The role of cytoskeleton-associated RNA-binding proteins in mRNA sorting during seed development in rice (*Oryza sativa L.*)

By

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The role of cytoskeleton-associated RNA-binding proteins in mRNA sorting during seed development in rice (*Oryza sativa L.*)

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By

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in memory of my teachers

Prof. J. Jayaraman

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R. Sami Subbu

DECLARATION

Certified that the work incorporated in this thesis entitled **"The role of cytoskeleton-associated RNA-binding proteins in mRNA sorting during seed development in rice (*Oryza sativa L.*)",** submitted by Mr. R. Sami Subbu was carried out under my supervision. The material obtained from other sources has been duly acknowledged in the thesis.

Vidya S. Gupta

Research supervisor

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LIST OF ABBREVIATIONS

bp	base pairs
mg	milligrams
µg	micrograms
ml	milliliters
µl	microliters
fmole	femto moles
U	units of enzyme
DTT	dithiothreitol
RPM	revolutions per minute
GTP	guanosine 5'-triphosphate
ATP	adenosine 5'-triphosphate
UTP	uridine 5'-triphosphate
CTP	cytidine 5'-triphospahte
SDS	sodium dodecyl sulphate
PAGE	polyacrylamide gel electrophoresis
Tris	tris-hydroxylmethyl amino methane
EDTA	ethylene diamine tetra acetic acid
UTR	untranslated region
MBP	myelin basic protein
GFP	green fluorescent protein
RBP	RNA binding protein
RRM	RNA recognition motif
RNP	ribonucleo protein
KH	hnRNAP K homolog
ER	endoplasmic reticulum
PB	protein body
c-ER	cisternal endoplasmic reticulum
PB-ER	protein body endoplasmic reticulum
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
UV	ultraviolet
HCA	hydrophobic cluster analysis
DAF	days after flowering
TCA	trichloroacetic acid
PMSF	phenylmethylsulfonyl fluoride
HRP	horseradish peroxidase
TBS	tris buffered saline
TBST	tris buffered saline triton
IPTG	isopropyl-β-D-thiogalactopyranoside

Chapter I

Cytoplasmic mRNA localization in eukaryotic cells

In an eukaryotic cell, synthesis and processing of macro molecules at appropriate place are ensured by a well-developed compartmental system. Transcription takes place in the nucleus and resulting RNA undergoes splicing process to yield mRNA. Processed mRNA is exported from the nucleus into the cytosol where it is translated either as a free or membrane bound polysomes. The cytoplasm crowded with sugars, amino acids, lipids, proteins, nucleic acids and other metabolites makes it difficult to passively diffuse the mRNA to its site of translation. Therefore, a concept of localized synthesis of protein has been put forth according to which mRNAs are transported to a specific domain of the cytoplasm, localized and are translated in a localization dependent manner (Wilhelm & Vale 1993, Curtis et al., 1995, St Johnston 1995). Fig. 1 depicts the central dogma of RNA transport.

Increasing number of evidences clearly indicate that mRNAs are localized at specific sites in the cytoplasm for efficient cellular processes. It is economically feasible for the cell to translate the transcripts at a site where the protein is actually required. It becomes easy for the system to control the translation of a specific transcript, when the translation is localized at a distinct region of the cytoplasm. Moreover, localization dependent translation enables translation of a specific transcript at appropriate site of cytoplasm thereby avoiding the translation of unwanted protein at that site. For instance, anterio-posterior axis formation in Drosophila egg cell is decided by the localization of specific set of transcripts at the appropriate poles of the oocytes and any failure in this process leads to abnormal development (St Johnston 1995). Besides, many copies of a protein can be translated from a single RNA molecule and hence local concentration of an appropriate protein can be achieved at the specific segment of the cytoplasm thereby ensuring an efficient post translational modifications. The multicellular organisms have, therefore, developed an efficient cellular mechanism to localize a specific transcript to an appropriate site in the cytoplasm to enable the normal differentiation process.

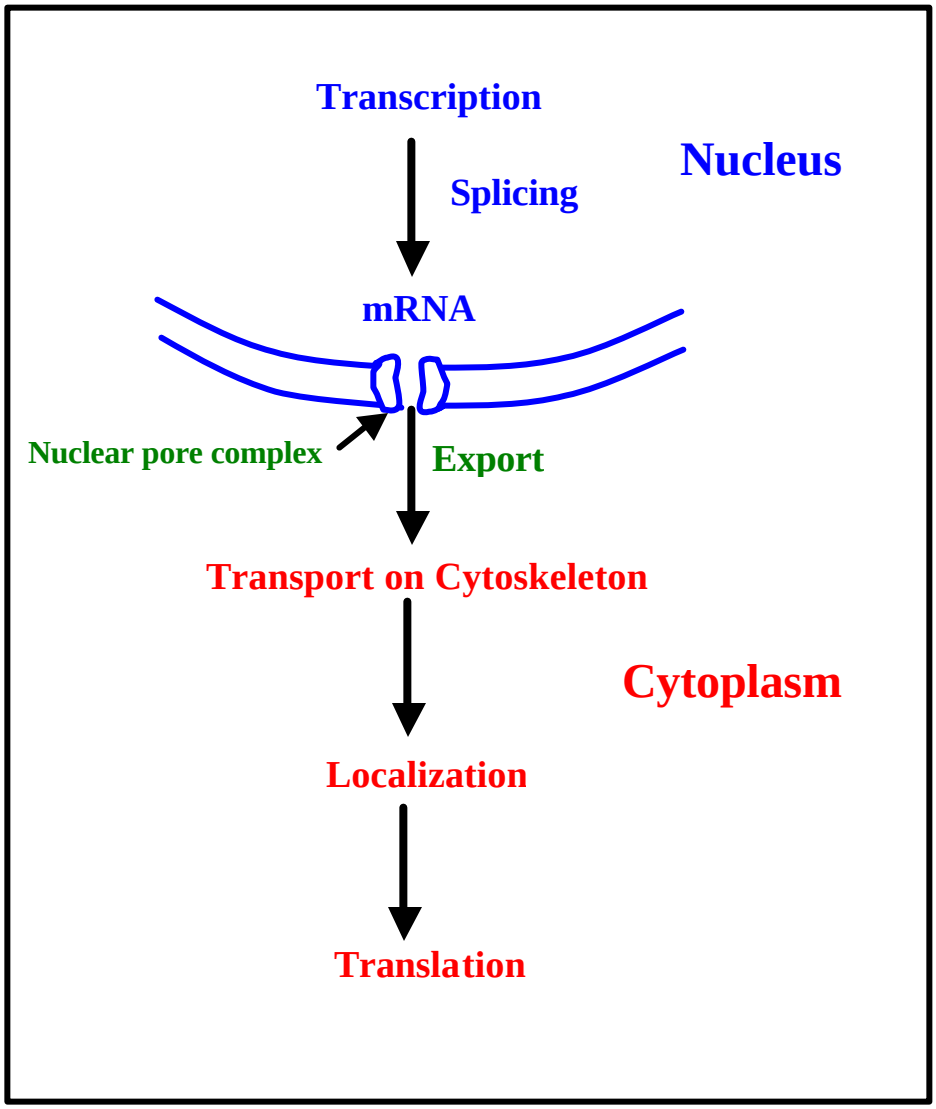

Fig. 1 A diagram showing central dogma of RNA transport

mRNAs are localized at distinct regions of cytoplasm:

Recent findings from animal and plant systems suggest that several mRNAs are localized at discrete sites in the cytoplasm rather than having random distribution (Wharton & Struhl 1989, Gavis & Lehman 1992, Melton 1987, Li et al., 1993, Bouget et al., 1996). One of the best examples of mRNA localization comes from Drosophila maternal mRNAs that control anterior-posterior axis formation. For instance, *bicoid* mRNA is localized at the anterior pole of the oocytes enabling formation of thorax region, whereas *oskar* (*osk*) and *nanos* (*nos*) mRNAs are localized at the posterior pole from where the abdomen differentiates (Driever 1993, Euphrussi et al., 1991, Kim-Ha et al., 1991, Lehmann & Nusslein-Volhardt 1991, Ephrussi & Lehmann 1992). Moreover, in Drosophila, blastoderm embryos are shown to have *fushi tarazu*, *hairy*, *bicoid* and *even-skipped* mRNAs in the apical compartment region (Davis & Ish-Horowicz 1991). Segregation of mRNAs to specific segment of the cytoplasm is not restricted to the fertilized egg cells, rather this seems to be a general cellular mechanism found in somatic cells as well. A diagrammatic representation of localization of various RNA species at different regions of the cytoplasm is shown in Fig. 2.

The best examples of localized transcripts at distinct sectors of the cytoplasm are β-actin mRNA and c-*myc* mRNA (Hesketh et al., 1994, Sundell & Singer 1990, Hill & Gunning 1993). Beta-actin mRNAs are localized at the peripheral cytoplasm. Although their function is not yet clearly understood, it is believed that they are involved in cell enlargement and motility. Alterations in growth hormone seem to affect the β-actin mRNA localization drastically. When myoblast cells were deprived of serum, it led to accumulation of β-actin mRNA around the perinuclear region while addition of serum led to localization of β-actin at peripheral cytoplasm (Latham et al., 1994, Hill et al., 1994). On the other hand, onco gene transcript c-*myc* is localized in the perinuclear cytoplasm (Hesketh et al., 1994). In case of neuronal cells, myelin basic protein (MBP) mRNAs are localized at the extremities of the long cell processes of both *in vivo* and cultured cells (Verity & Campagnoni 1988, Shiota et al., 1989, Barbarese 1991). During a brief period of oogenesis in Xenopus oocytes, Vg1 mRNA and its protein product are localized at the vegetal cortex, which is important for mesoderm induction (Thomsen & Melton 1993).

