lectins have a much lower affinity for this carbohydrate. Within the L-30 group, the binding specificities of RL-29

and HL-29 are very similar but, nevertheless, distinguishable (22,23).

Regulation of Expression and Subcellular Localization

Crittenden *et al.* (70) noted wide differences in the level of CBP35 in various tissues of mice and, for some tissues, between embryonic and adult stages. Ho and Springer (52) found that induction of cell-surface Mac-2 in mouse macrophages was dependent on a specific eliciting agent such as thioglycollate. Raz and Lotan (26) found that the expression of L-34 occurred concomitantly with neoplastic transformation of normal rat embryonal fibroblasts. Taken together, these and other observations indicate that the lectin is differentially expressed during development, stimulation, or change in growth status or potential of cells.

In quiescent cultures of 3T3 fibroblasts, CBP35 is found primarily in the cytoplasm, whereas in proliferating cultures, it increases in amount and is located predominantly in the nucleus. The addition of serum growth factors to quiescent 3T3 cells increases the expression of CBP35; this increase occurs early after stimulation, well before the onset of the first S-phase of the cell cycle (80,81). These conclusions are derived from as follows: (a) at the single cell level by immunofluorescence; (b) at the protein level by Western blotting; (c) at the mRNA level by Northern blotting; and (d) ot the transcriptional level of

the CBP35 gene in nuclear run-off experiments. At the mRNA accumulation and gene transcription levels, the increases in the expression of CBP35 occur even in the presence of cycloheximide. In this respect, the expression of CBP35 resembles that of c-fos, whose transcription is also activated in response to serum growth factors, independent of the synthesis of other proteins (82).

There is a single CBP35 gene in the normal mouse genome, based on sequence analysis of genomic clones (Jia S. and Wang J.L. unpublished results). This gene spans about 9 kb of genomic DNA and contains five exons and four introns. Upstream from the initiation site of transcription, putative TATA and CCAAT boxes are found at positions corresponding with similar sequences of other genes. There is also a putative serum response element, a 10-nucleotide consensus sequence known to bind serum-responsive transcription factors during the activation of c-fos and β-actin genes (83), about 200 nucleotides upstream from the same transcription start site. Finally, a polyadenylation signal is found in the 3′ untranslated region. These structural features may account for the regulation of expression of the CBP35 gene, particularly during serum-stimulated activation of quiescent cultures of 3T3 fibroblasts.

At the protein level, the expression of CBP35 was further analyzed by subcellular fractionation, two-dimensional gel electrophoresis to separate the isoelectric

variants, and immunoblotting (79). The phosphorylated (pI 8.2) form of the CBP35 is found both in the cytosol and nucleus, whereas the unmodified (pI 8.7) species is found exclusively in the nucleus. Quiescent populations express primarily the phosphorylated form, but it is at a low level and is located predominantly in the cytoplasm. Serum-stimulated cells have an increased level of the phosphorylated form, both in the cytosol and the nucleus, but the amount of the unmodified (pI 8.7) form increases dramatically and is all nuclear.

When quiescent young (passage 11) SL66 fibroblasts were treated with serum there was a dramatic increase in the expression of CBP35 (84). This was shown by immunoblotting studies and immunofluorescence analysis with anti-CBP35 antiserum. Two dimensional gel electrophoresis showed that these cells expressed both isoelectric forms of CBP35. In contrast older cultures of SL66 cells (passage 31-35) did not exhibit proliferation dependent expression of CBP35 (84). They also do not express the pI 8.7 form of the protein. The significance and mechanism of the differential expression and localization of the two isoelectric variants of CBP35 remain as some of the many intriguing, but unanswered, questions regarding this and related lectins.

CARBOHYDRATE STRUCTURES IN THE NUCLEUS

There has been little or no evidence that carbohydrate recognition occurs via the nuclear lectins discussed above.

For one thing, the endogenous carbohydrate ligand(s) to which the nuclear (and cytoplasmic) lectins can bind remains to be identified. Although the subject of nuclear and cytoplasmic glycosylation has been reviewed elsewhere (1,88), it is, nevertheless, important to consider several classes of candidates for these ligands. Just as the binding of neoglycoproteins to nuclear structures implicated lectins, the binding of the lectins to nuclear components has implicated nuclear glycoconjugates. These observations have been confirmed by the detection of nuclear glycoconjugates by other methods: a) determination of carbohydrate content of isolated nuclear components; and b) studies in which nuclear components were labelled with specific glycosyl transferases. These studies have been reviewed in detail elsewhere (1,8,85). In this section of my literature review, I will focus on studies in which the glycoconjugates having GlcNAc, Gal or Man containing saccharide moieties have examined.

Nuclear binding sites for plant lectins.

Studies of the binding of wheat germ agglutinin (WGA) to nuclear components provided some of the first evidence for GlcNAc containing glycoconjugates in the nucleus (86-88). There are WGA binding sites on the nuclear envelope that are associated with the nuclear pore complex (89-91). Fluorescently conjugated WGA strongly labels mitotic and interphase chromatin structures in cultured fibroblasts

(92). WGA has been shown to bind to polytene chromosomes isolated from *Drosophila* (93). Some of these chromatin associated WGA binding sites have been be found in many different components of the nucleus.

Glycoprotein that bear Gal or GalNAc containing carbohydrate moieties can be detected with soybean agglutinin (SBA) or *Ricinis communis* agglutinin (RCA) (85, 94-96). Transformation of rat liver induced the expression of RCA-binding proteins (two 95 kD polypeptides, and a 55 kD polypeptide) that were associated with the nuclear matrix (95). Kinoshita *et al.* have shown that RCA and SBA binding sites can be found in chromatin isolated from sea urchin embryo. Gastrulation increased the number of RCA binding sites in the chromatin, but did not modify the amount of SBA binding (96). These SBA and RCA binding sites have not been identified.

Man-containing glycoconjugates can be detected by the lectin concanavalin A (ConA). ConA binding sites are associated with the nuclear membranes (97,98). It has been suggested that this lectin staining was largely due to ConA binding to gp-210 in broken nuclei (99-102). Characterization of gp210, the most prominent ConA binding glycoprotein in the nucleus, showed that it is an integral membrane protein, and its carbohydrate moiety was in the lumen of the perinuclear space (101,102). Thus, gp210 was not a true nuclear glycoprotein. Chromatin isolated from a

number of different sources had ConA binding sites (92,103). These glycoproteins have not been further characterized.

Glycoproteins containing O-linked GlcNAc residues

Studies of nuclear WGA binding sites have led to the identification of a novel carbohydrate moiety; GlcNAc glycosidically linked to a polypeptide through a hydroxyl group (hereafter designated as O-GlcNAc). Proteins bearing O-GlcNAc moieties have been found in the cytoplasm and the nucleus of cells from mammalian sources (1,85-92,104,105, 108,122,123,127,130-132,135), *Drosophila* (93,105), cytomegalovirus (106) and the blood fluke *S. mansonii* (107). A list of glycoproteins containing this structure is provided in Table 3. Many of these proteins have been shown to bind to WGA (105-109, 122-127, 130-135) and can be labelled by UDP[^{3}H]-gal and galactosyl transferase, which specifically labels terminal GlcNAc residues (105-109, 122-126, 130-134).

An assay for UDP-GlcNAc: peptide GlcNAc transferase has led to the identification and purification of the enzyme (M_r ~115,000)(111, 112). A cytoplasmic N-acetylglucosaminidase (M_r ~224,000) was purified from extracts of human lymphocytes (113). These activities provide a novel pathway for glycosylation and de-glycosylation of polypeptides without involving the endoplasmic reticulum or Golgi apparatus.

Activation of lymphocytes by mitogens transiently increased the number of proteins possessing O-GlcNAc

Table 3. O-GlcNAc Modified Nuclear Proteins[a]

Name	Species	Tissue/Cell Type	M_r (in reducing SDS-PAGE)	References
Nucleoporin/p54	rat	liver	54,000	89,90,121
Nucleoporin/p58	rat	liver	58,000	89,90,121
Nucleoporin/p62	rat	liver	62,000	89,90,121
Nucleoporin	rat	liver	145,000	89,90
Nucleoporin	rat	liver	180,000	89,90
Nucleoporin	rat	liver	210,000	89,90
p43	human	HeLa	43,000	135
RNA Polymerase II	human	HeLa	214,000	132
Sp-1	human	HeLa	95,000	105,127
Sp-1	human	HeLa	105,000	105,127
CTF/NE	human	HeLa	60,000	105
CTF/NE	human	HeLa	62,000	105
AP-1	human	HeLa	39,000	105
AP-1	human	HeLa	47,000	105
AP-1	human	HeLa	52,000	105
AP-2	human	HeLa	52,000	105
AP-4	human	HeLa	40,000	105
SRF	human	HeLa	67,000	130
HNF-1	mouse	hepatocytes	80,000	126
PTF1	rat	acinar pancreas	75,000	131
Zeste	*Drosophila*	embryo	80,000	105
GAGA	*Drosophila*	embryo	67,000	105
GAGA	*Drosophila*	embryo	72,000	105
GAGA	*Drosophila*	embryo	80,000	105
Adf-1	*Drosophila*	K_c	37,000	105

[a] This table provides a comprehensive list of O-GlcNAc bearing glycoproteins that are found in the nucleus. Each polypeptide is listed separately.

modifications in the nucleus. There was also a transient decrease in the number of proteins having O-GlcNAc modifications in the cytoplasm (114). This suggests that the levels of O-GlcNAc moieties can be modulated in the same manner as phosphorylation.

The function of the O-GlcNAc modifications in these proteins is still not known. Several studies have shown that WGA can inhibit nucleocytoplasmic transport, suggesting O-GlcNAc plays a role in this transport process (108,109). Other studies have suggested that O-GlcNAc masks possible sites of protein phosphorylation (109,110,113,132), protects proteolytic cleavage sites (109,122,123), allows assembly into macromolecular complexes (128), or acts as a targeting signal for nuclear transport (109,129).

A distinct property of O-GlcNAc bearing glycoproteins is that the O-GlcNAc moieties are particularly immunogenic. An array of monoclonal antibodies was developed against rat liver nuclear pore complexes. These antibodies detected a set of proteins of 54-210 kD (Table 3). Each of these proteins contained 10-12 O-GlcNAc moieties. The epitopes that these proteins shared were the sites of O-GlcNAc modifications (89,90). Similarly a monoclonal antibody directed against nuclear pore complexes of Chinese hamster ovary cells identified a family of O-GlcNAc containing glycoproteins. These proteins also shared epitopes that are characterized by O-GlcNAc moieties (91).

The import of proteins into the nucleus can be divided into two steps: a) nuclear proteins bind to the periphery of the nuclear pore complex and b) proteins pass through the pore in an ATP-dependent manner (115-121). WGA and antibodies directed against components of the nuclear pore have been shown to inhibit this import phenomena (115-118) Control experiments using proteins that are smaller than the exclusion limit of the nuclear pore complex (30 kD), such as myoglobin, indicate that this inhibition cannot be explained by occlusion of the nuclear pore (115-119). Forbes and co-workers have developed a system with which GlcNAc bearing protein can be specifically removed from the nuclear pore. These O-GlcNAc bearing glycoproteins depleted nuclear pores maintain a normal morphology, but do not import protein. Addition of the depleted pore glycoproteins fully restores protein import (120). Further immunoaffinity fractionation of these nuclear pore glycoproteins demonstrated that a complex of three proteins, (p62, p58, and p54) was necessary to maintain nucleocytoplasmic transport of proteins (121).

Microinjection of antibodies directed against O-GlcNAc modified proteins of the nuclear pore complex can inhibit the export of RNA from the nucleus. Analysis of RNA transport in clam oocytes demonstrated that antibodies directed against p67, an O-GlcNAc bearing glycoprotein, in the nuclear pore complex and WGA produced a reduction in

total amount of RNA transported from the nucleus (88). Similarly, other antibodies against O-GlcNAc bearing nuclear pore complex glycoproteins reduced the export of 5S RNA and tRNA in a rat liver nuclei system (118). These data indicates that O-GlcNAc bearing glycoproteins are associated with the channels through which RNA is exported from the nucleus and may play a role in this transport process (88,118).

The best characterized and most abundant nuclear pore complex WGA binding protein is known as p62 (109,122,123). Each p62 polypeptide has 10-12 O-GlcNAc moieties (109, 122,123). Structural analysis of the polypeptide yielded a proteolytic fragment that contained an O-GlcNAc-modified serine. The sequence of this p62 peptide fragment was used to isolate a cDNA clone (124,125). The amino acid sequence deduced from the nucleotide sequence of this clone contains several serine rich domains that could be recognized by the GlcNAc transferase (114).

In addition to nuclear pore complex polypeptides, a second group of O-GlcNAc bearing glycoproteins is composed of RNA polymerase II transcription factors (105,127-131). Transcription factors for a number of human promoters (Sp-1, CTF/NE, AP-1, AP-2, AP-4, PTF1, HNF-1, and SRF) and *Drosophila* promoters (Zeste, GAGA and Adf-1) can be labeled with UDP-[^{3}H]Gal and galactosyl transferase. The AP-1 transcription factor consists of polypeptides known as c-

fos, c-jun and a fos related antigen each of which contains 10-12 O-GlcNAc moieties (105,127,130,131). In contrast, transcription factors for RNA polymerase I (UBF1) and RNA polymerase III (TFIIIA) did not contain O-GlcNAc moieties. This data implies that O-GlcNAc modifications may play a role in the activity of RNA polymerase II transcription factors (105).

Sp-1 was the first transcription factor that was shown to contain O-GlcNAc moieties. This protein consists of 2 polypeptides having molecular weights of 95 and 105 kD (105,128,129). The 105 kD polypeptide is derived by phosphorylation of the 95 kD form of the protein (128). The Sp-1 polypeptide has alternating glutamine-rich and serine-rich sequences in its amino terminal domain (129), which are thought to be the sites of the O-GlcNAc modifications. Treatment of Sp-1 with WGA reduced its transcriptional enhancing activity. A recombinant Sp-1 isolated from *E. coli*, which is unglycosylated, was 3-5x less active than glycosylated Sp-1 isolated from HeLa cells (105). Accordingly, the amino terminal domain may be responsible for the transcriptional enhancing activity of the protein (128,129). Curiously, labeling of Sp-1 with UDP-[^{3}H] galactose and galactosyl transferase did not affect its ability to enhance transcription (105). All of these observations support the notion that glycosylation protects the protein from a process that reduces its transcription

enhancing activity (105,128,129). This provides a fascinating insight into the role that O-GlcNAc glycosylation could play in this nuclear glycoprotein.

