This is to certify that the

dissertation entitled

Evidence for an Association Between Splicing Components: Galectin-3 and Polypeptide(s) of snRNA

presented by

SUNG-YUAN WANG

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in **Biochemistry**

Major professor

Date 5/8/95

EVIDENCE FOR AN ASSOCIATION BETWEEN SPLICING COMPONENTS: GALECTIN-3 AND POLYPEPTIDE(S) OF snRNP

By

Sung Yuan Wang

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1995

ABSTRACT

EVIDENCE FOR AN ASSOCIATION BETWEEN SPLICING COMPONENTS: GALECTIN-3 AND POLYPEPTIDE(S) OF snRNP

By

Sung Yuan Wang

Galectin-3 is a galactose/lactose-binding protein found in the nucleus and cytoplasm of a variety of cell types. Using a cell-free assay for pre-mRNA splicing, previous studies have demonstrated that galectin-3 is a required factor for spliceosome formation and splicing activity. Depletion of the lectin from a splicing competent extract resulted in a loss of both activities, which could be restored upon reconstitution with purified recombinant galectin-3.

The goal of this thesis was to demonstrate an association of galectin-3 with components of the splicing machinery; specifically, the Sm epitopes found on polypeptides of the small nuclear ribonucleoproteins (snRNPs). This was accomplished at two levels: (a) at the level of single cells, the immunofluorescence patterns of galectin-3 were shown to be similar to that of the snRNP Sm polypeptides under a variety of conditions and colocalization could be demonstrated in double labeling experiments; and (b) at the level of protein-protein interactions, it was shown that when anti-Mac 2, a monoclonal antibody specific for galectin-3, immunoprecipitates galectin-3 from nuclear extracts of HeLa cells and mouse 3T3 fibroblasts, the Sm B polypeptides are coprecipitated.

The immunofluorescence experiments were carried out with mouse 3T3 fibroblasts. The staining patterns for galectin-3 and snRNP Sm polypeptides were monitored in parallel and were found to behave in the same fashion: (a) both yielded diffuse distribution of fluorescence covering the entire nucleus, except for several nucleoli, in fixed and permeabilized cells; (b) using conditions of permeabilization and extraction that preserved a RNP-containing nuclear matrix, both yielded speckled staining patterns; (c) the fluorescence staining was lost for both galectin-3 and snRNP Sm polypeptides when permeabilized nuclear residues were treated with ribonuclease A, whereas neither staining was sensitive to deoxyribonuclease I treatment; and (d) during the various stages of mitosis, both galectin-3 and snRNP Sm polypeptides were found to be excluded from the area of chromosomal localization, condensation and separation.

Nuclear splicing extracts were subjected to immunoprecipitation with anti-Mac 2. Immunoblotting of the anti-Mac 2 precipitate with polyclonal rabbit anti-galectin-3 revealed the presence of the lectin. Immunoblotting of the same anti-Mac 2 precipitate with an autoimmune serum reactive with the snRNP B polypeptides showed that a fraction of the Sm B polypeptides in the nuclear extract was coprecipitated with the lectin by the monoclonal antibody. The specificity of this coimmunoprecipitation was analyzed by comparing the polypeptides precipitated by anti-Mac 2 versus those found in the corresponding precipitate fractions obtained with anti-Sm (positive control) and with anti-transferrin receptor, an isotype-matched monoclonal antibody (negative control). The coprecipitation of Sm B with galectin-3 by anti-Mac 2 was dependent on the presence of the lectin. Nuclear extracts depleted of galectin-3 by prior adsorption on a lactose affinity resin

failed to yield Sm B in the anti-Mac 2 precipitate. The coprecipitation was not affected by the addition of lactose; nor was it perturbed by prior treatment of the nuclear extract with ribonuclease. These results suggest that at least a fraction of the Sm B polypeptides in the splicing extract can interact with galectin-3 through protein-protein interactions.

In the course of these studies on the polypeptides precipitated by anti-Mac 2, we also analyzed the composition and identities of polypeptides bound to the affinity resin lactose-agarose under conditions used to deplete the nuclear extract of splicing activity. We found that, in addition to galectin-3, the bound fraction of the lactose-agarose contained at least three other polypeptides. Of particular interest was the identification of one of the other polypeptides to be galectin-1 (the prototype member of the galectin family), whose presence in the nucleus of HeLa cells was confirmed by confocal fluorescence microscopy. These findings provide an explanation for previous observations that while lactose-agarose can deplete nuclear extracts of splicing activity, anti-Mac 2 precipitation failed to yield the same effect. Together with the observation that galectin-1, as well as galectin-3, can alone reconstitute splicing activity in a lactose-agarose depleted extract, these results suggest that the activities of galectin-1 and galectin-3 are redundant in the cell nucleus.

To Yi Yu and Catherine

and

To My Parents

for their love, support and faith in me

I would lik
encouragement du
science and life. I
encouragement, s
both scientific and

I owe a sp
that he provided
members of my g
Revzin for their

Finally, I
colleagues Anan
Eric Arroyo, Sh
lab very special
Michigan State

ACKNOWLEDGMENT

I would like to express my sincere appreciation to Dr. John L. Wang for his advice and encouragement during my graduate study in his laboratory. I thank him for teaching me about science and life. I would also like to extend my gratitude to Mrs. Patty Voss for her encouragement, support and tolerance. I thank her for providing a conductive environment for both scientific and social growth.

I owe a special note of thanks to Dr. Ronald Patterson for his valuable advice and support that he provided me throughout the years. I would also like to express my appreciation to members of my guidance committee, Dr. Richard Anderson, Dr. Zachary Burton and Dr. Arnold Revzin for their advice and criticisms.

Finally, I wish to thank Dr. Melvin Schindler, Dr. Siu-Cheong Ho and my friends and colleagues Anandita, Mark Kadrofske, Yeou Guang Tsay, Dr. Jamie Laing, Dr. Neera Agrwal, Eric Arnoys, Sharon Grabski who shared both the ups and downs and who made my time in the lab very special. Knowing them has been a privilege and worth by itself the time I have spent at Michigan State.

TABLE OF CONTENTS

CHAPTE

LIST OF TABLES

Page

CHAPTER I:

LIST OF FIGURES

Page

CHAPTER I:

CHAPTER II:

CHAPTER III:

CHAPTER IV:

CHAPTER I

Literature Review

LITERATURE REVIEW ON GALECTINS

A) Carbohydrate Binding Proteins of Animal Cells

There are three main classes of proteins involved in protein-carbohydrate interactions (1): a) enzymes that use carbohydrates and glycoconjugates as substrates, such as glycosidases and glycosyl transferases; b) sugar-binding antibodies; and c) the carbohydrate binding protein (CBP) group. Carbohydrate binding proteins are defined as non-enzymatic and non-immune proteins which can selectively bind specific carbohydrate structures and are now referred to as lectins. Ricin, a toxic protein, was documented as the first lectin from a plant source (2). Since the initial identification, lectins have been found in a variety of organisms, in various tissues and cell types. More recently, much attention has been paid to animal lectins. Since carbohydrate structures such as glycoproteins and glycosaminoglycans have been found in the cytoplasm and the nucleus (3), there has been, in turn, greater interest in the intracellular localization of lectins.

Animal lectins have been classified based on the nature of their carbohydrate ligands, their involvement in biological processes, their subcellular localization, and their dependence on divalent cations. However, since the primary structures of many lectins have been determined, the shared sequence characteristic is now one of the most useful way to classify lectins. Based on their amino acid sequences, especially those of characteristic carbohydrate-recognition domains (CRD), most animal lectins can be classified into two major distinct families (4-6). One is the family of C-type lectins

which is found extracellularly or associated with plasma membrane and which requires calcium ions for their sugar-binding activity. The other one is the galectin family (7), consisting of soluble, metal-independent, β-galactoside-binding proteins formerly known as S-type or S-Lac lectins (Table 1).

B) C-Type Lectins

The C-type CRDs derive their name from the fact that they require calcium ions for sugar binding activity. The carbohydrates that their CRDs recognize are diverse, including simple carbohydrates such as galactose, *N*-acetylglucosamine, and complex glycoconjugates such as sialyl Lewis antigens (5). Although calcium ions are required for CRD binding to its ligand, the other domain(s) of C-type lectins is involved in many of the functions of the different proteins. All of the C-type lectins have primary structures typical of extracellular proteins or transmembrane proteins in that they exhibit a signal sequence for entry into the endoplasmic reticulum. C-type animal lectins are found in serum, extracellular matrix and membranes; thus they can be divided into insoluble and soluble groups (6).

The insoluble C-type lectin group includes those transmembrane receptors, such as a type-I receptor whose N-terminal region is extracellular and C-terminal region is cytoplasmic. This group includes selectins, mannose receptor and thrombomodulin. The selectins mediate the initial phase of adhesion between leukocytes and endothelia in a weak transient adhesion. A larger number of membrane-integrated C-type lectins are

Table 1. Comparison of C-type Lectins and Galectins

Properties	C-type Lectins	Galectins
Calcium requirement	Yes	No
Solubility	Variable	Soluble
Cys residues	Disulfides	Free thiols
Cellular localization	Extracellular Transmembrane	Intracellular Extracellular
Carbohydrate specificity	Various	β-galactosides

categorized into type-II receptors such as hepatocyte receptor and macrophage receptor. A type-II receptor has a cytoplasmic N-terminal region and an extracellular C-terminal domain. Some of the functions ascribed to these receptors include the removal of glycoproteins by endocytosis and phagocytosis. Soluble C-type lectins include collagen-like proteins such as the mannose-binding protein. This type of lectin is composed of an N-terminal collagenous domain and a C-terminal CRD. Other soluble C-type lectins include proteoglycan core proteins, snake venom lectins, etc.

C) Galectins

1) Structure of Galectins

S-type lectins which possess affinity for β-galactosides and show a significant sequence similarity in the carbohydrate-binding site are now renamed as galectins (7). The general designation of the genes encoding galectins is *LGALS* (lectin, galactoside-binding, soluble), and gene numbering is being kept consistent with the numbering of the proteins; so that *LGALS1* encodes galectin-1, and *LGALS2* encodes galectin-2, etc (7). The CRD of the galectin family is clearly distinguishable from the corresponding CRD of the calcium-dependent C-type lectin (4, 8). Unlike C-type lectins, galectins have been found both inside and outside of cells. They do not depend on cations for carbohydrate binding activity and are isolated usually as soluble proteins.

At present, seven galectins in this family have been studied (Table 2). Galectin-1 is isolated as a homodimer with monomer molecular weight of ~ 14 kDa. It is abundant

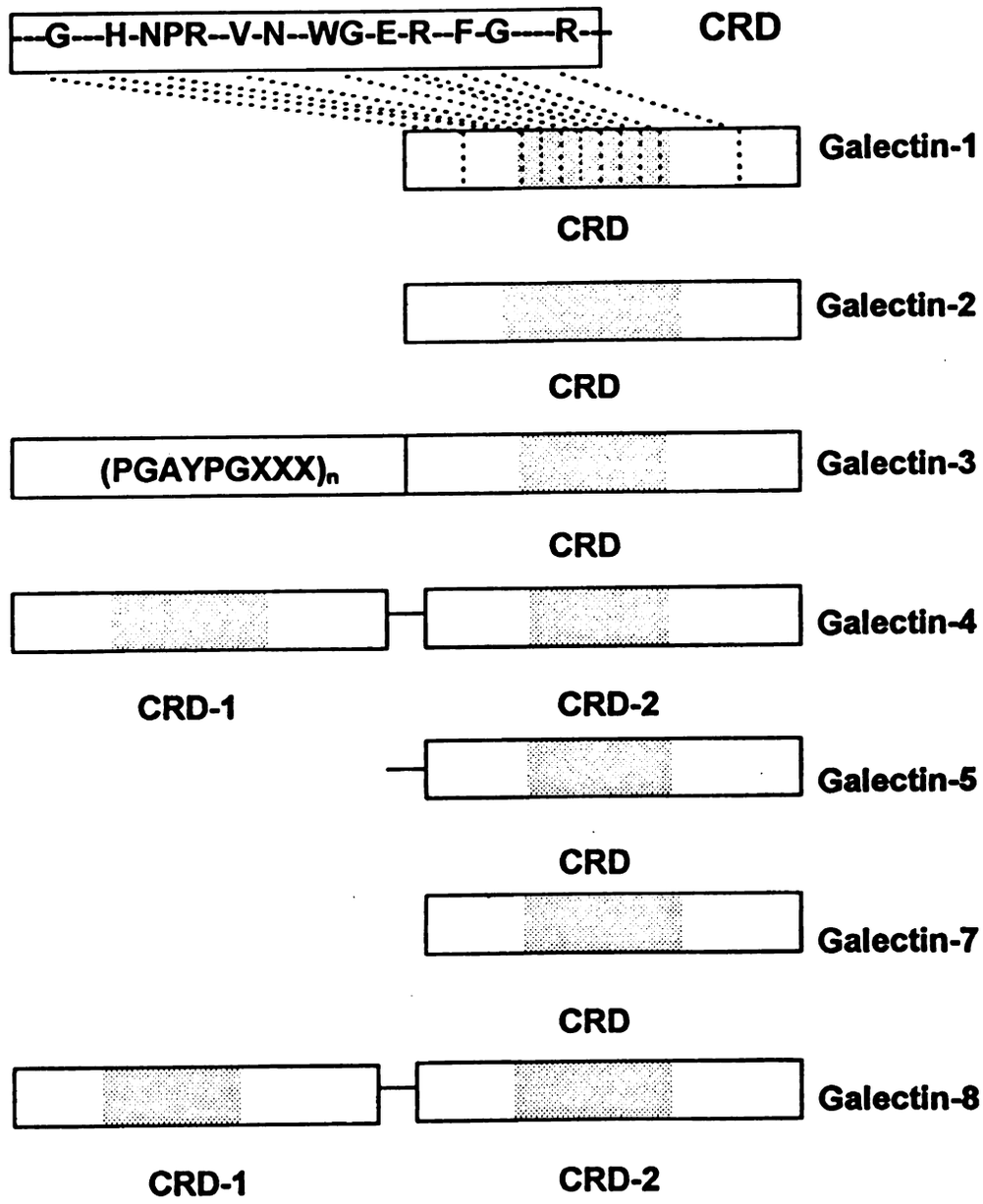
in smooth and skeletal muscle, but is also found in many other cell types. Galectin-2, originally found in human hepatoma, is isolated as a homodimer (9). Galectin-3, previously known as CBP35 was first isolated from mouse 3T3 fibroblasts (10, 11). It consists of a single polypeptide of ~ 33 kDa. Galectin-4 was found as an abundant rat intestinal 36 kDa lectin (12). It is homologous to a 32 kDa β-galactoside-binding protein from *Caenorhabditis elegans* (13). Galectin-5 was originally isolated from rat lung as a monomer of ~ 18 kDa (14). Galectin-7 is a 15 kDa monomeric protein found in human keratinocytes (15). Galectin-8 was originally cloned from rat liver (16) with a molecular weight of 34 kDa. Unlike galectin-4, which is abundant in the intestine galectin-8 was found in liver, kidney, cardiac muscle, lung and brain. In addition, there are invertebrate lectins whose CRDs fit the consensus sequence of the galectin CRDs but whose subfamily designations has not been decided. These include the sponge (17) and nematode (13) S-type lectins.

As implied by their nomenclature, the structures of all the galectins have at least one carbohydrate recognition domain (CRD), with conserved amino acid sequence between members of the family and homologues of the same member across different species (Figure 1). Thus, galectin-1 galectin-2, galectin-5 and galectin-7 each has one CRD, representative of the prototype of the family. Galectin-4 and galectin-8 represent tandem-repeat type with two CRDs in the structure. Each of the CRDs in galectin-4 shows about 25% sequence identity to vertebrate galectin-1, and has carbohydrate-binding activity. Sequence analysis also showed that galectin-8 exhibits about 34%

Table 2. Members of the Galectin Family

Galectin	Subunit Structure	Polypeptide M_r (SDS-PAGE)	Source	Chromosome Location
1	Homodimer	14,000	Various species, tissues/cell types	Human 22
2	Homodimer	14,000	Human	Human 22
3	Monomer	33,000	Various species, tissues/cell types	Human 13
4	Monomer	36,000	Rat (*C. elegan*)	-
5	Monomer	18,000	Rat Mouse	Mouse 11
6	?	?	?	?
7	Monomer	15,000	Human	Human 19
8	Monomer	34,000	Rat	-

Figure 1: Schematic diagram illustrating the domain context and organization of the various galectins.
The carbohydrate recognition domain(s) (CRD) in each galectin is highlighted by the shaded rectangle. Amino acid residues that are conserved among the various galectins are highlighted in the top. The sequence of a nine residue motif that is tandemly repeated (n times, depending on species) giving rise to a proline- and glycine-rich domain in galectin-3 is indicated.


---G---H-NPR--V-N--WG-E-R--F-G----R--
CRD
Galectin-1
CRD
Galectin-2
CRD
(PGAYPGXXX)n
Galectin-3
CRD
Galectin-4
CRD-1
CRD-2
Galectin-5
CRD
Galectin-7
CRD
Galectin-8
CRD-1
CRD-2

sequence identity to galectin-4. Galectin-6 has been mentioned (7), but its characterization has yet to be published.

2) Galectin-3: Chimera of Two Domains

The galectin-3 group includes proteins originally isolated from various sources which exhibit β-galactoside binding activity and which have been designated different names (Table 3). Genomic Southern blot analysis suggested that there is only one gene coding for the polypeptides of the galectin-3 group. The C-domain of galectin-3 polypeptide contains both hydrophilic and hydrophobic regions, as is characteristic of many globular proteins. This region is about 35% identical with several members of the galectin-1 group, and 14 amino acid residues in this region are highly conserved among galectin-3 and other members of galectin family (Figure 1). Consequently, the C-domain of galectin-3 is assumed to harbor the CRD. In contrast, the N-domain of galectin-3 polypeptide contains neither a highly hydrophilic nor a hydrophobic region. This domain includes a stretch of eight contiguous 9-residue repeat units having the sequence *Pro-Gly-Ala-Tyr-Pro-Gly* followed by three other amino acid residues. As a result, this stretch of the sequence is characterized by a high proportion of proline and glycine residues (18). Thus, galectin-3 is classified as a chimera-type galectin .

Study on the N-terminal domain of galectin-3 showed that it exhibits no apparent β-galactoside-sugar binding activity; in contrast, the C-terminal domain is sufficient for two independent transitions representing the thermal denaturations of the C-domain sugar-binding (19). Differential scanning calorimetry analysis of galectin-3 has yielded

Table 3. Previous Names for Galectin-3

Name	Mr (SDS-PAGE)	Species	Tissue/Cell type	Focus of interest
L-29	29,000	Rat, Human, Dog	Lung, Brain, Kidney	Non-classical secretory pathway
εBP	31,000	Rat, Human	Basophilic leukemia cell	IgE binding activity
Mac-2	32,000	Mouse, Human	Macrophage	Cell surface antigen
L-34	34,000	Mouse	Fibrosarcoma Melanoma	Cell surface lectin metastatic marker
CBP35	35,000	Mouse, Human	Lung, Fibroblast	Intracellular lectin
LBP	35,000	Mouse	Macrophage	Laminin binding activity

(~ 40 °C) and C-domain (~ 55 °C). The binding of lactose to the intact galectin-3 polypeptide and to the C-domain half molecule shifted the 55 °C transition to ~ 65 °C. These results suggest that ligand binding by galectin-3 is accompanied by a conformational change that significantly stabilizes the polypeptide against thermal denaturation.

Analysis of the isoelectric point (pI) of *E. coli* expressed recombinant galectin-3 showed that the pI value of unmodified polypeptide is 8.7, as determined by calculation from the deduced amino acid sequence and by isoelectric focusing gel electrophoresis analysis (20). However, when mouse 3T3 cell extract was subjected to two-dimensional gel electrophoresis and immunoblotting analysis for galectin-3, two spots corresponding to pI values of 8.7 and 8.2 were observed. The pI 8.2 species represents a post-translational modification of the pI 8.7 polypeptide by the addition of a single phosphate group.

3) Carbohydrate-Binding Properties of Galectins

All mammalian galectins recognize the same structural determinant on lactose and related β-galactosides (10, 12, 21-24); however, they share a higher affinity of binding for lactose than that for galactose (25-27). The affinity of galectin-3 for lactose binding was found to be 50-100 times greater than that for galactose. The critical positions of the lactose molecule include the hydroxyls at positions 4 and 6 of Gal and position 3 of Glc, since substitutions at any of these positions resulted in reduced binding activity.

Modification of lactose by incorporating an acetamido group at position 2 of glucose moiety to yield *N*-acetyllactosamine enhanced the binding of galectins.

The sequences responsible for carbohydrate binding are mainly encoded by three exons (28-30), for example exons II, III and IV for galectin-1 and IV, V and VI for galectin-3. Most of the residues directly interacting with the carbohydrate ligand are conserved among the galectins and encoded by a single exon. Mutation of some of these residues in exon III of galectin-1 resulted in impaired carbohydrate binding activity (31, 32). Site-directed mutagenesis on the CRD of galectin-1 showed that the conserved hydrophilic residues, such as *His44, Asn46, Arg48, Asn61, Glu71,* and *Arg73,* are important in carbohydrate recognition (32). Deletion of the N-terminal and C-terminal regions of galectin-1 also decreased the carbohydrate-binding affinity indicating that the other two exons may also be important for carbohydrate binding activity (31).

Recently, crystallographic studies of galectin-1 have shown that the lectin appears to form a dimer structure. Each of the monomer associates with one *N*-acetyllactosamine molecule (33, 34). The hydroxyl groups of the galactose molecule in the disaccharide play major roles in its interactions with the protein. The main determinant of the specificity for galectin-1 binding to carbohydrate resides on the hydroxyl at position 4 of the galactose, which interacts with the side chain of *Arg48* and *His44* through hydrogen bonds. Other residues involved in the carbohydrate binding include *Asn46*, which interacts with hydroxyls at positions 3 and 4 through a water molecule, and *Asn61* with hydroxyl at position 6. The interaction between the side chains of *Lys63* and *Trp68* also aligns the two residues to interact with the galactose ring. The hydroxyl group of

galactose at position 2 shows no interaction with the proteins. The GlcNAc moiety of the disaccharide also interacts with the protein, but the interaction is less extensive than that of the galactose portion. The GlcNAc-protein interactions are mediated through the contact of the hydroxyl at position 3 with *Arg73*, *Arg48* and *Glu71*. In addition, the N2 position of the *N*-acetyl group is involved in the interaction with *Arg73*, *Asp54* and *His52*. This may explain the enhanced binding of galectin to *N*-acetyllactosamine over that of lactose. A similar result has been found in the study of the crystal structure of N-acetyllactosamine and recombinant galectin-2 complex (35).

4) <u>Subcellular Localization of Galectins</u>

Most of the C-type lectins have been found to be localized either in a defined cellular compartment such as membrane-integrated receptors or exported extracellularly as soluble proteins. In contrast, the cellular localization of galectins is somewhat less clear cut. Galectin-1 polypeptides have been shown to be intracellular, though reports vary regarding the nuclear and cytoplasmic distribution. Immunofluorescence staining for galectin-1 on cryostat sections from adult chicken kidney (36), calf pancreas (37) have shown that the lectin is localized in cell nuclei as well as cytoplasm. Similarly, immunocytochemical studies in dorsal root ganglion neurons have shown that galectin-1 could be detected both in the nucleus and cytoplasm of the neurons (38). Immunoelectron microscopy has also localized galectin-1 in the nucleus of the epidermal cells from the intermediate layer of chick embryonic skin (39). However, immunofluorescence and ultrastructural studies have also led to explicit statements that anti-galectin-1 antibodies failed to label the cell nucleus. In a recent study,

immunofluorescence staining of Chinese hamster ovary cells by anti-galectin-1 antibodies, detected galectin-1 both intracellularly and extracellularly. However galectin-1 was absent from the nucleus (40).

Despite the intracellular localization, there is considerable evidence for the extracellular localization of galectin-1, though no typical secretory signal sequence has been found (8). Studies of export of muscle galectin-1 have shown that during myoblast differentiation the lectin becomes concentrated in evaginations of plasma membrane which pinch off or bud off to form labile lectin-rich extracellular vesicles (41, 42). The exported lectin can then interact with carbohydrate on laminin (43) and other extracellular glycoproteins (44). These observations suggest a possible mechanism for lectin export from the cytosol to the extracellular matrix. Galectin-1 is also found to be externalized upon injection of epinephrine without the involvement of secretion vesicle in *Xenopus laevis* skin cells (45).

Similarly, galectin-3 has been found on the cell surface and inside the cell. Most of the galectin-3 was localized intracellularly, especially in the nucleus as shown by immunofluorescence staining, though a small amount of the lectin was detected on the cell surface (46). Subcellular fractionation studies on rat basophilic leukemia cells showed that the majority of the rat galectin-3 is intracellular including in the nucleus (47). Other studies have also shown the intracellular and extracellular localization of galectin-3 in dorsal root ganglion neurons (38, 48), in murine fibrosarcoma cells and human carcinoma HeLa-S3 (49), and mouse macrophages (50). In Baby Hamster Kidney (BHK) cells, the majority of galectin-3 is found in the cytoplasm and small amounts are

deposited on the cell surface and substratum (51). In Madin-Darby canine kidney cells the lectin is expressed and secreted at the apical domain of the polarized cells (52), suggesting that apical secretion may be the possible way for galectin-3 externalization.