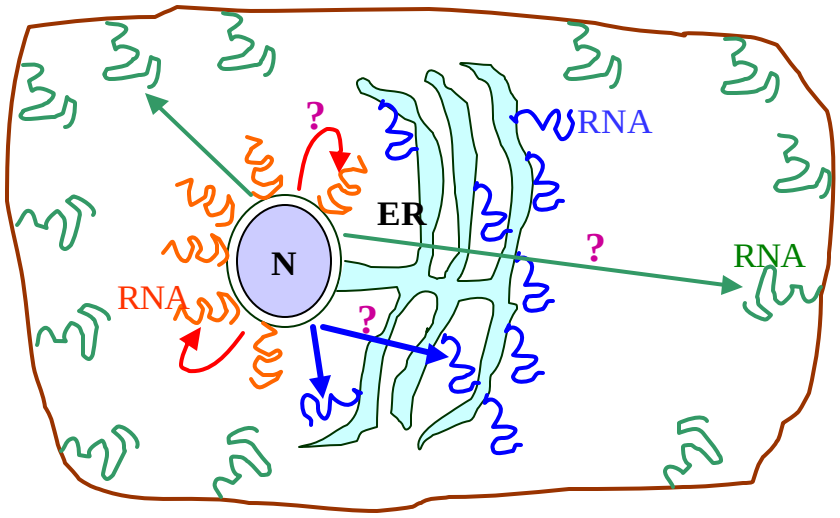

Fig. 2. A diagrammatic representation shows the localization of various RNA species at different regions of cytoplasm. RNAs in blue represent localization on ER membrane, green in pericytoplasmic region and red in perinuclear region. The arrows indicate the possible path of RNA transport.

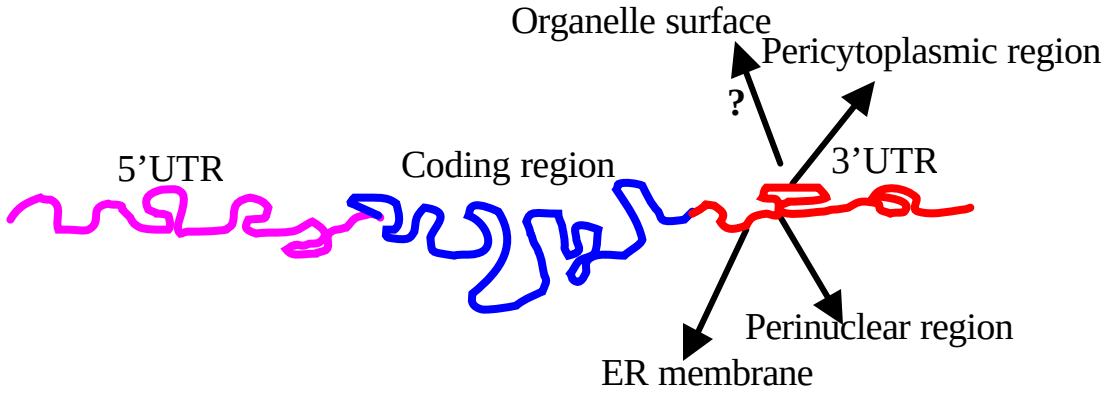

Fig. 3. A diagrammatic representation showing signal at 3'UTR responsible for localization of RNA at different regions of cytoplasm.

One of the few examples of mRNA localization in plants comes from rice endosperm cells where prolamine and glutelin mRNAs are differentially localized on different domains of the ER membrane (Li et al., 1993). *In situ* hybridization of rice endosperm tissue sections and electron microscopic observation revealed that prolamines are seven fold higher than glutelin transcripts on PB-ER fraction while glutelin transcripts are two fold higher than prolamine on cisternal-ER membrane. Further dot blot analysis using poly(A) RNA from PB-ER and c-ER supports the *in situ* localization observation that prolamine transcript dominate the PB-ER membrane whereas glutelin transcripts are more on c-ER membrane.

Signals responsible for mRNA localization:

A cognate signal sequence at the N-terminal region of the protein was proposed to segregate the protein to a specific endomembrane system (Blobel 1980). However, recent findings in mRNA localization studies lead us to postulate binding of a protein at a specific secondary structure of RNA leading to localization at discrete site. Indeed, evidences suggest that a specific region of 3'UTR in MBP mRNA is responsible for its localization at the myelin region of the mouse brain cells (Ainger et al., 1997). When fluorescent labeled truncated RNAs were microinjected into cultured mouse brain cells, it resulted in identification of two distinct regions namely RNA transport signal (RTS) and RNA localization region (RLR). When 3'UTR of MBP RNA was fused with globin coding sequence, it resulted in localization of globin coding sequences in the myelin region of neuronal cells (Ainger et al., 1997). Deletion of RLR while retaining RTS resulted in appearance of fluorescently labeled RNA in the perikaryon and process but was absent in the myelin region indicating that RTS was involved in transport process. RTS when attached to green fluorescent protein (GFP) led to appearance of fluorescent labeled RNA in the processes but not in myelin region. Frame shift mutation in the coding region of MBP did not affect the localization process indicating that the latter was independent of translation. Thus MBP RNA localization process can be clearly defined as a sequential process which includes granule formation, transport along the process and

localization at the myelin region. In case of Xenopus, Xcat-2 RNA is localized at the vegetal cortex of stage II oocyte (Melton 1987). Truncated Xcat-2 RNA when microinjected in oocytes resulted in identification of localization element located in 3'UTR. This finding was confirmed by fusing the 3'UTR of Xcat-2 with luciferase ORF where Xcat-2 3'UTR was sufficient to localize the luciferase at the vegetal region of stage IV oocytes (Zhou & King 1996). Localization signal at the 3'UTR has been diagrammatically shown in fig. 3.

In fibroblast cells, c-*myc* RNA localization was studied by transfecting with appropriate constructs followed by hybridization with labeled antisense RNA. The 3'UTR of c-*myc* when fused with β-globin ORF resulted in detection of β-globin ORF at the perinuclear cytoplasm (Veyrune et al., 1996). While transfection of cells with β-globin RNA without c-*myc* 3'UTR resulted in appearance of signal all over the cytoplasm similar to the results obtained with complete β-globin construct. These results indicate that β-globin does not contain the RNA sequence required for localization to perinuclear cytoplasm. Further deletion within the 3' UTR of c-*myc* mRNA indicated a 86 nucleotide region playing a vital role in localizing the reporter gene. Moreover, attachment of this 86 nucleotides to the β-globin coding sequences led to localization at the perinuclear cytoplasm. On sequence comparison within these 86 bases, a highly conserved sequence motif of AUUUA was found in a class of unstable mRNAs coding for various cytokines and oncoproteins. Mutation of this AUUUA sequence motif failed to localize the reporter gene at perinuclear cytoplasm (Veyrune et al., 1996). It has been documented that alpha and beta actin mRNAs are localized at perinuclear and preipheral cytoplasm, respectively. The 3'UTR of alpha- and beta-actin mRNAs when fused with β-galactosidase gene resulted in detection of β-galactosidase activity in the perinuclear region and in the pericytoplasmic region respectively. However, the 5'UTR did not show any localized activity (Kislauskis et al., 1993, Kislauskis et al., 1994). These findings suggest that distinct regions of mRNA contain signals that are responsible for localization. These sequences of mRNA tempt us to speculate that the recognition of RNA signal by a motor protein or by a RNA binding protein in association with kinesin like protein can translocate the RNA across the cytoplasm to the site of

localization/translation. Cytoplasm being a liquid can not act as a media to transport the molecules across the cells by any motor driven transport. Therefore, cytoskeleton is thought to play a vital role in transport of RNA molecules to the site of localization or translation as supported by Yisraeli et al., (1990) and Pokrywka & Stephenson (1991).

Role of cytoskeleton in RNA localization:

In a cell, a number of functions including transport of organelles and macromolecules, cell division, cell motility and cell enlargement are mediated through cytoskeletal elements (Kirkeeide et al., 1993, Small et al., 1999, Vidwans & O'Farrell 1999, Machesky & Schliwa 2000, Rogers & Gelfand 2000). Cytoskeleton, radiating from the nucleus towards the periphery of the cytoplasm, runs like cables across the cytoplasm and is made up of microfilaments and microtubules. Actin monomers form the microfilament whereas tubulins form microtubule. *In situ* hybridization and electron microscopic observation reveal that most of the polyadenylated RNAs are associated with the actin filaments in fibroblast cells (Singer et al., 1989). Moreover, by immunohistological methods, it is detected that translational machinery seems to be closely associated with the cytoskeletal components (Toh et al., 1980, Hesketh & Pryme 1991).

Important contribution in understanding cytoskeleton-RNA interaction was made by biochemical treatment of fibroblast cells coupled with digital imaging microscopy (Taneja et al., 1992). When fibrobalst cells were extracted with Triton-X100 and subjected to *in situ* hybridization resulted in 11% loss of poly(A) RNAs. When the ribosomes and nascent polypepetides were dissociated from mRNA using puromycin, there was loss of 50% poly(A) RNA whereas remaining RNAs were retained by the cytoskeleton (Taneja et al., 1992). Puromycin treatment results indicate that association of these poly(A) RNAs with cytoskeleton is independent of nascent polypeptide. Although colcemid treatment resulted in solubilizing the microtubule, it had no effect on nonrandom distribution of poly(A) RNA. However, a drastic change was detected when the cells were treated with cytochalasin D. A 40% loss of poly(A) RNA was observed by puromycin treatment alone and an additional 40% loss of poly(A) RNA was documented

for every low dosage of cytochalasin D treatment (Taneja et al., 1992). From these results, it can be speculated that puromycin and cytochalasin may interact with different subsets of poly(A) RNA (Taneja et al., 1992).

There are several approaches available to unravel the cytoskeletal components in a cell. Confocal microscopy has made a revolution in understanding the architecture of the cytoskeletal component in the cells. Pharmacological drugs have been often used to pinpoint the role of specific components of cytoskeleton in mRNA localization. Drugs like colchicin, a cytoskeletal inhibitor drug, disrupts the microtubules whereas cytochalasin disrupts the microfilaments. Such drugs are often used to study the role of cytoskeletal components in macromolecule transport.