One particularly interesting nuclear glycoprotein is associated with the pancreatic transcription factor (PTF1). PTF-1 exists in two forms (PTF1α and PTF1β)that can be resolved by electro-phoretic mobility shift assay. PTF1α and PTF1β are found in the cytoplasm. The nucleus contains only PTF1α. The major difference between these transcription factors is that PTF1α is associated a 75 kD O-GlcNAc containing glycoprotein (p75), that is not associated with PTF1β. Cross-linking assays demonstrated that p75 does not bind the PTF1 cognate sequence. It has not been determined how p75 associates with PTF1α (130).

The carboxyl terminus of the large subunit of RNA polymerase II contains a distinctive amino acid sequence (Tyr-Ser-Pro-Thr-Ser-Pro-Ser) that is repeated 52 times (131). An O-GlcNAc modification is found in this repeat (132). This sequence can also be extensively phosphorylated. The phosphorylated form of this protein does not possess an O-GlcNAc moiety and is more transcriptionally active (132, 133). This result suggests that modulation of the O-GlcNAc moiety as well as phosphorylation may regulate RNA polymerase II activity.

The antigen for an anti-nuclear antibody isolated from the sera of dogs having systemic lupus erythematosus has

been characterized as a 43 kD glycoprotein (p43). Cell fractionation and immunoprecipitation experiments have demonstrated that p43 is associated with hnRNP. The p43 polypeptide could be metabolically labelled with [^{14}C] GlcNAc. Lectin blotting experiments showed that p43 bound WGA but not ConA or the Fuc specific lectin, *Ulex europaeus* agglutinin (UEA), suggesting that this protein has an O-GlcNAc modification. These data are of particular interest as they are the first evidence of a glycoprotein in hnRNP, the nuclear fraction with which CBP35 is associated (134).

Many different nuclear proteins have O-GlcNAc modifications, as is summarized in Table 3. Most of these proteins are associated with the nuclear pore complex or RNA metabolism. As the activities that occur at the nuclear pore are not well understood, it is difficult to discern the role that the O-GlcNAc moieties may play in these activities. However the influence that O-GlcNAc moieties play in the RNA processing machinery are more clearly illustrated. In Sp-1, the O-GlcNAc moieties are needed to maintain transcriptional enhancing activity (105). In RNA polymerase II, the O-GlcNAc moieties suppress the activity of the protein possibly by preventing phosphorylation (132). Clearly, the O-GlcNAc modification must be capable of producing different effects in these different systems, in a manner analogous to phosphorylation as suggested by Kearse and Hart (114).

Other nuclear glycoproteins and glycoconjugates

Although the binding of plant lectins, including RCA, ConA, and UEA, is well established, the structures of the glycosylated moieties remain to be established. One SBA binding glycoprotein that is of considerable interest is the large T antigen of the SV-40 virus (57,66,135-137). This multipurpose protein is necessary for the replication of SV-40 in virus infected cells. Metabolic labeling experiments have shown that the SV-40 T antigen can incorporate both [^{3}H]Gal and [^{3}H]GlcNAc (66,135-137); this labeling has been shown to be resistant to tunicamycin treatment and endoglycosidase H digestion, suggesting that the saccharide is O-linked (66,137,138). Although originally isolated in the nucleus, consistent with its biological activities, a considerable amount of effort has been expended to show rigorously that the protein also exists at the cell surface. The SV-40 large T antigen is a prime example not only of a nuclear glycoprotein, but one in which there is a good example of extracellular and intracellular localization. From the perspective of our discussion on the S-type lectins, the SV-40 large T antigen is interesting because the disaccharide structure implicated would serve as a good ligand for the S-type lectins.

The high mobility group proteins (HMG) are abundant non-histone chromatin proteins named for their electrophoretic mobilities. They undergo a number of post-

translational modifications, including methylation, acetylation, phosphorylation, poly(ADP) ribosylation, and glycosylation (138). Metabolic labeling experiments indicate that the HMG proteins contain Man, GlcNAc, Glc, Gal, and Fuc (139). These proteins could also bind to the Fuc specific lectin UEA (139). Although it was suggested that this carbohydrate moiety play a role in the adhesion of the HMG proteins to the nuclear matrix (140), the precise structure of this carbohydrate remains to be established.

While there is a large body of evidence that the O-GlcNAC modified proteins are found in the nucleus, the list of nuclear glycoconjugates containing other saccharides is more limited. A significant weakness of these studies is that the structure of the saccharide moieties of these glycoproteins has not been determined. Structural studies would determine if these saccharide moieties are new structures, or have been previously identified. The question of how these proteins are synthesized could then be addressed. This would expand our knowledge of the glycoproteins found in the nucleus of the cell.

Finally, it should be noted that a number of lines of evidence indicate that glycosaminoglycans can be found in the nucleus (1). Recent studies of rat hepatocytes have indicated that there is a structurally unique nuclear heparan sulfate. This heparan sulfate is characterized by a large amount of an unusual sulfated glucuronosyl residue

that is not found in other cellular compartments. Normally, heparan sulfate is not sulfated until glucuronic acid is converted to iduronic acid. Quantitation showed that this nuclear heparan sulfate accounted for ~6% of the heparan sulfate in the cell (141).

The synthesis of this heparan sulfate has been studied in detail (142-147). Hepatocytes can transport exogenously added heparan sulfate proteoglycan to the nucleus (143,144). This uptake is mediated by a high affinity heparan sulfate receptor (145,146). Pulse chase studies showed this heparan sulfate appeared in the nucleus within 2 hours of synthesis (142). Heparan sulfate treatment of hepatocytes blocks cellular proliferation (143-147) and suppresses the activity of the the transcription factors c-fos and c-jun (147). This effect may be caused by heparan sulfate breaking the association of the transcription factors with DNA (147). These data suggests that nuclear heparan sulfate may play a role in regulating transcriptional activity of cell cycle dependent genes. These observations could open an interesting new chapter in the function of nuclear glycoconjugates.

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CHAPTER II

BIOCHEMICAL AND IMMUNOLOGICAL COMPARISONS OF CARBOHYDRATE BINDING PROTEIN 35 AND AN IgE-BINDING PROTEIN

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FOOTNOTES

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ABBREVIATIONS: εBP, IgE-binding protein; RBL,rat basophilic leukemia; CBP35, Carbohydrate Binding Protein (M_r ~35,000); ASF, asialofetuin; Gal-Sepharose, Sepharose derivatized with N-(ε-aminocaproyl)-D-galactosamine; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

ABSTRACT

The predicted amino acid sequence of Carbohydrate Binding Protein 35 (CBP35; Mr ~35,000), a galactose-specific lectin found in many mouse and human cells, has been compared to the predicted sequence of an IgE-binding protein (εBP) originally identified in rat basophilic leukemia cells. The sequences of the two proteins showed that: (a) 85% of the amino acid residues are identical; (b) the polypeptide chains are comprised of two distinct domains; and (c) highly conserved internal repetitive sequences are present. Consistent with these observations, antisera raised against CBP35 or against a synthetic peptide derived from the εBP sequence cross-reacted with both proteins. Moreover, fractionation of extracts of mouse 3T3 fibroblasts over an IgE-Sepharose affinity column showed that CBP35 bound to IgE; this binding was reversed by addition of lactose. Conversely, fractionation of extracts of rat basophilic leukemia cells over an affinity column of Sepharose derivatized with N-(ε-aminocaproyl)-D-galactosamine showed that εBP was a galactose-binding protein. Furthermore, εBP bound to IgE-Sepharose could be eluted by lactose. Finally, transcription and translation *in vitro* of the cDNA corresponding to εBP yielded a polypeptide containing carbohydrate-binding activity. All of these results strongly support the conclusion that CBP35 and εBP are respectively mouse and rat homologues.

INTRODUCTION

The IgE-binding protein designated εBP is a Mr ~31,000 polypeptide that was first identified as an in vivo (1) and in vitro (2) translation product of mRNA derived from rat basophilic leukemia (RBL) cells. This protein bound IgE and was reactive with antibodies raised against RBL proteins purified on an IgE-containing affinity column. This IgE-binding protein is also expressed in certain cell types known to possess IgE-specific receptors (2). The cDNA coding for this protein was cloned and the deduced amino acid sequence showed unique structural features of the polypeptide, including the delineation of two distinct domains and the presence of a highly conserved repetitive amino acid sequence (3). Recently, it was shown that mRNA that hybridizes to the εBP cDNA is produced in many rat tissues and cell lines in addition to RBL cells(4).

Carbohydrate Binding Protein 35 (CBP35) is a Mr ~35,000 polypeptide initially purified from extracts of cultured mouse 3T3 fibroblasts on the basis of its binding to galactose-containing glycoconjugates (5). It is produced by a number of adult and embryonic tissues (6). Subcellular localization studies of this protein have identified it in the nucleus (7), possibly as a component of the heterogeneous nuclear ribonucleoprotein complex (8).

Recently, the nucleotide sequence of a cDNA clone for CBP35 was determined (9,10); the deduced amino acid sequence

was strikingly similar to that of εBP, including the organization of the polypeptide chain into two distinct domains and the presence of a highly conserved repetitive sequence. These observations prompted us to carry out experiments to establish the relationship between εBP and CBP35. In the present communication, we report several lines of evidence that indicate εBP and CBP35 are the rat and mouse homologues, respectively, of the same protein.

MATERIALS AND METHODS

Cell Lines and Reagents. Extracts were prepared from RBL cells (11) (kindly provided by H. Metzger of the National Institutes of Health) and from Swiss mouse 3T3 fibroblasts (American Type Culture Collection, CCL92, Rockville, MD). The preparation of asialofetuin (ASF) Sepharose, its use in the purification of CBP35, and the generation of rabbit antiserum directed against CBP35 (anti-CBP35) have been described (5). Affinity columns were prepared from Sepharose 4B (Pharmacia, Uppsala, Sweden) derivatized with N-(ε-aminocaproyl)-D-galactosamine (Gal-Sepharose) (8). Mouse IgE derived from an IgE-secreting hybridoma (12) was conjugated to CNBr-activated Sepharose 4B to prepare IgE-Sepharose as previously described (2). Rabbit antisera designated anti-εBP 1 and 2 were raised against synthetic peptides 1: FNPRFNENNRRVIC and 2: CNTKQDNNWGREERQ derived from the deduced εBP amino acid sequence (4).

Comparison of the Amino Acid Sequence and Structural Features of the Polypeptides. The identification and nucleotide sequences of the cDNA clones for εBP (clone E8 cDNA) (2,3) and CBP35 (clone 1 cDNA) (9,10) have been reported. The deduced amino acid sequences were compared using the FASTP program (13) and analyzed in terms of hydrophilicity or hydropathy value (14).

Affinity Chromatography and Immunoblotting Analysis. RBL and 3T3 cell extracts were prepared as described previously (1,4-7). For immunoblotting analysis, ~10 μg of isolated proteins or proteins of total cell extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (15). The separated proteins were transferred to nitrocellulose paper (16) and immunoblotted with rabbit anti-εBP or anti-CBP35 as described (4,8). For affinity chromatography on ASF-Sepharose (5) or Gal-Sepharose (8), 100μl of the derivatized beads were packed into a Quik-Sep Micro Column (Isolabs, Akron, OH) equilibrated with PBS and 10 mM β-mercaptoethanol. RBL or 3T3 cell extracts were applied to the column and washed with 6 ml of PBS and 10 mM β-mercaptoethanol. The bound material was eluted with 100 μl of PBS, 0.3 M lactose and 10 mM β-mercaptoethanol. A similar procedure was used for affinity chromatography with IgE-Sepharose, except the washing was performed with 20 mM Tris, pH 7.5,1 mM EDTA, 150 mM NaCl, 1% Trasylol and 1%

Triton X-100 for purification of εBP and with PBS and 10 mM β-mercaptoethanol for purification of CBP35. The eluted materials were then subjected to SDS-PAGE and immunoblotting analysis.

***In Vitro* Transcription and Translation.** *In vitro* transcription and translation of the PST I- PVU II fragment from the cDNA clone for εBP (clone E8) has been described previously (3). The translation products were affinity purified on IgE-Sepharose or ASF-Sepharose as above. Material bound to the affinity support was eluted with 0.3 M lactose and was analyzed by SDS-PAGE and fluorography (EN3HANCE; New England Nuclear, Boston, MA).

RESULTS

Comparison of the Primary Structure of EBP and CBP35. The amino acid sequences of εBP (3) and CBP35 (10) were deduced from the nucleotide sequences determined for their respective cDNA clones (Fig. 1). The sequences were compared using the FASTP program (13). The open reading frame of the cDNA clone for εBP codes for 262 amino acids; 223 of these amino acids (85%) were identical to CBP35. Three gaps were introduced to optimize the alignment (Fig. 1). Of the 38 positions that had different amino acids, 10 of them occurred between residues 94 and 103. The remaining differences involved mostly conservative substitutions (e.g., Gly/Ala, Phe/Tyr, Arg/Lys, Val/Ala, etc.).

As previously reported, the polypeptide sequence of

FIGURE 1: **Comparison of the amino acid sequence of εBP and CBP35.** The amino acid sequences of εBP and CBP35 were deduced from the nucleotide sequences of cDNA clones E8 (3) and clone 1 (10), respectively. The numbering system is derived from the open reading frame of εBP cDNA. Two gaps, indicated by *, were introduced for optimal alignment. The sequence of CBP35 is indicated only at positions that differ from the εBP sequence.