A wide difference in the level of galectin-3 in various tissues of mice during developmental stages was noted (26). The induction of galectin-3 in mouse macrophages was found to be dependent on the addition of thioglycolate (50). The regulation of expression and subcellular localization of galectin-3 was studied in detail in mouse 3T3 fibroblasts. Galectin-3 was found primarily in the cytoplasm of quiescent cells; however, in proliferating cells, the expression level increased and is predominantly localized in the nucleus (53). In quiescent cells, the cytoplasmic galectin-3 was found to be phosphorylated (pI 8.2). Upon serum stimulation, the synchronized cells showed an increased level of the phosphorylated form (pI 8.2), both in the cytosol and the nucleus. The level of unmodified form (pI 8.7) also increased but could only be found in the nucleus of the stimulated cells (20). The significance and mechanism of the differential expression and localization of the two isoelectric variants of galectin-3 remain unclear. Nevertheless, one study on human colonic tissue specimens has shown that galectin-3 is concentrated in the nuclei of differentiated epithelial cells (54). The expression and nuclear localization changed significantly during the progression from normal mucosa to adenoma to carcinoma; the lectin is absent from the nuclei of carcinoma cells but still localized in the cytoplasm. These observations suggest that the exclusion of galectin-3 from the nucleus may be related to the neoplastic progression of colon cancer.

5) Functions of Galectins

It has long been suggested that animal lectins might function in modulating cell-cell and cell-extracellular matrix interactions. For example, galectin-1 could be involved in modulating transitory adhesions during embryonic cell movement (55), and may also play a role in the elaboration or organization of extracellular matrix components (56). In skeletal muscle, galectin-1 has been shown to either promote or inhibit cell adhesion. One study showed that galectin-1 binds to laminin and inhibits cell-matrix interactions (43); presumably, binding of galectin-1 to polylactosamine on laminin in myoblasts interferes with laminin recognition by the major laminin receptor integrin $\alpha_7\beta_1$. In contrast, a recent report has shown that that galectin-1 binds to laminin and promotes cell adhesion to laminin (57). Nevertheless, these results suggest that galectin-1 may have a role in muscle development. Other studies suggested that galectin-1 might participate in regulating cell proliferation (58, 59).

On the basis of the identification as a laminin-binding protein (see Table 3), it has been proposed that galectin-3, like galectin-1, might also play a role in cell adhesion (60). Some studies suggested that binding of galectin-3 for both IgE and IgE receptor can trigger activation of mast cells and basophils and play a role in inflammation (61). Two glycoproteins named M2BP-1 and M2BP-2 were identified from certain colon cancer cell lines by coprecipitation with galectin-3 (62). The interaction is mediated by CRD of galectin-3 and sugar moieties of the glycoproteins. The localization of M2BP-1 and M2BP-2 is not clear, but these glycoproteins may play a role in mediating galectin-3

function. The exclusion of galectin-3 from the nuclei of carcinoma cells may be related to the neoplastic progression of colon cancer (54).

Several lines of evidence suggest that galectin-3 may play a role in pre-mRNA splicing (63, 64). First, immunofluorescence staining and immunoblotting analyses have shown that ribonuclease A (RNase) treatment of permeablized cells can remove nuclear galectin-3 while DNase I failed to do so. In addition, fractionation of nucleoplasm derived from Swiss 3T3 cells on a cesium sulfate density gradient revealed galectin-3 in fractions with densities of 1.30 -1.32 g/ml , corresponding to the range of densities reported for hnRNP and snRNP. Finally, the most direct evidence for a role of galectin-3 in pre-mRNA splicing comes from experiments using a cell free assay. The nuclear extracts used for this assay contain galectin-3, as detected by immunoblotting. Addition of saccharides that bind to galectin-3 with high affinity inhibits the cell free splicing. Most persuasively, depletion of galectin-3 by affinity adsorption on a lactose column resulted in a concomitant loss of splicing activity, which could be restored by reconstitution with recombinant galectin-3.

LITERATURE REVIEW ON snRNP PARTICLES AND PRE-mRNA SPLICING

A) Structure and composition of snRNP

During and after transcription, the pre-mRNA molecules are processed and transported to the cytoplasm where translation occurs. For most of the RNA polymerase II transcripts, this process includes the addition of m^7G cap at the 5'-end, polyadenylation at the 3'-end, assembly with proteins to form hnRNP, splicing to remove noncoding introns and ligation of exons, and the exchange of hnRNP proteins for mRNA proteins. The catalytic elements required for pre-mRNA splicing reaction include snRNPs, non-snRNP splicing factors and probably many other factors. The steps in nuclear pre-mRNA splicing have been elucidated and are illustrated in Figure 2 (65). Pre-mRNA splicing proceeds through a two step mechanism involving two separate transesterification reactions. The first step is cleavage at the 5' splicing site generating free exon 1 by activating the 2'-OH at the branch site producing a lariat RNA; the second step involves another transesterification reaction at the 3' splice site to join the exons and remove the intron.

The catalytic elements, snRNPs, contain uridine-rich small nuclear RNA, a common set of core polypeptides and one or more snRNP-specific proteins. In mammalian cells, there are six major snRNAs (Table 4), named U1 through U6, ranging in size from 107 to 216 nucleotides (66). The major snRNPs are found located in the nucleoplasm, except for U3 which is localized in the nucleolus. Other minor snRNAs have also been characterized; however, this review will focus on the major snRNPs. The

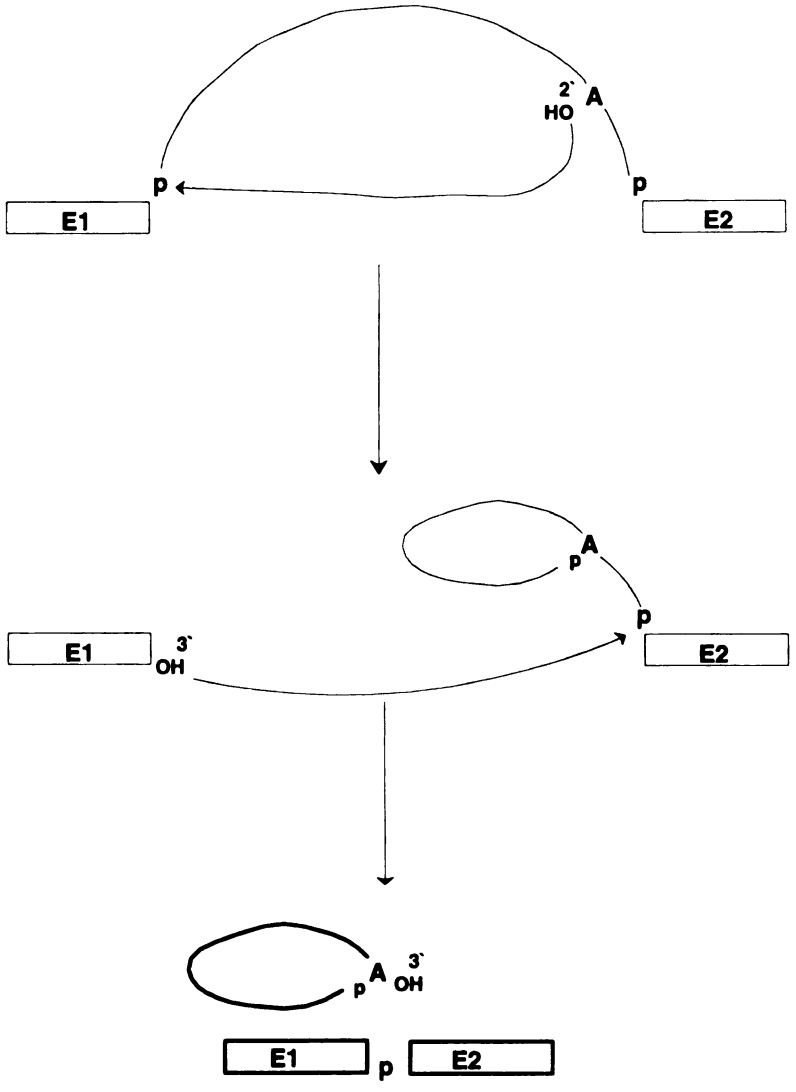


Figure 2: Schematic diagram illustrating the two major steps in pre-mRNA splicing.
Step 1 is a transesterification reaction leading to the formation of a lariat intermediate. Step 2 is another transesterification reaction leading to intron removal and exon joining. This scheme was modified from reference 65.

Table 4. Major Mammalian snRNAs

RNA	Size Nucleotides	RNA Polymerase	5' end Cap	Sm Binding Site
U1	165	II	m^3GpppAmUmA	AAUUUG
U2	189	II	m^3GpppAmUmC	GAUUUUUGG
U3	216	II	m^3GpppAmUmG	-
U4	139	II	m^3GpppAmGmC	AAUUUUUGG
U5	117	II	m^3GpppAmUmA	AAUUUUUG
U6	107	III	CH_3pppGUG	-

major snRNAs are transcribed by RNA polymerase II, except for U6 which is transcribed by RNA polymerase III (66, 67). Analysis of pre-mRNA intron regions has revealed some consensus sequences to be complementary to conserved internal sequences in U2 snRNA and at the 5'-end of U1 snRNA (68-70). U1 and U2 snRNPs recognize the consensus sequences at the 5' splice site and branch site in the initial steps in the splicing reaction (Figure 3). In the meantime U4 and U6 snRNAs bind to each other through an extended complementary region to form the U4/U6 snRNP complex (71) and then the U4/U6 snRNP particle associates with U5 snRNP (72). This tri-snRNP is then bound to the pre-mRNA/U1/U2 complex along with other components for intron removed. The snRNPs are either recycled for other splicing events or degraded.

Patients with systemic lupus erythematosus (SLE) often possess antibodies against two nuclear antigens called Sm and RNP. Anti-Sm antibodies can immunoprecipitate five major snRNAs, U1, U2, U4, U6 and U5 (Table 5), and minor snRNAs U7, U8, U9, U10, U11 and U12 (73). This was the first indication that different snRNP particles share identical or at least similar proteins. The major epitopes recognized by the anti-Sm class autoantibodies are antigenic determinants on the 28 kDa B and 16 kDa D polypeptides of the snRNP core proteins (Table 6). In addition to these shared proteins, each snRNA is found to be associated with one or more specific polypeptides to form a specific snRNP particle. The anti-(U1)RNP autoantibodies found commonly in SLE patients recognize determinants on the U1-specific proteins (73, 74); this antibody reacts mainly with a U1-specific 70 kDa protein, a 33 kDa A and a 22 kDa C protein (75-77) (Table 6).

Figure 3: Schematic diagram delineating the number and order of assembly of the intermediates in spliceosome formation.
The pre-mRNA substrate contains two exons (E1 and E2), a 5'-splicing site (5'SS), a 3' splicing site (3'SS), and a branch point (BP). The association of pre-mRNA with hnRNP leads to the formation of the ATP-independent H complex. In the presence of ATP, addition of U1 and U2 snRNPs results in complex A. The addition of a preassembled U4, U5, U6 snRNP complex to complex A results in complex B formation. An ATP-dependent reaction converts complex B to the spliceosome (complex C). After the product mRNA (E1 and E2 joined) is released, the lariat form of the intron is still associated with the snRNPs in a complex. While the snRNPs are recycled, the lariat intron is degraded. This scheme was modified from reference 65.

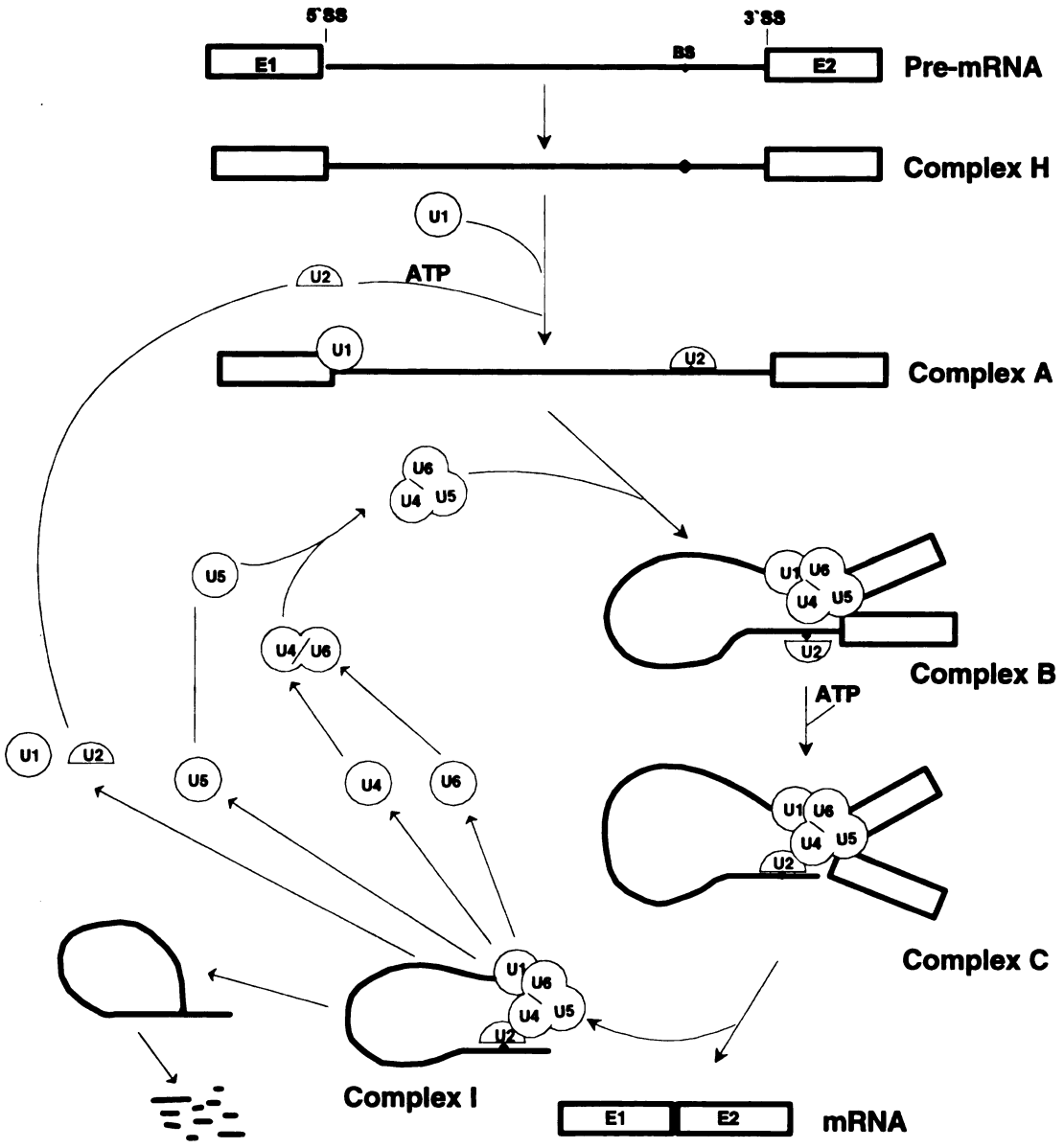

5'SS
3'SS
BS
E1
E2
Pre-mRNA
Complex H
U1
U2
ATP
Complex A
U6
U4
U5
Complex B
ATP
Complex C
Complex I
mRNA

Table 5. Major mammalian snRNP

snRNP	Protein Composition	snRNP Nuclear Localization	Antibodies to the snRNP
U1	Core Polypeptides U1 70K A C	Nucleoplasm	Anti-Sm Anti-m^3G Anti-(U1)RNP
U2	Core Polypeptides A' B"	Nucleoplasm	Anti-Sm Anti-m^3G Anti-(U2)RNP
U3	? 34 K	Nucleolus	Anti-(U3)RNP Anti-m^3G
U4/U6	Core Polypeptides	Nucleoplasm	Anti-Sm Anti-m^3G
U5	Core Polypeptides 100 K	Nucleoplasm	Anti-Sm Anti-m^3G

Table 6. Proteins of Mammalian snRNPs

Protein	M_r (SDS-PAGE)	Direct binding to snRNA	Antibodies	snRNP
Shared Core polypeptides				
B'	29,000	-	Anti-Sm	U1, U2, U4/U6, U5
B	28,000	-	Anti-Sm	U1, U2, U4/U6, U5
D'	18,000	-	Anti-Sm	U1, U2, U4/U6, U5
D	16,000	-	Anti-Sm	U1, U2, U4/U6, U5
E	13,000	Yes	-	U1, U2, U4/U6, U5
F	12,000	Yes	-	U1, U2, U4/U6, U5
G	11,000	Yes	-	U1, U2, U4/U6, U5
snRNP Specific Proteins				
U1 70K	70,000	Yes	Anti-(U1)RNP	U1
A	33,000	Yes	Anti-(U1)RNP	U1
C	22,000	-	Anti-(U1)RNP	U1
A'	32,000	Yes	Anti-(U2)RNP	U2
B"	28,000	Yes	Anti-(U2)RNP	U2
U3 34K	34,000	?	Anti-(U3)RNP	U3
U5 100K	100,000	?	-	U5

With the exception of U6 and the nucleolar snRNP U3, the major snRNPs share a common core of polypeptides, which include B (28 kDa), D' (18 kDa), D (16 kDa), E (13 kDa), F (12 kDa) and G (11 kDa) proteins (Tables 5 and 6). Some of these polypeptides recognize a conserved sequence motif on snRNAs called the Sm binding site (Table 4) and are assembled to form a $B_2D'_2D_2EFG$-containing snRNP particles (78, 79). In human cells, one B protein is replaced by a B' protein (80), but the B' protein is not found in non-primate mammals. Since U6 snRNA is base-paired with U4 snRNA, it could be isolated by antibodies against the core proteins, though it has no Sm binding site (71). At high ionic strength, only the D, E, F and G polypeptides remain associated with the snRNA. These polypeptides are tightly associated and some of the proteins bind to RNA to protect the Sm consensus sequences from RNase digestion (81). A similar RNase protection pattern was also found with particles containing the B protein. These observations suggest that B protein is associated with the snRNP particle through contact with other polypeptides, not by direct binding to snRNA. Consistent with this notion, biochemical studies of the assembly of snRNP particles have demonstrated that D, E, F and G proteins initially assemble to form a 6S particle; the 6S intermediate binds to snRNA followed by the association of two copies of B protein or one copy each of B and B' proteins (78, 79, 82). Binding of the core proteins to snRNA is directed by a sequence motif of AUnG (n = 2 - 6) (Table 4). This sequence motif is found in the single-stranded region of the 3' half of all the anti-Sm precipitable snRNAs U1, U2, U4, and U5 (81). Insertion of this sequence into a single-stranded heterologous RNA has been shown to

induce the assembly of the snRNP core proteins suggesting that this sequence is necessary and sufficient for core protein binding (81).

Besides the core polypeptides, U1 snRNP also contains the U1-specific 70 kDa protein, the 33 kDa A protein, and the 22 kDa C protein (Tables 5 and 6) (83-85). Sequence elements in the stem-loop structure at the 5'-end of U1 RNA are essential for binding of U1-specific proteins, U1 70 kDa and 33 kDa A (86-88). Purification of U2 snRNP has identified two unique proteins, a 32 kDa methionine-poor A' protein and a 28 kDa B" protein (84, 85). The U2-specific B" protein has been found to be homologous to the U1-specific A protein (89, 90). Both A' and B" proteins have been found to associate with the 3'-end stem-loop region of U2 RNA (91). Although the shared core proteins remain associated with the snRNA under rigorous isolation conditions, the association of the snRNP-specific proteins is sensitive to ionic strength during isolation. For example, there is no specific protein other than core proteins that could be isolated with the U4/U6 and U5 snRNPs from HeLa cell extracts prepared in 0.5 M NaCl (85). This suggested that the polypeptide composition of snRNP identified may depend on the isolation condition. Protein cross-linking experiments have shown that B and D proteins are directly associated with E, F and G core proteins but not with each other, and the U1 snRNP-specific U1 70 kDa and 33 kDa A polypeptides can be cross-linked to each other, but not to the core proteins (92, 93). These observations suggested that the U1 snRNP-specific proteins and the core proteins occupy different domains on U1 snRNA.

B) Nuclear Localization of the snRNP

Immunofluorescence localization studies using human autoantibodies (anti-Sm and anti-U1RNP) have shown that the major snRNPs are localized in the nucleoplasm, while U3 is confined to the nucleolus (94, 95). The snRNPs appear to be concentrated in 20-50 nuclear speckles with a diffuse distribution throughout the nucleoplasm, except for nucleoli (96). The diffuse nuclear staining may represent an excess population of snRNPs, or those snRNPs in transit to or from nascent transcripts, or snRNPs in transit to speckles from a cytoplasmic assembly site, and possibly those Sm antigens dissociated from snRNPs. Non-snRNP splicing factors such as SC-35 also have been localized to nuclear speckled structures (97). However, a splicing factor does not necessarily have to be localized to the speckled structure. The protein U2AF (U2 snRNP auxiliary factor) which facilitates U2 snRNP binding to the intron branch site was shown to be distributed throughout the nucleoplasm (98, 99). Fluorescence *in situ* hybridization and immuno-electron microscopy have revealed a close association between the newly transcribed *c-fos* RNA and the nuclear speckled structures (100). This result suggested that the nuclear speckled structures observed by immunofluorescence staining for certain snRNPs and non-snRNP splicing factors are indeed the sites for pre-mRNA splicing.

Immunoelectron microscopy studies of the distribution of splicing factor SC35 have shown that the speckled staining pattern corresponds to nuclear structures enriched in interchromatin granules and perichromatin fibrils (101, 102). The interchromatin granules with a diameter of 20-25 nm are linked together by thin fibrils (103). It has been shown that after inhibition of RNA polymerase II transcription, a stable population of

poly(A)$^+$RNA reorganizes into fewer large interchromatin granule clusters. Along with the reorganization of interchromatin granules, splicing factor SC35 could also be found in these regions (104). Perichromatin fibrils with a diameter of 3-5 nm are found at the peripheral regions of condensed chromatin and dispersed throughout the interchromatin spaces including on the surface of interchromatin granule clusters (103). In contrast to interchromatin granules, perichromatin fibrils could be rapidly labeled with [^{3}H]uridine, suggesting that these fibrils correspond to the structure containing newly transcribed RNA (103). Various components involved in splicing, snRNPs, hnRNP antigens and a non-snRNP splicing factor SC-35, have all been localized to these fibrils (103).

Detergent extraction of cells followed by fluorescence *in situ* hybridization studies have shown that nuclear poly(A)$^+$RNAs are concentrated primarily within several discrete transcript domains which often surround nucleoli in interphase nuclei. Moreover, poly(A)$^+$RNA localization in the cell nucleus is coincident with the Sm containing snRNP (105). Detergent extraction of cells has previously been shown to retain the nuclear matrix and most of the matrix-associated hnRNP (106). Comparison of nuclei with and without detergent extraction demonstrated that specific nuclear RNAs are unambiguously retained upon nuclear matrix preparation (107). Taken together, these results indicate that upon detergent extraction, these RNP molecules remain in the nucleus and suggest that poly(A)$^+$RNA and snRNP may associate tightly with subnuclear structures, and possibly, interchromatin granules and perichromatin fibrils may correspond to these subnuclear structures. These results provide support that these subnuclear structures are involved in pre-mRNA splicing.

Previous studies on the distribution of snRNPs have demonstrated that during mitosis, along with the breakdown of the nuclear envelope, the speckled structures observed in the interphase nucleus disappeared and the snRNPs are distributed diffusely throughout the cell except the region for condensed chromatin (108, 109). Immunofluorescence and immunoprecipitation analyses showed that during mitosis the snRNP particles still retain the structure and protein composition of the particles seen during interphase (108). The snRNP particles begin returning to the daughter nuclei after the chromatin begins decondensing in telophase, and they are returned quantitatively to the daughter nuclei during early G1 phase, at which time the speckled structures are reformed.

C) Cytoplasmic Assembly and Nuclear Import of the snRNP

The snRNAs are transcribed as precursors, with several nucleotides longer than the mature nuclear snRNA. These extra nucleotides of newly transcribed snRNA are removed and a 2,2,7-trimethyl-guanosine cap (m^3G cap) is added to the snRNA during maturation and snRNP assembly in the cytoplasm (110). The U6 snRNA is transcribed by polymerase III and is capped at the 5' end by a methyl group (Table 4) (111). The cap structure provides resistance to 5' exoribonuclease activity (112).

Kinetic studies of assembly of snRNP have shown that newly transcribed snRNA appears transiently in the cytoplasm. Analysis of cytoplasm generated from mouse fibroblasts by sucrose gradient centrifugation followed by immunoprecipitation with anti-Sm antibodies has identified a 6S snRNA-free protein intermediate which consists of the

D protein and the E, F, and G proteins. This 6S protein intermediate assembles with free snRNA and then with two copies of the B protein to form the snRNP particle. The mature snRNP is then transported back into the nucleus (Figure 4) (79, 80, 82). In contrast to the core polypeptides, some of the U1 and U2 specific proteins are restricted to the nucleus (Figure 4).