In Xenopus oocytes, differential effect of cytochalasin and colchicin on RNA localization have been documented. Treatment of oocytes with cytochalasin resulted in disruption of Vg1 mRNA localization at vegetal cortex. While colchicin treatment did not affect the already localized mRNAs but localization of newly made RNA was disrupted (Kloc & Etkin 1995, Yisraeli et al., 1990). These results indicate that the microtubules are necessary for the transport of the Vg1 RNA to the vegetal cortex while the microfilaments are important for the anchoring process. In Drosophila, *staufen* is involved in localization of *bicoid* mRNA at the anterior pole of the oocytes. To study the role of cytoskeleton in *bicoid* RNA localization, *bicoid* 3'UTR was injected in embryos and then treated with cytochalasin and colcemid. Treatment of embryos with cytochalasin B, an actin microfilament depolymerizing agent, did not affect the localization of Staufen particle to the extremities of the mitotic spindle. In contrast to this finding, embryos treated with microtubule destabilizing drug colcemid resulted in appearance of *bcd*-3'UTR-Staufen in a random fashion (Ferrandon et al., 1994). These results demonstrate that microtubules are required for localization of *bcd*-3'UTR-Staufen complex to the anterior pole and are independent of microfilaments.

RNA-binding proteins in mRNA sorting:

Advancement of research in cytoskeleton and mRNA localization using techniques like confocal microscopy and microinjection experiment suggest that RNA localization can be a motor driven transport rather than a passive diffusion. Recent findings indicate that RNA binding proteins, directly or indirectly associated with the cytoskeleton, bind to specific sequences of the mRNA and are involved in the RNA localization process. A classical example is Vera, that binds to 366 nucleotide region at the 3'UTR of Vg1 RNA named as Vg1 localization element (VgLE). Deletion analysis of VgLE indicated that four repeat motifs (E1-E4) were required for Vera binding. Moreover, deletion of regions which disrupted Vera binding also impaired localization (Deshler et al., 1997). Therefore, Vera binding to these specific sequences in Vg1 was interpreted as a prerequisite for localization at the vegetal cortex. Cloning of Vera cDNA has revealed that it is a homolog of Xenopus zip-code-binding protein (ZPB) encoding gene (Ross et al., 1997). The latter binds to a 54-nucleotide region at the 3'UTR of β-actin mRNA, which is localized at the periphery of the cytoplasm in fibroblast cells. Zip-code-binding protein failed to bind to a mutated version of β-actin mRNA and was also unable to get localized at the periphery (Ross et al., 1997).

Interestingly, Vera shares high homology with zip-code-binding protein, which is a microfilament associated protein while Vera is microtubule based protein (Deshler et al., 1998). Vg1 RNA binding protein (RBP) sequence indicates the presence of a RNA recognizing motif (RRM) and four tandem KH (hnRNP K homolog) domains (Vera & Vg1 RNA binding proteins are identical and were isolated by two different groups namely Deshler et al., 1998 and Havin et al., 1998, respectively). The RRM is a typical motif found in RNP1 (ribonucleo protein) and RNP2 whereas KH domain is found in a wide variety of RNA binding proteins including snRNA, pre-mRNA, mRNA and rRNA-binding proteins (Siomi & Dreyfuss 1997). A nuclear export motif was also noticed in Vg1 RBP (Havin et al., 1998) although, its functional role is not yet known. It would be interesting to study whether the process of RNA localization is initiated in nucleus itself by the interaction of RNA with an appropriate RNA-binding protein.

In Drosophila, genetic studies have shown that *staufen* gene is required to localize *bicoid* RNA to anterior pole and *oskar* RNA to posterior pole of oocyte (St Johnston et al., 1989, St Johnston et al., 1991, Ferrandon et al., 1994). Later, role of Staufen, a double stranded RNA binding protein, in localization of *bicoid* mRNA has been studied using microinjection and *in situ* localization techniques (Ferrandon et al., 1994). When *in vitro* transcribed *bicoid* 3'UTR microinjected in Drosophila embryos, the Staufen protein was recruited to the site of injection (Ferrandon et al., 1994). However, other injected RNAs such as *caudal* sense and antisense, *bicoid* full length antisense, yeast GAL4 and *bicoid* without 3'UTR failed to localize Staufen at the site of injection (Ferrandon et al., 1994). Similarly, when different mutant versions of *bicoid* 3'UTR were injected, it resulted in identification of three distinct regions, nucleotides between 211-360, 421-510 and 541-630 responsible for Staufen binding. Although, *in vitro*, Staufen binds to double stranded RNA, *in vivo*, Staufen-*bicoid*-3'UTR particle formation was efficient than non specific double stranded RNA and the movement to the cortex was observed when *bicoid* 3'UTR was injected and not the other nonspecific RNAs. Moreover, Staufen-*bicoid*-3'UTR particle was not affected by microfilament depolymerizing drug whereas microtubule depolymerizing drug treatment resulted in even distribution of Staufen-*bicoid*3'UTR particle (Ferrandon et al., 1994). It is therefore believed that microtubules are necessary for the Staufen mediated *bicoid* localization process.

Recently, a human homolog of Staufen was reported using influenza virus NS1 protein as bait in yeast two-hybrid system (Marion et al., 1999). The predicted protein sequence shares significant sequence similarity with the C-terminal region of the Drosophila Staufen protein. A recombinant hStaufen could strongly bind to 3'UTR of *bicoid* mRNA. Immunolocalization of *He La* cells transfected with hStaufen construct indicated strong signals around the nucleus radiating towards the periphery of the cell (Marion et al., 1999). Moreover, mild detergent treatment did not affect the localization of hStaufen, suggesting the association of hStaufen with cytoskeleton and membrane (Marion et al., 1999). Double labeling of sections with ER membrane markers indicated the localization of hStaufen on ER membrane (Marion et al., 1999) while sucrose density gradient experiment results showed that hStaufen co-sedimented with polysomes (Marion

et al., 1999). This fast sedimenting complex containing polysomes and hStaufen was sensitive to EDTA treatment. These evidences support the view that RNA binding proteins are involved in the process of mRNA transport in the cytoplasm. Yet another Staufen like protein was identified in mammalian neuronal system (Wickham et al., 1999). This protein also shares significant sequence similarity with the double stranded RNA binding domain of Staufen and microtubule-binding domain. RNA binding assays demonstrated strong binding of mammalian Staufen protein to *bicoid* RNA (Wickham et al., 1999). The binding was to double stranded structure in the RNA and was not to any specific sequence of RNA. Further it was shown that the mammalian Staufen protein could bind to microtubule but not to actin (Wickham et al., 1999). When mammalian cells were transfected with cDNA coding for a Staufen-HA fusion protein, the Staufen-HA was found to be associated with the rough endoplasmic reticulum (Wickham et al., 1999).

Convincing evidences are available to support a close association of mRNA to cytoskeleton. To add more to this list, recently a general RNA binding protein associated with cytoskeleton has been identified. Using a novel *in vivo* method, about 160 kD protein associated with cytoskeleton was identified in cultured PC12 cells (DeFranco et al., 1998). Radiolabeled RNA probes were incubated with the cell extract and subjected to electrophoretic mobility shift assay (EMSA) resulting in identification of several complexes (DeFranco et al., 1998). Among all complexes, complex 1 was noticed to be independent of the probe used. This complex was stable even at, as high as, 2 M KCl salt concentration indicating that the RNA-protein interaction was not electrostatic but was hydrophobic in nature (DeFranco et al., 1998). Moreover, single-stranded random RNA polymers were efficient competitors, whereas double stranded RNA polymers, single stranded/double stranded DNA were unable to compete the binding. Competition experiment with unlabeled RNAs confirmed that the complex 1 binding was length dependent and required a minimum of 120 nucleotides. When complex 1 was dissected using two-dimensional gel electrophoresis approach it resulted in identification of 160 kD protein. Molecular mass of this protein was identical to a 160 kD protein that was detected in the *in vivo* crosslinking experiment. When antibody for a cytoskeleton

protein MAP 1A was used in the EMSA, it resulted in supershift in complex 1 demonstrating the presence of cytoskeleton associated protein in this complex (DeFranco et al., 1998). This report further supports the view that RNA binding activity is associated with cytoskeleton.

Why translation of certain mRNAs requires localization?

In polarized egg cells, protein localization is mandatory to achieve a normal differentiation process. Translation of RNA at an unwanted site could be deleterious and hence transport of RNA to the appropriate site of translation and localization dependent translation have gained a great deal of importance. Moreover, from a single mRNA molecule many copies of a protein can be translated and thereby a local high concentration of a particular protein can be achieved. In somatic cells, post-translational modification can be made efficient when a particular protein is concentrated at a discrete site of cytoplasm. The best example of localization dependent translation was put forth by Gavis & Lehman (1994). In Drosophila embryo, although a significant amount of *nos* RNA was uniformly distributed through out the embryo, Nos protein is localized at the posterior pole. In a chimeric gene approach, *nos* coding region was fused with tubulin RNA 3'UTR and introduced into embryos that resulted in uniform distribution of Nos protein throughout the embryo. This was in contrast to *nos* wild type RNA whose protein was restricted to the posterior pole region (Gavis & Lehman 1994). From these results, it was concluded that unlocalized *nos* RNA is translationally suppressed and the element for translation suppression is possibly located at the 3'UTR.

In an another example, posterior pole determinant *osk* mRNA translation control is considered at three levels namely, translational repression, release from the repression and activation of translation (Kim-Ha et al., 1995). An ovarian RNA binding protein Bruno, which binds to multiple sites in 3'UTR region was identified as a repressor of *osk* mRNA (Kim-Ha et al., 1995). The *bicoid*, posterior determinant forms anterior-posterior gradient, while its counter part *cad* forms the concentration gradient in an opposite direction. It was shown that *bicoid* protein binds to *cad* mRNA and exerts translation control through *bcd*-binding region of *cad* mRNA (Rivera-Pomar et al., 1996). These

findings strongly support the model that translation of RNA is localization dependent and unlocalized RNAs are translationally repressed. Although these findings are from polarized egg cells where pattern formation is due to protein gradient, similar phenomenon can be proposed for somatic cells as well.