Figure 1.


cBP Met Ala Asp Gly Phe Ser Leu Asn Asp Ala Leu Ala Gly Ser Gly Asn Pro Asn Pro Arg Gly Trp Pro Gly Ala Trp Gly Asn Gln Pro 30
CBP35 Arg --- Ser --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- Gln --- Tyr --- --- --- --- --- --- --- --- ---
cBP Gly Ala Gly Gly Tyr Pro Gly Ala Ser Tyr Pro Gly Ala Tyr Pro Gly Gln Ala Pro Pro Gly Gly Tyr Pro Gly Gln Ala Pro Pro Ser 60
CBP35 --- --- --- --- --- --- --- --- Ala --- --- --- --- --- --- --- --- --- --- --- --- Ala --- --- --- --- --- --- --- --- Gly
cBP Ala Tyr Pro Gly Pro Thr Gly Pro Ser Ala Tyr Pro Gly Pro Thr Ala Pro Gly Ala Tyr Pro Gly Pro Thr Ala Pro Gly Ala Phe Pro 90
CBP35 --- --- --- --- Gln Ala Pro --- Tyr ---
cBP Gly Gln Pro Gly * Gly Pro Gly Ala Tyr Pro Ser * Ala Pro Gly Ala Tyr Pro Ser Ala Pro Gly Ala Tyr Pro Ala Thr Gly Pro 120
CBP35 --- --- --- Ala Pro --- Ala Phe Pro Gly Gln Pro Gly --- --- --- --- --- --- Gln Cys Ser --- Gly --- --- --- Ala --- ---
cBP Phe Gly Ala Pro Thr Gly Pro Leu Thr Val Pro Tyr Asp Met Pro Leu Pro Gly Gly Val Met Pro Arg Met Leu Ile Thr Ile Ile Gly 150
CBP35 Tyr --- Val --- Ala --- --- --- --- --- --- --- --- --- Leu --- --- --- --- --- --- --- --- --- --- --- --- --- --- Met ---
cDP Thr Val Lys Pro Asn Ala Asn Ser Ile Thr Leu Asn Phe Lys Lys Gly Asn Asp Ile Ala Phe His Phe Asn Pro Arg Phe Asn Glu Asn 180
CBP35 --- --- --- --- --- --- --- Arg --- Val --- Asp --- Arg Arg --- --- --- Val --- --- --- --- --- --- --- --- --- --- --- ---
cBP Asn Arg Arg Val Ile Val Cys Asn Thr Lys Gln Asp Asn Asn Trp Gly Arg Glu Glu Arg Gln Ser Ala Phe Pro Phe Glu Ser Gly Lys 210
CBP35 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- Lys --- --- --- --- --- --- --- --- --- --- --- --- --- ---
cBP Pro Phe Lys Ile Gln Val Leu Val Glu Ala Asp His Phe Lys Val Ala Val Asn Asp Val His Leu Leu Gln Tyr Asn His Arg Met Lys 240
CBP35 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- Ala --- --- --- --- --- --- --- --- --- --- --- ---
cBP Asn Leu Arg Glu Ile Ser Gln Leu Gly Ile Ile Gly Asp Ile Thr Leu Thr Ser Ala Ser His Ala Met Ile
CBP35 --- --- --- --- --- --- --- --- --- --- Ser --- --- --- --- --- --- --- --- --- Asn --- --- --- ---

both ϵBP (3) and CBP35 (10) can be delineated into two distinct domains. The COOH-terminal portion (residues 127-264) contains both hydrophilic and hydrophobic regions, typical of globular proteins, while the NH2-terminal portion (residues 1-126) by contrast exhibits neither a highly hydrophilic nor a hydrophobic nature. Likewise, ϵBP and CBP35 share a striking and unique structural feature within the NH2-terminal domain: there are tandem repeats of highly conserved sequence of nine amino acids (Tyr-Pro-Gly-X-X-X-Pro-Gly-Ala).

Both ϵBP and CBP35 lack potential N-linked carbohydrate attachment sites and do not contain a typical signal sequence or transmembrane segment. They differ, however, in one significant aspect in that ϵBP contains only one cysteine residue, whereas CBP35 has two cysteines, one of which is conserved relative to ϵBP.

Immunoblotting Analysis of Extracts of RBL and 3T3 Cells. Extracts of RBL and 3T3 cells were prepared and subjected to SDS-PAGE and immunoblotting analysis. When the nitrocellulose strip containing the separated 3T3 cell proteins was immunoblotted with anti-CBP35, a single band migrating at a position corresponding to a polypeptide of Mr ~35,000 was observed (Fig. 2A, lane 1). When an identical strip containing the separated 3T3 cell proteins was immunoblotted with anti-ϵBP2, a single band was also

FIGURE 2:A. **Immunoblotting patterns of antibodies directed against ϵBP and CBP35.** Extracts of mouse 3T3 fibroblasts were subjected to SDS-PAGE and immunoblotting with anti-CBP35 (Lane 1), anti-ϵBP (Lane 2) and a combination of anti-CBP35 and anti-ϵBP (Lane 3). Antibodies bound to protein antigens on the nitrocellulose strips were revealed by horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin. The numbers indicate the positions of molecular weight markers.

B. **Immunoblotting analysis of proteins bound to Gal-Sepharose and eluted with lactose.** Extracts of RBL cells were fractionated on Gal-Sepharose (100 μl of beads). The bound material was eluted with 0.3 M lactose and subjected to SDS-PAGE and immunoblotting: Lane 1, anti-ϵBP; Lane 2, anti-CBP35. Antibodies bound to protein antigens on the nitrocellulose strips were revealed by horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin. The arrow on the right indicates the position of migration of authentic CBP35 from 3T3 cells.

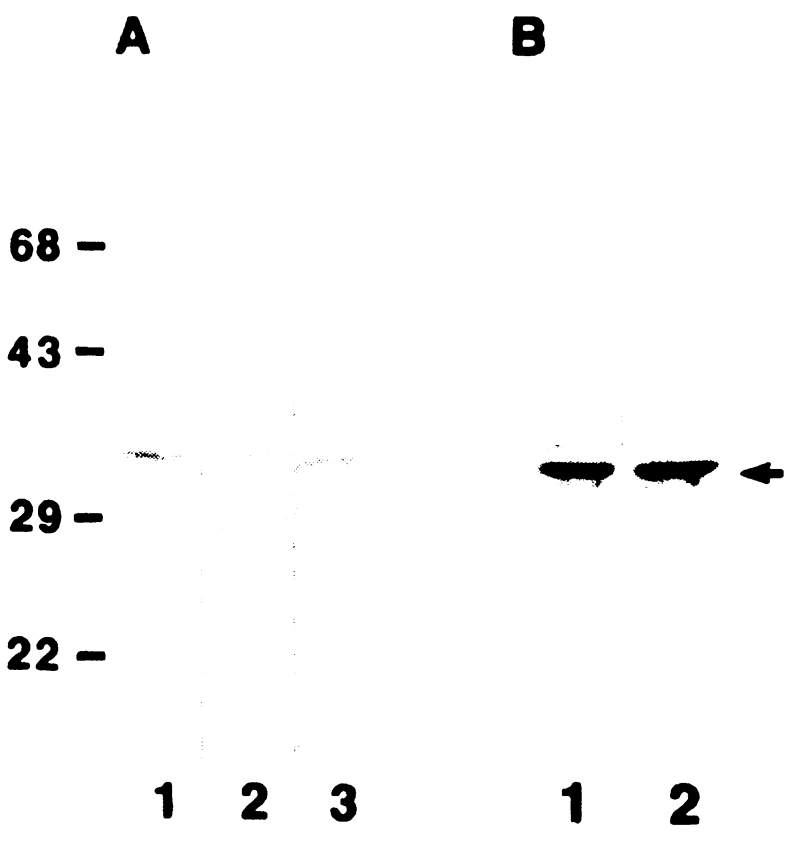

Figure 2.

observed (Fig. 2A, lane 2); this band co-migrated with that seen in the anti-CBP35 blot (Fig. 2A, lanes 1 and 2). Finally, when both anti-εBP2 and anti-CBP35 were used simultaneously to blot the 3T3 cell proteins, the same band and no additional band was observed (Fig. 2A, lane 3). These results suggest that both antibodies crossreacted with the same polypeptide in extracts of 3T3 cells. The same conclusion could be derived from analysis of extracts of RBL cells. In this case, blotting with anti-CBP35 or with anti-εBP2 each yielded polypeptides that co-migrated with the polypeptide revealed by blotting with a combination of anti-εBP2 and anti-CBP35 (data not shown).

Carbohydrate Binding Activity of εBP. In light of the similarity between εBP and CBP35, it was of interest to test whether εBP can bind to carbohydrates. Extracts of RBL cells were fractionated on a Gal-Sepharose column and the material bound to the column was eluted with 0.3 M lactose. Immunoblotting of this eluted fraction with anti-εBP2 yielded one predominant polypeptide band and a scarcely detectable band of slightly lower molecular weight (Fig. 2B, lane 1). Similar results were obtained when extracts of RBL cells were fractionated over an ASF-Sepharose column (data not shown). The bound material could also be eluted with lactose and immunoblotted with anti-εBP2. It should be noted that εBP, isolated from RBL extracts by saccharide-specific affinity chromatography, could be immunoblotted

with anti-CBP35 (Fig. 2B,lane 2). We conclude from these results that εBP, as originally identified (2) and characterized with an εBP-specific antibody (4), exhibits carbohydrate binding activity, which appears to be galactose-specific.

Binding of εBP and CBP35 to IgE and Elution by Saccharides. The binding of εBP to IgE in the absence or presence of lactose was then explored. When RBL extracts were adsorbed with IgE-Sepharose 4B and eluted with SDS, a protein of Mr 31,000 was isolated along with other proteins (Fig. 3A, lane 1). (It has been demonstrated previously (2) that two-cycle affinity purification is required to purify EBP from other contaminating proteins). Importantly, when the adsorbent was eluted with 0.3 M lactose, a single band of Mr 31,000 was detected (Fig. 3A, lane 2). In addition, the Mr 31,000 protein was nearly quantitatively eluted by lactose, as further treatment of the adsorbent with SDS did not yield additional protein (Fig. 3A, lane 3). Immunoblotting definitively established that the protein eluted from IgE-Sepharose by lactose is εBP, as it reacted with antibodies (anti-εBP2) specific for a peptide sequence of εBP (Fig.3B, lane 2), and it has the same apparent Mr as authentic εBP (Fig. 3B, lane 4). The immunoblot (Fig. 3B) also showed that lactose was as effective as SDS in eluting εBP from IgE-Sepharose (lane 2 vs. lane 1) and that lactose eluted εBP efficiently from the adsorbent (lane 3 vs. lane

FIGURE 3: **Electrophoretic and immunoblotting analysis of ϵBP and CBP35 bound to IgE Sepharose and eluted with lactose.** Extracts of RBL cells were fractionated on IgE-Sepharose (100 μl of beads). The bound material was eluted with SDS-PAGE sample buffer (15) or 0.3 M lactose and subjected to SDS-PAGE followed by silver staining (Panel A) or immunoblotting with anti-ϵBP2 (Panel B). For both Panels A and B: Lane 1, elution with SDS; Lane 2, elution with lactose; Lane 3, elution with SDS following elution with lactose. Lane 4 of Panel B is two-cycle affinity purified ϵBP as described previously (2). Antibodies bound to protein antigens on the nitrocellulose strips were revealed by ^{125}I-labelled goat anti-rabbit immunoglobulin, followed by autoradiography. Extracts of 3T3 cells (Panel C) were fractionated on IgE-Sepharose and the bound material was eluted with SDS-PAGE sample buffer (Lane 1) or 0.3 M lactose (Lane 2) and immunoblotted with anti-ϵBP as above. Lane 3 of Panel C is an ϵBP reference standard. The numbers indicate the positions of molecular weight markers. The arrows indicate the positions of ϵBP (Panels A and B) and CBP35 (Panel C).

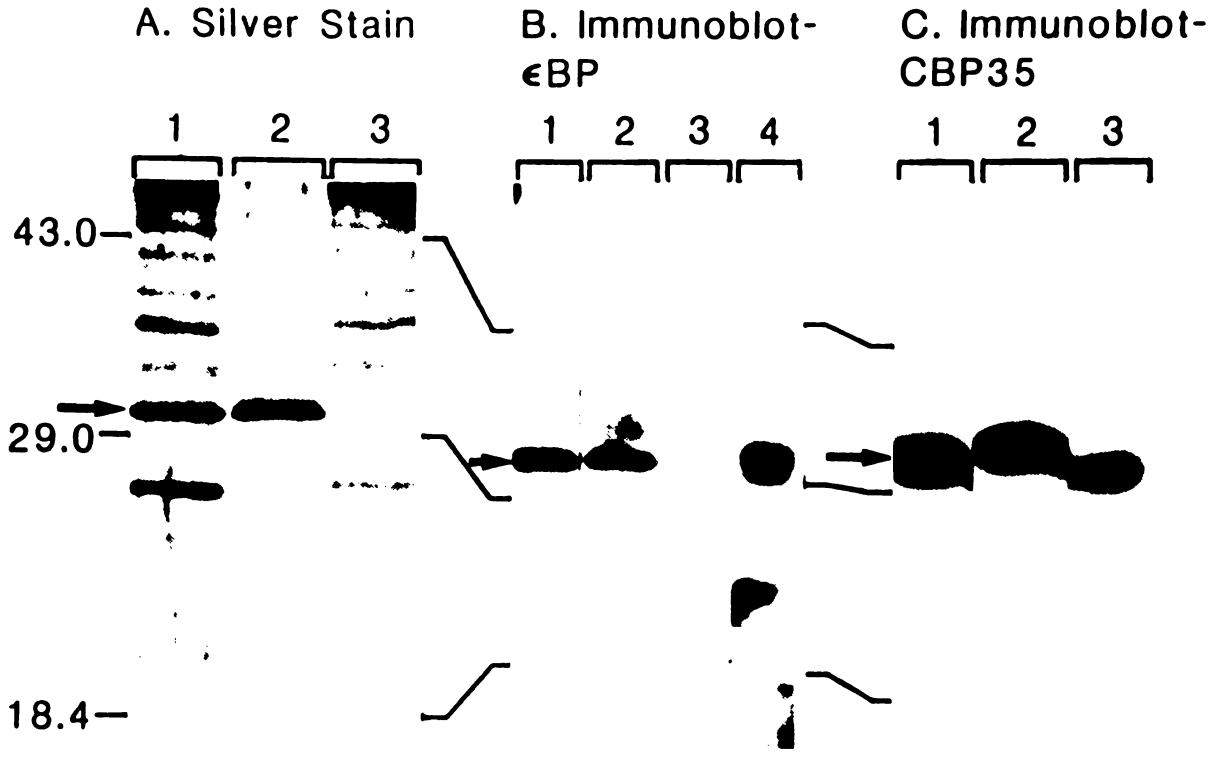
A. Silver Stain
B. Immunoblot-
εBP
C. Immunoblot-
CBP35
1 2 3
1 2 3 4
1 2 3
43.0
29.0
18.4

Figure 3.

1). Extracts of 3T3 fibroblasts were similarly fractionated on an affinity column containing IgE-Sepharose and eluted with either SDS or lactose. Upon immunoblotting with anti-εBP1, SDS-eluted material yielded a polypeptide band of Mr ~35,000 (Fig. 3C, lane 1), which was detected equivalently in a parallel blot with anti-CBP35 (data not shown). These results indicate that CBP35 binds to IgE in a manner similar to that demonstrated for εBP. More importantly, the interaction between CBP35 and IgE appears to be mediated via carbohydrate recognition since saccharides such as lactose can reverse the binding (Fig. 3C, lane 2). Side-by-side comparison of CBP35 with εBP (Fig. 3C, lane 3) indicates the apparent molecular weight of CBP35 is slightly higher than εBP.