The binding of core proteins to the snRNA appears necessary for transport of the snRNA into the nucleus. Mutation of the U2 snRNA Sm consensus sequences, preventing the core protein assembly with snRNA, resulted in the accumulation of the snRNA in the cytoplasm; however, removal of sequence responsible for binding of U2 specific protein does not prevent snRNA core protein assembly or nuclear localization of U2 snRNP. These results suggest that the snRNP nuclear localization signal may be generated by the association of core proteins and snRNA. However, nuclear localization of snRNP may also be affected by the snRNA structure. Both mutant U2 snRNA with 3' extension sequences and mature U2 snRNA could be immunoprecipitated by anti-Sm antibodies; however, mutant snRNA with 3'-end extension could only be found in the cytoplasm (113). Analysis of the 3'-end extension sequence has predicted an interaction between the 3' extension and the 5'-end stem-loop structure, but this does not prevent binding of core proteins. Similar results have also been obtained for mutant U1 snRNA carrying 3'-end extension sequence (114). These results indicated that core protein binding alone is not the only factor involved in snRNP nuclear import. Proper snRNA structure is also required for snRNP transport into the nucleus.

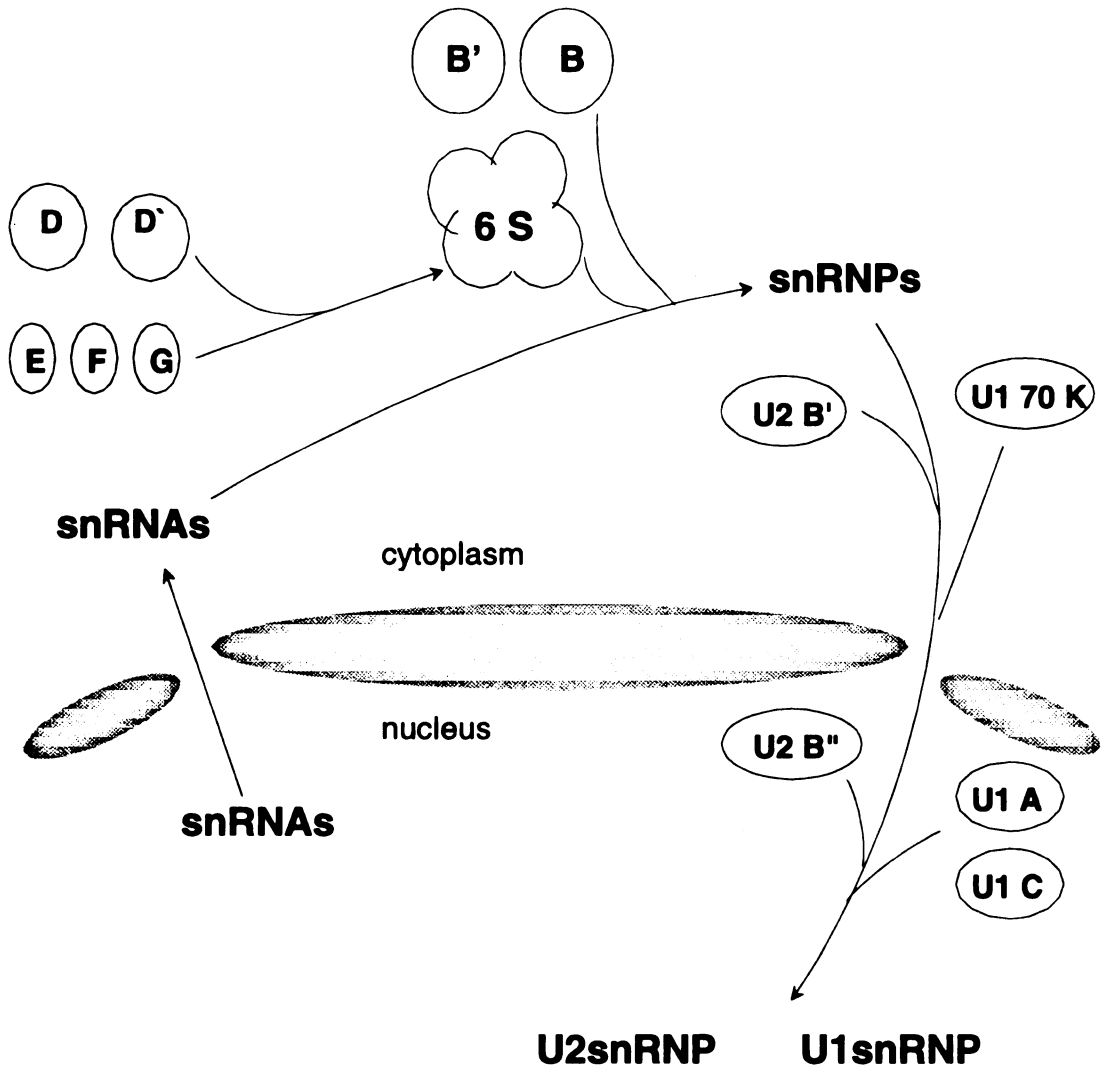


Figure 4: Schematic diagram illustrating the nuclearcytoplasmic shuttle in the assembly of the RNA and polypeptide components of U1 and U2 snRNPs.

The snRNAs transcribed in the nucleus are exported to the cytoplasm, where the core polypeptides B, D, and E-G are added to form snRNPs. Certain U1-specific and U2-specific polypeptides are also assembled on the respective RNPs. These are then translocated into the nucleus, where addition U-specific and U2-specific polypeptides are added. This scheme are modified from reference 79.

D) Non-snRNP Splicing Factors

A number of proteins essential for splicing activity are not associated with snRNPs. These proteins contain either a serine/arginine-rich (SR) domain or a RNA recognition motif (RRM). In mammalian cells, splicing factors SC-35 (115), ASF/SF2 (116), U2AF (117) and U1 70 kDa polypeptide (116) contain the RRM and a SR-rich domain (Figure 5). In *Drosophila*, the products of the *transformer (tra)* and *transformer 2 (tra2)* genes (118), which regulate *doublesex (dsx)* pre-mRNA splicing, also contain the SR-rich domain (119). It has been shown that *tra, tra2*, and members of the SR family are sufficient to commit *dsx* pre-mRNA to female-specific splicing, though individual SR proteins differ significantly in their ability to participate in commitment of splicing complex formation. More importantly, the localization signal for nuclear speckled structures may reside in the SR-rich domain (119). A monoclonal antibody which labels mammalian cells in a speckled pattern (120) has been used to identify a family of nuclear phosphoproteins that contain an SR-rich carboxyl terminal domain (121). SDS-PAGE showed that this SR protein family consists of at least five different proteins (20, 30, 40, 55, and 75 kDa). Except for the 20 kDa polypeptide, a repeated sequence that encompasses an RRM is found in all four SR proteins, and each of the four SR proteins individually can complement a splicing-deficient cytoplasmic S-100 extract. The 30 kDa band contains two distinct proteins, SRp30a and SRp30b which have been shown to be ASF/SF2 and SC35, respectively (122). Each individual SR protein initiated splicing of a different pre-mRNA with substrate specificity (123). These observations suggest that

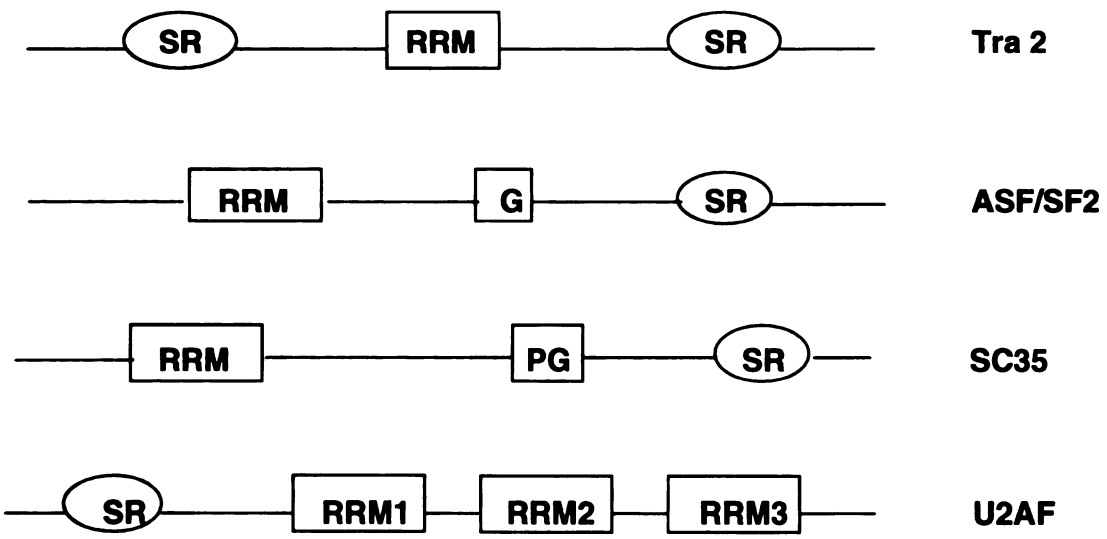


Figure 5: Schematic diagram summarizing the context and organization of specific domains in SR family of proteins.
The RRM box indicates the RNA recognition domain. The relative location of the serine/glycine rich domain (SR) is shown for each protein. PG and G indicate proline/glycine and glycine rich region, respectively.

specific SR proteins have distinct and essential roles in pre-mRNA splicing. One possible role is that SR proteins have different specificities for subclasses of pre-mRNAs, like *tra* and *tra2* gene products for *Drosophila* sex determination, and regulation of the levels of SR proteins in different cell types contributes to the regulation of cell-specific splice choices.

The non-snRNP splicing factor U2AF is required for the binding of U2 snRNP to the pre-mRNA intron branch site. Purified U2AF consists of two polypeptides of 35 kDa and 65 kDa. An *in vitro* splicing assay using cytoplasmic S-100 extract showed that U2AF is an essential splicing factor and all U2AF activity resides in the 65 kDa polypeptide (99). SC35 has been shown to be required for the splicing reaction, especially for the ATP-dependent complex formation (124). In the presence of ATP, SC35 is required for the interaction of U1 snRNP with both the 5' and 3' splice sites along with the U2 snRNP binding to the branch site. ASF/SF2 displays two activities in an *in vitro* splicing assay. It is required for the assembly of the first detectable ATP-dependent splicing complex (116), and is able to switch utilization of alternative 5' splice sites in a concentration-dependent manner (125). The SR domain in SF2/ASF is not required for alternative site selection, but is necessary for constitutive splicing, and the RRM motif is essential for alternative splicing (126). The alternative splicing activity of ASF/SF2 can be antagonized by hnRNP A1 protein (127). Both ASF/SF2 and hnRNP A1 have strand annealing activities, which may promote base pairing of snRNAs to the alternative splice sites in the intron.

Recently, the SR proteins have been shown to be able to complement splicing activity in reactions depleted of U1 snRNP, but can not restore splicing activity to either U2 snRNP or U4/U6 snRNP-depleted reactions (128). However, the concentration of SR proteins required to complement the U1 snRNP-depleted splicing reaction is about 10-fold greater than that of the endogenous concentration, and the activity of SR proteins to complement the U1 snRNP-depleted splicing reaction is dependent on the particular pre-mRNA. This observation suggests that U1 snRNA is not essential for catalysis of either of the transesterification steps in splicing, and since SR proteins are thought to bind to RNA in a sequence-specific manner, different pre-mRNAs might have differential activity for recognition by SR proteins.

Literature Cited

1. Goldstein, I. J. 1979. Carbohydrate-Protein Interaction. American Chemical Society. ACS Symposium Series; 88.
2. Sharon, N., and Lis, H. ed. 1989. Lectins, Chapman and Hall Ltd., London
3. Hart, G. W. Haltiwanger, T. S., Holt, G. D., and Kelly, W. G. 1989. Ann. Rev. Biochem. 58, 841-874.
4. Drickamer, K. 1988. J. Biol. Chem. 263, 9557-9560.
5. Drickamer, K., and Taylor, M. E. 1993. Annu. Rev. Cell Biol. 9, 237-264.
6. Hirabayashi, J. 1993. Trend in Glycosci. Glycotech. 5, 251-270.
7. Barondes, S. H., Castronovo, V., Cooper, D. N. W., Cummings, R. D., Drickamer, K., Feizi, T., Gitt, M. A., Hirabayashi, J., Hughes, R. C., Kasai, K., Leffler, H., Liu, F. -T., Lotan, R., Mercurio, A. M., Monsigny, M., Pillai, S., Poiries, F., Raz, A., Rigby, P., Rini, J. M., and Wang, J. L. 1994. Cell 76, 597-598.
8. Hirabayash, J., and Kasai, K. 1993 Glycobiolgy 3, 297-304.
9. Gitt, M. A., Massa, S., Leffler, H., and Barondes, S. H. 1992. J. Biol. Chem. 267, 10601-10606.
10. Roff, C. F., Rosevear, P. R., Wang, J. L., and Barker, T. 1983. Biochem. J. 211, 625-629.
11. Roff, C. F., and Wang, J. L. 1983. J. Biol. Chem. 258, 10657-10663.
12. Oda, Y., Herrmann, J., Gitt, M. A., Turck, C. W., Burlingame, A. L., Barondes, S. H., and Leffler, H. 1993. J. Biol. Chem. 268, 5929-5939.

13. Hirabayashi, J., Satoh, M., and Kasai, K. 1992. J. Biol. Chem. 267, 15485-15490.

14. Gitt, M. A., Wiser, M. F., Leffler, H., Herrmann, J. Xia, Y. R., Massa, S. M., Cooper, D. N. W., Lusis, A. J., and Barondes, S. H. 1995. J. Biol. Chem. 270, 5032-5038.

15. Madsen, P., Rasmussen, H. H., Flint, T., Gromov, P., Kruse, T. A., Honore, B., Vorum, H., and Celis, J. E. 1995. J. Biol. Chem. 270, 5823-5829.

16. Hadari, Y. R., Paz, K., Dekel, R., Mestrovie, T., Accili, D., and Zick, Y. 1995. J. Biol. Chem. 270, 3447-3453.

17. Pfeifer, K., Haasermann, M., Gamulin, V., Bretting, H., Fahrenholz, F., and Muller, W. E. G. 1993. Glycobiology 3, 179-184.

18. Jia, S and Wang, J. L. 1988. J. Biol. Chem. 263, 6009-6011.

19. Agrwal, N., Sun, Q., Wang, S. Y., and Wang, J. L. 1993. J. Biol. Chem. 268, 14932-14939.

20. Cowles, E. A., Agrwal, N., Anderson, R. L., and Wang, J. L. 1990. J. Biol. Chem. 265, 17706-17712.

21. Lee, R, T., Ichikawa, Y., Allen, H. J., and Lee, Y. C. 1990. J. Biol. Chem. 265, 7864-7871.

22. Abbott, W. M., Hounsell, E. F., and Feizi, T. 1988. Biochem. J. 252, 283-287.

23. Sato, S., and Hughes, R. C. 1992. J. Biol. Chem. 267, 6983-6990.

24. Knibbs, T. N., Agrwal, N., Wang, J. L., and Goldstein, I. J. 1993. J. Biol. Chem. 268, 14940-14947.

25. Leffler, H., and Barondes, S. H. 1986. J. Biol. Chem. 261, 10119-10126.

26. Crittenden, L. L., Roff, C. F., and Wang, J. L. 1984. Mol. Cell. Biol. 4, 1252-1259.

27. Sparrow, C. P., Leffler, H., and Barondes, S. H. 1987. J. Biol. Chem. 265, 7383-7390.

28. Gitt, M, A., and Barondes, S. H. 1991. Biochemistry 30, 82-89.

29. Gritzmacher, C. A., Mehl, V. S., and Liu, F. T. 1992. Biochemistry 31, 9533-9538.

30. Rosenberg, I. M., Iyer, R., Cherayil, B., Chiodino, C., and Pillai, S. 1993. J. Biol. Chem. 268, 12393-12400.

31. Abbott, W. M., and Feizi, T. 1991, J. Biol. Chem. 266, 5552-5557.

32. Hirabayashi, J., and Kasai, K. 1991. J. Boil. Chem. 266, 23648-23653.

33. Liao, D. I., Kapadia, G., Ahmed, H., Vasta, G. R., and Herzberg, O. 1994. Proc. Natl. Acad. Sci. USA 91, 1428-1432.

34. Boune, Y., Bolgiano, B., Liao, D. L., Stecker, G., Cantau, P. Herzberg, O., Feizi, T., and Cambillau, C. 1994. Str. Biol. 1, 863-870.

35. Lobsanov, Y. D., Gitt, M. A., Leffler, H., Barondes, S. H., and Rini, J. M. 1993. J. Biol. Chem. 268, 27034-27038.

36. Beyer, E. C., and Barondes, S. H. 1980. J. Supramol. Struct. 13, 219-227.

37. Childs, T. A., and Feizi, T. 1980. Cell Biol. Int. Rep. 4, 775.

38. Hynes, M. A., Gitt, M., Barondes, S. H., Jessell, T. M., and Buck, L. B. 1990. J. Neurosci. 10, 1004-1013.

39. Akimoto, Y, Kawakami, H., Oda, Y., Obinata, A., Endo, H., Kasai, K., and Hirano, H. 1992. Exp. Cell Res. 199, 293-304.

40. Cho, M., and Cummings, R. D. 1995. J. Biol. Chem. 270, 5207-5212.

41. Cooper, K. N. W., and Barondes, S. H. 1990. J. Cell Biol. 110, 1681-1691.

42. Harrison, F. L., and Wilson, T. J. G. 1992. J. Cell Sci. 101, 635-646.

43. Cooper, K. N. W., Massa, S. M., and Barondes, S. H. 1991. J. Biol. Chem. 115, 1437-1448.

44. Gu, M. J., Wang, W. W., Song, W. K., Cooper, D. N. W., and Kaufman, S. J. 1994. J. Cell Sci. 107, 175-181.

45. Bols, N. C., Roberson, M., Haywood-Reid, P. L., Cerra, R. F., and Barondes, S. H. 1986. J. Cell Biol. 102, 492-499.

46. Moutsatsos, I. K., Davis, J. M., and Wang, J. L. 1986. J. Cell Biol. 102, 447-483.

47. Gritzmacher, C. A., Robertson, M. W., and Liu, F. T. 1988 J. Immunol. 141, 2801-2806.

48. Regan, L. J., Dodd, J., Barondes, S. H., and Jessell, T. M. 1986. Proc. Natl. Acad. Sci. U. S. A. 83, 2248-2252.

49. Raz, A., and Lotan, R. 1987. Cancer Metastasis Rev. 6, 433-452.

50. Cherayil, B. J., Weiner, S. J., and Pillai. S. 1989. J. Exp. Med. 170, 1959-1972.

51. Sato, S., Burdett, I., and Hughes, R. C. 1993. Exp. Cell Res. 207, 8-18.

52. Lindstedt, R., Apodaca, G., Barondes, S. H., Mostov, K. E., and Leffler, H. 1992. J. Biol. Chem. 268, 11750-11757.

53. Moutsatsos, I. K., Wade, M., Schindler, M., and Wang. J. L. 1987. Proc. Natl. Acad. Sci. USA 84, 6452-6456.

54. Lotz, M. M., Andrews, C. W., Jr., Korzelius, C. A., Lee, E. C., Steele, G. D., Jr.,Clarke, A., and Mercurio, A. M. 1993. Proc. Natl. Acad. Sci. USA. 90, 3466-3470.

55. Zalike, S. E., Thomson, L. W., and Ledsham, I. M. 1987. J. Cell Sci. 88, 483-493.

56. Catt, J. W., and Harrison, F. L. 1985. J. Cell Sci. 73, 347-359.

57. Zhou, Q., and Cummings, R. D. 1993. Arch. Biochem. Biophys. 300, 6-17.

58. Yamaoka, L., Ohno, S., Kawasaki, H., and Suzuki, K. 1991. Biochem. Biophys. Res. Commun. 179, 272-279.

59. Wells, V., and Mallucci, L. 1991. Cell 64, 91-97.

60. Woo, H. J., Lotz, M. M., Jung, J. U, and Mercurio, A. M. 1991, J. Biol Chem. 266, 18419-18422.

61. Frigeri, L. G., Zuberi, T. I., and Liu, F. T. 1993. Biochemistry 32, 7644-7649.

62. Rosenberg, I., Cherayil, B. J., Isselbacher, K. J., and Pilai, S. 1991. J. Biol. Chem. 266, 18731-18736.

63. Laing, J. G., and Wang. J. L. 1988. Biochemistry 27, 5329-5334.

64. Dagher, S. F., Wang, J. L., and Patterson, R. J. 1995. Proc. Natl. Acad. Sci. USA 92, 1213-1217.

Sharp, P. A. 1994. Cell 77, 805-815.

Guthrie, C., and Patterson, B. 1988. Ann. Rev. Genet. 22, 387-419.

67. Kunkel, G. R., Maser, T. L., Calvet, J. P., and Pederson, T. 1986. Proc. Natl. Acad. Sci. USA 83, 8575-8579.

68. Moore, M. J., and Sharp, P. A. 1993. Nature 365, 364-368.

69. Lerner, M. R., Boyle, J. A., Mount, S. M., Wolin, S. L., and Steitz, J. A. 1980. Nature 283, 220-224.

70. Parker, R., Sillciano, P. G., and Guthrie, C. 1987. Cell 49, 229-239.

71. Hashimoto, C., and Steitz, J. A., 1984 Nucl. Acids Res. 12, 3283-3293.

72. Konarska, M. M., and Sharp, P. A. 1987.Cell 49, 763-774.

73. Lerner, M. R., and Steitz, J. A. 1979. Proc. Natl. Acad. Sci. USA 76, 5495-5499.

74. Williams, D, G. Stocks, M. R., Smith, P. R., and Maini, R. N. 1986. Immunol. 58, 495-500

75. White, P. J., and Hoch, S. O. 1981. Biochem. Biophys. Res. commun. 102, 365-371.

76. White, P. J., Billings, P. B., and Hoch, S. O. 1982. J. Immunol. 128, 2751-2756.

77. Pettersson, I., Hinterbergwer, M., Mimori, T., Gottlieb, E., and Steitz, J. A. 1984. J. Biol. Chem. 259, 5907-5914.

78. Feeney, R. J., Sauterer, R, A., Feeney, J. L., and Zieve, G. W. 1989. J. Biol. Chem. 264, 5776-5783.

Fisher, D. E., Conner, G. E., Reeves, N. H., Wisniewolski, R., and Blobel, G. 1985. Cell 42, 751-758.

Rokeach, L. A., Jannatipour, M., Haselby, J. A., and Hoch, S. O. 1989. J. Biol. Chem. 264, 5024-5030.

81. Liautard, J. P., Sri-Widada, J., Brunel, C., and Jeanteur, P., 1982. J. Mol. Biol. 162, 623-643.

82. Sauterer, R. A., Goyal, A., and Zieve, G. W.1990. J. Biol. Chem. 265, 1048-1058.

83. Hinterberger, M., Pettersson, I., and Steitz, J. A. 1983 J. Biol. Chem. 258, 2604-2613.

84. Kinlaw, C. S., Roberson, B. L., and Berget, S. M. 1983. 258, 7181-7189.

85. Bringmann, P., and Luhrmann, R. 1986. EMBO J. 5, 3509-3516.

86. Hamm, K., van Santen, R. A., Spritz, R. A., and Mattaj, I. W. 1988. Mol. Cell. Biol. 8, 4781-4791.

87. Patton, J. R., and Pederson, T., 1988. Proc. Natl. Acad. Sci. USA 85, 747-751.

88. Patton, J. R., Habets, W., van Venrooij, W., and Pederson, T. 1989. Mol. Cell. Biol. 8, 3360-3368.

89. Habet, W. J., Sillekens, P. T. G., Hoet, M. H., Schalken, J. A., Boebroek, A. J., Leunissen, J. A., van de Ven, W. J. M., and van Venrooij, W. I. 1987. Proc. Natl. Acad. Sci. USA 84, 2421-2425.

90. Sillekens, P. T., Habets, W. J., Beijer, R. P., and van Venrooij, W. I. 1987. EMBO J. 6, 3841-3848.

91. Mattaj, I. W., and De Robertis, M. 1985. Cell 40, 111-118.

92. Harris, S. G., Hoch, S. O., and Smith, H. C. 1988. Biochemistry 27, 4595-4600.

93. Luhrmann, R. snRNP Proteins. 1988. in Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles. Birnstiel, M. L. ed. Springer-Verlag, New York.

94. Nyman, U., Halman, H., Hadlaczly, G., Petterson, I., Sharp, G., and Ringertz, N. R. 1986. J. Cell Biol. 102, 137-144.