Storage protein mRNA localization in rice endosperm cells:

Rice, unlike many other cereals, expresses both prolamine and globulin type seed storage proteins during seed development. Translation of these storage proteins takes place on ER membrane. Depending on the nature of the storage protein, either it is retained in the ER lumen (prolamine protein body) or it is transported to vacuoles (glutelin) via golgi (Okita & Rogers 1996). Rice endosperm cells, therefore, form a model system to study the transport and localization of mRNA and their differential distribution on different domains of the ER membrane. Subcellular fractionation study indicates that polyadenylated mRNA obtained from the PB fraction resulted in synthesis of prolamine polypeptides in an *in vitro* translation system (Yamagata et al., 1986). Whereas, the microsomal fraction contains glutelin transcripts two folds over prolamine (Kim et al., 1993). Differential distribution of prolamine and glutelin mRNAs onto different domains of the ER membrane has been well documented using fine resolution *in situ* localization at electron microscope level (Li et al., 1993). *In situ* hybridization of ultra thin section of rice endosperm tissue with prolamine and glutelin probes has revealed seven fold of prolamine transcripts over glutelin on PB-ER while two fold of glutelin transcripts over prolamine on c-ER (Li et al., 1993). However, the molecular mechanisms of this localization process are still obscure. Biochemical and subcellular fractionation analysis have brought out some information on prolamine RNA localization (Muench et al., 1998). Nonionic detergent and salt treatment of isolated protein body indicates that the association of prolamine polysomes with the PB is detergent resistant but salt sensitive. Therefore, the association could be either through mRNA or ribosome but not due to nascent polypeptide. NaF treatment of rice seeds followed by membrane-stripped PB isolation and sucrose density gradient analysis has revealed that ribosome free prolamine RNAs remain associated with membrane-stripped PB (Muench et al., 1998). This suggests that ribosome free prolamine RNA interacts with the RNA binding

activity near or on the PB surface. Further, salt treatment of NaF treated membrane-stripped PBs indicates that high salt concentration (300 mM NaCl) is required to release the RNA from the RNA binding activity associated with PBs. When rice seed extract prepared in low salt buffer was subjected to sucrose density gradient and the fractions of the gradients were analyzed by immuno blot and RNA dot blot. The results indicate that most of the prolamine RNA, prolamine protein, actin and tubulin appear in the same fraction or sedimented together. High salt treatment of this fraction results in solubilization of actin and tubulin demonstrating the association of prolamine mRNA not only to ER membrane but to cytoskeleton as well. It appears that the presence of RNA binding activity associated with cytoskeleton is possibly involved in prolamine RNA localization onto PBs (Muench et al., 1998).

Genesis of thesis:

Our laboratory at National Chemical Laboratory (NCL) has been engaged in research on seed storage proteins and their regulation during rice seed development. I joined this laboratory to work on prolamine and glutelin gene regulation in rice endosperm cells. During the tenure, I was selected to work with Prof. Thomas Okita at Institute of Biological Chemistry, Washington State University, Pullman, USA for two years under the graduate fellowship from the Rockefeller Foundation, USA. At Prof. Okita's laboratory, I worked on RNA binding proteins associated with cytoskeleton. I returned to NCL, India and completed studies on differential distribution of message specific RNA binding proteins on ER membrane domains of rice endosperm cells.

Specific objectives of my Ph. D. work were as follows:

1. **To isolate and purify cytoskeleton-associated RNA-binding protein(s).**
2. **To study their binding activity to prolamine & glutelin RNAs.**
3. **To assess their interaction with cytoskeleton.**
4. **To isolate corresponding cDNAs for the purified RNA-binding protein.**
5. **To characterize specific RNA-binding proteins associated with ER-membrane**

The work carried out to fulfill these objectives has been organized in subsequent chapters:

Chapter 2: A cytoskeleton associated RNA-binding protein binds to untranslated regions of prolamine mRNA and to poly(A)

Chapter 3: Rp120: A cytoskeleton associated tudor domain protein binds to mRNA

Chapter 4: Message specific RNA binding proteins associated with ER membrane in rice endosperm cells

Chapter 5: General discussion

Summary

References

Chapter II

A cytoskeleton associated RNA-binding protein binds to untranslated regions of prolamine mRNA and to poly(A)

ABSTRACT

Prolamine mRNAs are not randomly translated on the extensive endoplasmic reticulum (ER) system in developing rice endosperm. Instead, they are localized on the surface of the prolamine protein bodies (PBs). Recent findings suggest that prolamine polysomes are attached not only to the surface of the prolamine PB-ER but also to the cytoskeleton which is associated with this organelle. To uncover the molecular and cellular basis for localization of prolamine mRNAs in rice endosperm cells, we obtained cytoskeleton-PB enriched fraction, fractionated on poly(U)-Sepharose and studied the cytoskeleton associated RNA binding activities. Using RNA-protein UV cross-linking assay, a dominant 40 kD RNA-binding protein associated with the cytoskeleton-PB enriched fraction was identified. This activity binds to the 3' and 5' untranslated regions of prolamine mRNA and the 3' untranslated region of glutelin mRNA but not to their coding sequences. Binding activity is readily competed by polyadenylic acid indicating that the binding recognition site is adenine-rich.

INTRODUCTION

During seed development, plants accumulate storage proteins that are used as carbon and nitrogen source to support post-germinative growth and development. In general, plants accumulate one of the two types of storage proteins either globulins or prolamines. Rice is an exception to this general rule as it accumulates both storage protein types and, moreover, stores these proteins into separate compartments within the endomembrane system (Krishnan et al., 1986, Yamagata et al., 1982). The dominant storage proteins in rice are the glutelins, proteins homologous to the 11S globulins, which are stored in a vacuole-like compartment. Prolamines, the alcohol-soluble proteins typically accumulated by cereals, are also present in rice but only at about one-third of the glutelin levels. The prolamines are directly sequestered and assembled within the ER lumen to form the prolamine protein body (PB).

Because prolamines are stored in the endomembrane system and glutelins are processed in the ER lumen, their mRNAs are translated on the rough ER. Recent evidence indicates that the storage protein mRNAs are not distributed on the rough ER in a stochastic fashion, but instead, distributed to specific ER subdomains. Polyadenylated RNA, isolated from a purified prolamine protein body fraction, synthesized only prolamine polypeptides suggesting that prolamine transcripts were the predominant mRNA species in this fraction (Yamagata et al., 1982). In contrast, RNA obtained from a microsomal membrane fraction enriched for rough ER vesicles contained more than 2-fold greater levels of glutelin transcripts than prolamine transcripts (Kim et al.,1993). Having two distinct endomembrane systems, where two different types of transcripts are translated, makes the rice endosperm cells the most attractive model system to study the transport and localization of mRNAs. These preliminary results were confirmed by Li et al., (1993) who unequivocally demonstrated that the prolamine transcripts are highly enriched on the ER membranes that bound the prolamine protein bodies (PB-ER) whereas glutelin transcripts predominate on the cisternal ER. These observations suggest that rice mRNAs, especially the prolamine mRNAs, are localized to distinct PB-ER subdomain.

The exact mechanism for the targeting of prolamine RNAs to PB-ER is not known but current evidences in other biological systems indicate that the mRNAs are localized by recognition of specific RNA signals and the active transport and targeting of the mRNA to a destination site within the cell (Hesketh 1996, Hovland et al., 1996, Wilhelm and Vale 1993). For instance, c-myc and myelin basic protein (MBP) mRNAs are localized to specific domains of the cytoplasm because of the presence of specific cis-elements located in the 3' untranslated regions (3'UTR) (Ainger et al., 1997, Veyrune et al., 1996). When the c-myc mRNA 3'UTR was attached to the globin coding region, it led to localization of globin RNA to the perinuclear cytoplasm, however, globin coding region alone could not get localized at the perinuclear region. When modified RNAs with different deletion in the 3'UTR of MBP mRNA were injected into cultured mouse brain cells that resulted in identification of transport and localization element at the 3'UTR. These results clearly demonstrate that a specific sequence motif or secondary structure is responsible for localizing mRNAs at the site of translation.

The presence of multiple cis-sequences in the 3'UTR for RNA transport and localization infers the existence of cognate trans-acting RNA binding proteins that recognize these RNA signals. Indeed, both biochemical and genetic evidences support such a view. Mutation in the gene *staufen* causes mislocalization of several maternal RNAs during Drosophila embryo development (Ephrussi et al., 1991, Kim-Ha et al., 1991). *Staufen* encodes a RNA-binding protein that binds specifically to the 3'UTR of *bicoid* mRNA and forms a ribonucleoprotein particle which moves in a microtubule dependent manner (Ferrandon et al., 1994). In Xenopus oocytes, *Vg1* mRNA is transported to specific ER subdomains in the vegetal cortex by microtubules and then anchored to these sites by microfilaments (Yisraeli et al., 1990). The localization of *Vg1* RNA has been suggested to be mediated by Vera, a 75 kD ER-associated protein, that specifically binds to the 3'UTR of *Vg1* RNA (Deshler et al., 1997). Indeed cytoskeleton is thought to play an important role not only in active transport of the mRNA but also their anchoring to the destination site. In fact such view has been confirmed by using the pharmacological agents that disrupt the cytoskeletal elements.

Recent biochemical (Muench et al., 1998) and indirect-fluorescence microscopic (Muench and Okita, unpublished observations) evidences suggest the role of cytoskeleton in transport of prolamine mRNA associated with the prolamine PBs. Prolamine polysomes remain attached to membrane-stripped PBs under conditions that stabilize the cytoskeleton which is readily observed around the prolamine PBs by fluorescence microscopy similar to that observed in maize endosperm (Clore at al 1996). These polysomes are readily released from the cytoskeleton-PB complex under conditions (high ionic strength) which disrupt the cytoskeleton (Muench et al., 1998). Treatment of developing seeds with NaF (a potent inhibitor of translation initiation but not elongation) results in the stochiometric recovery of non-ribosome associated prolamine RNAs with the membrane-stripped PBs (Muench et al., 1998). These findings suggest that the polysomes are attached via the mRNA although the possible involvement of the ribosome in this process cannot be entirely ruled out.