***In Vitro* Transcription and Translation of E8 cDNA Yields a Carbohydrate Binding Protein.** In previous studies, mRNA synthesized *in vitro* from E8 cDNA and translated in a cell-free system yielded εBP which was specifically immunoadsorbed by IgE-Sepharose (3). In the present study, the in vitro transcribed-translated product was fractionated on IgE-Sepharose and the bound material was eluted with lactose. Upon SDS-PAGE and fluorography, a single radioactive polypeptide (Mr ~30,000) was observed (Fig. 4, lane 2) similar in quantity to that eluted with SDS (Fig. 4, lane 1). These results demonstrate the carbohydrate binding activity of the polypeptide derived from *in vitro*

FIGURE 4: **Fractionation on IgE-Sepharose or ASF-Sepharose of εBP derived from *in vitro* transcription of cDNA.** Clone E8 was transcribed and translated in the presence of [^{35}S]-methionine. The translation product was then adsorbed on IgE-Sepharose (Lane 1 and 2) or ASF-Sepharose (Lane 3). The bound material was eluted with SDS-PAGE sample buffer (Lane 1 and 3) or 0.3 M lactose (Lane 2) and subjected to SDS-PAGE and fluorography. The numbers on the right indicate the positions of molecular weight markers.

Figure 4.

transcription and translation of the E8 cDNA. Moreover, the *in vitro* transcription-translation product could also be fractionated on an ASF-Sepharose column, yielding the same radioactive polypeptide (Fig. 4, lane 3).

DISCUSSION

The key conclusion of the present study is that ϵBP and CBP35 are homologous proteins in rat and mouse cells, respectively. Evidence supporting this are the following: (a) the primary structures of the polypeptide chains are 85% identical; (b) ϵBP binds to carbohydrates; and (c) both ϵBP and CBP35 bind to IgE and their binding could be reversed by specific saccharides. Previous analysis showed that the mRNA species corresponding to the proteins are similar in size: ϵBP mRNA is ~1.1 kb (2,4), while CBP35 mRNA is ~1.3 kb (9). Furthermore, the subcellular locations of ϵBP and CBP35 are identical (4,7).

A hydropathy analysis of the primary structures of ϵBP and CBP35 clearly indicates a two domain structure in both proteins. This appears to be a recurring motif in the primary sequence of two families of animal cell lectins (17): (a) the calcium-dependent (C-type) family of which the asialoglyprotein receptor is an example, and (b) the thiol-dependent (S-type) family of which CBP35 is a member. The COOH-terminal domain (residues 127-264 in Fig. 1) of ϵBP has significant homology with the sequences of a number of β-D-galactoside specific S-type lectins (18-24). This could

provide the structural basis for the present observation that εBP interacts with galactose-containing glycoconjugates.

The NH2-terminal domains (residues 1-126 in Fig. 1) of εBP and CBP35 contain tandem homologous repeats. In the sequence of CBP35, this region was found to be homologous to certain proteins of the heterogeneous nuclear ribonucleoprotein (hnRNP) complex (10). Consistent with this observation, immunoblotting and immunofluorescence studies have shown that CBP35 could be found in the nucleus of 3T3 cells (7,8). Likewise, subcellular fractionation studies with anti-εBP show that the majority of the antigenic determinant is found intracellularly, including the nucleus (4).

Although εBP and CBP35 share significant sequence similarity and biological properties, they have one notable difference: while CBP35 requires reducing conditions for its carbohydrate-binding activity, εBP retains its IgE-binding activity in the absence of any reducing agent. This is probably related to the fact that CBP35 contains two cysteines in contrast to just one found in εBP. Thus, reducing conditions may be necessary to maintain both cysteine residues in the reduced state for the carbohydrate binding function of CBP35.

Various carbohydrate and IgE-binding proteins studied by different laboratories and assigned different names maybe

identical or closely related proteins. Another laboratory has identified IgE-binding proteins of Mr 30,000-33,000 (25). Whether they also interact with carbohydrate determinants is presently unknown. Also, RL-29, a protein which is isolated from rat lung tissue, may be identical to εBP based on its molecular weight (Mr ~30,000) and carbohydrate binding specificity (26,27).

The role of endogenous lectins in the immune system is beginning to be revealed. Low affinity IgE receptor (FcεR-II) on lymphocytes, macrophages and eosinophils has been shown to have marked homology with mannose-binding lectins (28-30) and is now grouped into the C-type lectin family. Therefore, endogenous lectins may play important roles in the IgE system. For example, the higher carbohydrate content of IgE (13%) as compared to IgG (3%) may allow specific discrimination of the two isotypes by lectins. Indeed, we have demonstrated previously that εBP binds specifically to IgE and not IgG (2,3) The COOH-terminal domain of εBP had previously been shown (3) to contain regions of 26 and 52 residues which are homologous with the α_1 domain of the Fcγ receptor (FcγR-II) on mouse lymphocytes and macrophages (31). Since at least the 26-residue stretch falls within the region of extensive identity with the lectins, the possibility exists that Fcγ receptor(s) may also bind to carbohydrate.

We have clearly demonstrated that εBP has a

carbohydrate binding activity and its binding to IgE can be reversed by disaccharides. However, it has not been determined whether the binding is solely dependent on carbohydrate determinants on IgE. We have noticed that more εBP consistently bound to IgE-Sepharose as compared to ASF-Sepharose (e.g., see Fig. 4). As stated above, lymphocyte FcεR-II has significant sequence similarity with mannose-binding lectins. However, it has been shown that carbohydrates on IgE are not essential for binding to FcεR-Il, as the receptor recognizes IgE treated with N-glycosidase and unglycosylated IgE fragments expressed in E. coli (32). Further examination of the binding specificity of εBP will be important to elucidate the role of εBP in the IgE system.

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CHAPTER III

IDENTIFICATION OF CARBOHYDRATE BINDING PROTEIN 35 IN HETEROGENOUS NUCLEAR RIBONUCLEOPROTEIN COMPLEX

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FOOTNOTES

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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 MgCl2, 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.

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 fractions 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.

INTRODUCTION

Carbohydrate binding protein 35 (CBP35, Mr 35000)[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 (Mr 35000) 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 (v/v)), 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 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 to combinations of the extraction steps were then subjected or 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 1300g 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 (1300g, 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 (1300g, 10 min), and the extracted material was processed for immunoblotting.

Fractionation 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 (1300g, 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 (1300g, 10 min) (Fey et al., 1986). These nuclei were ruptured by sonication (4 x 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 4500g 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 112000g 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 x 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 40x 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 3T3 Cells Treated with DNase I and RNase A. Serum-starved quiescent 3T3 cells were stimulated by the addition of serum; after 16 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

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 subjected to enzyme treatment(s). After the treatment(s) the cells were fixed in 3.7% formaldehyde for 15 minutes prior to staining and light microscopy. Row 1, phase contrast microscopy. Row 2, immunofluorescence staining with anti-CBP35 (1:10 dilution of antiserum) and rhodamine-conjugated goat anti-rabbit immunoglobulin (1:30 dilution) observed using a 580 nm barrier filter. Row 3, fluorescence staining with the DNA-specific dye Hoechst 33258 (10 μg/ml) observed 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 a control. Column b, 3T3 cells permeabilized as above, were incubated in TKM buffer containing DNase I (100 μg/ml) and 1 mM vanadyl adenosine 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.

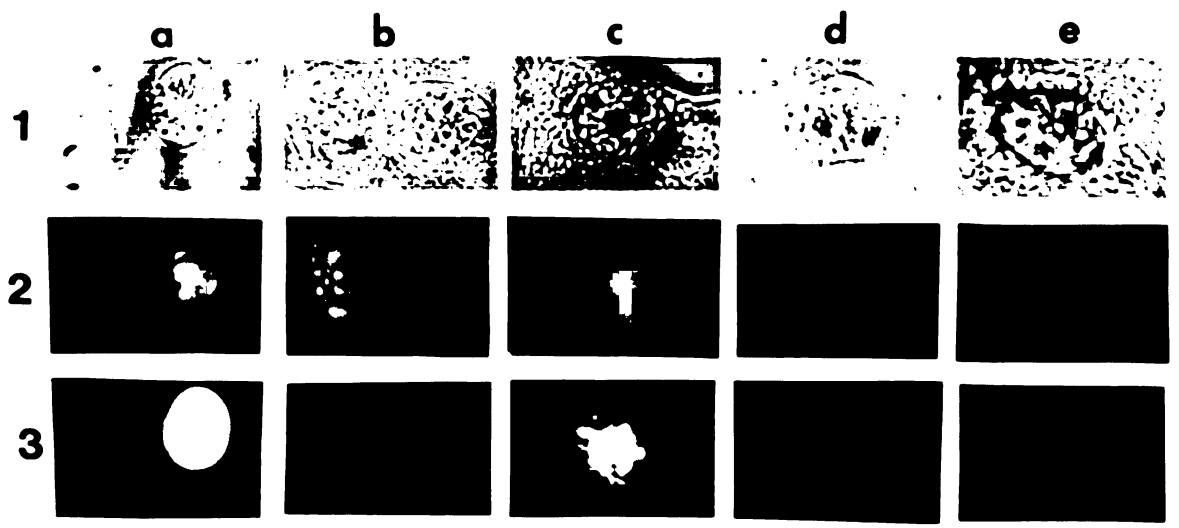

Figure 1.

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 Released By DNase I and RNase A. In conjunction with these extraction procedures, we have monitored the proteins released 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 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

Figure 2. Immunoblotting analysis of the supernatant fractions derive from various treatments of 3T3 cells. Lane 1, the supernatant derived from intact 3T3 cells incubated in TKM containing 1 mM vanadyl adenosine, 250 mM ammonium sulfate, and 0.5 % Triton X-100. Lane 2, supernatant fraction derived from treatment of permeabilized 3T3 cells with TKM 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 treating permeabilized 3T3 cells with TKM buffer containing 1mM vanadyl adenosine and 100 mM lactose. The samples were then analyzed on SDS-PAGE (12.5% acrylamide) and immunoblotted with rabbit anti-CBP35 (1:250 dilution of antiserum) and horseradish peroxidase goat anti-rabbit immunoglobulin (1:1500 dilution). The arrows on the left indicate the positions of migration of molecular weight markers: ovalbumin (M_r 43000); carbonic anhydrase (M_r 31000); and soybean trypsin inhibitor (M_r 22000). The arrow on the right indicate the position of migration of authentic CBP35.

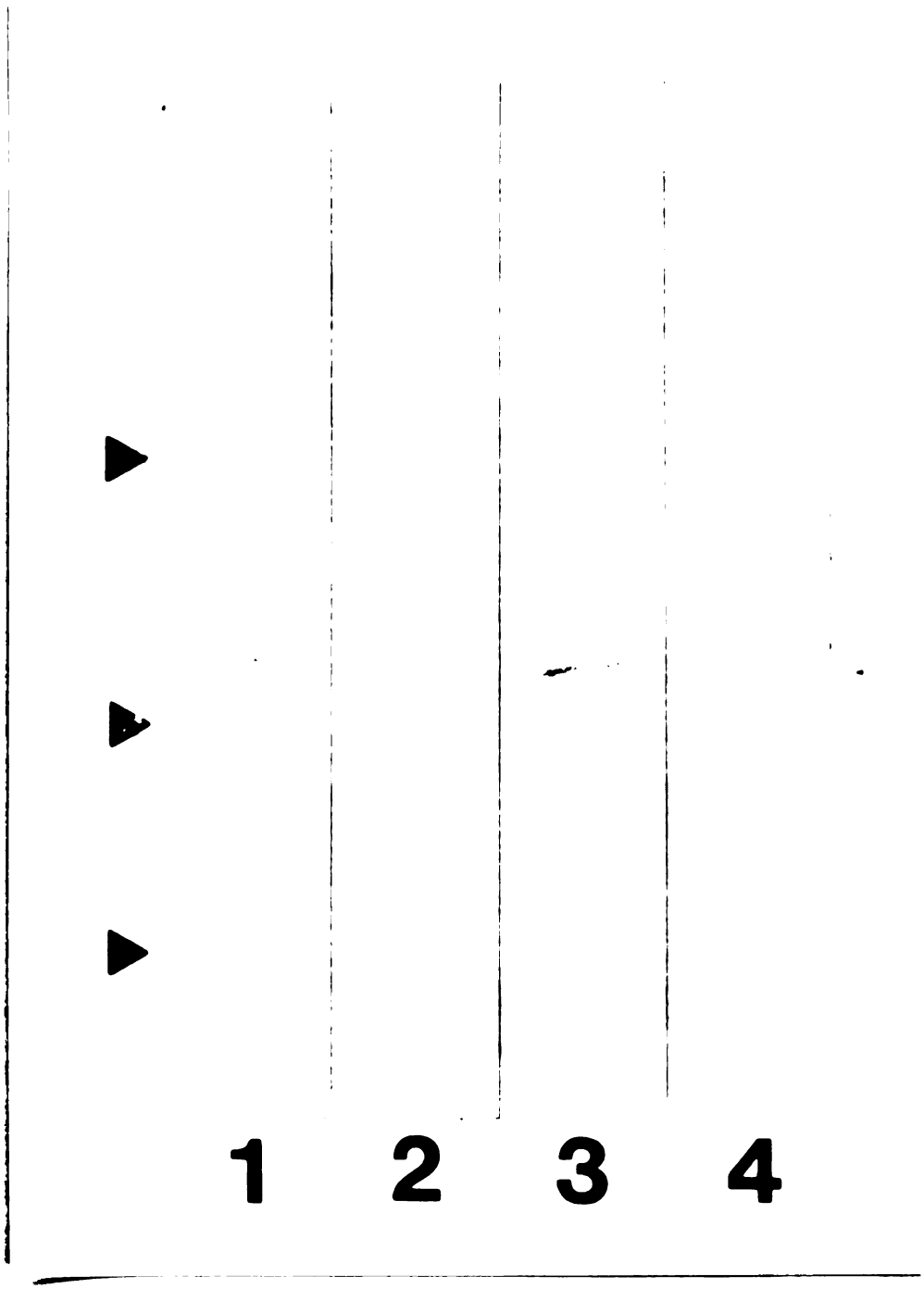

Figure 2.

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 (Figure 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

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.