95. Spector, D. L., 1984. Biol. Cell. 51, 109-112.

96. Spector, D.L. 1990. Proc. Natl. Acad. Sci. USA 87, 147-151.

97. Fu, X. D., Maniatis, T. 1990. Nature 343, 437-441,

98. Carmo-Fonseca, M., Tollervey, D., Pepperkok, R., Barabinok S. M. L., Merdes, A., Brunner, C., Zamore, P. D., Green, M. R., Hurt, E., and Lamond, A. I. 1991.EMBO J. 10, 195-206,

99. Zamore, P. D., and Green, M. R. 1991. EMBO J. 10, 207-214,

100. Huang, S., and Spector, D. L. 1991. Genes Develop. 5, 2288-2302,

101. Spector,D. L., Fu, X. D., and Maniatis, T. EMBO J. 1991. 10, 3467-3481,

102. Carmo-Fonseca, M., Pepperkok, T., Carvalho, M. T., and Lamond, A. I. 1992 J. Cell Biol. 117, 1-14,

103. Spector, D. L. 1993. Ann. Rev. Cell Biol. 9, 265-313,

104. Huang, S., Deerinck, T. J., Ellisman, M. H., and Spector S. L. 1994. J. Cell Biol. 126, 877-899,

105. Carter, K. C., Taneja, K. L., and Lawrence, J. B. 1991. J. Cell Biol. 115, 1191-1202.

106. Fey, E. G., Krochmalnic, G., and Penman S. 1986. J. Cell Biol. 102, 1654-1665.

107. Xing, Y., and Lawrence, J. B. 1991. J. Cell Biol. 112, 1055-1063.

108. Reuter, R., Appel, B., Rinke, J., and Luhrmann, R. 1985. Exp. Cell Res. 159, 63-79.

109. Spector, D. L., and Smith, H. C. 1986. Exp. Cell Res. 163, 87-94.

110. Mattaj, I. W. 1986. Cell 46, 905-911.

111. Singh, R., and Reddy, T., 1989. Proc. Natl. Acad. Sci. USA 86, 8280-8283.

112. Murthy, K. G. K., Park, P., and Manley, J. L. 1991. Nucl. Acids. Res. 19, 2685-2692.

113. Konings, D. A. M. and Mattaj, I. W. 1987. Exp. Cell Res. 172, 329-339.

114. Neuman De Vegvar, H. E., and Dahlberg, J. E. 1990. Mol. Cell. Biol. 10, 3365-3375.

115. Fu, X. D., and Maniatis, T. 1992. Science 256, 535-538.

116. Krainer, A. R., Mayeda, A., Kozak, K., and Binns, G. 1991. Cell 66, 383-394.

117. Zamore, P. D., Patton, J. G., and Green, M. R. 1992. Nature 355, 609-614.

118. Tian M., and Maniatis, T. 1993. Cell 74, 105-114.

119. Li, H., and Bingham, P. M. 1991. Cell 67, 335-342.

120. Roth, M. B., Murphy, G., and Gall, J. G. 1990. J. Cell Biol. 111, 2217-2223.

121. Zahler, A. M., Lane, W. S., Stolk, J. A., and Roth, M. B. 1992 Genes Develop. 6, 837-847.

122. Zahler, A. M., Neugebauer, K. M., Lane, W. S., and Roth M. B. 1993. Science 260, 219-222.

123. Fu, X. D. 1993. Nature 365, 82-85.

124. Fu, X. D., and Maniatis, T. 1992. Proc. Natl. Acad. Sci. USA 89, 1725-1729.

125. Zuo, P., and Manley, J. L. 1993. EMBO J. 12, 4727-4737.

126. Caceres, J. F., and Krainer, A. R. 1993. EMBO J. 12, 4715-4726.

127. Mayeda, A., and Krainer, A. R. 1992. Cell 68, 365-375.

128. Crispino, J. D., Blencowe, B. J., and Sharp, P. A. 1994. Science 265, 1866-1869.

CHAPTER II

Similarities in the Nuclear Matrix Localization of Galectin-3 and Small Nuclear Ribonucleoproteins (snRNP): Evidence from a Comparative Immunofluorescence Analysis

SUMMARY

Galectin-3 is a galactose/lactose-specific lectin identified as a required factor in pre-mRNA splicing assayed in a cell-free system. In the present study, a comparative analysis of the immunofluorescence staining patterns was carried out using an antiserum specific for galectin-3 and human autoimmune sera reactive against the Sm antigens of small nuclear ribonucleoproteins (snRNP). Using mouse 3T3 fibroblasts, fixed with paraformaldehyde and permeabilized with Triton X-100, both anti-galectin-3 and anti-Sm yielded intense staining covering the entire nucleus, with the exception of ~5 circles devoid of fluorescence. These "black holes" most probably represent the nucleoli within the nucleus. Permeabilization without fixation, followed by extraction with 0.25 M ammonium sulfate prior to fixation and staining yielded speckled patterns for both anti-galectin-3 and anti-Sm. Thus, differences in description of staining patterns, such as diffuse versus speckled, reflect, for the most part, quantitative differences in either the antigen, the antibody or both rather than intrinsic differences in the localization of the two antigens. The nuclear staining was lost for both anti-galectin-3 and anti-Sm when permeabilized 3T3 cells were treated with ribonuclease A while parallel treatment with deoxyribonuclease I failed to yield the same effect.

During the mitotic phase of the cell cycle, when chromosomal condensation and nuclear envelope breakdown begins, galectin-3 can be found predominantly around the area of the nucleus but a band along the axis of the chromosomes is totally devoid of galectin-3. Thus, it appears that galectin-3 is found throughout the cell during mitosis except where

chromosomal DNA is located. Essentially the same results are obtained with anti-Sm staining. These immunofluorescence data suggest that both galectin-3 and the snRNPs are colocalized on the RNP-containing nuclear matrix. This notion is supported by double immunofluorescence staining, which indicate that there is a direct one-to-one correspondence between the speckled patterns obtained with anti-galectin-3 and with anti-Sm.

INTRODUCTION

Galectin-3 is a galactose/lactose-specific carbohydrate-binding protein found in both the cytoplasm and nucleus of a wide variety of cells (1). The amino acid sequence of the polypeptide, deduced from the nucleotide sequence of a cDNA clone (2), exhibited structural features, such as tandem repeats of a sequence motif rich in proline and glycine residues, similar to proteins found in the core polypeptides of heterogeneous nuclear ribonucleoprotein complex (hnRNP) and in spliceosomes (3, 4). Using a cell-free assay for pre-mRNA splicing, it was recently demonstrated that galectin-3 constituted a required factor in spliceosome formation and mRNA processing (5).

The subcellular localization of several splicing factors has been studied by immunocytochemistry and by *in situ* hybridization. Immunofluorescence studies using antibodies specific for the small nuclear ribonucleoproteins (snRNP) and non-snRNP splicing factors, such as SC35, have shown that these components are distributed in a speckled pattern in interphase nuclei (see reference 6 for review). *In situ* hybridization with oligonucleotide probes complementary to the major spliceosomal snRNAs resulted in a similar observation (7, 8).

The demonstration of a role for galectin-3 in cell-free pre-mRNA splicing called to question the subnuclear distribution of the protein, relative to the other known components of RNA splicing. Our initial immunofluorescence studies on galectin-3 showed intense staining of the nucleus, covering the entire organelle, and some staining of the cytoplasm (9). Subsequent studies have found varied labeling patterns, ranging from diffuse

distribution of fluorescence to distinct punctate staining (10, 11). Thus, the present study was undertaken with three main goals in mind. First, we wanted to clarify the issue of the apparent discrepancy in the labeling patterns of splicing factors by a direct comparison between the immunofluorescence yielded by anti-galectin-3 versus an autoimmune antibody reactive against polypeptides of the snRNPs. We found that qualitative differences in the description of the labeling patterns (diffuse versus speckled) may reflect quantitative differences in the antigen, antibody or both. Second, although we had previously reported the sensitivity of the nuclear staining of anti-galectin-3 to ribonuclease treatment (11), implicating anchorage of the protein to a RNA-containing matrix, no direct comparison with a known splicing factor was available. We now report the results of such a study. Finally, we wished to follow the subcellular localization of galectin-3, along with a known splicing factor, through the entire cell cycle. In the present study, we found that both galectin-3 and snRNPs were excluded from the segregating chromosomes during mitosis. Combined with the biochemical data to be reported in Chapter III, the various lines of evidence strongly suggest that galectin-3 is associated with the storage/assembly sites of splicing factors.

MATERIALS AND METHODS

Cell Culture. Mouse 3T3 fibroblasts were obtained from American Type Culture Collection (Rockville, MD). The cells were grown as monolayers in Dulbecco modified Eagle's Medium containing 10% calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 10% CO_2. Cells grown at a density less than 5 x 10^4 cells/cm^2 were proliferative (sparse culture); above this density, the cells were confluent. In some experiments, cells at low density were arrested by removal of serum and maintenance in medium containing 0.2% calf serum for 48 hours (quiescent cells). Upon readdition of serum (10%), the cells were reactivated (10).

Antibodies. In the present study, antiserum against galectin-3 was derived from rabbit #33. This Flemish Giant rabbit was immunized with recombinant galectin-3, purified from an *E. coli* expression system. The details for the production of recombinant galectin-3, the generation of the antisera, and the characterization of its specificity have been previously reported (12). Antiserum directed against RAP30 (13) was a gift of Dr. Zachary Burton (Michigan State University). Human autoimmune serum reactive with the Sm antigens of snRNPs (ENA anti-Sm) was purchased from The Binding Site (San Diego, CA).

Immunofluorescence Microscopy. Mouse 3T3 fibroblasts were seeded onto coverslips (22 x 22 cm), which were then placed in 6 -well (8 cm^2/well) cluster dishes. The cells were washed twice (4 ml/well) with phosphate-buffered saline (PBS, 140 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4). Cells were fixed by incubation (20 minutes at room temperature) in 4% paraformaldehyde in PBS (2 ml/well),

followed by washing twice with PBS (10 minutes each, on a rotating platform). The residual aldehyde groups were blocked by 0.1 M glycine in PBS (4 ml/well; 20 minutes at room temperature). After removal of the glycine-PBS solution, cells were permeabilized with 0.5% Triton X-100 in PBS (2 ml/well; 4 minutes at room temperature). The cells were again washed twice with PBS. The cells were then incubated with antiserum, with appropriate dilution, for one hour at room temperature. After washing three times in T-TBS (10 mM Tris pH 7.5, 500 mM NaCl, 0.05% Tween 20) (15 minutes each), the cells were incubated with the secondary antibody, at appropriate dilution. Fluorescein-conjugated or rhodamine-conjugated goat anti-rabbit immunoglobulin (BMB, Indianapolis, IN) was used to detect rabbit anti-galectin-3 and rabbit anti-RAP30 binding and fluorescein-conjugated or rhodamine-conjugated goat anti-human immunoglobulin was used to detect human autoimmune ENA anti-Sm binding. After immunostaining, cells were counterstained for DNA with 4',6-diamidino-2-phenylindole-2-HCl (DAPI) at a concentration of 1 μg/ml (14). Finally, the coverslips were washed three times in T-TBS (15 minutes each) before mounting with Perma-Fluor on glass microscope slides.

Effects of Permeabilization, Extraction, and Nuclease Treatment. In some experiments, 3T3 cells were permeabilized (0.5% Triton X-100 in 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 10 mM 1,4-piperazinediethanesulfonic acid, pH 7.4; 10 minutes at 4 °C) without prior fixation. The permeabilized cells were washed with PBS twice (30 seconds each) and were either extracted with 0.25 M ammonium sulfate for 10 min at room temperature (15) or were digested with ribonuclease A (RNase; Sigma; 100 μg/ml) or deoxyribonuclease I (DNase; BMB; 100 U/ml) at 37 °C for 1 hour. The treated cells were

washed with PBS twice (30 seconds each), and cells were fixed by incubation (20 minutes at room temperature) in 4% paraformaldehyde in PBS (2 ml/well), followed by washing twice with PBS (10 minutes each, on a rotating platform). The residual aldehyde groups were blocked by 0.1 M glycine in PBS (4 ml/well; 20 minutes at room temperature). After removal of the glycine-PBS solution, the cells were then stained with antiserum as described above.

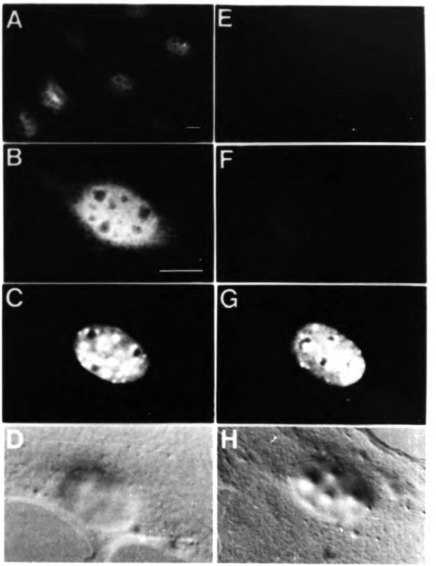
RESULTS

Immunofluorescence Analysis of Galectin-3. We had previously reported the development of polyclonal rabbit antisera against recombinant galectin-3, purified from an *E. coli* expression system (12). One antiserum (#33) and its preimmune control serum were used in experiments throughout this entire study. This antiserum immunoblots a single polypeptide (M_r 33,000) in extracts of mouse 3T3 fibroblasts. Moreover, immunoblotting analysis of purified NH_2- and COOH-terminal domains of galectin-3 indicated that the principal epitopes recognized by anti-serum #33 were localized within the NH_2-terminal half of the polypeptide (12). These results obviate any complication in the interpretation of the staining patterns, particularly in terms of the possibility that the antibody reagent might cross-react with the carbohydrate recognition domain of the members of the galectin family, which exhibit sequence homology with each other (1).

Mouse 3T3 fibroblasts, fixed with paraformaldehyde and permeabilized with Triton X-100, yielded both nuclear and cytoplasmic staining with anti-galectin-3. This was observed both in low magnification micrographs showing a field containing several cells (Figure 1A) as well as high magnification micrographs showing a single cell (Figure 1B). Parallel analysis with the corresponding preimmune serum showed negligible staining (Figure 1E and Figure 1F). In general, the staining appeared to be diffuse, covering the entire nucleus with the exception of ~5 circles devoid of fluorescence (Figure 1B). These "black holes" were also observed when the same cells are counterstained with the DNA-

Figure 1: Immunofluorescence staining of 3T3 cells after fixation with paraformaldehyde (4%) and permeabilization with Triton X-100 (0.5%).

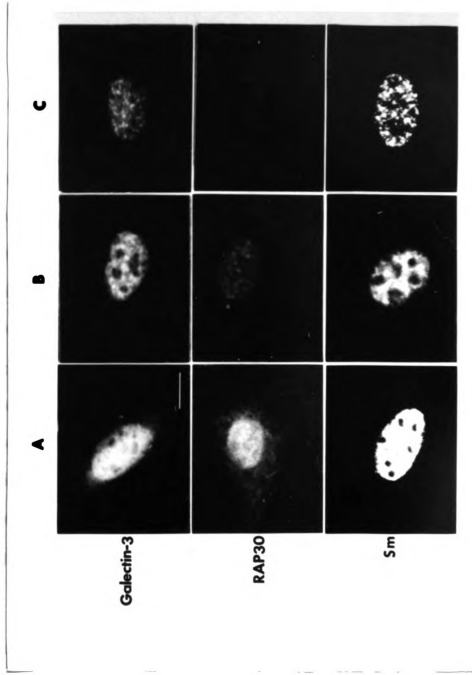
A, E: low magnification showing fields containing several cells; B-D, F-H: high magnification showing single cells. A and B, rabbit anti-galectin-3 (1:150 dilution of antiserum #33); E and F, preimmune serum from the same rabbit #33 (1:150 dilution). The binding of the primary antibody was detected by rhodamine-conjugated goat anti-rabbit immunoglobulin. The cells in B and F are shown in DAPI counterstaining and phase contrast micrographs in C, G and D, H, respectively. (Bar, 50 μm)


A
E
B
F
C
G
D
H

specific dye DAPI (Figure 1C and Figure 1G). In phase contrast micrographs of the same cells, these "black holes" correspond to phase dense structures in the nucleus (Figure 1D and Figure 1H). Moreover, the "black holes" were seen in cells treated with antibodies directed against two other nuclear proteins:(a) RAP30, a 30 kDa component of the general transcription factor, TFIIF; and (b) Sm, an epitope defined on proteins B and D of snRNPs (Figure 2, column A). On the basis of these results and comparison with previously published staining patterns of snRNPs and the non-snRNP spliceosome component SC35 (14, 16), we believe the "black holes" correspond to nucleoli. Thus, the nuclear distribution of galectin-3 appears to be diffuse throughout the nucleoplasm, excluding the nucleoli.

Immunofluorescence of Permeabilized Cells after Salt Extraction or Nuclease Digestion. When 3T3 cells were permeabilized with Triton X-100 (without fixation), then fixed and stained with antibodies against galectin-3, Sm, and RAP30, the labeling patterns (Figure 2, column B) were qualitatively different from those obtained with cells that were initially fixed prior to permeabilization (Figure 2, column A). First, cytoplasmic staining was lost from the galectin-3 and RAP30 samples. Second, there was reduction in the staining intensity in all three samples. Finally, there appeared to be a general smearing of stained structures, yielding a diffuse distribution of fluorescence and accentuating the nucleolar "black holes" (galectin-3 and Sm). The most striking labeling patterns were obtained, however, when the permeabilized cells were extracted with 0.25 M ammonium sulfate prior to fixation and staining. This procedure extracts the majority of the nonhistone nuclear proteins, leaving chromatin, nuclear matrix, and associated RNAs (15, 17). In all

Figure 2: Comparison of the immunofluorescence staining pattern of 3T3 cells.
(A) Cells were fixed with paraformaldehyde (4%) prior to Triton X-100 (0.5%) permeabilization; (B) cells were permeabilized first and then fixed; (C) cells were permeabilized, extracted with 0.25 M ammonium sulfate, and then fixed. The residue from each sequence was then incubated with rabbit anti-galectin-3 (1:150 dilution of antiserum #33), rabbit anti-RAP30 (1:150 dilution), and human autoimmune serum ENA anti-Sm (1:500 dilution). The binding of the primary antibody was detected by rhodamine-conjugated goat anti-rabbit immunoglobulin or by rhodamine-conjugated goat anti-human immunoglobulin. (Bar, 50 μm)


A
B
C
Galectin-3
RAP30
Sm

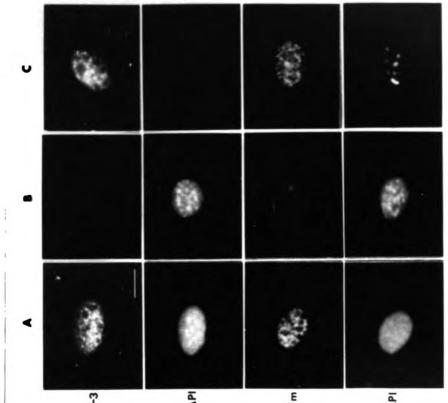
cases except RAP30 (which has lost all of its staining), distinct speckled patterns were observed (Figure 2, column C).

It should be noted that differences between the diffuse staining pattern seen in column A of Figure 2 and the speckled pattern seen in column C of the same figure (for galectin-3 and Sm) probably reflect, at least in part, quantitative differences in either the antigen, the antibody, or both. With high titer antiserum and high levels of antigen (e.g. in fixed and permeabilized cells of column A), the staining intensity is so strong that it covers the entire nucleus, obscuring even the nucleolar "black holes" (Figure 2, column A, anti-galectin-3 staining). With loss of antigen from the nuclei during the permeabilization and extraction procedures, the staining of discrete structures/regions becomes more distinct, giving rise to a speckled pattern (Figure 2, column C, anti-galectin-3 staining). Alternatively, the same staining protocol could yield, with two different antisera against the same antigen, either diffuse or speckled patterns. In this connection, we have also observed variations in the DAPI staining of DNA, ranging from a completely fluorescent nucleus, diffuse fluorescence except for nucleolar "black holes", to punctate patterns (see, for example, DAPI staining in Figure 3 below).

The speckled staining pattern observed for galectin-3 and Sm (Figure 2, column C) suggests that these antigens are associated with subnuclear structures. Therefore, the effects of RNase and DNase on the immunofluorescence patterns of Triton X-100 permeabilized cells (Figure 2, column B) were studied. RNase digestion resulted in drastic reduction in the intensity of galectin-3 staining (Figure 3, columns A and B). There appeared to be some

Figure 3: Effect of enzyme treatments on the intranuclear staining pattern for galectin-3 and the Sm antigen of snRNPs.

(A) Triton X-100 (0.5%) permeabilized cells; (B) Triton X-100 permeabilized cells treated with RNase (100 µg/ml) for 60 minutes at room temperature; (C) Triton X-100 permeabilized cells treated with DNase (100 U/ml) for 20 minutes at room temperature. The enzyme-treated cells were then fixed and incubated with rabbit anti-galectin-3 (1:150 dilution) or with ENA anti-Sm (1:500 dilution) and counterstained with DAPI. The binding of the primary antibody was detected by rhodamine-conjugated goat anti-rabbit immunoglobulin or rhodamine-conjugated goat anti-human immunoglobulin. (Bar, 50 µm)


A
B
C
Galectin-3
DAPI
Sm
DAPI

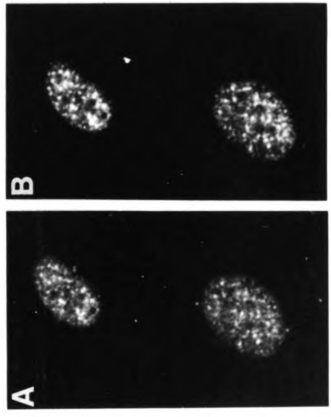
rearrangement of nuclear organization, as manifested by the DAPI staining. On the other hand, DNase digestion completely removed the DAPI fluorescence, while the galectin-3 staining was retained (Figure 3, column C). Essentially the same results were obtained with anti-Sm staining: drastic reduction of intensity in RNase-treated samples and little or no difference in staining in the DNase-treated samples. We conclude from these results that nuclear staining of galectin-3 (and Sm) was sensitive to RNase but not to DNase, implicating the association of the proteins with RNP structures.

Permeabilized 3T3 cells were extracted with 0.25 M ammonium sulfate, fixed, and then sequentially labeled with anti-Sm and anti-galectin-3. Figure 4A shows the speckled immunofluorescence pattern due to anti-galectin-3, detected with fluorescein-conjugated goat anti-rabbit immunoglobulin. Figure 4B shows the immunofluorescence pattern, over the same two cells as those shown in Figure 4A, due to anti-Sm, detected with rhodamine-conjugated goat anti-human immunoglobulin. There was a direct (one-to-one) correspondence between the two speckled patterns in each cell, suggesting the co-localization of the two polypeptides.

Immunofluorescence Analysis as a Function of the Cell Cycle. We had previously shown that the addition of serum growth factors to quiescent cultures of 3T3 fibroblasts resulted in an elevation of the expression of the galectin-3 gene. This stimulation was observed: (a) at the level of transcription rate of the gene as assayed by nuclear run-off experiments (18); (b) at the level of accumulation of the mRNA as

Figure 4: Double immunofluorescence staining for galectin-3 and Sm antigen of snRNPs in the same cells.

3T3 cells were permeabilized (0.5% Triton X-100), extracted with 0.25 M ammonium sulfate, and then fixed. The residue was incubated sequentially with human autoimmune serum ENA anti-Sm (1:500 dilution), followed by rabbit anti-galectin-3 (1:150 dilution of antiserum #33). Panel A shows the staining pattern of anti-galectin-3, revealed by fluorescein-conjugated goat anti-rabbit immunoglobulin. Panel B shows the staining pattern of anti-Sm over the identical cells, revealed by rhodamine-conjugated goat anti-human immunoglobulin.


B
A

determined on Northern blots (18); and (c) at the level of the protein as measured on immunoblots (19). At the level of single cells, this elevation of galectin-3 expression is manifested by an increase in the general level of fluorescence in both the cytoplasm and the nucleus, when assayed in fixed and permeabilized cells. Serum-starved cultures of 3T3 cells show weak labeling with anti-galectin-3 (Figure 5). Upon serum stimulation, there is intense nuclear labeling, as well as a noticeable increase in cytoplasmic fluorescence. The first wave of DNA synthesis was observed between 16 and 24 hours following serum addition to the quiescent cultures (10). Thus, the increase in the expression and nuclear localization of galectin-3, as revealed by immunofluorescence, can be observed well before the onset of the S-phase of the first cell cycle. In fact, the most intense nuclear staining was observed at 16 hours, followed by a decrease in the staining intensity and partitioning of the protein between the nuclear and cytoplasmic compartments (Figure 5). This prompted us to analyze the staining patterns of galectin-3 during the later portion of the cell cycle in some detail.

Cells from cultures between 20 and 24 hours post serum stimulation were fixed, permeabilized, and stained with anti-galectin-3 (Figure 6). The position of chromosomal DNA was simultaneously followed by DAPI staining while the presence of the nuclear membrane was observed by phase contrast microscopy. In prophase, when chromosomal condensation and nuclear envelope breakdown begins, galectin-3 can be found predominantly around the area of the nucleus but there appears to be a band along the axis of the chromosomes where galectin-3 is excluded (Figure 6A). This band, devoid of galectin-3, is accentuated in metaphase; chromosomes aligned at the metaphase plate, as

Figure 5: Immunofluorescence staining patterns of galectin-3 in 3T3 cells as a function of time following serum stimulation.

Cells were subjected to serum starvation (48 hours at 0.2% calf serum) and restimulated by the addition of calf serum (10%). Panels A-E represent staining patterns observed at 0, 8, 16, 32, and 40 hours after serum addition, respectively. The cells were fixed, permeabilized, and incubated with rabbit anti-galectin-3 (1:150 dilution of antiserum #33). The binding of the primary antibody was detected by rhodamine-conjugated goat anti-rabbit immunoglobulin.