The attachment of prolamine polysomes through mRNA sequences suggests the involvement of RNA-binding activities associated with the cytoskeleton. In this study, cytoskeleton-PB enriched extract was obtained and it was fractionated on poly(U)-Sepharose to purify RNA binding activities. Using RNA-protein UV crosslinking assays under stringent conditions, a dominant 40 kD RNA-binding protein was identified. This RNA binding activity interacted with 3'UTR and weakly with 5'UTR but not with the coding region of prolamine mRNA. Binding activity was also observed with the glutelin 3'UTR but not with glutelin coding sequence. Binding to these storage protein sequences was competed by poly(A), suggesting that 40 kD RNA binding activity is a related member of a family of poly(A) RNA binding proteins. A possible role of the 40 kD RNA binding activity in prolamine mRNA localization and translation is discussed.

MATERIALS AND METHODS

Plasmid DNA isolation:

Single bacterial colony was inoculated in 25 ml LB medium containing 100 µg/ml ampicillin and incubated for over night at 37^{o}C in an orbital shaker at 300 RPM. Cells were harvested by centrifuging at 5000 RPM for 7 min at 4^{o}C. Plasmid DNA was

isolated by alkaline lysis method as per the protocol of Sambrook et al., (1989) and was further purified by cesium chloride ultra centrifugation. Plasmid pellet was suspended in 0.8 ml of TE buffer to which 35 µl of 10% Sarkosyl and 5 µl of 10 mg/ml proteinase K were added followed by incubation at 55°C for 15 mins. To this plasmid solution, 0.75 g of cesium chloride and 10 µl of ethidium bromide were added and the mixture was centrifuged at 110,000 RPM using tabletop ultra centrifuge. Plasmid band was removed using a pipetman and extracted with water saturated n-butanol to remove the ethidium bromide. To this cesium chloride plasmid mix, 3 volumes of TE was added followed by 1/2 volume of 7.5 M ammonium acetate and two volumes of ethanol. It was left on ice for 15 mins and centrifuged at 10,000 RPM using a microcentrifuge. The pellet was washed with 70% ethanol, dried using N_2 gas and suspended in TE or sterile water. Concentration of DNA was estimated spectrophotometrically.

Plasmid DNA construction:

Immunoscreening of rice developing seed cDNA library with prolamine antibodies resulted in identification of cDNA prolamine 7 (Kim & Okita 1988). Predicted polypeptide of prolamine 7 cDNA indicates that it shares high similarity with other prolamines. For this study, the prolamine 3'UTR was obtained by digesting prolamine 7 cDNA (Kim & Okita 1988) with *Xba*I. The resulting 287 bp fragment, which contains 70 bases of the coding region, 111 bases of the 3'UTR and 106 adenine residues of the poly(A) tail, was then cloned at the *Xba*I site of Bluescript plasmid to give pSam1. This plasmid was used as a template to amplify the 3'UTR excluding the poly(A) tail by using the T7 sequencing primer as the sense primer and 5'AAATATGAAAGGCAACTTTATTTCTATTTATAT as the antisense primer in a standard PCR experiment. The reaction contained 1X PCR reaction buffer, 25 mM $MgCl_2$, 2.5 mM dNTPs and 1 U of Taq polymerase. The reaction conditions were: template denaturation at 94°C for 1 min, primer annealing at 55°C for 30 sec, polymerization at 72°C for 2 min, amplification was carried out for 35 cycles in a MJ thermal cycler. The resulting amplified DNA fragment was then used as template for transcription reaction using T7 RNA polymerase. The 449 nucleotide coding region was amplified by PCR using primers, 5'CAGCTCGACAGCATTTGAAGATCATTTTCGT

and 5'TCAAGCTTAATGGTGATGGTGATGGTGCAAGACACCGCCA. The PCR product was cloned in Bluescript plasmid and named pDGM3. The 204 nucleotide 5'UTR was obtained by PCR amplification. The primers used in the PCR were (5'primer: 5'TACGGGCCCGGATCCTGCTTCTTCCCGTCCTC and the 3' primer 5'GACCTCGAGCGGGACACTAGATCTTTTCGT) and cloned in Bluescript plasmid and named pDGM4.

Using mono specific antibodies to acidic subunit of glutelin storage protein, a cDNA of 1.8 kb namely pG22 was isolated and characterized (Okita et al., 1989). Nucleotide sequence comparisons revealed that pG22 belongs to Gt3 class of glutelins. In the current study, the glutelin 3'UTR was obtained by digesting pG22 (Okita et al., 1989) with *Eco* RI which resulted in the release of a 325 bp fragment containing 161 bp coding region, 135 bp 3'UTR and 29 bases of the poly(A) tail which was cloned in Bluescript plasmid to give pSamG1. The remaining digested plasmid DNA containing a 1362 bp fragment which include glutelin 5'UTR and most of the coding region was self-ligated to give pSamG2 and used in transcription of glutelin to yield 5'UTR along with coding region. The ligated plasmid was subjected to electroporation using XL1 Blue bacterial cells according to the manufacturer's protocol (Clone Tech). Transformed cells were plated on LB plate containing ampicillin, X-gal and IPTG. Presence of insert in recombinant cells was confirmed by isolating the plasmid followed by restriction digestion and DNA sequencing. To obtain the 5'UTR (actually 30 bp 5'UTR and 91 bp coding region) transcript of glutelin, pG22 plasmid was digested with *Spe*I and transcribed from the T3 promoter.

***In vitro* transcription:**

Plasmid DNAs were linearized with *Bam*HI, *Hind*III, *Eco*RI or *Spe*I depending on the plasmid and used as a template for RNA transcription. ^{32}P-labeled RNA transcripts were synthesized using the Ambion Maxiscript kit in the presence of 100 µCi of ^{32}P-UTP, 1 mM each of (ATP, GTP, CTP) 40 µM UTP and 1 unit of RNase inhibitase (5'-3' Inc.). The reaction was terminated by adding 1 unit of RNase-free DNase (Promega). Incorporation of ^{32}P-UTP was estimated by precipitating a small sample of the *in vitro*

transcription reaction with 10% TCA, collecting the precipitate by filtration on glass fiber filters and washing it extensively with 2.5% TCA followed by ethanol. The amount of radioactivity incorporated was measured by liquid scintillation counting. To ensure that the bulk of the synthesized RNA was full length, a small sample of the reaction was resolved by urea PAGE (Ambion protocol) and analyzed by autoradiography. The reaction containing radiolabeled RNA was extracted with phenol followed by chloroform and then precipitated by two volumes of ethanol in the presence of 4 µg of yeast tRNA. The pellet was washed with 70% ethanol, dried and suspended in appropriate volume of RNase free water and used in RNA-protein UV cross-linking assays. Unlabeled RNA for competition experiments was made using Ambion Megascript kit according to the manufacturer's protocol.

Purification of RNA-binding proteins from cytoskeleton-PB enriched fraction:

Milky stage developing rice seeds (12 – 15 DAF) were ground in cytoskeleton stabilizing buffer (Abe & Davies 1991) consisting of 5 mM HEPES-NaOH pH 7.5, 10 mM MgOAc, 2 mM EGTA, 1 mM PMSF, 1 mM DTT, 200 mM sucrose and 1% Triton X-100. The seed extract was passed through miracloth and then centrifuged at 2500 RPM for 10 min. The pellet containing mainly protein bodies with attached cytoskeleton and starch grains was collected and subjected to 60 to 80% sucrose density gradient centrifugation according to Muench et al., (1998). The cytoskeleton-PB heavy fraction which appeared at about 65% sucrose concentration was collected and suspended in high salt buffer containing 200 mM Tris-HCl, pH 8.5, 500 mM KOAc, 2 mM EGTA to disrupt the cytoskeleton (Muench et al., 1998) and then centrifuged at 49,000 RPM for 45 min in order to remove ribosomes, polysomes and protein bodies. The supernatant was dialyzed against poly(U) Sepharose column loading buffer containing 20 mM HEPES-NaoH pH 7.5, 100 mM KCl, 1 mM DTT and 10% glycerol. Dialyzed protein was loaded on a poly(U) Sepharose column (Pharmacia), washed with two volumes of column loading buffer, and the bound protein was eluted with 300 mM KCl followed by 1 M KCl. Both fractions were dialyzed in the column loading buffer and subjected to RNA-protein UV cross-linking assay.

RNA-protein UV cross-linking assay:

The reaction mixture contained 20 mM HEPES, pH 7.5, 100 mM KCl, 1 mM DTT, 10% glycerol, 20 fmole of ^{32}P-labeled RNA, 4 µg of tRNA, 1 unit of RNase inhibitase (5'-3' Inc.) and either 1-5 µg of protein from the 0.3M or 1.0 M poly(U) Sepharose fractions or 20 µg of crude cytoskeleton-PB fraction in 35 µl volume. The reaction mixture was incubated at 4^{o}C for 10 min followed by addition of 5 mg/ml final concentration of heparin and incubated for additional 10 min. In competition experiments, both radiolabeled and unlabeled RNAs were added to the reaction, mixed well and left on ice for 5 min before adding the protein. The reaction mixture was transferred onto a parafilm and exposed to UV light for 7 min (168000 µJ/cm^2 using Ultralum UV crosslinker). The reaction mixtures were then treated with 100 units of RNase T1 and 20 µg of RNase A1 at 37^{o} C for 25 min and then subjected to SDS-PAGE. After electrophoresis, the gels were dried and exposed to X-ray film for 14 to 24 hours at -80 ^{o} C.