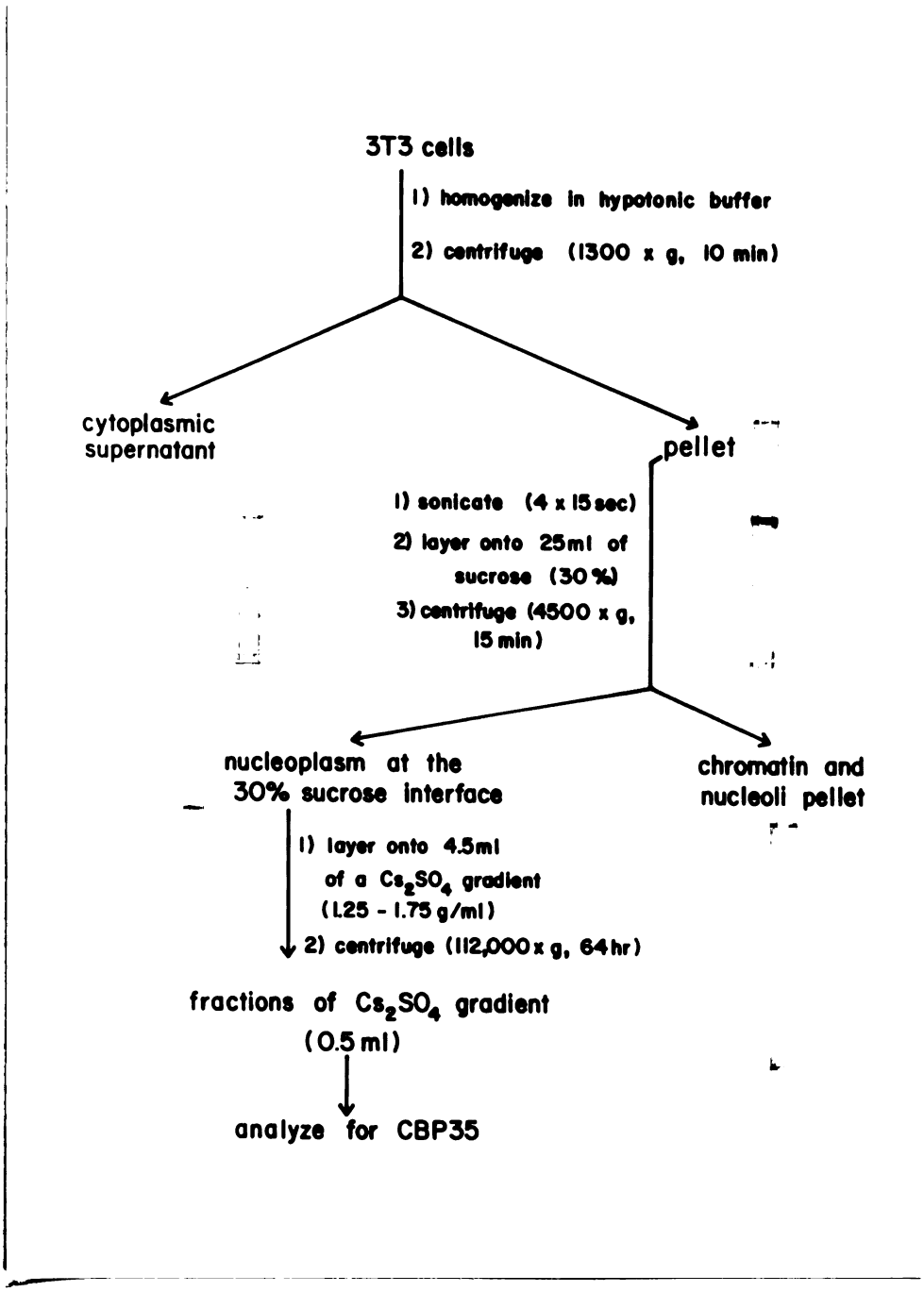
3T3 cells
1) homogenize in hypotonic buffer
2) centrifuge (1300 x g, 10 min)
cytoplasmic supernatant
pellet
1) sonicate (4 x 15 sec)
2) layer onto 25ml of sucrose (30%)
3) centrifuge (4500 x g, 15 min)
nucleoplasm at the 30% sucrose interface
chromatin and nucleoli pellet
1) layer onto 4.5ml of a Cs_2SO_4 gradient (1.25 - 1.75 g/ml)
2) centrifuge (112,000 x g, 64 hr)
fractions of Cs_2SO_4 gradient (0.5 ml)
analyze for CBP35

Figure 3.

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 from these labeled cells was fractionated on the Cs_2SO_4 gradient. (b) The density of the individual fractions 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.

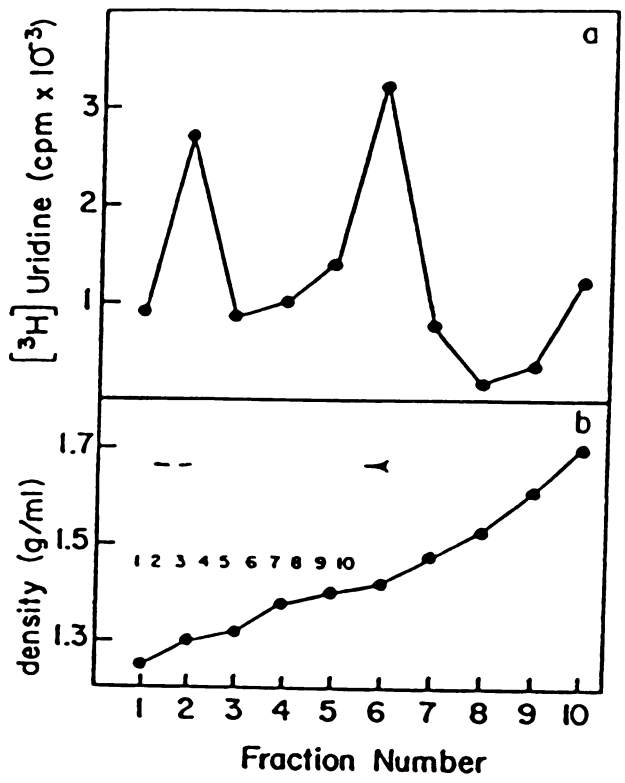
a
[3H] Uridine (cpm x 10-3)
3
2
1
b
density (g/ml)
1.7
1.5
1.3
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10
Fraction Number

Figure 4.

the presence of [^{3}H]uridine for 30 min to pulse-label the RNA species. The nucleoplasmic material derived from these labeled 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 (-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) 68000; (b) 53000; (c) 43000;(d) 37000; (e) 35000; (f) 34000; and (g) 32000 (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

Figure 5. SDS-PAGE analysis of the polypeptide compositions of the fractions derived from nucleoplasm subjected to Cs_2SO_4 gradient sedimentation and from nucleoplasm subjected to affinity chromatography on a 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 did bind to the affinity column (Figure 6) and was 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 position of migration of molecular weight markers.

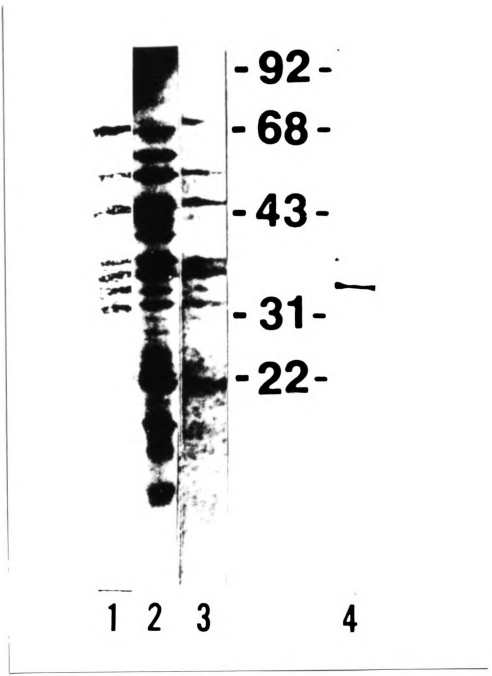

Figure 5.

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~35000).

Isolation of hnRNP by Saccharide Specific 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 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) Mr 68000; (b) Mr 53000; (c) Mr 44000; (d) Mr 37000; (e) Mr 35500; (f) Mr 34000; (g) Mr 32000; and (h) Mr 22000. 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;

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 was fractionated on the affinity column (0.8x 3 cm) equilibrated in 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 either 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 the 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.

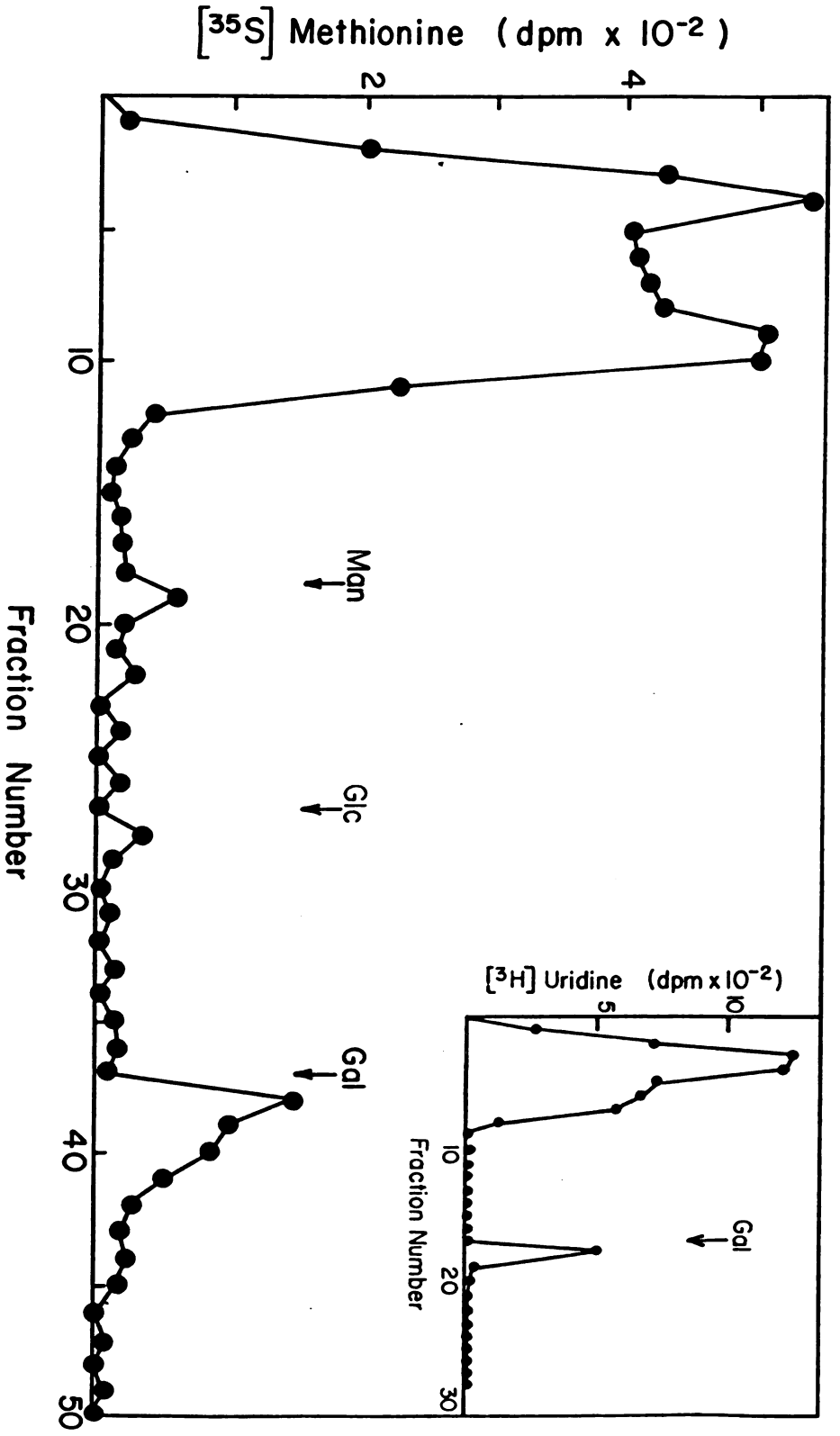
[35S] Methionine (dpm x 10-2)
2
4
10
20
30
40
50
Man
Glc
Gal
Fraction Number
[3H] Uridine (dpm x 10-2)
5
10
10
20
30
Gal
Fraction Number

Figure 6.

Celis et al.,1986). The lone exception is the Mr 22000 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 (Mr 10000-25000), 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 (Mr ~32000), group B proteins (Mr ~35000), group C proteins (Mr ~37000), group D proteins (Mr ~ 44000), and the Mr 53000 and 68000 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 analysisof 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 Seve 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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CHAPTER IV

Perturbations of the Carbohydrate Binding Protein 35 — Ribonucleoprotein Complexes in the Nucleus*

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Running Title: Carbohydrate Binding Protein 35 and Ribonucleoprotein Complexes

FOOTNOTES

* This work was supported by grants GM-38740 and GM-27203 from the National Institutes of Health.

[1] The abbreviations used are: CBP35, Carbohydrate Binding Protein 35 (M_r ~ 35,000); rCBP35, recombinant CBP35; hnRNP, heterogeneous nuclear ribonucleoprotein complex; snRNP, small nuclear ribonucleoprotein particle; M2, the Mac-2 antigen; TR, transferrin receptor; RNase A, ribonuclease A; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate; TBS, Tris-buffered saline (20 mM Tris-HCl, 140 mM NaCl, pH 7.5).

SUMMARY

Nucleoplasm derived from mouse 3T3 fibroblasts was subjected to immunoprecipitation by antibodies directed against Carbohydrate Binding Protein 35 (CBP35). This immunoprecipitate contained not only CBP35 but also the small nuclear ribonucleoprotein particle (snRNP)-associated B polypeptide (Sm-B), which is a target of anti-Sm autoimmune sera. Conversely, immunoprecipitation of nucleoplasm with anti-Sm autoimmune sera also co-precipitated CBP35. Sm-B and CBP35 were not cross-reactive with their respective antibodies. Moreover, co-precipitation was not observed if nucleoplasm was first treated with ribonuclease A. Thus, it appears that CBP35 and Sm-B were associated via a ribonucleoprotein complex, a notion supported by their positions of sedimentation on cesium sulfate gradients.

These results implicating the association of CBP35 with snRNP and previous studies suggesting a similar association with heterogeneous nuclear ribonucleoprotein complex (hnRNP) provoke the possibility that the lectin may be a component of the spliceosome that processes pre-mRNA. Saccharides and antibodies that bind to CBP35 inhibit pre-mRNA splicing, in an *in vitro* pre-mRNA splicing assay, while control reagents failed to yield the same effect.

INTRODUCTION

Carbohydrate-binding Protein 35 (CBP35;[1] M_r ~ 35,000), a lectin endogenous to cultured 3T3 fibroblasts and most mouse tissues, was isolated initially on the basis of its ability to bind to galactose (Gal) and Gal-containing glycoconjugates (1-3). The amino acid sequence (264 residues), deduced from the nucleotide sequence of a cDNA clone (4), showed that the CBP35 polypeptide consisted of two domains: an NH_2-terminal half that contains repeats of the sequence Pro-Gly-Ala-Tyr-Pro-Gly followed by three other amino acids (Pro-, Gly-rich domain) and a COOH-terminal carbohydrate recognition domain that is homologous to other galactose-binding proteins classified as S-type lectins (5). Amino acid sequence information on several other proteins, originally isolated from other sources and designated different names, showed that they must be identical to (within the same species) or homologous (between species) with CBP35. These include: (a) L-34 (6), HL-29 (7), and RL-29 (8), which were isolated on the basis of their Gal-specific carbohydrate-binding activities from mouse, human, and rat cells, respectively; and (b) Laminin Binding Protein (9), Mac-2 (M2) (10), and IgE-binding Protein (11), which were tested for carbohydrate-binding activity because of their sequence identity/homology to CBP35, with the results showing Gal-specific binding.