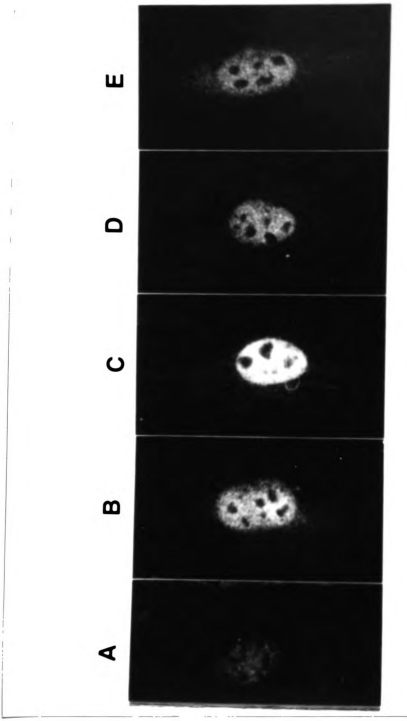
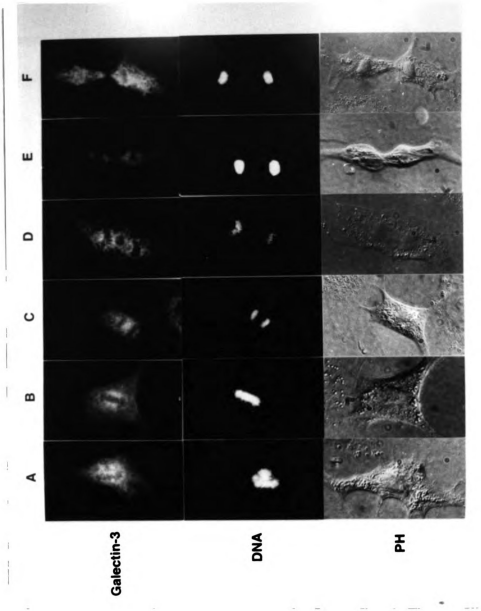

A
B
C
D
E

Figure 6: Analysis of the immnofluorescence staining pattern of galectin-3 as 3T3 cells undergo mitosis. Cells from cultures between 20 and 24 hours post serum stimulation were fixed, permeabilized, and incubated with rabbit anti-galectin-3 (1:150 dilution of antiserum #33). The binding of the primary antibody was detected by rhodamine-conjugated goat anti-rabbit immunoglobulin. The position of chromosomal DNA was followed by counterstaining with DAPI. Cells representing various stages of mitosis are shown: (A) prophase; (B) metaphase; (C) anaphase; (D) telophase; (E) cytokinesis; and (F) two newly formed daughter cells. The same cells are shown under phase contrast microscopy (PH).

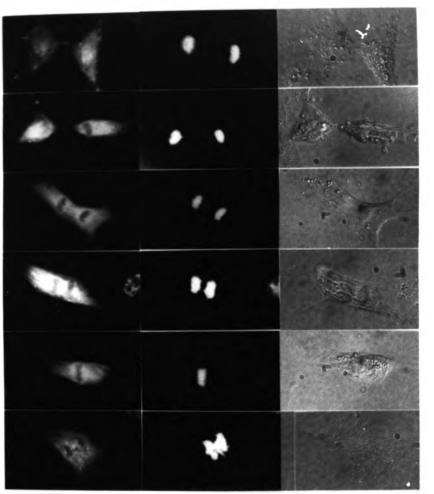

A
B
C
D
E
F
Galectin-3
DNA
PH

revealed by DAPI staining, appears as a dark band in the immunofluorescence photograph (Figure 6B). As the chromosomes separate in anaphase (Figure 6C), and decondense in telophase (Figure 6D), galectin-3 is found throughout the cell except where chromosomal DNA is located. As the nuclear envelope reforms around the decondensing chromosomes and the cell undergoes cytokinesis (Figure 6E), galectin-3 appears to be excluded from the nucleus. This cytoplasmic localization of galectin-3 persists in the newly formed daughter cells (Figure 6F). Thus, galectin-3 must be retranslocated into the nucleus as the cells enter the G1 phase of the next cell cycle and cells from cultures 32 hours post serum stimulation yielded staining patterns similar to those from cultures 8 hours after serum addition (Figure 5).

Essentially the same results are observed when the Sm antigen of snRNPs are stained with autoimmune serum (Figure 7). The prophase through cytokinesis stages of mitosis are again discerned from DAPI staining of DNA. Like galectin-3, the staining of Sm was excluded from the position of chromosomes. After separation of the daughter cells, Sm was found throughout the cytoplasm and must be retransported into the nucleus.

Figure 7: Analysis of the immunofluorescence staining pattern of the Sm antigens as 3T3 cells undergo mitosis.

Cells from cultures between 20 and 24 hours post serum stimulation were fixed, permeabilized and incubated with human autoimmune serum ENA anti-Sm (1:500 dilution). The binding of the primary antibody was detected by rhodamine-conjugated goat anti-human immunoglobulin. The position of chromosomal DNA was followed by counterstaining with DAPI. Cells representing various stages of mitosis are shown: (A) prophase; (B) metaphase; (C) anaphase; (D) telophase; (E) cytokinesis; and (F) two newly formed daughter cells. The same cells are shown under phase contrast microscopy (PH).


A
B
C
D
E
F
Sm
DNA
PH

DISCUSSION

The experiments documented in this study lead to two main conclusions. At the technical/language level, it calls for caution in interpreting the qualitative description of immunofluorescence patterns, such as diffuse, speckled, and punctate, derived from separate studies, even from the same laboratory. Thus, the initial reports of galectin-3 localization, with intense fluorescence over the entire nucleus (10), and snRNP proteins, with a speckled pattern (6, 20) were taken to imply that the two sets of proteins localized to different subcompartments of the nucleus. We have shown in the present study, however, that with high levels of endogenous protein antigen and a high titer antibody, one can observe intense labeling of the nucleus, diffuse over the entire organelle (except for "black holes") for both anti-galectin-3 and anti-Sm. By lowering the amount of antigenic target, accomplished in the present study by extraction with ammonium sulfate, one can obtain a speckled pattern for both anti-galectin-3 and anti-Sm. It appears, therefore, that rigorous conclusions regarding similar versus different localization of specific protein antigens can be best made by direct comparisons of their immunofluorescence patterns in parallel assays, or more ideally, by double labeling experiments.

When such a double immunofluorescence was carried out with anti-galectin-3 and anti-Sm, there was a one-to-one correspondence between the two speckled patterns yielded by the two antibodies. Therefore, the second major conclusion, at the conceptual/scientific level, is that galectin-3 and Sm are colocalized in the nuclear matrix. Studies at the light microscopy level (21), as well as at the ultrastructural level (22, 23), have identified an RNP network which contains components of the nuclear matrix. Moreover, the use of RNase

inhibitors and ammonium sulfate extraction have significantly improved the preservation of the RNP network in nuclear matrix preparations (17, 24). The nuclear matrix has been proposed to play an important role in many activities, such as DNA replication, transcription, and RNA processing (23). In particular, the interaction of newly synthesized RNA with the nuclear matrix has been demonstrated by Xing and Lawrence (15), who showed that Epstein-Barr virus primary transcripts were found in the nuclear matrix, and by Huang et al. (25), who demonstrated that the majority of poly $(A)^+$RNA is closely associated with the matrix.

The speckled patterns observed for immunofluorescence of snRNP antigens and non-snRNP splicing factors have been found to correspond, at the ultrastructural level, to interchromatin granule clusters and perichromatin fibrils (14, 26). Interchromatin granule clusters are not labeled with short pulses of [^{3}H]uridine. Perichromatin fibrils, however, do label with [^{3}H]uridine (27) and anti-RNA polymerase II antibodies (28), suggesting that they represent nascent transcripts at the sites of mRNA synthesis (29) and early events of pre-mRNA processing (30).

Galectin-3 has recently been identified as a factor required for the splicing of pre-mRNA in a cell-free assay (5). Indeed, electron microscopic analysis, using the same anti-galectin-3 antibody preparation of this study (antiserum #33), yielded immunogold labeling of interchromatic spaces and at the borders of condensed chromatin (perichromatic fibrils) (31). This localization of galectin-3 to interchromatic spaces and perichromatic fibrils is similar to the reported distribution of the non-snRNP splicing factors U2AF (30) and SC35

(14, 20), respectively. In addition, immunoelectron microscopy has also localized hnRNP proteins mostly to the perichromatic fibrils (26).

Finally, it should be noted that our ultrastructural studies have also shown that there was little or no anti-galectin-3 labeling of chromatin. Thus, galectin-3 does not appear to be a DNA-associated protein. This conclusion is consistent with our present immunofluorescence evidence that galectin-3 is excluded from the location of chromosome during mitosis.

REFERENCES

1. Barondes, S. H., Cooper, D. N. W., Gitt, M. A., and Leffler, H. 1994, J. Biol. Chem. 269, 20807-20810.
2. Jia, S., and Wang, J. L. 1988. J. Biol. Chem. 263, 6009-6011.
3. Dreyfuss, G., Matunis, M. J., Pinol-Roma, S., and Brud, G. C. 1993. Ann. Rev. Biochem. 62, 289-321.
4. Bennett,M., and Reed, T. 1993. Science 262, 105-108.
5. Dagher, S. F., Wang, J. L., and Patterson, R. J. 1995. Proc. Natl. Acad. Sci. USA 92, 1213-1217.
6. Spector, D. L. 1993. Ann. Rev. Cell Biol. 9, 265-313.
7. Carmo-Fonesca, M., Pepperkok, R., Sproat, B. S., Ansorge, W., Swanson, M. S., and Lamond, A. I. 1991. EMBO J. 10, 1863-1873..
8. Huang, S., and Spector, D. L. 1992. Proc. Natl. Acad. Sci. USA 89, 305-308.
9. Moutsatsos, I. K., Davis, J. M., and Wang, J. L. 1986. J. Cell Biol. 102, 447-483.
10. Moutsatsos, I. K., Wade, M., Shindler, M., and Wang. J. L. 1987. Proc. Natl. Acad. Sci. USA 84, 6452-6456.
11. Laing, J. G., and Wang. J. L. 1988. Biochemistry 27, 5329-5334.
12. Agrwal, N., Sun, Q., Wang, S. Y., and Wang, J. L. 1993. J. Biol. Chem. 268, 14932-14939.
13. Burton, Z. F., Killeen, M., Sopta, M., Ortolan, L. G., and Greenblatt, J. 1988. Mol. Cell Biol. 8, 1602-1613.

14. Spector, D. L., Fu, X.-D., and Maniatis, T. 1991. EMBO J. 10, 3467-3481.

15. Xing, Y., and Lawrence, J. B. 1991. J. Cell Biol. 112, 1055- 1063.

16. Spector, D. L. 1990. Proc. Natl. Acad. Sci. USA 87, 147-151.

17. Fey, E. G., Krochmalnic, G., and Penman, S. 1986. J. Cell Biol. 102, 1654-1665.

18. Agrwal, N., Wang, J. L., and Voss, P. G. 1989. J. Biol. Chem. 264, 17236-17242.

19. Cowles, E. A., Agrwal, N., Anderson, R. L., and Wang, J. L. 1990. J. Biol. Chem. 265, 17706-17712.

20. Jimenez-Garcia, L. F., and Spector, D. L. 1993. Cell 73, 47-59.

21. Smetana, K., Steele, W. J., and Busch, H. 1963. Exp. Cell Res. 31, 198-201.

22. Berezney, R., and Coffey, D.S. 1974. Biochem. Biophys. Res. Commun. 60, 1410-1419.

23. Berezney, R. 1991. J. Cell. Biochem. 47, 109-123.

24. Belgrader, P., Siegel, A. J., and Berezney, R. 1991. J. Cell Sci. 98, 281-291.

25. Huang, S, Deerinck, T. J., Ellisman, M. H. and Spector, D. L. 1994. J. Cell Biol. 126, 877-899.

26. Fakan, S., Leser, G., and Martin, T. E. 1984. J. Cell Biol. 98, 358-363.

27. Fakan, S., and Puvion, E. 1980. Int. Rev. Cytol. 65, 255-299.

28. Spector, D. L., O'Keefe, R. T., and Jimenez-Garcia, L. F. 1993. Cold Spring Harbor Symp. Quant. Biol. 58, 799-805.

29. Fakan, S., Puvion, E., and Spohr, G. 1976. Exp. Cell Res. 99, 155-164.

30. Zhang, G., Taneja, K. L., Singer, R. H., and Green, M. R. 1994. Nature 372, 809-812.

31. Hubert, M., Wang, S.-Y., Wang, J. L., Seve, A.-P., and Hubert, J. 1995. Exp. Cell Res., submitted for publication.

CHAPTER III

Coimmunoprecipitation of Sm Polypeptides of snRNP with Galectin-3 by a Monoclonal Antibody Directed against the Lectin

SUMMARY

In previous studies, it had been shown that the galactose/lactose-binding protein, designated galectin-3, was a required factor in the splicing of pre-mRNA as assayed in a cell-free system and that its nuclear localization, as detected by immunofluorescence microscopy, was similar to the distribution of the small nuclear ribonucleoproteins (snRNPs). In the present study, nuclear splicing extracts derived from HeLa cells were subjected to immunoprecipitation with anti-Mac 2, a monoclonal antibody specifically directed against galectin-3. Immunoblotting of the anti-Mac 2 precipitate with polyclonal rabbit anti-galectin-3 revealed the presence of the lectin. Immunoblotting of the same precipitate with an autoimmune serum reactive with the snRNP B (Sm B) and D (Sm D) polypeptides revealed that a fraction of the Sm B in the nuclear extract was coprecipitated with the lectin by the monoclonal antibody. Finally, RNA components, one of which corresponded in mobility to U2 snRNA, could also be identified in the anti-Mac 2 precipitate. The specificity of this coimmunoprecipitation was ascertained by comparing the results obtained with anti-Mac 2 versus those observed with precipitation by autoimmune anti-Sm (positive control) and by an isotype-matched monoclonal antibody against the transferrin receptor (negative control). Moreover, the coprecipitation of Sm B with galectin-3 by anti-Mac 2 was dependent on the presence of the lectin; nuclear extracts depleted of galectin-3 by prior adsorption on a lactose affinity resin failed to yield Sm B in the immunoprecipitate of anti-Mac 2. The coprecipitation of Sm B by anti-Mac 2 was not perturbed by the addition of lactose or by prior treatment of the nuclear extract with

ribonuclease. These results suggest that at least a fraction of the Sm B polypeptides, possibly on U2 snRNP, may interact with galectin-3 through protein-protein interactions.

INTRODUCTION

Galectin-3 is a galactose/lactose (Lac)-binding protein found in the nucleus and cytoplasm of a variety of cell types (1). It has been implicated as a required factor for pre-mRNA splicing in a cell-free assay (2). Nuclear extracts capable of carrying out in vitro splicing contain galectin-3, as revealed by immunoblotting. When these splicing competent extracts are subjected to affinity adsorption on Lac-containing beads, the unbound fraction is depleted of the lectin. Concomitant with the depletion of galectin-3, the unbound fraction also loses the *in vitro* splicing activity. Reconstitution of the depleted extract with recombinant galectin-3, purified from an *E. coli* expression system, restores the splicing activity in a dose-dependent fashion.

In the course of these studies, it was also observed that in a gel mobility shift assay, splicing competent nuclear extracts yielded both ATP-independent H complex, as well as ATP-dependent, higher order complexes (designated A, A', and B) intermediate in the formation of the spliceosome. In contrast, nuclear extracts depleted of galectin-3 and splicing activity yielded only the ATP-independent H complex (2). These results suggested that galectin-3 may interact with components that constitute the spliceosome. Coupled with the demonstration that the association of galectin-3 with the cell nucleus was sensitive to ribonuclease (RNase) treatment (reference 3 and chapter II), the possibility is raised that the lectin may interact with a RNA-containing component. However, the sequence of galectin-3 exhibits no obvious RNA recognition motif(s) (4). Nor have attempts to demonstrate an

association of galectin-3 with RNA, using cross-linking by irradiation with ultraviolet light, yielded any evidence for direct galectin-3-RNA interactions (S. F. Dagher and R. J. Patterson, unpublished observations).

One approach to discern if galectin-3 interacts with any components of the splicesome is to determine if immunoprecipitation of galectin-3 using a highly specific monoclonal antibody can simultaneously coprecipitate known spliceosomal components. We now report that, indeed, the Sm B (and perhaps Sm D as well) protein of nuclear small ribonucleoprotein (snRNP) particles is precipitated by a galectin-3 specific monoclonal antibody. This coimmunoprecipitation is dependent on the presence of galectin-3 in the mixture subjected to the immunoprecipitation protocol.

MATERIALS AND METHODS

Cell Cultures. Mouse 3T3 fibroblasts were obtained from American Type Culture Collection (Rockville, MD). The cells were grown as monolayers in Dulbecco modified Eagle's Medium containing 10% calf serum, 100 U/ml penicillin and 100 U/ml streptomycin at 37 °C in a humidified atmosphere of 10% CO_2. Cells grown at a density less than 5 x 10^4 cells /cm^2 were proliferative and were harvested to generate nuclear extracts. In some experiments, cells at low density were arrested by removal of serum and maintenance in medium containing 0.2% calf serum for 48 hr (quiescent cells). Upon readdition of serum (10%), the cells were reactivated and were cultured for another 16 hours (5).

HeLa S3 cells were cultured in spinner flasks in Minimum Essential Medium containing 5% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. They were grown to 4 to 6 x 10^5 cells per ml prior to harvesting for nuclear extract preparation.

Antibodies and Affinity Columns. A rat monoclonal antibody was developed against the Mac 2 antigen (6), which has been shown to be galectin-3 (7). The hybridoma line producing this monoclonal antibody (M3/38.1.2.8.HL.2) was obtained from the American Type Culture Collection (TIB 66) (Rockville, MD). The hybridoma cells were cultured in serum-free medium (RPMI 1640 containing Nutridoma SP (BMB, IN)). After centrifugation to pellet the cells, supernatants from the cultures were pooled, subjected to

ammonium sulfate precipitation (45% of saturation), dialyzed against phosphate-buffered saline (PBS; 0.13 M NaCl, 5 mM sodium phosphate, pH 7.5) exhaustively, and stored in aliquots at a concentration of 25 μg/ml. This antibody preparation, hereafter designated as anti-Mac 2, consists of rat $IgG_{2a}\kappa$.

An isotype matched rat monoclonal antibody ($IgG_{2a}\kappa$), directed against the transferrin receptor (T_fR), was used as a control for anti-Mac 2. The hybridoma (R17 217.1.3) producing the rat anti-T_fR was also obtained from American Type Culture Collection (TIB 219) and cultured in serum-free medium. Anti-T_fR was isolated in the same fashion as described for anti-Mac 2.

Polyclonal rabbit anti-galectin-3 antiserum was obtained from rabbit #32. This New Zealand White rabbit was immunized with recombinant galectin-3. The characterization of the specificity of this antiserum had previously been documented (8). Human autoimmune serum reactive with the Sm antigens of snRNPs (ENA anti-Sm) was purchased from The Binding Site (San Diego, CA). Lactose immobilized on 6% beaded agarose (Lac-agarose) was purchased from Sigma (St. Louis, Mo), as was agarose derivatized with cellobiose (Cello-agarose) used as a control. Sepharose CL4B and protein G-agarose beads were purchased from Pierce (Rockford, IL)

Buffers. Buffers used for nuclear extract preparation are designated as follows: (i) buffer A contains 10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM NaCl, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5 mM dithiothreitol (DTT); (ii)

buffer C contains 20 mM HEPES pH 7.9, 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT; and (iii) buffer D contains 20 mM HEPES pH 7.9, 20% (v/v) glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT. Both DTT and PMSF were added fresh to the buffers just before use.

Buffers used for nucleoplasm preparation and cesium sulfate gradient sedimentation are designated as follows: (i) RSB contains 10 mM Tris , pH 7.5, 10 mM NaCl, 1.5 mM $MgCl_2$; and (ii) cesium sulfate stock solutions which contain cesium sulfate in RSB with final densities of 1.20 and 1.75 g/ml.

Washing buffers for immunoprecipitation and Lac depletion are designated as: (i) buffer 1 containing 10 mM Tris, pH 7.5, 150 mM NaCl, 0.5% NP-40, 0.2 mM EDTA; and (ii) buffer 2 containing 10 mM Tris, pH 7.5, 500 mM NaCl, 0.5% NP-40, 0.2 mM EDTA; and (iii) buffer 3 containing 10 mM Tris, pH 7.5.

Nuclear Extract Preparation. Nuclear extract was prepared according to the procedure of Dignam et al. (9). Briefly, mouse 3T3 cells cultured as monolayers were washed once with PBS containing 0.2% EDTA at 37 °C. The cultures were then incubated for 3 minutes with PBS containing 0.1% trypsin and 0.2% EDTA. Cells detached from the flasks were then harvested and washed twice with ice-cold PBS. Human HeLa cells grown in spinner flasks were harvested from cell culture media and washed twice with ice-cold PBS. In some experiments, 3T3 cells and HeLa cells were metabolically labeled with 100 μCi/ml of [^{35}S]methionine (NEN, MA) for 12-16 hours.

The volumes of the packed cell pellets were determined. The harvested cells were then suspended in five volumes of ice-cold buffer A and allowed to stand on ice for 10 minutes. The cells were collected by centrifugation and suspended in two volumes of buffer A and lysed by 10 strokes in a Dounce homogenizer at 4 °C. The homogenate was checked for cell lysis under a microscope and then centrifuged at 4 °C for 10 minutes at 1,500 x g to pellet nuclei. The supernatant was carefully decanted and the pellet was subjected to a second centrifugation for 20 minutes at 25,000 x g to remove residual cytoplasmic material and this pellet was designated as crude nuclei. The crude nuclei were resuspended in 3 ml of buffer C per 10^9 cells and then homogenized with 10 strokes in a Dounce homogenizer. The nuclei suspension was stirred gently with a magnetic stirring bar for 30 minutes and then centrifuged for 30 minutes at 25,000 x g. The resulting clear supernatant was designated as high salt nuclear extract and frozen at -70 °C. The high salt nuclear extract was dialyzed against 50 volumes of buffer D twice prior to the subjected experiments. In some experiments, the high salt nuclear extract was dialyzed against buffer D containing 0.3 M lactose.

Immunoprecipitation and Lac-depletion of Nuclear Extract. Nuclear extract was precleared by incubating with Sepharose beads for 1 hour at 4 °C. The unbound fraction of this preclearing step was subjected to immunoprecipitation or Lac-agarose adsorption. ENA anti-Sm antibody (10 µl) and 5.0 µg of anti-Mac 2 antibody were preabsorbed with a 50 µl suspension of protein G-agarose beads at room temperature for 30 minutes, and the antibody bound beads were washed three times with the buffer D. In

a typical precipitation, 50 µl of the precleared nuclear extract was incubated with the anti-Sm or anti-Mac 2 bound protein G beads for 1 hour at 4 °C. In Lac-agarose depletion experiment, 50 µl of Lac-agarose beads was used instead. The bound fractions were washed three time with buffer D. The material remaining bound to the beads after the washing steps was eluted with SDS-PAGE sample buffer (10) and subjected to SDS-PAGE (12.5% acrylamide; 200 volts (V) for 30 minutes, followed by 300 V for 2 hours), followed by immunoblotting analysis.

For immunoblotting analysis, ENA anti-Sm was used at 1:10,000 dilution; rabbit anti-galectin-3 was used at 1:10,000 dilution; and rat anti-Mac 2 was used at 1:5,000 dilution. All three primary antibodies were diluted in TBS (10 mM Tris, 0.5 M NaCl, pH 7.5). Horseradish peroxidase conjugated goat anti-human, sheep anti-rabbit, and goat anti-rat antibodies (BMB) were used at 1:5,000 dilution in T-TBS (TBS containing 0.05% Tween 20) as secondary antibody. Proteins resolved on SDS-PAGE were electrophoretically transferred to Immobilon-P (Millipore) membrane in a transfer buffer containing 0.025 M Tris, 0.19 M glycine and 20% methanol for 3 hour at 50 milliamp (mA), 9 hours at 100 mA and 3 hour at 200 mA sequentially; the membrane was briefly rinsed twice with T-TBS and blocked with 5% dry milk in T-TBS for at least 1 hour at room temperature. After briefly being washed with T-TBS twice, the membrane was then incubated with primary antibody for 1 hour at room temperature. The membrane was then rinsed with T-TBS twice and washed extensively with T-TBS (15 minutes once, then 10 minutes 4 times). The washed membrane was incubated with horseradish peroxidase

conjugated secondary antibody at room temperature for 1 hour. The blot was rinsed and washed as described above. Proteins on the membrane were detected by ECL Western blotting detection reagents (Amersham, England) and revealed by exposing the luminescence to a sheet of autoradiography film (Hyperfilm-ECL (Amersham)).

Immunoprecipitation and Lac-agarose adsorption were also carried out on [^{35}S]methionine labeled nuclear extracts at high ionic strength (in buffer C). The same procedure was used, as described above, except the bound fractions were subjected to the following washing steps: (i) twice with buffer 1; (ii) twice with buffer 2; and (iii) twice with buffer 3. The material remaining bound to the beads were then analyzed by SDS-PAGE as detailed above and the radioactive polypeptides were revealed by fluorography.