RESULTS

Purification of RNA-binding proteins from cytoskeleton-PB enriched fraction:

It has been reported earlier that prolamine polysomes are not only attached to the ER but also to the cytoskeleton. Membrane-stripped PB-cytoskeleton fraction from NaF treated seeds when extracted with different concentration of NaCl, released prolamine RNA (Muench et al., 1998). Indeed, fluorescence microscopy of endosperm sections treated with phalloidin conjugated with the fluorescent probe rhodamine showed that the PBs are enmeshed in actin-containing microfilaments (Muench and Okita, unpublished). At least one interaction between the prolamine polysomes and cytoskeleton is possibly through the mRNA sequences, an observation that infers the existence of RNA binding activity. In view of this, we decided to purify and characterize the RNA-binding protein activities that are associated with the cytoskeleton-PB fraction.

Milky stage developing rice seeds were chosen for isolation of cytoskeleton fraction since prolamine transcription is maximum at this stage (Kim & Okita 1988). The developing rice seeds were homogenized in low ionic strength buffer containing Triton X-100 to solubilize membranes and then subjected to low speed centrifugation (2500 RPM) to obtain a crude cytoskeleton-PB fraction. The latter was then purified by subjecting to 60-80% sucrose density gradient (Muench et al., 1998). The purified cytoskeleton-PB fraction was collected from the sucrose gradient and then treated with high salt buffer containing Triton X-100 to dissociate microfilaments and microtubules and to release the prolamine polysomes and cytoskeleton-associated proteins. Polysomes and PBs were removed by centrifuging at 49,000 RPM for 45 min and then the extract was dialyzed in column loading buffer to reduce the salt concentration. The dialyzed extract was fractionated on a poly(U) Sepharose column, a technique commonly employed to affinity purify RNA-binding proteins (Kuhn & Pieler 1996, Pellizzoni et al., 1996). Different poly(U) Sepharose fractions namely flow through, 300 mM KCl, 1 M KCl and the enriched cytoskeleton-PB extract were analyzed by RNA protein UV cross-linking assay under stringent conditions in the presence of 100 mM KCl, yeast tRNA and heparin (up to 10 mg/ml) which eliminated non-specific ionic interactions. Under these conditions, BSA was unable to cross-link to the ^{32}P-labeled prolamine RNA (Fig. 1).

When the cytoskeleton-PB enriched extract was subjected to RNA-protein UV cross-linking assay, a 35 kD polypeptide band was readily observed (Fig. 1). This 35 kD binding activity was sensitive to proteinase K treatment. Various fractions from poly(U) Sepharose were then tested for RNA binding activity. The poly(U) Sepharose flow-through contained no detectable RNA binding activity. Several RNA binding activities, however, were observed in the 300 mM KCl elution fraction with proximate sizes of about 120 kD, 100 kD, 75 kD and 40 kD although none of the binding activities were prominent. The 1 M KCl poly(U) Sepharose fraction contained a prominent 40 kD and a minor 35 kD RNA binding activities. The 40 kD protein was not observed in the crude cytoskeleton-PB extract when equivalent amounts of proteins were analyzed for RNA binding property. In order to rule out the possibility of inhibition of 40 kD protein in the crude fraction, 40 kD binding activity was assayed with varying amounts of crude

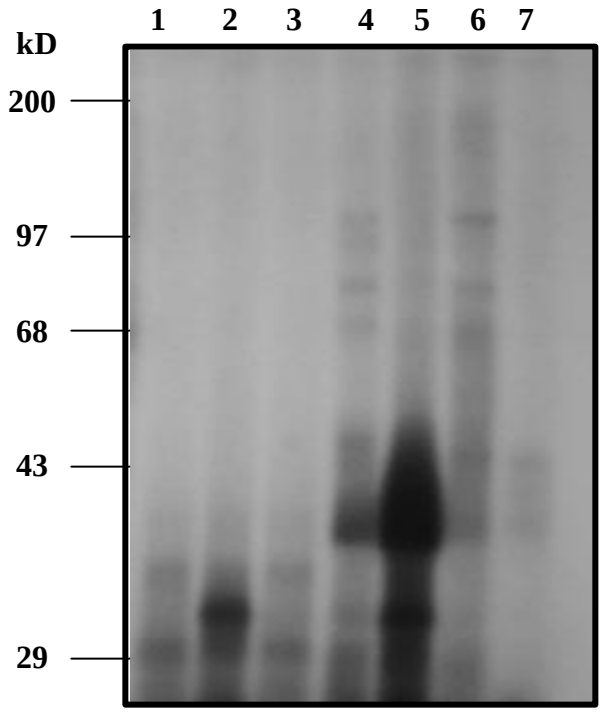

Fig. 1

Fig. 1. RNA binding activities as assessed by UV cross-linking of ^{32}P-labeled prolamine RNA to protein (20 µg) from cytoskeleton-PB enriched extract (lanes 1 and 2) and from the flow-through (10 µg) (lane 3), 0.3 M KCl (lane 4, 1 µg) and 1.0 M KCl (lane 5, 1 µg) of poly(U)-Sepharose column fractions, followed by Heparin 300 mM elution (lane 6) and Heparin 1 M elution (lane 7). Lane 1 contains a cross-linking reaction with cytoskeleton-PB, cross-linked product pretreated with proteinase K before loading onto SDS-PAGE. A 120 kD protein which appears in Heparin 300 mM KCl fraction is discussed in the next chapter.

cytoskeleton-PB extract, however, no significant effect of crude extract on 40 kD binding activity was observed. Possible reason for not observing the 40 kD binding activity in the crude cytoskeleton-PB, may be due to low quantity of 40 kD protein in the crude cytoskeleton-PB fraction. As it got enriched several fold in 1 M KCl fraction of Poly(U) Sepharose, a prominent band was seen. The 35 kD binding activity did not show any sequence specificity (Fig.2). Hence the 40 kD protein was subjected to further study.

Mapping of 40 kD protein binding site on prolamine and glutelin mRNAs:

To determine the sequence specificity of the 40 kD protein binding to prolamine mRNA, binding reactions with ^{32}P-labeled prolamine RNA were conducted in the presence of excess amounts of unlabeled RNA sequences containing prolamine 3'UTR, 5'UTR or coding region. Binding activity to the ^{32}P-labeled prolamine RNA was unaffected by the presence of the coding and 5'UTR sequences even at a 400-fold molar excess. In contrast, a 100 molar excess of unlabeled RNA containing the 3'UTR efficiently competed the binding activity to ^{32}P-labeled prolamine RNA. The 287 nucleotide 3'UTR RNA fragment contained 70 bases of the coding region, 111 bases of the 3'UTR and 106 adenine residues of the poly(A) tail. Since the binding activity was not competed by RNA containing the intact coding region and 5'UTR indicating that the 40 kD polypeptide specifically binds to the 3'UTR and/or poly(A) of prolamine RNA.

The binding specificity was also evaluated by directly using ^{32}P-labeled RNA sequences containing 3'UTR, 5'UTR and coding sequences in a crosslinking reaction. The 40 kD polypeptide binds to RNA containing the 3'UTR but not to ^{32}P-labeled RNAs containing the coding or antisense 3'UTR sequences. The binding activity, however, was detected with the ^{32}P-labeled 5'UTR though the interaction was much less than that observed with the intact prolamine mRNA or 3'UTR sequences (Fig.2C).

RNA protein binding assays showed that the 40 kD polypeptide also interacted with glutelin mRNA sequences. Similar to the prolamine RNA binding results, excess of cold glutelin 5'UTR and coding sequences did not compete out the binding of 40 kD to ^{32}P-labeled glutelin mRNA sequences (Fig. 2B), although binding activity was abolished

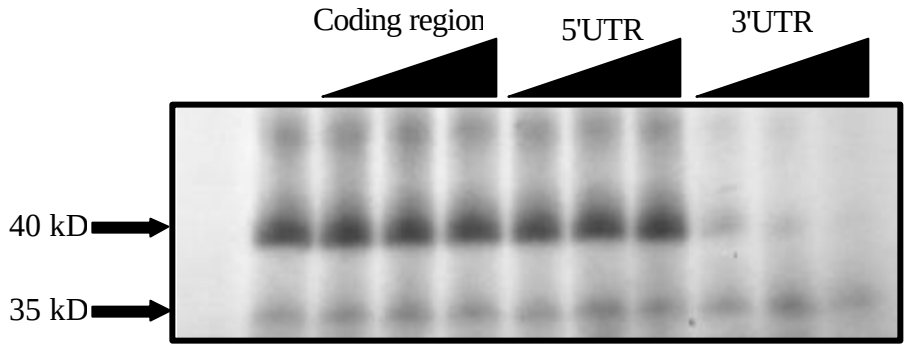

Fig. 2a

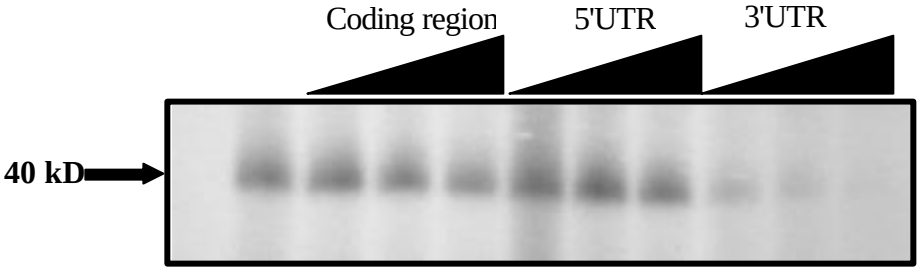

Fig. 2b

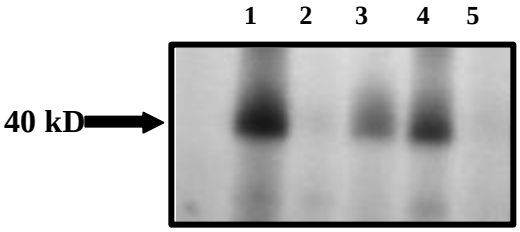

Fig. 2c

Fig. 2. Mapping of the 40 kD binding site on prolamine and glutelin RNAs. RNA-protein UV cross-linking assays were conducted with 1 M poly(U) Sepharose fraction (1 µg) by incubating ^{32}P-labeled prolamine (2a) or glutelin (2b) RNAs in the absence (lane 1) or presence of 100, 200 & 400 molar excess of unlabeled coding region (lanes 2, 3 and 4, respectively), of unlabeled 5'UTR region (lanes 5, 6 and 7, respectively), or unlabeled 3'UTR-poly(A) (lanes 8, 9, and 10, respectively).