Although most of the studies on this protein under its various names were from the perspective of its extracellular

localization (cell surface and medium), the predominant proportion of the CBP35 was found to be intracellular in mouse 3T3 fibroblasts (12). Immunofluorescence staining of fixed and permeabilized cells showed prominent labeling of the nucleus and variable staining of the cytoplasm in proliferating cultures of 3T3 cells. In previous studies (13), several lines of evidence suggested that CBP35 was associated with ribonucleoprotein (RNP) complexes in the nucleoplasm: (a) CBP35 was released from permeabilized nuclei by treatment with ribonuclease A (RNase A) but not by similar treatment with deoxyribonuclease I; (b) when nucleoplasm was fractionated by density gradient centrifugation, immunoblotting analysis localized the lectin in fractions with densities corresponding to those reported for heterogeneous nuclear RNP (hnRNP) (1.30-1.35 g/ml on cesium sulfate gradients and 40S on sucrose gradients); and (c) fractionation of nucleoplasm on Gal-Sepharose beads resulted in the binding of the lectin as well as RNA and a set of polypeptides whose molecular weights matched those reported for the proteins in hnRNP.

In the course of these studies, we had also obtained evidence for the association of CBP35 with the small nuclear ribonucleoprotein particles (snRNPs). The association of CBP35 with hnRNP and snRNP indicated that the lectin may be a component of the spliceosome and suggested specific tests of its role in the processing of pre-mRNA. In the present paper,

we report the perturbation of pre-mRNA splicing in a cell-free assay by carbohydrate ligands and antibody reagents that bind CBP35.

EXPERIMENTAL PROCEDURES

Cell Culture and Antibody Reagents

Antibody directed against CBP35 was generated in Flemish giant female rabbits. Monoclonal antibodies directed against M2 and Transferrin Receptor (TR) were isolated from hybridomas M3/38 (14) and R17 217 (15) purchased from American Type Cultures Collection. Autoimmune sera directed against the Sm antigen (ENA anti-Sm) was purchased from the Binding Site.

HeLa cells were cultured in spinner bottles with Dulbecco modified Eagle's Medium containing 5% (v/v) fetal calf serum (16). Swiss 3T3 fibroblasts were cultured in the same medium, 10% calf serum (v/v) (17). The cells were synchronized by serum starvation (48 h in medium containing 0.2% calf serum). The medium was then changed to Dulbecco modified Eagle's medium containing 10% serum (13). The cells were incubated in this medium for 16 h and used for the isolation of nucleoplasm.

Fractionation of 3T3 Cell Nucleoplasm

After serum stimulation, the cells were harvested in 0.25 % trypsin and were resuspended in 20 mM Tris-HCl, pH 7.5, 10 mM NaCl, 10 mM $MgCl_2$ (Tris buffer). 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 (15 min, 1330 g). The nuclei were then ruptured by sonication (4 x 15s), and the nucleoplasmic material was separated from chromatin by layering the sonicate onto 25 ml of 30% sucrose in Tris buffer and centrifuging for 15 min at 4500 g in a Sorvall HB-4 rotor. The nucleoplasm was removed from the 30% sucrose interface (18,19).

Nucleoplasm was layered onto a preformed cesium sulfate gradient (1.2-1.75 g/ml), which was then centrifuged at 155,187 g for 40 h in a Beckman SW50.1 rotor (18,19). The resulting gradients were collected in a Beckman fraction recovery system (0.4 ml/fraction). The density of these fractions was determined from the weight of 10 μl of each fraction. Twenty-five microliter of anti-M2-bound Protein A Trisacryl (Pierce) were added to each fraction. Each fraction was then incubated overnight at 4°C with shaking. The beads were subsequently pelleted and the supernatant was removed. The pellets were washed with 1 ml of 10 mM Tris, 20 mM NaCl, 5 mM $MgCl_2$, pH 7.5. Control gradients, in which nucleoplasm that had been digested with micrococcal nuclease (50 μg/ml, 30°C, 30 min) or recombinant CBP35 (rCBP35) (20) were subjected to centrifugation, were analyzed in parallel.

Immunoprecipitation

Antibody-bound affinity resins were prepared by incubating, for 2 h at 4°C with shaking, 25 μl of protein A Sepharose CL-4B (Pharmacia) with the following antibodies: a) 1 μg anti-M2 antibody and 10 μg rabbit anti-rat immunoglobulin

(Sigma); b) 10 μl ENA anti-Sm serum; and c) 1 μg anti-TR and 10 μg rabbit anti-rat immunoglobulin. The beads were pelleted and the supernatant was removed. The beads were then washed four times (by centrifugation and resuspension) with 1 ml phosphate-buffered saline (10 mM sodium phosphate, 140 mM NaCl, and 4 mM KCl, pH 7.5) (21,22).

Nucleoplasm (1 ml) was added to an Eppendorf tube containing 25 μl normal rabbit immunoglobulin-bound Protein A Sepharose CL-4B. This was incubated overnight at 4°C with shaking. The beads were pelleted in a microfuge, and the supernatant (preclear unbound) was removed. One-third of this material was digested with RNase A (25 μg/ml, 37°C, 30 min). Aliquots of the untreated preclear unbound were added to tubes containing 25 μl of Protein A Sepharose CL-4B beads bound with: a) anti-M2; b) ENA anti-Sm; or c) anti-TR. Aliquots of the RNase A-treated preclear unbound were added to corresponding tubes containing: a) anti-M2 or b) ENA anti-Sm. These tubes were then incubated at 4°C for 2 h with shaking. The beads were pelleted in a microfuge and the supernatants were removed (21-23). These beads were washed four times with 0.5 ml phosphate-buffered saline. The washes were pooled with the supernatant to form the unbound fraction. The unbound material was concentrated by ethanol precipitation. Bound and unbound material was solubilized by heating in sample buffer and analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE).

SDS-PAGE and Immunoblotting

Proteins were separated on a 12.5% polyacrylamide gel in the presence of SDS (24). The proteins were electrophoretically transferred (1 h at 25 V) to Immobilon-P (Millipore) (25). The membrane was then incubated for 2 h at 37°C in TBS (20 mM Tris-HCl, 140 mM NaCl, pH 7.5) containing 2% gelatin. Incubation with primary antibody (1:1000 dilution of polyclonal rabbit anti-CBP35 or 1:100 dilution of ENA anti-Sm serum (26) in TBS containing 1% gelatin) was carried out for 2 h at room temperature. The blot was then washed three times in TBS containing 0.5% Tween-20, followed by incubation with secondary antibody (1:5000 dilution of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (BMB) or 1:3000 dilution of alkaline phosphatase-conjugated goat anti-human immunoglobulin) in TBS containing 0.5% Tween-20. The membrane was washed two times with TBS containing 0.5% Tween-20 and two times with water and, finally, developed with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate as substrates.

***In vitro* Splicing System**

Human β-globin pre-mRNA was produced by SP6 RNA polymerase transcription of SP64-H$\beta\Delta$6 after linearization with BamHI (27). Transcription was carried out in the presence of 0.5 mM 7-methyl-guanosine-(5)triphospho-(5)guanosine (7-

meGpppG) (28) and [^{32}P]UTP. Nuclear extracts were prepared from exponentially growing HeLa cells by the procedure of Dignam *et al.* (29). Splicing reactions were performed in volumes of 10 or 25 μl, 50-60% of which was nuclear extract (28). For perturbation studies, the nuclear extracts were incubated with the saccharides or antibodies on ice for 30 min, and splicing was initiated by the addition of polyvinyl alcohol and ^{32}P-labeled HβGΔ6. Splicing was performed at 30°C for 60-90 min.

Saccharides were purchased from Aldrich and blood group A tetrasaccharide-human serum albumin conjugate was obtained from Biocarb. Saccharides added to the splicing reaction were dissolved in 20 mM Hepes, pH 7.9. These solutions were prepared fresh on the day of use. Antibodies were dialyzed against 20 mM Hepes, pH 7.9 and were concentrated by centrifugation in a Centricon 10 filter. The protein concentrations were determined by the Bradford protein assay (30).

RNA was isolated from splicing reactions after proteinase K digestion by phenol:chloroform extraction, followed by ethanol precipitation. The RNA was analyzed on a denaturing 10% polyacrylamide, 8.3 M urea gel. Following autoradiography, the intensities of bands were quantitated using a BioImage Visage 110 densitometer. Splicing was defined as the intensity of the mRNA product band (exon 1-exon 2) divided by the sum of the intensities of all bands (pre-

mRNA, unligated intermediates, and ligated mRNA). The inhibitory effect of different reagents was calculated by comparing the amount of splicing in the treated reactions to the amount of splicing in an untreated control.

RESULTS

Ribonucleoprotein Complexes Containing CBP35 and the Sm Antigens of snRNP

Mouse 3T3 fibroblasts were synchronized by serum starvation and reactivated by the addition of serum. Nucleoplasm derived from these cells, 16 h post-serum addition (13), was subjected to centrifugation on a Cs_2SO_4 gradient that ranged in density from 1.20 to 1.75 g/ml (Fig. 1). After concentration of the individual fractions, immunoblotting with anti-CBP35 showed that the lectin could be found in fractions 3 through 6 (1.28-1.35 g/ml). These results are consistent with our previous observations on the position of migration of CBP35 when nucleoplasm of 3T3 fibroblasts was subjected to Cs_2SO_4 gradient centrifugation (13).

The human autoimmune serum, ENA anti-Sm, recognizes the Sm antigens of the core polypeptides (B, D, and E) of the uracil-rich snRNPs (31). When the individual fractions of the Cs_2SO_4 gradient were subjected to immunoblotting with ENA anti-Sm, polypeptide bands corresponding to Sm-B (M_r ~ 29,000), Sm-D (M_r ~ 16,000), and Sm-E (M_r ~ 13,000) could be observed in fractions 4-10. These fractions covered a range of densities from 1.30 to 1.53 g/ml. Therefore, at least

Figure 1: Profiles of the density and immunoblotting patterns of the individual fractions when nucleoplasm derived from serum-stimulated (16 h after addition of 10% calf serum) 3T3 fibroblasts was fractionated on a Cs_2SO_4 gradient. The density was determined by weighing 10 μl aliquot of each fraction. For immunoblotting, individual fractions were concentrated, subjected to SDS-PAGE (12.5% acrylamide), and transferred onto Immobilon-P membrane. The nitrocellulose membrane was immunoblotted for CBP35 using rabbit anti-CBP35 (1:1000 dilution of antiserum) and for the Sm antigens using ENA anti-Sm (1:100 dilution of autoimmune serum), followed by alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin and alkaline phosphatase-conjugated goat anti-human immunoglobulin. The arrows on the left indicate the positions of migration expected for CBP35 (M_r ~ 35,000), Sm-B (M_r ~ 29,000), Sm-D (M_r ~ 16,000), and Sm-E (M_r ~ 13,000).

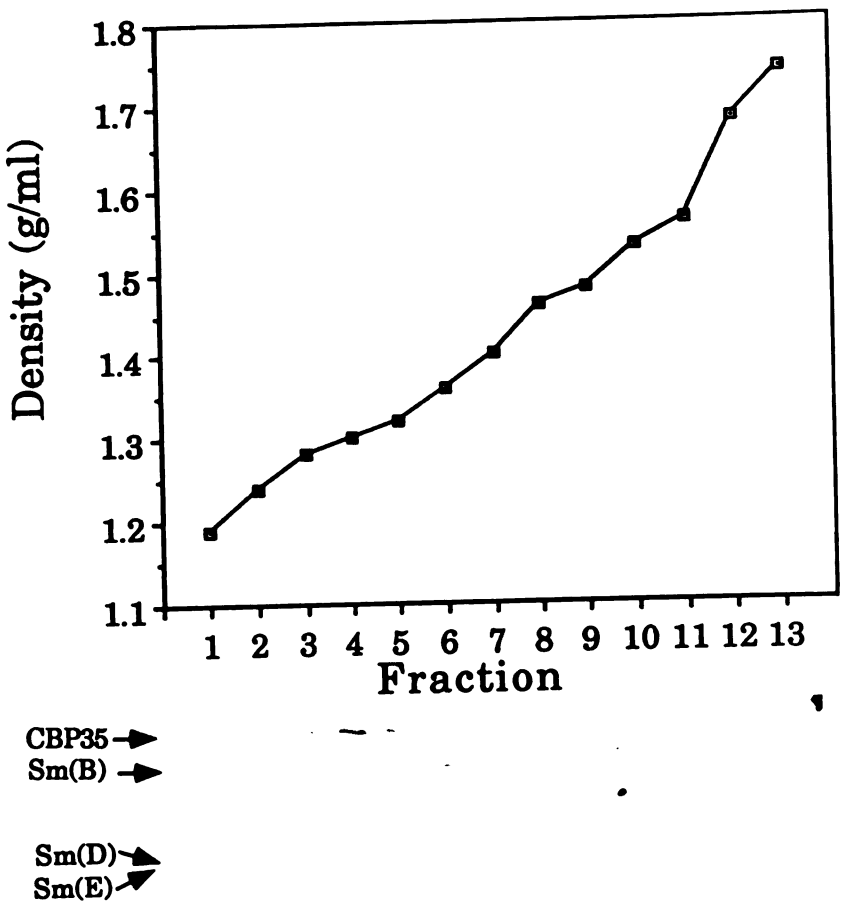

Density (g/ml)
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1 2 3 4 5 6 7 8 9 10 11 12 13
Fraction
CBP35
Sm(B)
Sm(D)
Sm(E)

Figure 1.

three of the fractions that contained CBP35 also contained the Sm antigens (fractions 4-6, Fig. 1).

Control experiments were carried out on parallel gradients. rCBP35, expressed and purified from *E. coli* (20), was found in fractions 1 and 2 (1.18 - 1.24 g/ml), indicating that the CBP35 polypeptide alone did not enter the Cs_2SO_4 gradient. In addition, when nucleoplasm was first treated with RNase A (50 μg/ml, 30°C, 30 min), the position of migration shifted to the top of the gradient. These results indicate that CBP35 of the nucleoplasm was associated with a ribonucleoprotein complex, which may contain the uracil-rich snRNPs.