Analysis of RNA Components in Bound Fractions of Immunoprecipitate and Lac-Adsorption. HeLa cells were cultured as described above and labeled with 50 μCi/ml of $^{32}PO_4$ (NEN, MA) for 12 hours. Nuclear extract generated from these ^{32}P-labeled cells was subjected to immunoprecipitation and Lac-agarose adsorption. After the washing steps, the bound fraction was resuspended in 100 μl of TE (10 mM Tris, pH 8.0, 10 mM EDTA) containing 0.3 M sodium acetate (NaOAc, pH 5.2) and 1 μg/ml glycogen. RNA was extracted with 100 μl of phenol. The aqueous phase was recovered and the residual phenol was extracted by adding 100 μl of chloroform/isoamyl alcohol (24:1, v/v). RNAs in the aqueous phase were precipitated with 600 μl of ethanol at - 70 °C overnight. The RNA pellet was rinsed once with ice-cold ethanol (70%) and air dried.

The extracted RNAs were subjected to electrophoresis in a polyacrylamide-urea gel (8% acrylamide (acrylamide/bisacrylamide 38:1; 8.3 M urea)). The gel was dried and the resolved RNAs were revealed by autoradiography.

Cesium Sulfate Gradient Fractionation and Immunoprecipitation.

Nucleoplasm was prepared from 3T3 cells 16 hours post serum stimulation of quiescent cultures (48 hours in 0.2% calf serum, followed by 16 hours in 10% calf serum). The harvested cells were resuspended in RSB and kept on ice for 15 minutes to allow the cells to swell. The cells were lysed through 20 strokes in a Dounce homogenizer and the nuclei were collected by centrifugation at 1,500 x g for 5 minutes at 4 °C. The nuclear pellet was resuspended in RSB containing 2 mM PMSF and 2 mM vanadyl-ribonucleoside complex (BRL/GIBCO, MD) and ruptured by sonication for 15 seconds four times. The material was then layered on a 30% sucrose/RSB and centrifuged at 4,500 x g for 15 minutes at 4 °C in a Sorvall HB-4 rotor (DuPont, DE) to remove chromatin and other insoluble material (11, 12). The resulting opaque interface of the 30% sucrose cushion was collected as nucleoplasmic material and used for cesium sulfate gradient fractionation.

Preformed linear cesium sulfate gradients (4.5 ml) were prepared in cellulose nitrate tubes (Beckman, CA) from stock solutions having initial densities of 1.20 g/ml and 1.75 g/ml in RSB. Samples of nucleoplasm (0.5 ml) were layered and the gradients were centrifuged at 112,000 x g for 60 hours at 15 °C in a SW50.1 rotor (Beckman, CA). These gradients were then fractionated with a Beckman fraction recovery system (0.5 ml

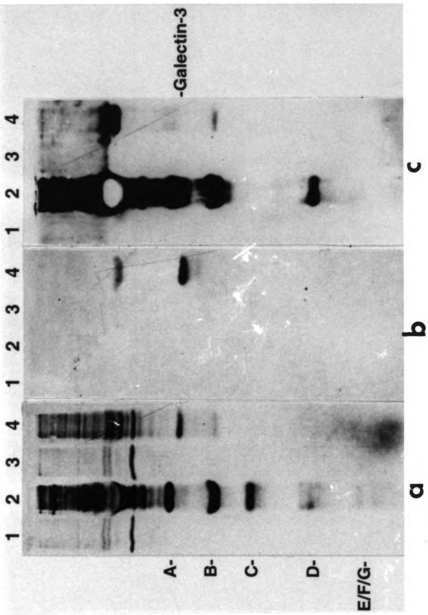
per fraction) and the profile of densities of the gradient was determined by weighing 100 μl portions of individual fraction at room temperature (11, 12). The individual fractions were diluted with two volumes of RSB and then subjected to immunoprecipitation by ENA anti-Sm antibody or anti-Mac 2 antibody. The immunoprecipitates were washed three times with RSB, subjected to SDS-PAGE, and western blotting by anti-Sm or rabbit anti-galectin-3.

RESULTS

Immunoprecipitation of Nuclear Extracts of 3T3 Cells by Autoimmune Anti-Sm and by Monoclonal Anti-Mac 2. Nuclear extracts were prepared from mouse 3T3 fibroblasts that had been metabolically labeled with [^{35}S]methionine. The extract was passed over a Sepharose column and the unbound fraction was then subjected to immunoprecipitation with ENA anti-Sm. The bound fraction of both the pre-clearing column (Sepharose) and the anti-Sm column were subjected to SDS-PAGE. Fluorographic analysis of the [^{35}S]methionine labeled polypeptides revealed that the pre-clearing column bound one predominant polypeptide of M_r 43,000 (Figure 1a, lane 1); this polypeptide has been identified to be actin, on the basis of immunoblotting with an antiserum raised against calf thymus actin (data not shown). The immunoprecipitate of the anti-Sm-protein G-agarose beads contained many radiolabeled bands, including polypeptides that correspond in molecular weight to the snRNP polypeptides A (33 kDa), B (28 kDa), C (22 kDa), D (18 kDa), and E/F/G (11-13 kDa) (Figure 1a, lane 2). When the same material (bound fraction of the anti-Sm precipitate) was subjected to immunoblotting with ENA anti-Sm, prominent immunoreactive bands were observed for Sm B and Sm D, as well as a conglomerate of high molecular weight bands (Figure 1c, lane 2). These results are consistent with the reported properties of this autoimmune serum (13, 14), whose principal epitopes are found on core polypeptides B and D, with some reactivity against U1 snRNP, which contains its specific polypeptides, A and C. In any case, the results illustrate that although anti-Sm

Figure 1: Immunoprecipitation of nuclear extracts of 3T3 cells by autoimmune anti-Sm and by monoclonal anti-Mac 2.

Nuclear extracts were prepared from mouse 3T3 fibroblasts metabolically labeled with [^{35}S]methionine. The extracts were subjected to immunoprecipitation and the polypeptides in the immunoprecipitates were resolved by SDS-PAGE (12.5% acrylamide), followed by fluorography or immunoblotting. (a) Fluorogram revealing the radioactive polypeptides; (b) Immunoblot with rabbit anti-galectin-3; and (c) Immunoblot with ENA anti-Sm. In panels (a)-(c), immunoprecipitations were performed with: (1) Sepharose; (2) ENA-anti-Sm protein G-agarose; (3) Sepharose; and (4) anti-Mac 2 protein G-agarose. The positions of migration of the snRNP-associated proteins A-G are highlighted on the left and the position of migration of galectin-3 is highlighted on the right.


1 2 3 4
1 2 3 4
1 2 3 4
-Galectin-3
A-
B-
C-
D-
E/F/G-
a
b
c

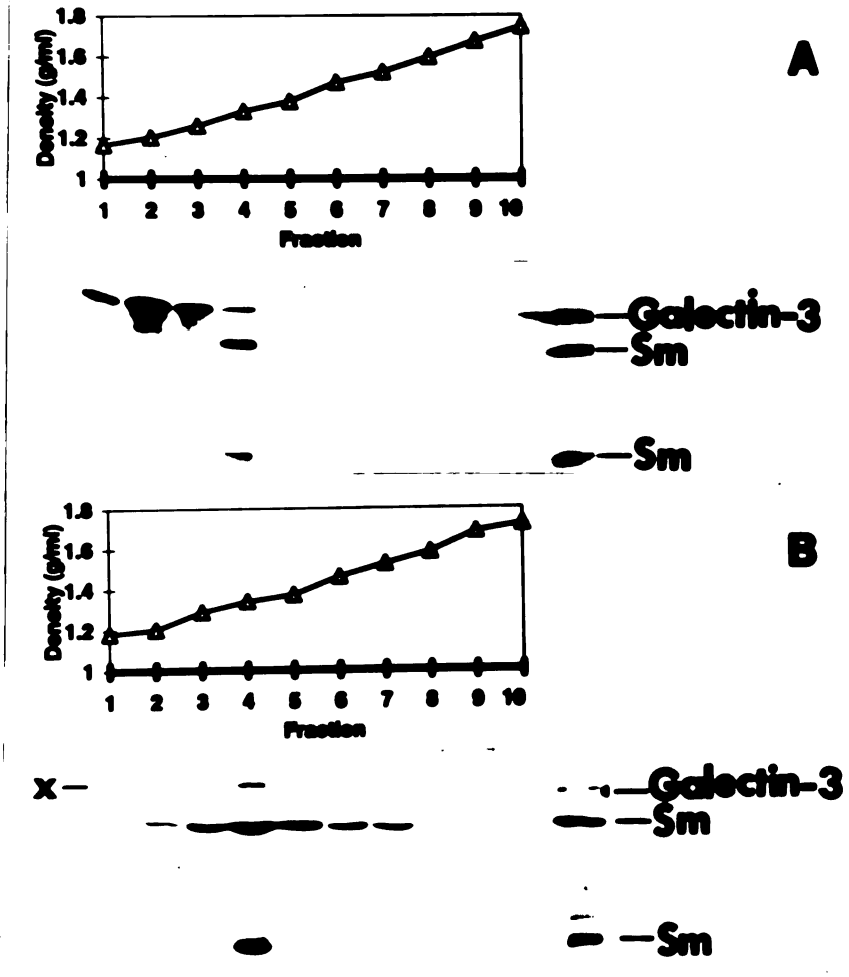
immunoblots Sm B and Sm D, the entire complement of snRNP polypeptides, A through G, is immunoprecipitated by the autoimmune serum as an RNP complex.

The same [^{35}S]methionine labeled nuclear extract was subjected to pre-clearing and immunoprecipitation with anti-Mac 2, a monoclonal antibody reactive against galectin-3 (7). The bound fraction of the pre-clearing column (Sepharose) again showed actin to be the predominant polypeptide (Figure 1a, lane 3). On the other hand, the anti-Mac 2 immunoprecipitate yielded many radiolabeled polypeptides on SDS-PAGE and fluorography (Figure 1a, lane 4). Two of these polypeptides, at the low molecular weight end, can be identified as galectin-3 (33 kDa) (Figure 1b, lane 4) and Sm B (Figure 1c, lane 4), on the basis of immunoblotting with a rabbit anti-galectin-3 antiserum (Figure 1, panel b) and ENA anti-Sm (Figure 1, panel c), respectively. These results are provocative in two respects: (a) when anti-Mac 2 immunoprecipitates galectin-3 (Figure 1a and 1b, lane 4), it coimmunoprecipitates a small fraction of the nucleoplasmic Sm B molecules (Figure 1a and 1c, lane 4); and (b) in contrast, when anti-Sm immunoprecipitates the snRNPs, no galectin-3 is apparently coprecipitated (Figure 1a and 1b, lane 2).

These conclusions are corroborated by experiments in which nuclear RNPs are first enriched by cesium sulfate gradient sedimentation. The gradient used ranged in density from 1.18 g/ml at the top to 1.75 g/ml at the bottom. Naked protein, such as recombinant galectin-3 ($\rho \cong 1.20$ g/ml) collected at the top of the gradient. Nucleoplasm from 3T3 cells was fractionated over such a gradient and the individual gradient fractions were subjected to

Figure 2: Profiles of the density and immunoprecipitation patterns of the individual fractions derived from cesium sulfate gradient sedimentation of nucleoplasm.

Nucleoplasm was prepared from serum-stimulated (16 hours after addition of 10% calf serum to quiescent cultures) 3T3 fibroblasts and subjected to fractionation on a cesium sulfate gradient. The density was determined by weighing 100 µl aliquot of each fraction. The individual fractions were diluted with RSB and subjected to immunoprecipitation with: (A) anti-Mac 2; and (B) ENA anti-Sm. For both (A) and (B), the polypeptides in the immunoprecipitates were resolved by SDS-PAGE (12.5% acrylamide) and then subjected to immunoblotting, in sequence, with anti-galectin-3 and ENA anti-Sm. The positions of migration of galectin-3, as well as Sm B and Sm D polypeptides of snRNP, are revealed by immunoblotting of nucleoplasm with both antibodies and are highlighted on the right. The letter X on the left designates a polypeptide that is observed in anti-Sm immunoprecipitates immunoblotted by anti-galectin-3. Its position of migration is distinct from that of galectin-3.


A
Density (g/ml)
1.8
1.6
1.4
1.2
1
1 2 3 4 5 6 7 8 9 10
Fraction
Galectin-3
Sm
Sm
B
Density (g/ml)
1.8
1.6
1.4
1.2
1
1 2 3 4 5 6 7 8 9 10
Fraction
X
Galectin-3
Sm
Sm

immunoprecipi

with anti-galec

indeed, the ma

However, there

fraction 4 (~1.

anti-Mac 2 hac

in the anti-Mac

When

anti-Sm (Figu

galectin-3, Sm

from 1.20 g/m

prominent Sm

with anti-gale

previously (Fi

In the

anti-Sm and i

2B, fraction 4

distinct, from

polypeptide is

immunoblotte

HeLa cell sys

immunoprecipitation with anti-Mac 2 (Figure 2A), followed by sequential immunoblotting with anti-galectin-3 and anti-Sm. Immunoblotting with anti-galectin-3 revealed that, indeed, the majority of the galectin-3 was found in fractions 1 and 2 (~1.20 g/ml). However, there was a population of galectin-3 found in the anti-Mac 2 precipitate of fraction 4 (~1.34 g/ml). This fraction, upon immunoblotting with anti-Sm, revealed that anti-Mac 2 had, again, coprecipitated Sm B (Figure 2A). In this case, Sm D was also found in the anti-Mac 2 immunoprecipitate.

When the individual gradient fractions were subjected to immunoprecipitation with anti-Sm (Figure 2B), followed by sequential immunoblotting with anti-Sm and anti-galectin-3, Sm B was found spread over many fractions of the gradient, ranging in density from 1.20 g/ml to 1.50 g/ml. The fraction with the majority of Sm was fraction 4, where prominent Sm B and Sm D were revealed by immunoblotting (Figure 2B). Immunoblotting with anti-galectin-3 failed to reveal the lectin in the anti-Sm precipitate, as was found previously (Figure 1a and 1b, lane 2).

In the course of these studies, we observed a band that was immunoprecipitated by anti-Sm and immunoblotted by anti-galectin-3. This band is labeled as band X in Figure 2B, fraction 4. The mobility of this band on SDS-PAGE was slightly slower and therefore, distinct, from that of galectin-3 (33 kDa) (Figure 2B). Although the identity of this polypeptide is not known, the phenomenon of its being immunoprecipitated by anti-Sm and immunoblotted by anti-galectin-3 has been reproducibly observed, in both the 3T3 and the HeLa cell systems (see Figure 3 and Figure 4).

Immu

carried out on

extracts were

extracts used

splicing extra

was observed

Western blot

(Figure 3b, l

Sm B is actu

extract was

immunopre

3a, lane 5).

revealed th

Sev

served as t

revealed th

anti-Sm im

galectin-3

however, a

Sm immu

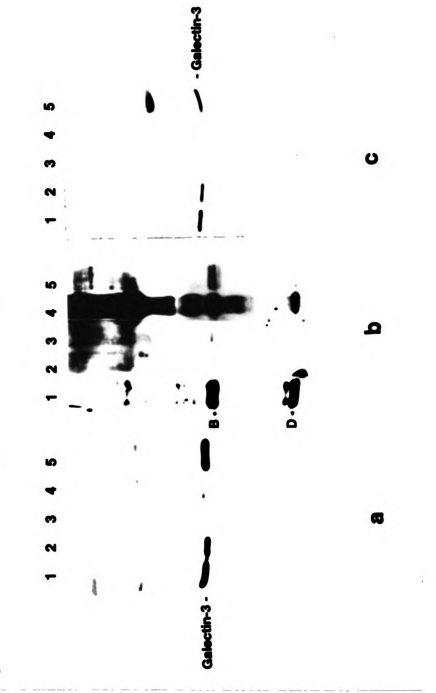
isotyped m

Immunoprecipitation of HeLa Cell Splicing Extracts. A similar analysis was carried out on nuclear extracts derived from human HeLa cells. In this case, the nuclear extracts were prepared according to the procedure of Dignam et al (9), equivalent to the extracts used to demonstrate a role for galectin-3 in a cell-free splicing assay (2). When splicing extracts were Western blotted with anti-galectin-3, a single polypeptide (29 kDa) was observed (Figure 3a, lane 1), corresponding to human galectin-3. When subjected to Western blotting with ENA anti-Sm, this nuclear extract yielded two sets of doublets (Figure 3b, lane 1), corresponding to Sm B (28 kDa) and Sm D (18 kDa). In human cells, Sm B is actually a doublet, Sm B/B'; Sm D is also a doublet, Sm D/D' (15). The splicing extract was immunoprecipitated with anti-Mac 2. Western blotting of this immunoprecipitate with anti-galectin-3 yielded the expected galectin-3 polypeptide (Figure 3a, lane 5). Western blotting of the same anti-Mac 2 immunoprecipitate with anti-Sm revealed the Sm B doublet prominently, as well as traces of Sm D (Figure 3b, lane 5).

Several crucial controls were performed in parallel. First, anti-Sm precipitation served as the positive control. Western blotting of the anti-Sm precipitate with anti-Sm revealed the expected Sm B and Sm D bands (Figure 3b, lane 4). Western blotting of the anti-Sm immunoprecipitate with anti-galectin-3 failed to reveal a band that matched human galectin-3 exactly (Figure 3a, lane 4). As noted previously in Figure 2B (fraction #4), however, a polypeptide designated as band X was observed in anti-galectin-3 blots of anti-Sm immunoprecipitates (Figure 3a, lane 4). Second, the monoclonal antibody anti-T_fR was isotyped matched to anti-Mac 2 and served as a negative control. The immunoprecipitates

Figure 3: Immunoprecipitation of HeLa cell splicing extracts by autoimmune anti-Sm and by monoclonal anti-Mac 2.

Splicing extracts were prepared from HeLa cells according to the procedure of Dignam et al. (9). The extracts were subjected to affinity adsorption or immunoprecipitation in Buffer D and the polypeptide in the bound fractions were resolved by SDS-PAGE (12.5% acrylamide), followed by immunoblotting. (a) Immunoblotting with rabbit anti-galectin-3 (#32); (b) Immunoblotting with ENA anti-Sm; and (c) Immunoblotting with rat anti-Mac 2. In panels (a)-(c), the samples in each lane were: (1) HeLa cell splicing extract; (2) bound fraction of the Lac-agarose; (3) rat anti-T_fR precipitate; (4) human anti-Sm precipitate; and (5) rat anti-Mac 2 precipitate. The positions of migration of Sm B, Sm D, as well as galectin-3, are highlighted.


Galectin-3
1 2 3 4 5
B
D
Galectin-3
1 2 3 4 5
1 2 3 4 5
a
b
c

of anti-T_fR f

(Figure 3a, l

precipitate g

anti-galectin

antigens we

samples. (i)

anti-Sm pre

anti-Mac 2.

polypeptide

was due to

failed to rec

or in the an

Eff

anti-Mac 2

corroborate

conditions

C), were th

galectin re

adsorption

agarose unb

galectin-3 s

of anti-T_fR failed to yield any prominent bands when immunoblotted with anti-galectin-3 (Figure 3a, lane 3) and anti-Sm (Figure 3b, lane 3). Third, Lac-agarose was used to precipitate galectin-3 through saccharide-binding. Western blotting of this precipitate with anti-galectin-3 revealed the 29 kDa polypeptide of the lectin (Figure 3a, lane 2) but no Sm antigens were observed upon blotting with anti-Sm (Figure 3b, lane 2). Finally, all of the samples, (i) splicing extract; (ii) Lac-agarose bound fraction; (iii) anti-T_fR precipitate; (iv) anti-Sm precipitate; and (v) anti-Mac 2 precipitate, were subjected to Western blotting with anti-Mac 2. This was done to test whether anti-Mac 2 interacted directly with the Sm B polypeptide or whether the observed coprecipitation by anti-Mac 2 of Sm B with galectin-3 was due to interactions between the two polypeptides. The results showed that anti-Mac 2 failed to recognize the Sm B (and Sm D) polypeptide in splicing extracts (Figure 3c, lane 1) or in the anti-Sm precipitate where Sm B and Sm D are enriched (Figure 3c, lane 4).

Effect of Galectin-depletion on Coimmunoprecipitation. The conclusion that anti-Mac 2 can precipitate Sm B only through the latter's interaction with galectin-3 was corroborated by experiments in which the lectin was depleted from the splicing extract. The conditions used for depletion, adsorption on Lac-agarose under high ionic strength (Buffer C), were the same as those used to show the concomitant loss of splicing activity with galectin removal from the splicing extract (2). The nuclear extract was subjected to adsorption on cellobiose-agarose in parallel as a control. Both Lac-agarose and cellobiose-agarose unbound fractions were dialyzed against buffer D. Immunoblotting with anti-galectin-3 showed that the unbound fraction of the cellobiose adsorption contained galectin-

3 (Figure 4

In contrast,

4, lanes 3 a

Sm. The u

doublets (F

derived fro

lane 2). In

found in th

to the concl

precipitatio

Thus, all of

between ga

Coi

Ionic Stren

(0.3 M). T

binding of

beads revea

galectin-3 (

little or no

Sm B and S

lane 4). Mo

3 (Figure 4a, lane 1), which can be immunoprecipitated by anti-Mac 2 (Figure 4a, lane 2). In contrast, the corresponding fractions of the Lac adsorption showed no galectin-3 (Figure 4, lanes 3 and 4). The same fractions were subjected to Western blotting with ENA anti-Sm. The unbound fraction of both the cellobiose and Lac adsorptions yielded the Sm B doublets (Figure 4b, lanes 1 and 3). However, only the Sm B in the unbound fraction derived from the cellobiose beads could be immunoprecipitated by anti-Mac 2 (Figure 4b, lane 2). In contrast, the Sm B in the unbound fraction derived from the Lac beads was not found in the anti-Mac 2 precipitate (Figure 4b, lane 4). These results provide strong support to the conclusion that anti-Mac 2 does not recognize Sm B directly and that the observed precipitation of Sm B by the monoclonal antibody depended on the presence of galectin-3. Thus, all of the coimmunoprecipitation data are interpreted to implicate an interaction between galectin-3 and the Sm B polypeptide(s).

Coimmunoprecipitation Experiments in the Presence of Lactose and at High Ionic Strength. The experiment shown in Figure 3 was repeated in the presence of Lac (0.3 M). The presence of the Lac in the HeLa nuclear extract completely inhibited the binding of galectin-3 to the Lac-agarose beads. The bound fraction of the Lac-agarose beads revealed neither galectin-3 nor Sm polypeptides upon Western blotting with anti-galectin-3 (Figure 5a, lane 2) or with anti-Sm (Figure 5b, lane 2). The presence of Lac had little or no effect on the immunoprecipitation by anti-Sm. Western blot by anti-Sm revealed Sm B and Sm D (Figure 5b, lane 4), just as was observed in the absence of Lac (Figure 3b, lane 4). Most importantly, Western blotting of the anti-Mac 2 precipitate showed galectin-3

Figure 4: The effect of galectin-3 depletion on the immunoprecipitation of Sm B by anti-Mac 2.
Splicing extracts were prepared from HeLa cells in buffer C and the extracts were subjected to affinity adsorption with cellobiose-agarose or Lac-agarose. The unbound fractions of these adsorptions were then subjected to immunoprecipitation with anti-Mac 2 and the polypeptides in the bound fraction were resolved by SDS-PAGE (12.5% acrylamide), followed by immunoblotting. (a)Immunoblotting with rabbit anti-galectin-3; (b)Immunoblotting with ENA anti-Sm. In panels (a) and (b), samples in each lane were: (1)unbound fraction of the cellobiose adsorption; (2)bound fraction of anti-Mac 2 precipitation of the material that did not bind cellobiose; (3)unbound fraction of the Lac adsorption; (4)bound fraction of anti-Mac 2 precipitation of the material that did not bind Lac. The positions of migration of galectin-3 and Sm B are highlighted.