Fig. 2c depicts RNA-protein UV crosslinking when 1 M KCl poly(U) Sepharose fraction was incubated with ^{32}P-labeled prolamine RNA (lane 1), with ^{32}P-labeled prolamine coding region (lane 2), ^{32}P-labeled prolamine 5'UTR (lane 3), ^{32}P-labeled prolamine 3'UTR lacking the poly(A) (lane 4) and ^{32}P-labeled prolamine 3'UTR antisense (lane 5).

in the presence of 100-molar excess of unlabeled glutelin RNA fragment containing the 3' end mRNA sequences which included 161 bp coding region, 135 bp 3'UTR and 29 bases of the poly(A) tail. These results indicate that the 40 kD protein binds to 3'UTR and/or poly(A) sequences of the prolamine and glutelin mRNAs but not to the coding sequences of these storage protein mRNAs.

Effect of homopolymer RNA on binding activity:

Most of the mRNAs are polyadenylated at the 3'end. Since the 40 kD protein seems to have high affinity for the poly(A), we decided to use other homopolymers apart from poly(A) in the crosslinking experiment. To study the effect of different homopolymer RNAs on the binding activity of 40 kD protein to prolamine RNA, reactions containing ^{32}P-labeled prolamine RNA were incubated with different concentrations of poly(A), poly(U), poly(G) and poly(C). The results obtained showed that the 40 kD binding activity was strongly competed by poly(A) even at 0.01 mg/ml final concentration while other homopolymer RNAs were only mildly effective at the higher concentrations (0.2 mg/ml) in competing for RNA binding (Fig. 3b).

Role of poly(A) in 3'UTR binding of 40 kD protein:

Since the 40 kD protein seemed to have high affinity for the poly(A), it was decided to study whether the poly(A) plays any role in binding to the 3'UTR of prolamine. The RNA-protein UV cross-linking assays were performed with ^{32}P-labeled prolamine and glutelin 3'UTRs with or without poly(A) sequences at the 3'end. Binding activity was readily observed with the prolamine 3'UTR irrespective of the presence of poly(A) tail although removal of poly(A) tail reduced the binding activity slightly (Fig. 3B). Binding activity with the prolamine 3'UTR sequences alone was effectively competed by unlabeled poly(A). Likewise, binding activity was also seen for glutelin 3'UTR without poly(A) tail although it was much less than that with 3'UTR+poly(A). These results indicated that the 40 kD protein bound to A-rich sequences present in 3'UTR sequences of both prolamine and glutelin mRNA transcripts. Moreover poly(A)

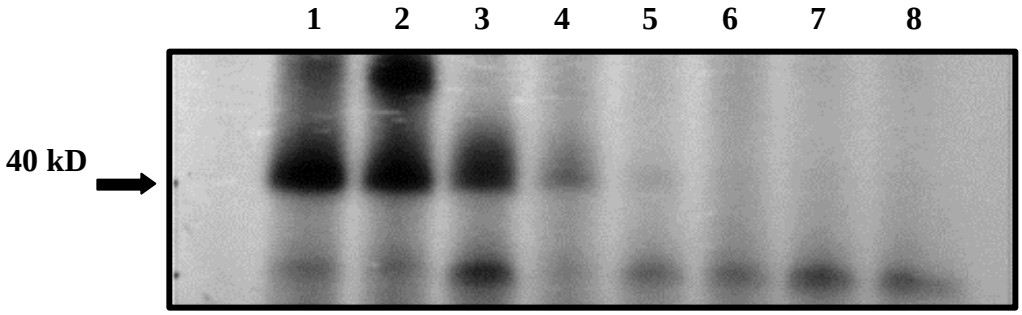

Fig. 3a

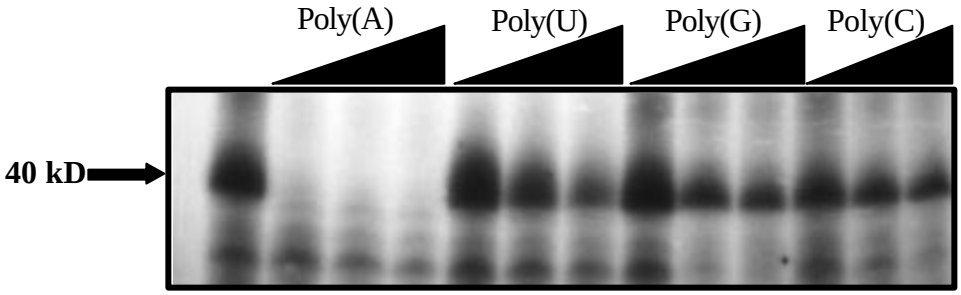

Fig. 3b

Fig. 3. Competition RNA-protein UV cross-linking assays in the presence of homopolymer RNAs.

3a. 1 M KCl poly(U)-Sepharose fraction was incubated with ^{32}P-labeled prolamine 3'UTR (lane 1) or ^{32}P-labeled prolamine 3'UTR without poly(A) tail (lane 3), glutelin 3'UTR (lane 2) or glutelin 3'UTR without poly(A) tail (lane 4), ^{32}P-labeled prolamine 3'UTR incubated with 1 and 5 µg/ml poly(A) (lanes 5 and 6 respectively), ^{32}P-labeled prolamine 3'UTR without poly(A) tail was incubated with 1 and 5 µg/ml poly(A) (lanes 7 and 8 respectively).

3b. 1M KCl Poly(U) Sepharose fraction was incubated with ^{32}P-labeled prolamine RNA (lane 1) and 0.01, 0.1 and 0.2 mg/ml of poly(A) (lanes 2, 3 and 4 respectively), of poly(U) (lanes 5, 6 and 7 respectively), of poly(G) (lanes 8, 9 and 10 respectively), and of poly(C) (lanes 11, 12 and 13 respectively).

sequences possibly assist in binding to 3'UTR strongly, since removal of poly(A) tail resulted in reduced binding activity. To find out if there is any conserved sequence motif, prolamine 5'UTR, 3'UTR, glutelin 3'UTR, Xenopus PABP consensus sequence and psbA 5'leader sequence were aligned which revealed conservation of four adenosine residues (Fig 4). In case of Xenopus poly(A) binding protein, it has been shown that this adenosine stretch is important for binding activity (Kuhn & Pieler 1996).

DISCUSSION

It has been documented that prolamine mRNAs are preferentially localized to the surface of prolamine PBs (Li et al., 1993). Moreover, it has been shown that membrane-stripped PBs retain prolamine polysomes through a possible interaction with cytoskeleton (Muench et al., 1998), presumably by a cytoskeleton-associated RNA-binding activity. To obtain direct evidence for such an activity, we purified an enriched cytoskeleton-PB fraction by sucrose density gradient centrifugation and obtained a high-salt extract of proteins associated with this fraction. This extract (about 60 mg) of cytoskeleton-PB associated proteins was then resolved by poly(U)-Sepharose into three fractions which consisted of flow through (40 mg) , 0.3 M KCl (10 mg) and 1 M KCl elution fraction (5 mg). All these fractions were analyzed for their RNA binding activities using ^{32}P-labeled prolamine and glutelin mRNA sequences by RNA-protein UV crosslinking technique.

While several RNA binding activities were readily observed in 300 mM KCl elution fraction, all bands appeared quite weak on the autoradiogram under stringent binding conditions. Weak signals may be due to binding of proteins to RNA sequences which are less rich in U residues and shorter in length, since the *in vitro* transcription was carried out in the presence of ^{32}P-UTP. A prominent RNA binding activity at 40 kD, however, was readily observed in 1 M KCl elution fraction even in the presence of excess quantity of heparin, a sulfated anionic polysaccharide commonly employed to eliminate non-specific RNA-protein interactions. Results from competition experiments indicated that the 40 kD protein specifically binds to the 3'UTR with or without poly(A) sequences of both prolamine and glutelin mRNAs. This is interesting to note because direct binding

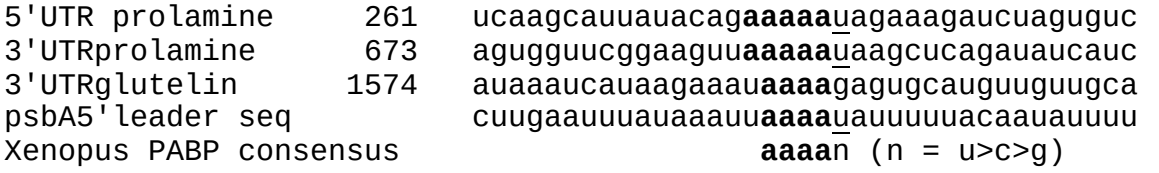

Fig. 4

Fig. 4. Comparison of 5'UTR and 3'UTR sequences of rice prolamine 7 mRNA and 3'UTR sequence of G22 rice glutelin mRNA sequence. Also shown is the minimal preferred binding sites for Xenopus PABP (Kuhn & Pieler 1996) and *C. reinhardtii* psbA mRNA RB47 (Danon & Mayfield 1991)

experiment results show that this protein also binds to the 5'UTR of prolamine RNA but at reduced levels of binding. The 40 kD binding activity to prolamine 3'UTR sequences in the presence or absence of the poly(A) tail were strongly competed by poly(A), while poly(U), poly(G) and poly(C) had a only slight effect on binding activity. These findings suggest that the 40 kD binding activity interacts with RNA sequences rich in adenosine residues. Interestingly, all three of these mRNA segments share a common sequence motif of 4 to 5 consecutive adenosine residues (Fig. 4) which may serve as the binding sequence. This would be similar to Xenopus poly(A)-binding protein (PABP), which requires a minimum stretch of four adenosine residues at the 5'-portion of the RNA substrate (Kuhn & Pieler 1996).