Co-immunoprecipitation of CBP35 and Sm Antigens from the Nucleoplasm

Co-immunoprecipitation analyses were carried out to test whether CBP35 and the Sm antigens might be found in the same ribonucleoproteins complex. Nucleoplasm from 3T3 cells was incubated with protein A beads (Fig. 2A). The material that did not bind to these beads (the "preclear unbound" fraction) was separated into two parts. One part was directly subjected to immunoprecipitation with ENA anti-Sm, with anti-M2 (rat monoclonal antibody reactive with CBP35), and with anti-TR (isotype matched rat monoclonal antibody for control). The second part of the "preclear unbound" fraction was digested with RNase A (25 μg/ml, 37°C, 30 min) prior to immunoprecipitation. The immunoprecipitated pellet (bound fraction)

Figure 2: (A) Summary of the protocol used to test for co-immunoprecipitation of the Sm antigen B polypeptide and CBP35 from nucleoplasm derived from serum-stimulated (16 h after addition of 10% calf serum) 3T3 fibroblasts. Nucleoplasm was first precleared by incubation with protein A-Sepharose and normal rabbit immunoglobulin. The supernatant (unbound fraction from preclear) was separated into two parts. One part was subjected to immunoprecipitation with anti-TR, anti-Sm, or anti-M2. The other part was treated with RNase A (25 μg/ml, 37^{o}C, 30 min) prior to immunoprecipitation. Material precipitated (bound fraction) and material remaining in the supernatant (unbound fraction) of each immunoprecipitation was subjected to SDS-PAGE and immunoblotting with anti-M2 for CBP35 (B) or with ENA anti-Sm for Sm-B (C).

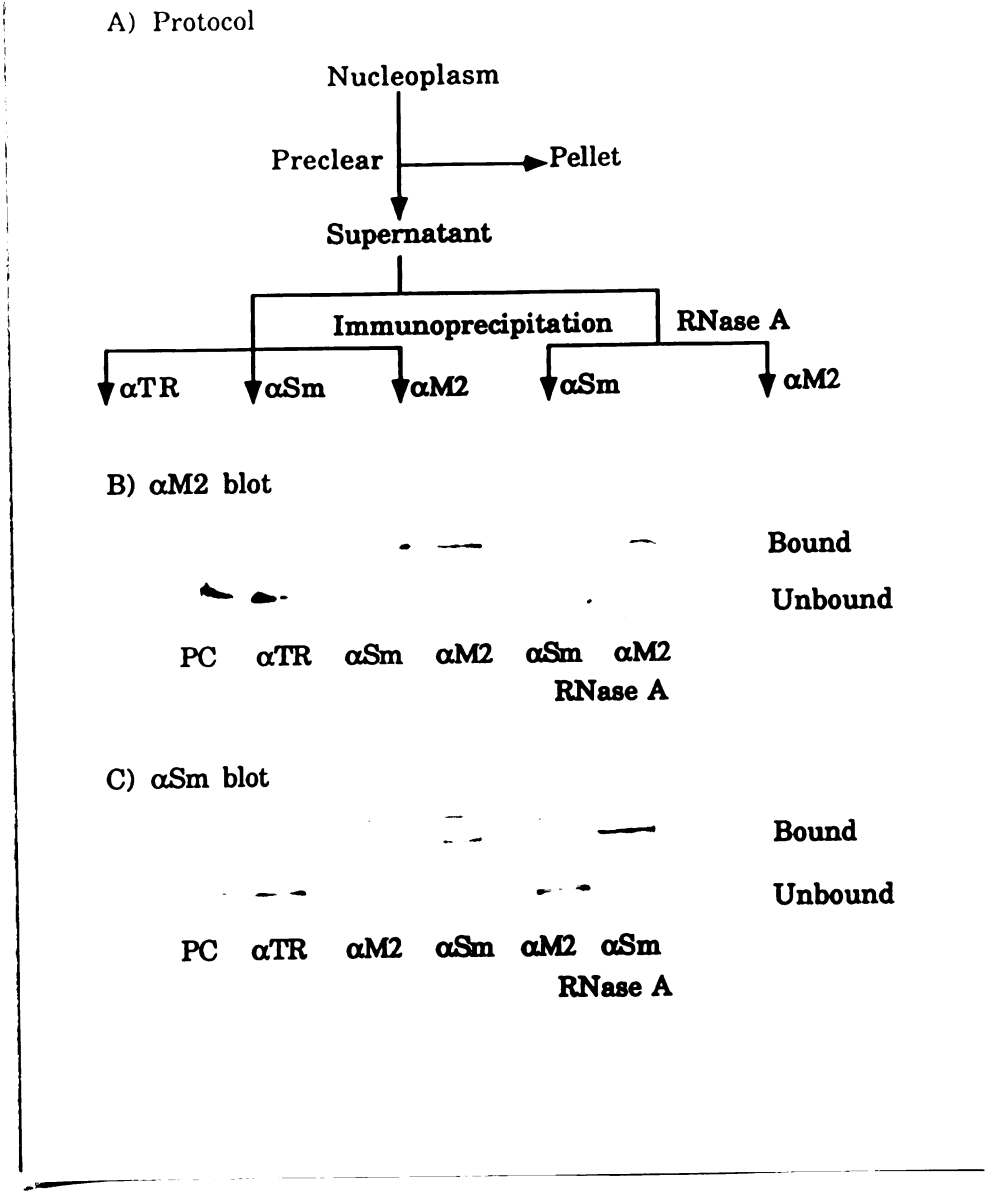

A) Protocol
Nucleoplasm
Preclear
Pellet
Supernatant
Immunoprecipitation
RNase A
αTR
αSm
αM2
αSm
αM2
B) αM2 blot
Bound
Unbound
PC
αTR
αSm
αM2
αSm
αM2
RNase A
C) αSm blot
Bound
Unbound
PC
αTR
αM2
αSm
αM2
αSm
RNase A

Figure 2.

as well as the supernatant (unbound fraction) were then analyzed by SDS-PAGE and immunoblotting with rat anti-M2 (Fig. 2B) and ENA anti-Sm (Fig. 2C).

CBP35 was found in the precleared unbound fraction, the material subjected to immunoprecipitation (Fig. 2B). It was also observed in the unbound fraction, but not in the bound fraction, after immunoprecipitation with the control antibody, rat anti-TR. In contrast, CBP35 was found in the bound fractions of both the immunoprecipitation by ENA anti-Sm, as well as by rat anti-M2. However, if nucleoplasm was first digested with RNase before the immunoprecipitation, CBP35 was found only in the anti-M2-bound fraction. In this case, ENA anti-Sm failed to immunoprecipitate CBP35 (Fig. 2B).

These results indicate that CBP35 was immunoprecipitated by ENA anti-Sm via its association with a RNP that also contained the Sm antigens, rather than via cross-reaction with the autoimmune antibody. Additional lines of evidence in support of this notion include: (a) ENA anti-Sm failed to immunoblot rCBP35 after SDS-PAGE; (b) when rCBP35 was subjected to immunoprecipitation with ENA anti-Sm, Western blot analysis of the bound and unbound fractions with anti-CBP35 revealed the lectin to be exclusively in the unbound fraction.

Essentially the same results were obtained when the bound and unbound fractions of the various immunoprecipitations were subjected to immunoblotting for the Sm antigen (Fig. 2C). Sm

antigen was found in the unbound fractions of the preclear and anti-TR precipitations. In the absence of RNase treatment, most of the Sm immunoreactive material was found in the bound fraction of the anti-M2 precipitate; a small but detectable amount of Sm could be observed in the unbound fraction. When ENA anti-Sm was used for immunoprecipitation, the bound fraction contained all of the Sm antigen. Prior RNase treatment yielded the corresponding result: Sm was found in the unbound fraction with anti-M2 and the bound fraction with ENA anti-Sm (Fig. 2C).

Effect of Saccharides in a Cell-Free Splicing Assay

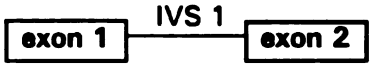
The Cs_2SO_4 gradient and co-immunoprecipitation results strongly suggest that CBP35 and Sm antigens are associated with the same ribonucleoprotein complex. Coupled with our previous identification of CBP35 with the hnRNP complex in the nucleoplasm (13), these results raise the possibility that the lectin was localized on a spliceosome assembly that carries out the processing of intron-containing pre-mRNAs in the nucleus. Therefore, ligands or antibodies that bind to CBP35 were tested for their ability to perturb a cell-free splicing assay.

The pre-mRNA substrate used in our experiments is an SP6 transcript of a human β-globin gene construct containing two exons separated by an intervening sequence (27). Nuclear extract derived from HeLa cells and ^{32}P-labeled pre-mRNA were mixed for a 60 min reaction at 30°C; the RNA components of the

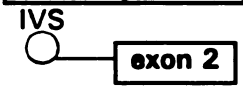
splicing mixture were extracted and resolved on a 10% acrylamide-8.3 M urea gel system. Both major steps of the splicing reaction, cleavage at the 3'-end of exon 1 to yield free exon 1 and lariat-exon 2 and ligation of exons 1 and 2 to form the final product exon 1-exon 2, can be discriminated by this gel system (Fig. 3, lane 1). In a typical assay, our preparations of the nuclear extract were capable of converting 20-40% of the pre-mRNA into the final mRNA product. Immunoblotting of these HeLa nuclear extracts with anti-CBP35 yielded the human homolog of the lectin (M_r ~ 29,000).

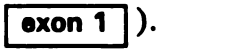
The nuclear extract was preincubated with various saccharides (100 mM) for 30 min prior to being mixed with the pre-mRNA substrate. Under these conditions, Lac inhibited the *in vitro* splicing system. There were much lower levels of the mRNA product, as well as each of the intermediates, observable in the gels (Fig. 3, lane 2). Similar results were also obtained with thiodigalactoside, which binds to CBP35 (Fig. 3, lane 3). In contrast, maltose, glucose, mannose, and *myo*-inositol did not inhibit the splicing (Fig. 3, lanes 4-7).

The autoradiograms of the acrylamide gels were subjected to densitometric scanning. Using the intensity of the product band (exon 1-exon 2) relative to the sum of the intensities of all the bands (highlighted at extreme right) as a measure of the extent of the reaction, the inhibitory action of various saccharides could be compared on a quantitative basis. For example, the dose-response curve for Lac showed that the

Figure 3: **Effects of various reagents on the cell-free splicing of pre-mRNA, as analyzed on 10% polyacrylamide-8.3 M urea gels.** The HeLa cell nuclear extract was incubated in the absence or presence of various reagents (100 mM) for 30 min. ^{32}P-Labeled HβGΔ6 pre-mRNA was added for a 60 min reaction at 30°C. The RNA components were extracted, electrophoresed, and subjected to autoradiography. Lanes: (1) control; (2) lactose; (3) thiodigalactoside; (4) maltose; (5) glucose; (6) mannose; (7) *myo*-inositol. The symbols on the right indicate the position of migration of the starting substrate (

), final product (

), as well as the intermediates of the splicing reaction (

and 

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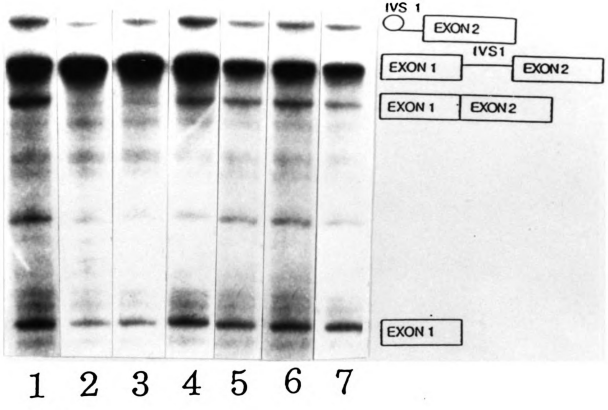

Figure 3.

concentration required for half-maximal inhibition was ~ 55 mM (Fig. 4 and Table I). Sucrose was used as a disaccharide control for the dose-response analysis. Although ~ 30% inhibition was observed (using the criteria described above), no concentration of sucrose tested could yield the degree of inhibition seen with Lac. In contrast to the results obtained with Lac, the monosaccharide ligand of CBP35, Gal, did not show inhibition of splicing at a concentration as high as 150 mM (Table I). Similarly, Glc, Man, GlcNAc, ribose and *myo*-inositol all failed to inhibit, up to a concentration of 150 mM.

Blood group A tetrasaccharide binds to HL-29 (human homolog of CBP35) with an affinity 32 times that for Lac (32). The effect of A-tetrasaccharide on the present assay was tested using a human serum albumin conjugate that contains 14 such saccharide moieties. Greater than 80% inhibition was observed in the concentration range 170-340 μM (Fig. 5, lanes 4 and 5; Table I). Control experiments using bovine serum albumin at 170-340 μM showed little or no inhibition (Fig. 5, lanes 2 and 3).

Effects of Antibodies Reactive with CBP35

When the nuclear extract was preincubated with anti-M2 prior to assays of *in vitro* splicing, there was inhibition of the splicing reaction (Fig. 6). The isotype-matched rat monoclonal antibody, anti-TR receptor, did not yield the same effect. Moreover, the effect of anti-M2 can be partially

Figure 4: Comparison of the dose-response curves of the effects of sucrose (□) versus lactose (◆) on the cell-free splicing assay. HeLa cell nuclear extract was preincubated for 30 min with various concentrations of the disaccharides. ^{32}P-Labeled HβGΔ6 pre-mRNA was then added for a 60 min reaction at 30°C. The RNA components were analyzed by 10% polyacrylamide-8.3 M urea gel electrophoresis, followed by autoradiography. The extent of the reaction was determined by densitometric scanning of the intensity of the band representing the product mRNA.

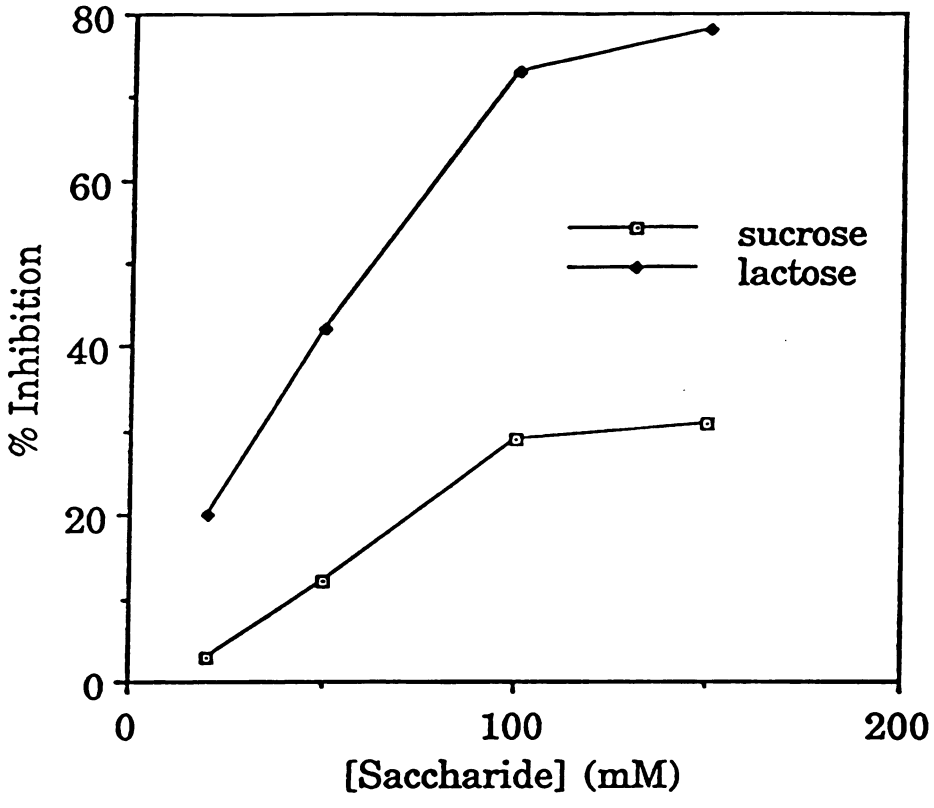
80
60
40
20
0
% Inhibition
0
100
200
[Saccharide] (mM)
sucrose
lactose

Figure 4.