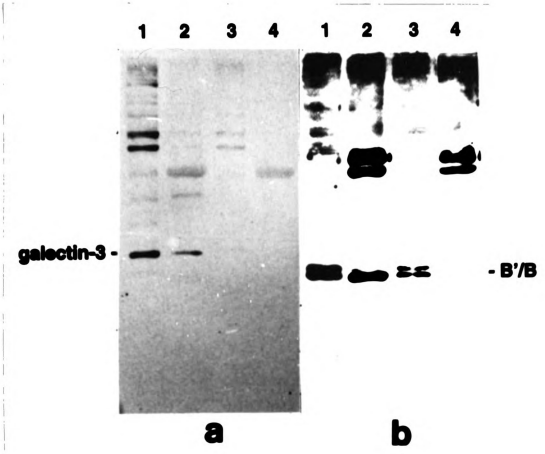
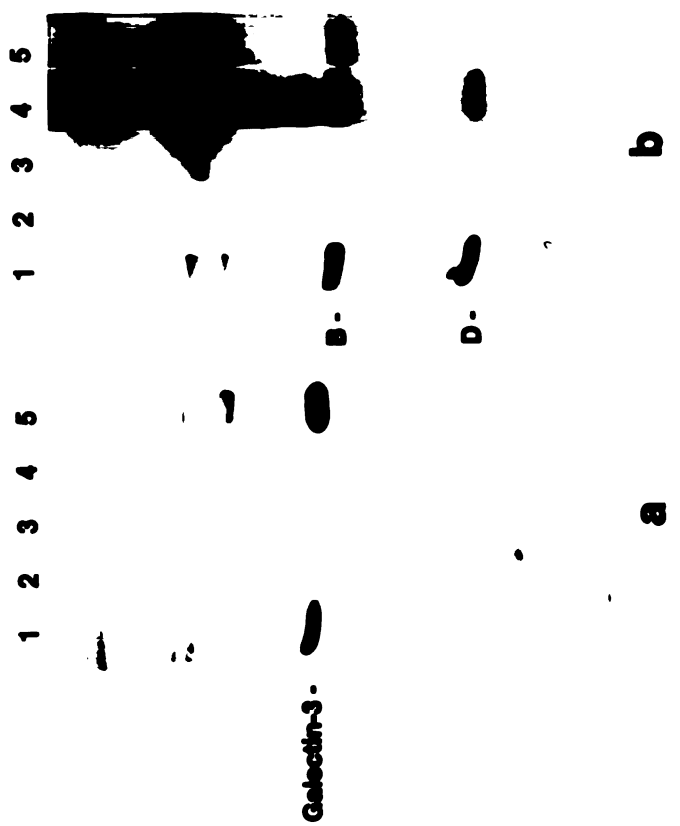

1 2 3 4 1 2 3 4
galectin-3 -
- B'/B
a
b

Figure 5: Immunoprecipitation in the presence of lactose (Lac) of HeLa cell splicing extract by autoimmune anti-Sm and by monoclonal anti-Mac 2.
Splicing extracts were prepared from HeLa cells according to the procedure of Dignam et al. (9). Lac was included to a final concentration of 0.3 M in buffer D during dialysis and the extracts were subjected to affinity adsorption or immunoprecipitation in Buffer D and the polypeptides in the bound fractions were resolved by SDS-PAGE (12.5% acrylamide), followed by immunoblotting. (a) Immunoblotting with rabbit anti-galectin-3; (b) Immunoblotting with ENA anti-Sm. In panels (a) and (b), samples in each lane were: (1) HeLa cell splicing extract; (2) bound fraction of Lac-agarose; (3) rat anti-TfR precipitate; (4) human anti-Sm precipitate; and (5) rat anti-Mac 2 precipitate. The positions of migration of Sm B, Sm D, as well as galectin-3, are highlighted.


1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
Galectin-3
a
b

(Figure 5a, lan

respectively.

immunoprecip

with galectin

In co

galectin 3 b

experimen

the galecti

experimen

[^{35}S]meth

specific a

fraction

(data no

Similar

The bo

polype

(14 kD

chapte

polyp

(Figu

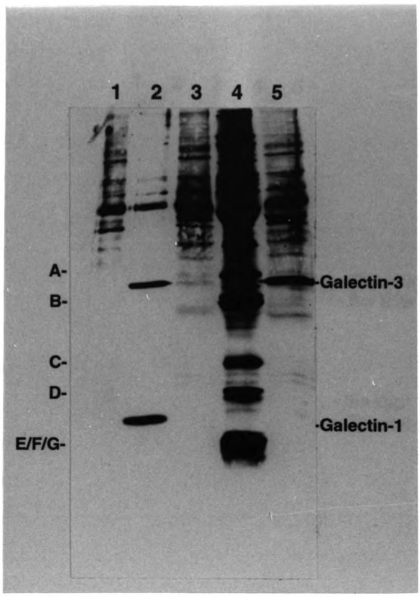
(Figure 5a, lane 5) and Sm B and Sm D (Figure 5b, lane 5) with anti-galectin-3 and anti-Sm, respectively. These results indicate that Lac-binding did not perturb the immunoprecipitation of galectin-3 by anti-Mac 2 and the coprecipitation of Sm B and Sm D with galectin-3 by the monoclonal antibody.

In contrast to this lack of an effect by Lac, the coimmunoprecipitation of Sm B with galectin 3 by anti-Mac 2 appears to be sensitive to high salt. The immunoprecipitation experiments were carried out in buffer C, corresponding to the conditions used to deplete the galectins from the splicing extract by Lac-affinity chromatography (2). In this experiment, the nuclear extracts were prepared from HeLa cells metabolically labeled with [^{35}S]methionine, such that polypeptides can be analyzed independent of reactivity with specific antibodies.

When the labeled nuclear extract was pre-cleared with Sepharose beads, the bound fraction, upon SDS-PAGE and fluorography, yielded a polypeptide corresponding to actin (data not shown), as well as polypeptides of higher molecular weight (Figure 6, lane 1). Similar results were obtained with precipitation by anti-T_fR control beads (Figure 6, lane 3). The bound fraction of the Lac-agarose precipitation revealed three predominant polypeptides (Figure 6, lane 2): (a) p50 (50 kDa); (b) galectin-3 (29 kDa); and (c) galectin-1 (14 kDa). The identities and analyses of these polypeptides will be addressed in the next chapter. The bound fraction of the anti-Sm beads yielded many bands, including polypeptides that correspond in molecular weight to the A-G polypeptides of snRNPs (Figure 6, lane 4). Finally, the anti-Mac 2 precipitate yielded a prominent polypeptide

Figure 6: Immunoprecipitation under high ionic strength of HeLa cell splicing extract by autoimmune anti-Sm and by monoclonal anti-Mac2.

Splicing extracts were prepared from HeLa cells metabolically labeled with [^{35}S]methionine. The extracts were subjected to affinity adsorption or immunoprecipitation in buffer C and the polypeptides in the bound fractions were resolved by SDS-PAGE (12.5% acrylamide), followed by fluorography. The samples in each lane were: (1) bound fraction of "pre-clearing" Sepharose; (2) bound fraction of Lac-agarose; (3) rat anti-T_fR precipitate; (4) human anti-Sm precipitate; and (5) rat anti-Mac 2 precipitate. The positions of migration of the snRNP-associated proteins A-G are highlighted on the left and the positions of migration of galectin-1 and galectin-3 are highlighted on the right.


1
2
3
4
5
A-
B-
C-
D-
E/F/G-
Galectin-3
Galectin-1

corresponding

bands, also ob

importantly, th

D) (Figure 6, l

of Sm B with

Analy

Sm B, normal

components ca

prepared from

polyacrylamid

revealed by au

tRNAs and U

probably corr

(labeled as M

correspondin

the basis of t

lane 4). The

decrease in t

band M, and

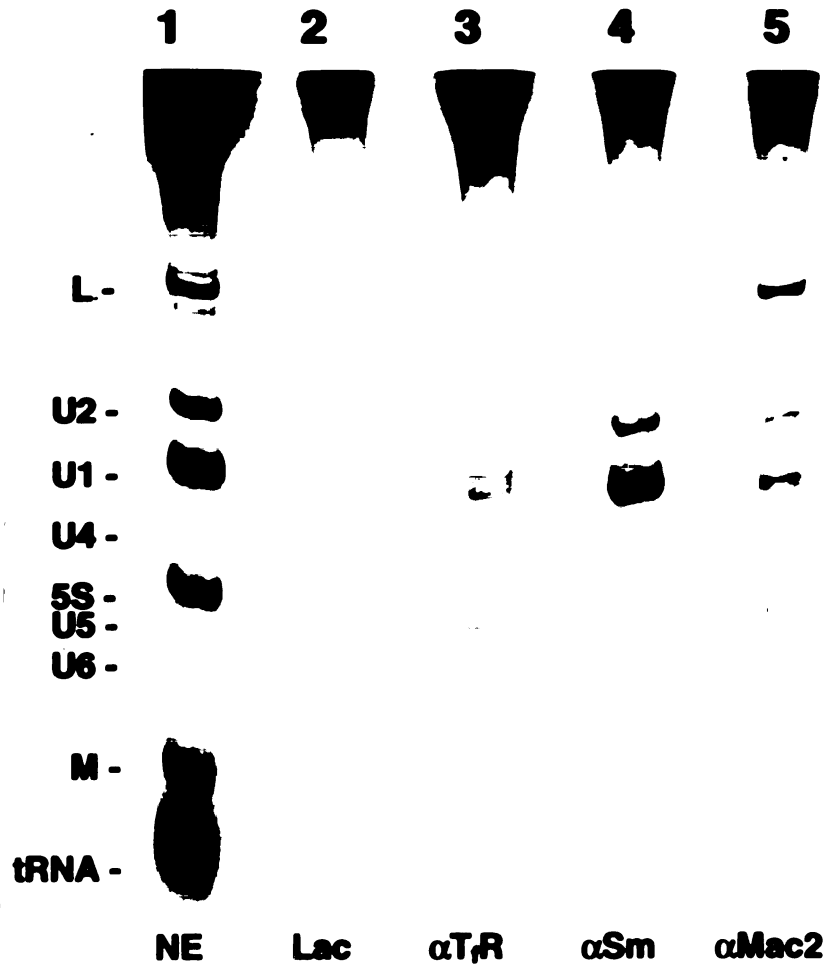
adsorption (

corresponding to galectin-3 (Figure 6, lane 5), as well as certain higher molecular weight bands, also observed as non-specific adsorptions to control beads (Figure 6, lane 3). More importantly, there was little intensity corresponding to a radioactive band for Sm B (or Sm D) (Figure 6, lane 5). These results indicate that the coimmunoprecipitation by anti-Mac 2 of Sm B with galectin-3 was sensitive to high ionic strength.

Analysis of the Anti-Mac 2 Precipitate for RNA. The coimmunoprecipitation of Sm B, normally found as snRNPs, by anti-Mac 2 prompted the question of whether RNA components can also be identified in the immunoprecipitates. Thus, nuclear extracts were prepared from HeLa cells that had been labeled with $^{32}PO_4$. When resolved on polyacrylamide-urea gels, the following low molecular weight RNA components were revealed by autoradiography (Figure 7, lane 1): (a) two prominent bands corresponding to tRNAs and U1 snRNA; (b) three bands of intermediate abundance, two of which most probably correspond to 5S rRNA and U2 snRNA while the third remains unidentified (labeled as M); (c) a band toward the top of the gel, labeled L; and (d) minor species corresponding to U4, U5, and U6 snRNAs. Some of these components were identified on the basis of the ^{32}P-labeled RNA components observed in the anti-Sm precipitate (Figure 7, lane 4). There were intense bands for U2 and U1 snRNAs, while there was a significant decrease in the intensity (relative to unfractionated nuclear extracts of band L, 5S rRNA, band M, and tRNAs. None of the RNA bands are enhanced in the bound fraction of the Lac adsorption (Figure 7, lane 2). This was not surprising inasmuch as Lac-agarose bound

Figure 7: Analysis of the RNA components when HeLa cell splicing extracts are immunoprecipitated by autoimmune anti-Sm and by monoclonal anti-Mac 2.

Splicing extracts were prepared from HeLa cells labeled with $^{32}PO_4$. The extracts were subjected to affinity adsorption or immunoprecipitation in buffer D and the RNA components in the bound fractions were resolved by polyacrylamide-urea gel (8% acrylamide, 8.3 M urea), followed by autoradiography. (1) HeLa cell splicing extract; (2) bound fraction of the Lac-agarose; (3) rat anti-T_fR precipitate; (4) human anti-Sm precipitate; and (5) rat anti-Mac 2 precipitate. The positions of migration of U1, U2, U3, U4, and U6 snRNAs, 5S rRNA, and tRNAs are indicated on the left. In addition, two bands corresponding unidentified RNA species are also highlighted as band L and band M.


1
2
3
4
5
L -
U2 -
U1 -
U4 -
5S -
U5 -
U6 -
M -
tRNA -
NE
Lac
αT,R
αSm
αMac2

galectin-3 but did not coprecipitate Sm components (Figure 3b, lane 2). Similar results were also observed in the bound fraction of the control anti-T_fR precipitate (Figure 7, lane 3). Thus, the faint bands observed in the bound fractions of the Lac-agarose and anti-T_fR precipitates most probably reflect nonspecific adsorptions of the snRNAs components on the respective beads. Note that the nonspecific binding was most pronounced for the U1 snRNA and 5S rRNA; there were drastic reductions in the intensities of band L, U2 snRNA, band M, and, in particular, tRNAs

When the bound fraction of the anti-Mac 2 precipitate (Figure 7, lane 5) was compared to the negative control anti-T_fR (Figure 7, lane 3) and to the positive control anti-Sm (Figure 7, lane 4), two bands were strikingly noteworthy: (a) The band labeled L was almost as intense as that seen in the original nuclear extract, certainly enhanced over the background seen in the other lanes (Figure 7, lane 5 versus lanes 2 and 3); and (b) The band corresponding to U2 snRNA was also apparently enriched, relative to background controls and comparable to the positive control in anti-Sm (Figure 7, lane 5 versus lane 4). The intensities of the U1 snRNA, 5S rRNA, and tRNAs were comparable to those found in the other precipitates. Thus, it appears that the anti-Mac 2 precipitates preferentially enhanced U2 snRNA and band L. The identity of band L remains to be determined; on the basis of size markers, it is estimated that the position of migration of band L corresponds to a nucleotide of approximately 300 base pairs.

To test whether the coprecipitation of Sm B by anti-Mac 2 was dependent on the presence of RNA, nuclear extracts were treated with RNase (100 μg/ml, 1 hour at 37 °C)

and then subjected to immunoprecipitation by anti-Sm and anti-Mac 2. RNase-treated nuclear extract immunoprecipitated by anti-Sm yielded Sm B and Sm D on anti-Sm blots (Figure 8b, lane 2), but no observable galectin-3 (Figure 3a, lane 2) as described previously. More importantly, the results showed that even after RNase treatment, anti-Mac 2 can immunoprecipitate both galectin-3 (Figure 8a, lane 3), as well as Sm B (Figure 8b, lane 3).

Figure 8: The effect of RNase treatment on the immunoprecipitation of Sm B by anti-Mac 2.
Splicing extracts were prepared from HeLa cells in buffer D and the extracts were treated with RNase (100 µg/ml, 30 minutes, 37 °C). The RNase digested extracts were then subjected to immunoprecipitation with ENA anti-Sm or with rat anti-Mac 2 and the polypeptides in the bound fractions were resolved by SDS-PAGE (12.5% acrylamide), followed by immunoblotting. (a) Immunoblotting with rabbit anti-galectin-3; (b)Immunoblotting with ENA anti-Sm. In panels (a) and (b), samples in each lane were: (1) nuclear splicing extract; (2) human anti-Sm precipitate of RNase-treated extract; and (3) rat anti-Mac 2 precipitate of RNase-treated extracted. The positions of migration of galectin-3 and Sm B are highlighted.


1 2 3
galectin-3 -
1 2 3
- B'/B
a
b

DISCUSSION

The experiments presented in this study document the following key points:(a) When nuclear extracts are subjected to immunoprecipitation with anti-Mac 2, a monoclonal antibody specific for galectin-3, a fraction of the snRNP B proteins are specifically coprecipitated with the lectin; (b) RNA components, one tentatively identified as U2 snRNA and another of approximately 300 nucleotides, are also found in the anti-Mac 2 precipitate; and (c) The coimmunoprecipitation is dependent on the presence of galectin-3 but is perturbed neither by Lac/ligand-binding nor by prior RNase treatment of the extract. There are several peculiar features of the observed coimmunoprecipitation that require discussion.

First, the coimmunoprecipitation does not appear to be reciprocal. Although anti-Mac 2 always coprecipitates some Sm B with galectin-3 from nuclear extracts of both human HeLa cells and mouse 3T3 fibroblasts, the lectin is not detected in the anti-Sm precipitates of the same extracts. One possible explanation is that anti-Mac 2 precipitates only those snRNPs bearing galectin-3 and this population represents a very minor fraction of the total nuclear snRNPs. Thus, when anti-Sm precipitates snRNPs, the lectin coprecipitated would constitute a barely detectable portion of the polypeptides present in the precipitate. An alternative explanation is that the binding of galectin-3 to Sm B and the binding of the particular autoimmune serum (ENA anti-Sm) are mutually exclusive. On this basis, the immunoprecipitation of Sm B by anti-Sm releases galectin-3 from the

complex and thus precludes the lectin from being detected in the precipitate fraction. Finally, we note that there is a minor polypeptide that is reproducibly immunoprecipitated by anti-Sm and subsequently immunoblotted by polyclonal anti-galectin-3. Although the mobility (molecular weight) of this band X is close to that of galectin-3, it is nevertheless distinct from the major population of galectin-3 molecules. It is possible that this represents a post-translationally modified form of galectin-3 and only this form of the lectin can associate with the snRNP complexes.

Second, it is interesting to note that although the presence of Lac in the nuclear extract does not inhibit the coprecipitation by anti-Mac 2 of Sm B with galectin-3, passing the nuclear extract over a Lac-agarose column resulted in the adsorption of only of galectin-3. No Sm B could be detected in the bound fraction of the Lac affinity beads. One possible explanation of these observations may be the different effects of univalent ligands versus multivalent ligands on the conformation of galectin-3. The binding of saccharides such as Lac to galectin-3 results in a conformational change, as detected by an increase in the transition temperature of thermal denaturation (8) and by alterations in the intrinsic fluorescence spectrum (16). This structural change does not result, however, in self-association (17,18). On the other hand, when galectin-3 is presented with a multivalent ligand (e.g. polylactosamine on laminin (19) or oligosaccharides on immunoglobulin E (17)), the resulting conformational change is sufficient to expose sites for protein-protein interactions such that there is self-association of the galectin-3 molecules and cooperative binding to the multivalent carbohydrates (17, 19). In the present context, the binding of

galectin-3 to the multivalent Lac-agarose beads would result in self-association to the exclusion of protein-protein interactions such as with Sm B. An alternative explanation is that a minor fraction of posttranslationally modified galectin-3 molecules (represented by band X discussed above) is bound to Sm B and this complex, at low ionic strength conditions of buffer D, binds neither free Lac nor Lac-agarose. At high ionic strength (buffer C), however, the complex is dissociated and thus Lac-agarose can still deplete all the galectin-3 molecules, including band X. The depleted extract no longer exhibits coimmunoprecipitation of Sm B by anti-Mac 2 (this work) or splicing activity (2).

The analysis of these various possibilities would be greatly facilitated by the rigorous identification and characterization of the RNA species in the anti-Mac 2 precipitate. For example, we could attempt to isolate the particular RNP fraction containing both galectin-3 and Sm B using antisense oligonucleotides rather than by anti-Sm immunoprecipitation, thus obviating difficulties associated with dealing with a majority of RNP complexes that do not bear galectin-3. Unfortunately, the identification of the RNA species in the immunoprecipitated fraction has been less than definitive. Anti-Mac 2 reproducibly enriches band L and a band matching in mobility to that of U2 snRNA. For example, U1 snRNA and tRNA are the two major RNA species found in unfractionated nuclear extract. In the anti-Mac 2 precipitate, however, the intensities of band L and U2 snRNA are equal or greater than these RNA species. The identity of band L is not known. Its position of migration indicates that it contains approximately 300 nucleotides, thus precluding it from being one of the minor snRNAs. (The minor snRNAs, U7 through U14,

range in size from 60 to 150 nucleotides (20)). The estimate of 300 nucleotides for band L puts it in the range of the 7S small RNAs, 7SL (303 nucleotides) and 7SK (330 nucleotides) (21, 22). The 7SL RNA has been identified as the RNA component of signal recognition particle, which functions in protein translocation across and integration into the membrane of the endoplasmic reticulum (23). Although this function associates the RNP with polysomes or microsomal membranes of the cytoplasm, it should be noted that the 7SL RNA has been detected in nuclear fractions (13, 24). In fact, in the original identification of snRNAs by Lerner and Steitz (13), autoimmune sera precipitation of U1, U2, U4, U5 and U6 snRNAs also included a RNA species with a mobility of 7S RNA. The significance of the presence of 7S/7SL RNA in the nuclear fraction is not known; it may simply represent newly transcribed RNA species on their way out of the nucleus.

In any case, the immunoprecipitation of Sm by anti-Mac 2, while stringently dependent on the presence of galectin-3, does not appear to be affected by RNase treatment of the nuclear extract. The results suggest that the interaction of Sm B with galectin-3 involves direct or indirect protein-protein interactions. This would be consistent with the notion that neither Sm B (25) nor galectin-3 (26) binds to RNA directly.

In previous studies (2), it was shown that when nuclear extracts were depleted of galectins by Lac-agarose adsorption, the formation of virtually all of the ATP-dependent splicing complexes was inhibited. The depleted extracts, when reconstituted with recombinant galectin-3, regained the ability to form higher order complexes by the addition of the snRNPs. The present identification of an association between galectin-3 and Sm B

(and possibly U2 snRNP) suggests that the role of the complex may be to organize the ATP-independent H complex for accepting the snRNPs.

REFERENCES

1. Barondes, S.H., Cooper, D.N.W., Gitt, M.A., and Leffler, H. 1994. J. Biol. Chem. 269, 20807-20810.
2. Dagher, S.F., Wang, J.L., and Patterson, R.J. 1995. Proc. Natl. Acad. Sci. USA 92, 1213-1217.
3. Laing, J.G. and Wang, J.L. 1988. Biochemistry 27, 5329-5334.
4. Jia, S. and Wang, J.L. 1988. J. Biol. Chem. 263, 6009-6011.
5. Moutsatsos, I.K., Wade, M., Schindler, M. and Wang, J.L. 1987. Proc. Natl. Acad. Sci. USA 84, 6452-6456.
6. Ho, M.K. and Springer, T.A. 1982. J. Immunol. 128, 1221-1228.
7. Cherayil, B.J., Weiner, S.J. and Pillai, S. 1989. J. Exp. Med. 170, 1959-1972.
8. Agrwal, N, Sun, Q., Wang, S.-Y., and Wang, J.L. 1993. J. Biol. Chem. 268, 14932-14939.
9. Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. 1981. Nucleic Acids Res. 11, 1475-1489.
10. Laemmli, U.K. 1970. Nature 227, 680-685.
11. Calvet, J. P., and Pederson, T. 1979. J. Mol. Biol. 122, 361-378.
12. Mayrand, S., and Pederson, T. 1983. Mol. Cell. Biol. 3, 161-171.
13. Lerner, M. R., and Steitz, J. A. 1979. Proc. Natl. Acad. Sci. USA 76, 5495-5499.
14. Williams, D, G. Stocks, M. R., Smith, P. R., and Maini, R. N. 1986. Immunol. 58, 495-500

15. Rokeach, L. A., Jannatipour, M., Haselby, J. A., and Hoch, S. O. J. Biol. Chem. 264, 5024-5030.

16. Mehul, B., Bawumia, S., Martin, S. R., and Hughes, R. C. 1994. J. Biol. Chem. 269, 18250-18258.

17. Hsu, D.K., Zuberi, R.I. and Liu, F.T. 1992. J. Biol. Chem. 267, 14167-14174.

18. Wang, J.L., Anandita, Wang, S.-Y., and Agrwal, N. 1993. in Lectins: Biology, Biochemistry and Clinical Biochemistry, Vol. 9 (P. Chakrabarti, J. Basu, and M. Kundu, eds.) M/S Wiley Eastern, Ltd., New Delhi, pp. 89-99.

19. Massa, S.M., Cooper, D.N.W., Leffler, H. and Barondes, S.H. 1993. Biochemistry 32, 260-267.

20. Shanab, G. M., Maxwell, E. S. 1992. Eur. J. Biochem. 206, 391-400.

21. Ullu, E., Murphy, S. and Melli, M. 1982. Cell 29, 195-201.

22. Ullu, E. and Melli, M. 1982. Nucleic Acids Res. 10, 2209-2223.

23. Walter, P. and Blobel, G. 1982. Nature 299, 691-698.

24. Reddy, R., Li, W.-Y., Henning, D., Choi, Y.C., Nohga, K., and Busch, H. 1981. J. Biol. Chem. 256, 8452-8457.

25. Liautard, J.P., Sri-Widala, J., Brunel, C. and Jenaeur, P. 1982. J. Mol. Biol. 162, 623-643.

26. Dagher, S.F. 1994. Ph.D. thesis, Michigan State University

CHAPTER IV

Nuclear and Cytoplasmic Localization of Galectins: Evidence from Laser Scanning Confocal Microscopy

SUMMARY

We have analyzed the composition and identities of the polypeptides bound to lactose agarose when splicing extracts of HeLa cells are subjected to affinity adsorption under conditions used to deplete splicing activity. We found that, in addition to galectin-3, the bound fraction of the lactose-agarose contained at least three other polypeptides. On the basis of immunoblotting, two of these were identified to be actin and galectin-1. The presence of galectin-1 in the nucleus of HeLa cells was confirmed by laser scanning confocal fluorescence microscopy.

INTRODUCTION

The galectins comprise of a family of galactose (Gal)/lactose (Lac)-specific carbohydrate-binding proteins (1). At present, this family consists of eight members, designated galectin-1 through galectin-8 (see Table 2, Chapter I). As implied by their nomenclature, the structures of all the galectins have at least one carbohydrate recognition domain (CRD), with conserved amino acid sequence between members of the family and between homologues of the same member across different species. Thus, galectin-1, -2, -5, and -7 each has one CRD, the prototype of the family (2; see also Figure 1, Chapter I). Galectin-4 and galectin-8 have tandem repeats of two CRDs (see Figure 1, Chapter I). Finally, galectin-3 has two domains: the amino terminal domain is rich in proline and glycine residues, with limited homology to certain polypeptides of the heterogeneous nuclear ribonucleoprotein complex (hnRNP) while the carboxyl terminal domain contains the CRD. Thus, galectin-3 exhibits a chimeric fusion of two distinct domains (2; see also Figure 1, Chapter I).

Previous studies had shown that galectin-3 was localized in the nucleus and cytoplasm (3) and that its level of expression (4) and nuclear localization (5) was dependent on the proliferative state of the cell. On the basis of the limited sequence homology to proteins of the hnRNP (6) and on the basis of experiments showing that the nuclear localization of the protein was ribonuclease sensitive (7), experiments were performed to test for a role of galectin-3 in nuclear pre-mRNA processing. Several key findings were made (8): (a) nuclear extracts capable of carrying out splicing in a cell-free assay contain

galectin-3; (b) nuclear extracts depleted of galectin-3 by affinity adsorption on Lac-agarose become deficient in splicing; (c) the activity of the Lac-agarose depleted extract can be reconstituted by addition of purified recombinant galectin-3; and (d) saccharides that bind galectin-3 with high affinity inhibit the splicing reaction. These results strongly suggest that galectin-3 may be a splicing factor/regulator.

During the course of these studies, however, Sue Dagher and Ron Patterson made another striking observation (9). While Lac-agarose can deplete the splicing activity, a monoclonal antibody specific for galectin-3 (the anti-Mac 2 antibody (10, 11)) failed to do so, despite the fact that >95% of the galectin-3 polypeptide had been removed from the extract. Moreover, the splicing activity of the galectin-3 depleted extract, derived from the anti-Mac 2 adsorption, was still sensitive to inhibition by Lac. This suggests that other Lac-binding proteins may be present in the splicing extract and they can be depleted, along with galectin-3, by Lac-agarose but not by anti-Mac 2. Thus, it seemed important to define the components that are bound by the Lac-agarose column and to test the role of each or combination of these components in terms of the reconstitution of splicing activity.

In the meantime, I had been attempting to test the hypothesis, by coimmunoprecipitation studies reported in Chapter III, that galectin-3 interacted with components of the spliceosome machinery. In comparing the polypeptides found in the bound fractions of anti-Mac 2, anti-T_fR, anti-Sm, and Lac-agarose precipitations (see for example, Figure 6, Chapter III), it became apparent that at least two other polypeptides, besides galectin-3, was adsorbed by the Lac affinity resin. Thus, it seemed possible to provide an explanation to Sue Dagher's observations (9) if we could definitively identify

these polypeptides as Lac-binding proteins and document their nuclear localization. The results of these studies are now reported in this chapter.

MATERIALS AND METHODS

Antibodies and Affinity columns. Polyclonal rabbit anti-galectin-3 antiserum was obtained from rabbit #33 (10). Polyclonal rabbit anti-galectin-1 antibody was a gift of Dr. S. H. Barondes (University of California, San Francisco). Rabbit antiserum against lactose dehydrogenase (LDH) was a gift from Dr. John Wilson (Michigan State University). The derivatization and characterization of rabbit anti-calf thymus actin have been reported (11, 12). ENA anti-Sm antibody was purchased from The Binding Site (San Diego, CA). Lactose immobilized on 6% beaded agarose (Lac-agarose) was purchased from Sigma (St. Louis, Mo). Sepharose CL4B beads was obtained from Pierce (Rockford, IL)

Lac-agarose Affinity Adsorption. Nuclear extract was prepared according to Dignam et. al.(15) as described in Chapter 3. The [^{35}S]methionine labeled nuclear extract (in buffer C, 20 mM HEPES pH 7.9, 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT) was precleared by incubating with Sepharose beads for 1 hour at 4 °C. The unbound fraction of this preclearing step was subjected to Lac-agarose adsorption. Precleared nuclear extract (50 µl) was incubated with 50 µl of Lac-agarose beads (pre-washed with buffer C twice) for 1 hour at 4 °C. The Lac-agarose were washed: (i) twice with buffer 1 (10 mM Tris, pH 7.5, 150 mM NaCl, 0.5% NP-40, 0.2 mM EDTA); (ii) twice with buffer 2 (10 mM Tris, pH 7.5, 500 mM NaCl, 0.5% NP-40, 0.2 mM EDTA); and (iii) twice with buffer 3 (10 mM Tris, pH 7.5). The material remaining bound to the beads were subjected to SDS-PAGE (12.5%

acrylamide) as detailed in Chapter 3 and the proteins were revealed by fluorography and by immunoblotting.

For immunoblotting analysis, rabbit anti-galectin-3, anti-galectin-1 and anti-actin were used at 1:500 dilution. All three primary antibodies were diluted in TBS (10 mM Tris, 0.5 M NaCl, pH 7.5) containing 0.2% gelatin. Horseradish peroxidase conjugated sheep anti-rabbit antibodies (BMB) were used at 1:2,000 dilution in T-TBS (TBS containing 0.05% Tween 20) as secondary antibody. Proteins resolved on a SDS-PAGE were electrophoretically transferred to Immobilon-P (Millipore) membrane as described in Chapter III. The membrane was briefly rinsed twice with T-TBS and blocked in 2% gelatin/TBS for at least 1 hour at room temperature. After briefly washing with T-TBS twice, the membrane was incubated with primary antibody for 1 hour at room temperature. The membrane was then rinsed with T-TBS twice and washed extensively with T-TBS (15 minutes once, then 10 minutes 4 times). The washed membrane was incubated with horseradish peroxidase conjugated secondary antibody at room temperature for 1 hour. The blot was rinsed and washed as described above, and then developed with 4-chloro-1-naphthol.

Immunofluorescence Staining and Laser Scanning Confocal Microscopy.

HeLa cells grown as monolayers were obtained from American Type Culture Collection (Rockville, MD). They were seeded onto coverslips (22 x 22 cm) in 6-well (8 cm^2/well) cluster dishes. The cells were washed twice (4 ml/well) with phosphate-buffered saline (PBS, 0.14 M NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4). Cells were fixed by incubation (20 minutes at room temperature) in 4% paraformaldehyde in PBS

(2 ml/well), followed by washing twice with PBS (10 minutes each, on a rotating platform). The residual aldehyde groups were blocked by 0.1 M glycine in PBS (4 ml/well; 20 minutes at room temperature). After removal of the glycine-PBS solution, cells were permeabilized with 0.5% Triton X-100 in PBS (2 ml/well; 4 minutes at room temperature). The cells were again washed twice with PBS. The cells were then incubated with antiserum (anti-galectin-3, 1:150; anti-galectin-1, 1:150; anti-LDH, 1:150; and ENA anti-Sm, 1:500) for one hour at room temperature. After washing three times in T-TBS (10 mM Tris pH 7.5, 0.5 M NaCl, 0.05% Tween 20) (15 minutes each), the cells were incubated with the secondary antibody for one hour. Fluorescein-conjugated goat anti-rabbit immunoglobulin (BMB) (1:50) was used to detect rabbit anti-galectin-3, rabbit anti-galectin-1, and rabbit anti-LDH binding and fluorescein-conjugated goat anti-human immunoglobulin (BMB) (1:100) was used to detect human autoimmune ENA anti-Sm binding. Finally, the coverslips were washed three times in T-TBS (15 minutes each) before mounting with Perma-Fluor on glass microscope slides.

A Meridian Instruments (Okemos, MI) Insight bilateral laser scanning confocal microscope was used with an argon ion laser as the excitation source. An 100 x objective lens and laser power of 50 mWatts were used for scanning the image. One photomultiplier was used to detect fluorescence emission of fluorescein (530 nm). Images (360 x 360 pixels) were collected from 9 consecutive focal planes as with an increment of 0.5 μm for each step in the *x* and *y* directions (16).

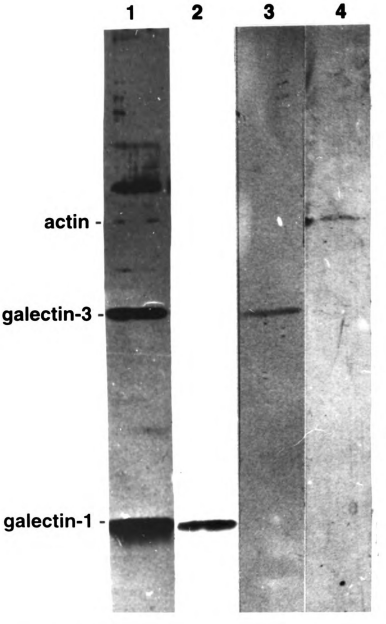
RESULTS

Polypeptides Bound by Lac-agarose. Nuclear extracts were prepared from HeLa cells metabolically labeled with [^{35}S]methionine. The extract was "precleared" by sepharose beads to remove components that would bind nonspecifically to the beads. The unbound fraction of the "preclearing" step was then subjected to binding with Lac-agarose. The conditions for these experiments (buffer C) were identical to those used to deplete splicing extracts of galectins and splicing activity (8, 9). The material bound to the beads was subjected to SDS-PAGE, followed by fluorography and immunoblotting. Five polypeptides were found in the bound fraction of the Lac-agarose adsorption with molecular weights of approximately: (a) 70,000; (b) 50,000; (c) 43,000; (d) 30,000; and (e) 15,000 (Figure 1, lane 1).

Three of these bands have been identified on the basis of immunoblotting analysis. First, a rabbit anti-calf thymus actin antiserum (reactive predominantly with non-muscle actins (13, 14)) immunoblotted the 43 kDa band (Figure 1, lane 4), suggesting it to be actin. This would be consistent with the observation that this polypeptide was also bound to the preclearing sepharose beads in a nonspecific fashion (see, for example, Figure 6, Chapter III). Second, polyclonal rabbit anti-galectin-3 identified the 29 kDa band as galectin-3 (Figure 1, lane 3), as was reported previously (8) that under these conditions (high ionic strength of buffer C), Lac-agarose depleted splicing extracts of galectin-3 with concomitant loss of splicing activity. Finally, the 14 kDa band was identified as galectin-1, on the basis

Figure 1: The composition and identities of polypeptides in the bound fraction of Lac-agarose adsorption.

Nuclear extracts were prepared, following the procedures of Dignam et al. (15), from HeLa cells metabolically labeled with [^{35}S]methionine. The nuclear extracts, in buffer C, were subjected to affinity adsorption on Lac-agarose beads. The bound fraction was then subjected to SDS-PAGE (12.5% acrylamide), followed by fluorography or by immunoblotting with various antibodies. Lane 1: Radioactive polypeptides revealed by fluorography. Lane 2: Immunoblot with anti-galectin-1. Lane 3: Immunoblot with anti-galectin-3. Lane 4: Immunoblot with anti-actin. The positions of migration of authentic protein markers are highlighted on the left.


1
2
3
4
actin -
galectin-3 -
galectin-1 -

of immunoblotting with affinity purified anti-galectin-1 antibodies (Figure 1, lane 2). Thus, it appears that Lac-agarose was indeed depleting more than one Lac-binding protein from the nuclear extract (i.e. galectin-1, in addition to galectin-3).

The identities of the other prominent band (M_r 50,000) and minor band (M_r 70,000) are not known. That the prominent band represents a bona fide Lac-binding protein is suggested by preliminary experiments showing its specific elution from the Lac-agarose beads by the addition of Lac. Neither of these two bands reacted with any of the anti-galectin antibodies available to us, including an antiserum against rat intestinal galectin-4.

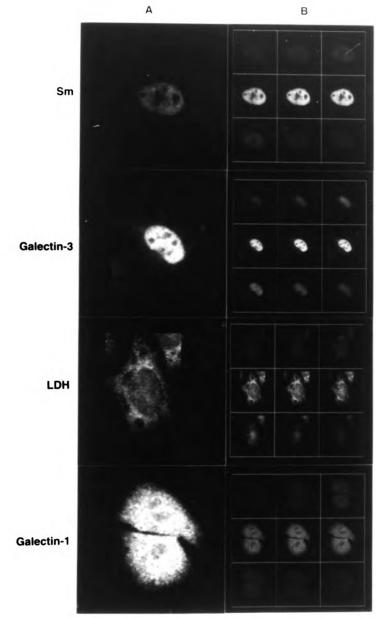
Nuclear and Cytoplasmic Localization of Galectin-1: Evidence from Laser Scanning Confocal Microscopy. The identification of galectin-1 in nuclear extracts of HeLa cells and its adsorption onto Lac affinity resins provided an explanation to the observation that while Lac-agarose can deplete the splicing activity, anti-Mac 2, a monoclonal antibody specifically reactive with galectin-3, failed to do so. Coupled with the demonstration that either galectin-1 or galectin-3 alone is sufficient to reconstitute splicing activity in a Lac-agarose depleted extract (9), the results would suggest that the activities of galectin-1 and galectin-3 in the nucleus may be redundant. On the other hand, however, the notion that galectin-1 can be found in the nucleus is controversial, particularly since, in the original publication reporting the purification of the chicken homologue, there was an explicit statement indicating that anti-chicken heart galectin-1 failed to label the cell nucleus of chicken embryo fibroblasts under immunofluorescence (17). The main difficulty in the analysis of galectin-1 is that, unlike galectin-3, the predominant portion of the former lectin is found in the cytoplasm. As a result, a strong fluorescent antibody probe staining the

cytoplasm surrounding the nucleus sometimes results in an image containing fluorescent flare that exceeds the resolution of the light microscope. To circumvent this difficulty, we took advantage of the availability of a laser scanning confocal microscope to analyze the staining pattern obtained with anti-galectin-1.

HeLa cells cultured on coverslips were fixed with paraformaldehyde, permeabilized with Triton X-100 and stained with various antibodies plus fluorescein-conjugated second antibody. The fluorescence staining was visualized by laser confocal microscopy, collecting images through 9 consecutive focal planes. In this study, ENA anti-Sm was used as a positive control for nuclear staining (Figure 2). In a plane above the cell nucleus, there was little fluorescence. Through the middle sections, the plane of focus cuts through the nucleus and yielded intense staining (Figure 2, column B). One of the middle sections is enlarged in column A (Figure 2), showing diffuse distribution of fluorescence throughout the nucleus, except for the "black holes" (see Figure 2, Chapter 2). Similarly, anti-galectin-3 yielded, through the middle sections, bright nuclear staining with the exception of the "black holes." Thus, the subcellular distribution of galectin-3 and the Sm epitopes of snRNPs are very similar, a conclusion documented in Chapter II. Staining for LDH, serving as a negative control, showed that the enzyme was predominantly cytoplasmic. With the enlarged middle section (Figure 2, column A), the anti-LDH yielded a distinct pattern, showing that the majority of fluorescence in the cytoplasm.

The staining for galectin-1 yielded sections that resembled neither those obtained with anti-Sm or anti-galectin-3 nor that seen with anti-LDH. Due to high levels of the lectin

Figure 2: The subcellular localization of galectin-1 as revealed by laser scanning confocal fluorescence microscopy.
The staining of snRNP Sm polypeptides and galectin-3 and the staining of LDH provided reference patterns for nuclear and cytoplasmic localizations, respectively. For each staining, images (360 x 360 pixels) were collected from 9 consecutive sections focal planes, with the increment of 0.5 μm for each step in the x and y directions. They are displayed serially in column B; column A provides an enlarged view of one middle section (#5).


A
B
Sm
Galectin-3
LDH
Galectin-1

in the cytoplasm, the middle sections yielded generally diffuse fluorescence throughout both the cytoplasm and the nucleus. The areas devoid of fluorescence are not nuclei; rather, they correspond to the "black holes" observed with anti-Sm or anti-galectin-3 staining.

DISCUSSION

The previous observations on the difference in the ability of Lac-agarose versus anti-Mac 2 to deplete splicing activity from nuclear extracts of HeLa cells suggested: (a) in addition to galectin-3, there must be other Lac-binding proteins in splicing competent extracts; (b) depletion of a single lectin from such an extract cannot remove the splicing activity; and (c) the splicing activity is lost only when all the Lac-binding proteins are adsorbed on the Lac-agarose resin. Coupled with the demonstration that either galectin-1 or galectin-3 can reconstitute the splicing activity in a depleted extract (9), these results suggest that there may be multiple members of the galectin family in the nucleus and that their activities may be redundant. Indeed, the results of this series of experiments have identified at least galectin-1 to be present, in addition to galectin-3, in the bound fraction of the Lac-agarose resin when nuclear extracts are depleted of splicing activity. The localization of galectin-1 to the nucleus was also confirmed by confocal immunofluorescence microscopy.

Previous comparisons of the immunofluorescence staining of live cells versus cells fixed with formaldehyde followed by permeabilization indicate that galectin-1 is found predominantly in the intracellular compartment. Within the cells, the staining is mostly cytoplasmic. Observations and interpretations pertaining to the nuclear localization of galectin-1 are somewhat more difficult to establish.

First, immunofluorescence and ultrastructural studies have led to explicit statements that anti-galectin-1 antibodies failed to label the cell nucleus. In chicken embryo

fibroblasts, anti-chicken heart lectin (chicken galectin-1) staining was described to be localized only to the cytoplasm (17). When 17-day-old chicken embryonic keratinized epidermis was stained with gold-labeled anti-galectin-1 under electron microscopy, gold particles were found around desmosomes, tonafilament bundles, and the intercellular space, while the cell nucleus was free of the particles (18).

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 regarding the intracellular distribution of galectin-1. Dorsal root ganglion neurons were subjected to immunofluorescence with anti-galectin-1 and with a monoclonal antibody directed against a lactoseries glycoconjugate (19). Although the intracellular distribution of the lectin was not discussed in that particular report, the same investigators have more recently concluded, on the basis of the previously published data, that galectin-1 could be detected in both the nucleus and cytoplasm of the neurons (20). Similarly, reassessment of previously published immunocytochemical studies in non-neuronal cells has suggested the presence of rat galectin-1 in nuclei and cytoplasm (21).

Finally, there are studies that specifically show the localization of galectin-1 in the nucleus as well as the cytoplasm. Cryostat sections of tissues subjected to immunofluorescence showed labeling of both nuclei and cytoplasm in anti-chicken galectin-1 staining of adult chicken kidney (22) and anti-bovine galectin-1 staining of calf pancreas (23). A monoclonal antibody, designated 36/8, was generated against bovine heart galectin-1 and stained the nuclei and cytoplasm of lymphoblastoid and leukemic cells (24). This monoclonal antibody immunoblotted polypeptides of apparent molecular mass 13, 36, 65,

80, and 130 kDa 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 (25) and, therefore, it is not clear whether the nuclear staining component is a galectin or some other polypeptide bearing this epitope sequence. At the ultrastructural level, it has been reported that anti-galectin-1 labels the nucleus of epidermal cells of the intermediate layer of a stratified epithelium, the chick embryonic skin (26).

On the basis of these previous reports, it seems apparent that the localization of galectin-1 has been somewhat controversial. It was important to establish, therefore, that galectin-1 can indeed be found in the cell nucleus. The criteria used in the present study consisted of parallel labeling studies with antibody reagents directed against known nuclear components (e.g. anti-Sm) and an enzyme generally accepted as a cytosolic marker (e.g. anti-LDH). The staining patterns obtained with anti-galectin-1 under confocal immunofluorescence analysis resembled neither anti-Sm nor anti-LDH. Rather, the anti-galectin-1 staining represented a composite of the labeling patterns of nuclear and cytoplasmic controls, suggesting that the lectin is found in both compartments of the cell.

REFERENCES

1. Barondes, S. H., Cooper, D. N. W., Gitt, M. A., and Leffler, H. 1994. J. Biol. Chem. 269, 20807-20810.
2. Hirabayashi, J. and Kasai, K. 1993. Glycobiology 3, 297-304.
3. Moutsatsos, I. K., Davis, J. M. and Wang, J. L. 1986. J. Cell Biol. 102, 477-483.
4. Agrwal, N., Wang, J. L. and Voss, P. G. 1989. J. Biol. Chem. 246, 17236-17242.
5. Moutsatsos, I. K., Wade, M., Schindler, M. and Wang, J. L. 1987. Proc. Natl. Acad. Sci. USA 84, 6452-6456.
6. Jia, S. and Wang, J. L. 1988. J. Biol. Chem. 263, 6009-6011.
7. Laing, J. G. and Wang, J. L. 1988. Biochemistry 27, 5329-5334.
8. Dagher, S. F., Wang, J. L. and Patterson, R. J. 1995. Proc. Natl. Acad. Sci. USA 92, 1213-1217.
9. Dagher, S. F. 1994. Ph.D. thesis, Michigan State University.
10. Ho, M. K. and Springer, T. A. 1982. J. Immunol. 128, 1221-1228.
11. Cherayil, B. J., Weiner, S. J., and Pillai, S. 1989. J. Exp. Med. 170, 1959-1972.
12. Agrwal, N., Sun, Q., Wang, S. Y., and Wang, J. L., 1993. J. Biol. Chem. 268, 14932-14939.
13. Metcalf, T. N., Szabo, L. J., Schubert, K. R., and Wang, J. L. 1984. Protoplasma 120, 91-99.
14. Villanueva, M. A., Ho, S. -C. and Wang, J. L. 1990. Arch. Biochem. Biophys. 277, 35-41.

15. Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. 1981. Nucleic Acids Res. 11, 1475-1489.

16. Wade, M. H., Defeijter, A. W., Frame, M. K., and Schindler, M. 1993. Bioanlyt. Instrumen. 37, 117-141.

17. Barak-Briles, E., Gregory, W., Fletcher, P. and Kornfeld, S. 1979. J. Cell Biol. 81, 528-537.

18. Hirano, H., Akimoto, Y., Kawakami, H., Oda, Y. and Kasai, K. 1988. in Proceedings of the Kagoshima International Symposium of Glycoconjugates in Medicine, Professional Postgraduate Service International, Tokyo, pp. 8-13.

19. Regan, L. J., Dodd, J., Barondes, S. H., and Jessell, T. M. 1986. Proc. Natl. Acad. Sci. USA 83, 2248-2252.

20. Hynes, M. A., Gitt, M., Barondes, S. H., Jessell, T. M. and Buck, L. B. 1990. J. Neurosci. 10, 1004-1013.

21. Barondes, S. H. 1988. Trends Biochem. Sci. 13, 480-482.

22. Beyer, E. C. and Barondes, S. H. 1980. J. Supramol. Struct. 13, 219-227.

23. Childs, R. A. and Feizi, T. 1980. Cell Biol. Int. Rep. 4, 775.

24. Carding, S. R., Thorpe, S. J., Thorpe, R. and Feizi, T. 1985. Biochem. Biophys. Res. Commun. 127, 680-686.

25. Abbott, W. M., Mellor, A., Edwards, Y. and Feizi, T. 1989. Biochem. J. 259, 283-290.

26. Akimoto, Y., Kawakami, H., Oda, Y., Obinata, A., Endo, H., Kasai, K., and Hirano, H. 1992. Exp. Cell Res. 199, 297-304.

CHAPTER V

Concluding Statement

Removal of intron and ligation of exons is one of the most important steps for pre-mRNA processing. Many catalytic elements have been elucidated for their involvement in splicing machinery. Previous studies have shown that galectin-3 is a required factor for spliceosome formation and splicing activity using a cell-free assay for pre-mRNA splicing. However, the subnuclear localization and the RNP components which galectin-3 is involved in pre-mRNA splicing remain unclear.

To address these questions, I have developed two levels of study to demonstrate that galectin-3 is associated with components of the splicing machinery, specifically, the Sm epitopes found on polypeptides of the snRNPs. At the single cell level, the immunofluorescence patterns of galectin-3 were shown to be similar to that of the snRNP Sm polypeptides during the course of cell cycle and under a variety of conditions. Double labeling experiments also demonstrated that galectin-3 colocalized with Sm antigens in the cell nucleus. At the biochemical level of protein-protein interaction, I have shown that a monoclonal antibody anti-Mac 2, which is specific for galectin-3, could coimmunoprecipitate a fraction of Sm B polypeptides from nuclear extracts of HeLa cells and mouse 3T3 fibroblasts. Nuclear extracts depleted of galectin-3 by prior adsorption on a lactose affinity resin failed to yield Sm B in the anti-Mac 2 precipitate. These results demonstrate that the coprecipitation of Sm B with galectin-3 by anti-Mac 2 was dependent

demonstrate that the coprecipitation of Sm B with galectin-3 by anti-Mac 2 was dependent on the presence of the lectin. The coprecipitation was not affected by the addition of lactose; nor was it perturbed by prior treatment of the nuclear extract with ribonuclease. Taken together, these results suggest that at least a fraction of the Sm B polypeptides in the splicing extract can interact with galectin-3 through protein-protein interactions. Identification of the RNP component with which galectin-3 is associated should be the next level of study to address the function of galectin-3 in the splicing machinery.

I have also analyzed the composition and identities of polypeptides bound to the affinity resin lactose-agarose under conditions used to deplete the nuclear extract of splicing activity. In addition to galectin-3, the bound fraction of the lactose-agarose also contains galectin-1, whose presence in the nucleus of HeLa cells was confirmed by confocal fluorescence microscopy. These findings provide an explanation for previous observations that while lactose-agarose can deplete nuclear extracts of splicing activity, anti-Mac 2 precipitation failed to yield the same effect. Together with the observation that galectin-1, as well as galectin-3, can alone reconstitute splicing activity in a lactose-agarose depleted extract, these results suggest that the activities of galectin-1 and galectin-3 are redundant in the cell nucleus.