TABLE 1. Inhibition of Splicing by Various Reagents

Reagent	Concentration Required for 50% Inhibition (mM)
A tetrasaccharide-HSA	< 0.17
lactose	55
thiodigalactoside	90
sucrose	> 150
N-acetylglucosamine	> 150
galactose	> 150
glucose	> 150
mannose	> 150
ribose	> 150
myo-inositol	> 150

Figure 5: **Effects of blood group A tetrasaccharide-human serum albumin conjugate on the cell-free splicing of pre-mRNA, as analyzed on 10% polyacrylamide-8.3 M urea gels. The protocol followed that described in the legend to Figure 3. Lanes: (1) control; (2) and (3) 170 μM and 340 μM, respectively, of bovine serum albumin; (4) and (5) 170 μM and 340 μM, respectively, of a human serum albumin conjugate containing 14 of blood group A tetrasaccharide groups.**

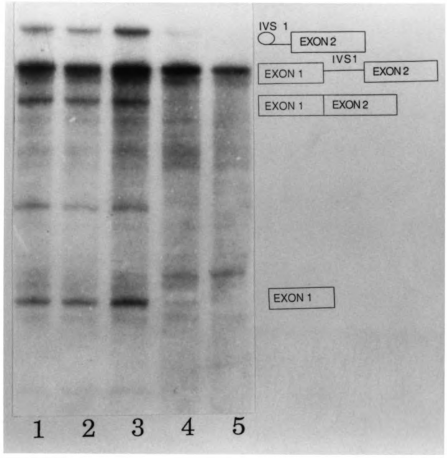

Figure 5.

Figure 6: Comparison of the dose-response curves of the effects of anti-M2 (□) and anti-TR (♦) on the cell-free splicing assay. The protocol followed that described in the legend to Figure 4.

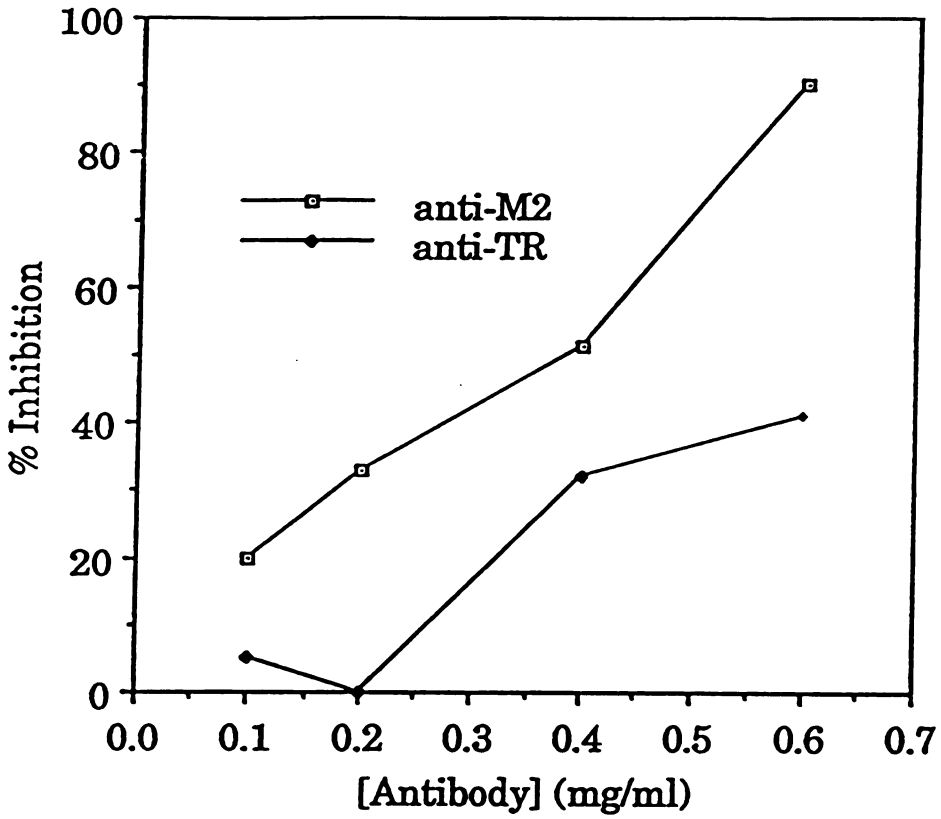
100
80
60
40
20
0
% Inhibition
anti-M2
anti-TR
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
[Antibody] (mg/ml)

Figure 6.

reversed by the inclusion of rCBP35 during the preincubation with anti-M2. These results, coupled with the saccharide inhibition of *in vitro* splicing, suggest that perturbation of CBP35 through binding of ligands or antibodies may affect the activities of the RNP complex with which the lectin is associated.

DISCUSSION

In previous studies, we had accumulated evidence that nucleoplasmic CBP35 was associated with RNP complexes. In particular, when nucleoplasm from 3T3 fibroblasts was fractionated by density gradient sedimentation, immunoblotting analysis yielded CBP35 in fractions with densities corresponding to those reported for hnRNP: 1.30-1.35 g/ml on cesium sulfate gradients and 40S on sucrose gradients. We have now extended these observations to include the snRNP as well. On cesium sulfate gradients, snRNPs containing the Sm antigen fractionated over a range of densities, from 1.30 to 1.53 g/ml. Three of these fractions (1.3-1.35 g/ml) also contained CBP35, while the other four fractions (1.40-1.53 g/ml) were devoid of the lectin. The Sm antigens are protein components of the U1, U2, U4, U5, and U6 snRNPs (31). On cesium chloride gradients (33), the U2, U4, and U6 snRNPs sediment to positions of high density (> 1.4 g/ml), while the U1 and U5 snRNPs can be found in the range of densities where CBP35 has been identified on cesium sulfate gradients (1.3-1.4 g/ml).

Of the polypeptides on U1 and U5 snRNPs, those similar in molecular weight to CBP35 include: (a) the B polypeptide (M_r ~ 29,000) common to U1 and U5; (b) the U1-specific A polypeptide (M_r ~ 33,000); and (c) the U5-specific M_r ~ 33,000 polypeptide. The amino acid sequences of the B (and related B') core polypeptide and the U1-specific A polypeptide have been derived from their respective cDNA clones (34,35). These sequences indicated that the A and B polypeptides were distinct from CBP35. Consistent with this notion, there was no apparent cross-reaction between rCBP35 and ENA anti-Sm or between the B polypeptide and anti-M2. At present, there is no structural information available on the M_r ~ 33,000 polypeptide identified on U5 snRNP (36); therefore, the formal possibility that CBP35 might be this polypeptide remains open.

Besides co-localization of CBP35 and the Sm antigen in certain fractions of the cesium sulfate gradients, they can also be co-immunoprecipitated by their respective antibodies. It is quite striking that ENA anti-Sm was able to co-immunoprecipitate the majority, if not all, of the CBP35 in the nucleoplasm. Similarly, most of the Sm immunoreactive material was found in the bound fraction of the anti-M2 precipitate, although a small amount of Sm could be detected in the unbound fraction. This is distinct from the results of the cesium sulfate gradients, in which there were clearly fractions of the gradient that contained the Sm antigen but no CBP35. It should be noted, however, that the

immunoprecipitations were carried out in "native" conditions in which many of the uracil-rich snRNPs could be complexed together on the same particle as CBP35, while the ionic conditions of the cesium sulfate gradients may have dissociated such a complex. In either case, there must necessarily remain specific protein-RNA interactions (and possibly protein-protein interactions as well) because both the co-immunoprecipitations and the positions of sedimentation on cesium sulfate gradients were altered when nucleoplasm was first treated with nucleases.

The association of CBP35 with hnRNP and snRNP is most readily explained in terms of the localization of the lectin on a spliceosome, which carries out the processing of intron-containing pre-mRNAs in the nucleus. Consistent with this notion, we have found that a rat monoclonal antibody reactive with CBP35 (anti-M2) inhibited splicing in a cell-free assay, whereas an isotype-matched control rat monoclonal antibody (anti-TR) showed much less effect at the same concentration. Antibody inhibition of *in vitro* splicing of pre-mRNAs has been demonstrated for autoimmune sera specific for the snRNPs (37), monoclonal antibody directed against polypeptides of hnRNP (38), as well as a monoclonal antibody reactive against the specific splicing factor SC-35 (39).

Carbohydrate ligands that bind to CBP35 also inhibit the splicing reaction *in vitro*. The specificity of such an effect is demonstrated by a comparison of the dose-response curves of

Lac versus sucrose. Half maximal inhibition for Lac was observed at ~ 55 mM, whereas the corresponding value for sucrose could not be determined in our assay (> 150 mM). Human serum albumin containing covalently conjugated groups of blood group A tetrasaccharide was ~ 300 times more potent in the inhibition of cell-free splicing. This effect can most readily be ascribed to several factors, including: (a) A tetrasaccharide has an affinity for the human homolog of CBP35 (HL-29) ~ 30 times higher than that of Lac (32); and (b) the 14-fold increase in the valence (number of A tetrasaccharide groups coupled) of the protein carrier.

It should be noted that there is less than perfect correlation between the affinity of a saccharide for CBP35 and its effect on the cell-free splicing assay. First, CBP35 was initially purified on the basis of its Gal binding activity. Yet, Gal failed to inhibit the splicing reaction. One rationalization of this negative result may be that the affinity of Gal for CBP35 is some 60-100 times lower than that of Lac (32). What is of greater concern, however, is the observation that the concentration required for half-maximal inhibition of the splicing assay for thiodigalactoside is 2-fold higher than that for Lac. In saccharide-binding assays, it was shown that thiodigalactoside in fact has a higher affinity for CBP35 than Lac (32). These results call for caution in interpreting the saccharide and antibody inhibition data to implicate a role of CBP35 in the splicing of pre-mRNA.

For one thing, we have yet to identify a glycoconjugate ligand of CBP35 inside the nucleus or on an RNP. Therefore, it is not clear that the carbohydrate-binding activity of the lectin is used in the RNP complex with which CBP35 is associated. For another, the saccharide and antibody inhibition of splicing represent, at best, perturbation experiments. It remains to be demonstrated that nuclear extracts depleted of CBP35 will be devoid of splicing activity and that such inactive extracts can be complemented with purified CBP35 to restore the splicing activity.

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Chapter V

Conclusions

As is detailed in the literature review (Chapter I) the L-30 lectins are found intracellularly and extracellularly. It has been demonstrated that ~95% of CBP35 is found within the cell (1). Previous studies had shown that CBP35 is found in the cytoplasm and the nucleus (1), and that CBP35 appears in the nucleus during the G_1 phase of the cell cycle (2).

The focus of this dissertation has been to study the CBP35 found in the nucleus. These led to the conclusion that the CBP35 found in the nucleus was associated with a ribonucleoprotein complex (RNP), possibly the spliceosome. This conclusion was based on the following observations (presented in Chapter III and IV of this thesis): ribonuclease A digestion released CBP35 from the nucleus, but deoxyribonuclease I digestion failed to do so; CBP35 sedimented at densities characteristic of the RNP on density gradient centrifugation (1.30-1.32 g/ml on a cesium sulfate gradient, 40S on a sucrose gradient); and finally fractionation of nucleoplasm by carbohydrate affinity

chromatography, or immunoprecipitation with anti-CBP35 antibody yielded a fraction that contained both CBP35 and RNA. Analysis of the anti-CBP35 immunoprecipitate by immunoblotting showed that the that the Sm(B) protein of the snRNPs were detected using autoimmune anti-Sm sera. Conversely autoimmune sera directed against the Sm antigen, immunoprecipitated CBP35, and the Sm(B) protein. All of these results suggest that nucleoplasmic CBP35 with RNP, in particular with hnRNP and snRNP. This suggests that CBP35 may be associated with the spliceosome, and suggested specific tests for its physiological activity.

The spliceosome is known to be the site processing of precursor RNA. Our results indicate that agents directed against CBP35 can perturb splicing activity. The agents that effect splicing activity include saccharide ligands for CBP35 (lactose, thiodigalactoside, and A tetrasaccharide-HSA complex) and a monoclonal antibody directed against CBP35 (anti-Mac2). Control reagent that did not bind to CBP35 did not effect splicing . While these results are consistent with the conclusion that CBP35 plays a role in splicing, they do not prove this.

In order to determine if CBP35 plays a role in splicing, it will be necessary to specifically remove CBP35 from a splicing extract, and to assess the splicing activity of this extract after depletion. If depletion of CBP35 inhibits splicing, it will be necessary to readd CBP35, and see if the added protein replenishes the splicing activity in the extract. This would determine if CBP35 is required for pre-mRNA splicing, and could open an exciting chapter in lectin research.

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ADDENDUM

In order to more clearly define the roles played by the different authors who contributed work to this dissertation, I have listed the parties who were responsible for the individual experiments.

Chapter II was derived from a collaborative effort between Dr. Fu-Tong Liu's laboratory in the Medical Biology Institute, La Jolla, California, and Dr. John Wang's laboratory at Michigan State University. It is reproduced with the permission of the the American Society for Biochemistry and Molecular Biology, from the Journal of Biological Chemistry (1989) 264:1907-1910. The data presented in Figures 1 and 2 were produced in East Lansing. The data presented in Figures 3 and 4 were derived from experiments performed in La Jolla, with reagents provided by our laboratory.

Chapter III was previously published in Biochemistry (1988) 27: 5329-5334, and is reproduced with permission from the American Chemical Society. All of the work in this chapter was performed in our laboratory.

Chapter IV was a result of a collaborative project between the laboratory of Drs. Ronald Patterson and Elizabeth Werner in the Department of Microbiology at Michigan State University and Dr. John Wang's laboratory in the Department of Biochemistry. The data presented in Figures 1 and 2 were produced in our laboratory. The data in Figures 3 and 5 were obtained by Dr. Patterson. Finally, the data reported in Figures 4 and 6, and in Table 1 were derived from experiments performed in Dr. Patterson's laboratory; I carried out the quantitative analysis of the data.