Although the 40 kD binding activity binds to poly(A) sequences, it is much smaller in size than the typical 70-73 kD PABPs and is capable of interacting with the prolamine 3'UTR and 5'UTR sequences and glutelin 3'UTR sequences. A small amount of rice PABP are found in this cytoskeleton-PB extract (the bulk of the PABP is located in the cytoplasm) and we do not expect this RNA binding activity to be detected since ^{32}P-UTP was used to radiolabel the RNA substrates. The 40 kD binding activity studied here may be related to a recently described 47 kD chloroplast protein or ELAV proteins. The 47 kD chloroplast protein, which regulates translation of the chloroplast psbA mRNA by binding to its 5'UTR sequences, contains structural motifs similar to that observed for the poly(A) binding protein indicating that it is a related member of this large family of RNA-binding proteins (Yohn et al., 1998). ELAV proteins are a group of RNA binding proteins that have specificity to AU rich sequences and show an unique property of binding to long poly(A) (Ma et al., 1997).

The activity of the 40 kD RNA binding protein varies depending on the RNA substrate tested. The strongest binding activity was evident with prolamine 3'UTR (without poly(A) tail) followed by a weaker interaction with prolamine 5'UTR followed, in turn, by a very weak interaction with glutelin 3'UTR (without poly(A) tail). The extent of binding activity likely depends on the sequence context of the adenine-rich

motif as suggested by the sequence specificity of the Xenopus PABP and the 47 kD chloroplast *psb* binding protein. Both of these RNA-binding proteins prefer A-rich motifs containing a uridine residue at the 3'end of the adenosine stretch (Danon & Mayfield 1991, Kuhn & Pieler 1996). A uridine nucleotide is found at the 3' end of the A-rich motif of the prolamine 3'UTR and 5'UTR whereas a G residue is found in the glutelin 3'UTR motif (Fig. 4).

The role of 40 kD RNA binding protein during rice endosperm development is not known. It binds equally well to prolamine and glutelin 3'UTRs containing poly(A) tail. A much stronger binding to the prolamine 3'UTR was observed compared to glutelin 3'UTR when the poly(A) tails were removed from these sequences. Interestingly, the binding properties to prolamine and glutelin 3'UTR sequences alone (without the poly(A) tail) are consistent with the relative levels of these mRNA sequences associated with the surface of prolamine PBs. As demonstrated by Li et al., (1993), prolamine mRNAs are present at 7 to 10-fold excess over glutelin transcripts on the PB-ER. Further studies are required to confirm the association of this 40 kD protein to the cytoskeleton and its possible role in localizing storage protein mRNAs to specific ER subdomains during rice endosperm development.

Chapter III

Rp120 : A cytoskeleton associated tudor domain protein binds to mRNA

ABSTRACT

Biochemical and cytological evidences reveal the possible role of cytoskeleton in RNA localization process in rice endosperm cells. In order to look for direct evidence, RNA binding protein, Rp120, was purified from the cytoskeleton-enriched fraction by column chromatography. RNA protein UV crosslinking results indicate that Rp120 bound to the 3'UTR of prolamine RNA and to glutelin and elongation factor mRNAs. Subcellular fractionation of rice endosperm tissue demonstrated that Rp120 was associated with the cytoskeleton at low salt conditions while at high salt condition Rp120 dissociated from the cytoskeleton. A significant portion of Rp120 was associated with endoplasmic reticulum as revealed by immunoblot analysis. Transmission electron microscopic observations indicate the Rp120 association with endoplasmic reticulum. Immunoblot results showed that Rp120 was expressed only in seeds and the protein was detected much early in the seed development. Antibodies for Rp120 were raised in rabbit and a partial cDNA encoding 875 amino acids of 120 kD protein was isolated. Predicted amino acid sequence of Rp120 reveal that it contains Drosophila tudor domain and nucleic acid binding motifs and a P-loop like sequence motif.

INTRODUCTION

Rice endosperm cells synthesize and accumulate two major classes of storage proteins namely prolamines and glutelins. In addition to the alcohol-soluble prolamines, the dominant storage protein accumulated by cereals, rice also stores glutelin, a storage protein homologous to the 11S globulins. The mRNAs for the prolamines and glutelins are translated on rough endoplasmic reticulum membranes. Subcellular fractionation studies suggest that these storage protein mRNAs are not randomly distributed on ER membrane complex but rather differentially distributed onto different domains of the ER membrane. Prolamine transcripts can be readily recovered from prolamine protein body (PB) enriched fraction whereas glutelin transcripts predominate on microsomal membrane fractions (Yamagata et al., 1982, Kim et al., 1993). These initial observations have been confirmed by Li et al., (1993). Messenger RNA quantitation and high-resolution *in situ* hybridization demonstrate that prolamine transcripts are localized on the PB-ER, while glutelin transcripts predominate on the cisternal-ER (Li et al., 1993).

The transport and localization of RNAs to specific intracellular regions of the cell is now a well-established phenomenon in both embryonic and somatic cell types as well as in plants. In rice endosperm cells segregation of prolamine and glutelin storage protein mRNAs to distinct ER subdomains is an attractive model system to study the transport and localization of mRNAs especially since these transcripts are abundantly expressed in the seed. The mechanism(s) by which prolamine mRNAs are localized to the prolamine PBs has not been determined. However, the available evidence supports the view that the RNA, and not the coded protein, is responsible for RNA localization. Detergent and high salt treatment of a membrane-stripped PB fraction indicates that the polysomes are bound to the PB surface by a detergent resistant but in a salt sensitive manner. Moreover, this association of polysomes to membrane-stripped PBs has been resistant to puromycin treatment indicating that the nascent polypeptide chain is not involved. Ribosome run-off experiments suggest that the prolamine polysomes are bound by RNA and/or ribosome receptor, possibly associated with the cytoskeleton

(Muench et al., 1998). Confocal microscopic studies have shown that the PBs are closely associated with both microtubules and microfilaments (Muench & Okita unpublished).

The localization of prolamine RNAs to the PB-ER by RNA-based mechanism infers the existence of RNA binding proteins that recognize one or more "zip code" signals within the prolamine RNA sequence. We, therefore, undertook a study to identify the types of RNA binding activities in protein body-cytoskeleton enriched fraction. As described in chapter II, RNA protein UV crosslinking experiments showed that purified fractions obtained by poly(U)-Sepharose and/or heparin-agarose chromatography contained several RNA binding activities. One of these RNA binding activities was a 40 kD RNA binding protein which recognized an A-rich region of 5'UTR and 3'UTR (untranslated region) of prolamine RNA as well as the 3' UTR of glutelin mRNA (Sami-Subbu et al., 2000). Here, we report the isolation and characterization of a second RNA binding activity, Rp120, from the cytoskeleton-PB-enriched fraction.

MATERIALS AND METHODS

Rp120 purification

The cytoskeleton-protein body enriched fraction from the milky stage rice seeds (cultivar M109) was obtained by the protocol stated elsewhere (Davies et al., 1991) with slight modifications as mentioned in the previous chapter. Milky stage rice seeds (~80 g) were ground in 100 ml of cytoskeleton stabilizing buffer consisting of 5 mM HEPES (pH 7.5), 10 mM MgOAc, 2 mM EGTA, 1 mM PMSF, 1 mM DTT, 200 mM sucrose and 1% Triton X-100. The extract was passed through miracloth and centrifuged at 2000 RPM for 10 min using SS34 rotor in a Sorval centrifuge. The pellet enriched in cytoskeleton, PB and starch grains was loaded on a 60-80% sucrose density gradient. Step gradient contained 14 ml each of 80% and 60% sucrose. After loading the endosperm extract, the gradient was spun at 27 K for 90 min using SW28 rotor of Beckman. The cytoskeleton-PB fraction appeared at about 65% in the sucrose gradient and was collected using a spatula and suspended in 25 ml of high salt buffer containing 200 mM Tris-HCl (pH 8.5), 500 mM KOAc, 2 mM EGTA and 50 mM MgOAc to dissolve the cytoskeletal network. This high salt extract was then centrifuged at 49 K RPM for 45 min using SW50 rotor to

remove the polysomes and PBs. The supernatant was dialyzed against poly(U) Sepharose column loading buffer containing 20 mM HEPES (pH 7.5), 100 mM KCl, 1 mM DTT and 10% glycerol. About one gram of poly(U) Sepharose was incubated in 10 ml of column loading buffer and packed in a small column (5 ml column volume). About 56 mg of cytoskeleton-PB extract was loaded on Poly(U) Sepharose and bound proteins were eluted with 300 mM KCl (10 mg) and 1 M KCl (4 mg). Poly(U) Sepharose 300 mM KCl fraction was dialyzed in column loading buffer and loaded (10 mg protein) onto a heparin column. Column was washed with loading buffer 2 to 3 volumes of the size of the column. Bound proteins (1.5 mg) were eluted with 300 mM and 1 M KCl. All the fractions obtained from the column chromatography and the crude cytoskeleton fraction were subjected to RNA-protein UV crosslinking assay.

To raise antibodies against Rp120, heparin 300 mM KCl fraction was subjected to SDS-PAGE. Rp120 region from the acrylamide gel was cut as a strip and crushed well. The slurry containing acrylamide and Rp120 were mixed with an adjuvant and injected in bunny rabbit. Initially about 75 μg was injected followed by 30 μg as booster dose after a week. After 3 weeks rabbit was bleeded and the serum was obtained by spinning out the red and white blood cells.

RNA-protein UV crosslinking assay:

Prolamine7, glutelin and EF1 alpha cDNA plasmids were linearized with either *Bam*HI or with appropriate restriction enzyme to yield complete RNA in the transcription. After the restriction digestion the sample was extracted once with phenol followed by chloroform. DNA was precipitated with 2 volumes of ethanol. One micro gram of purified template was used in each transcription reaction.

Radiolabeled prolamine transcript was made using Ambion maxiscript transcription kit with slight modifications. In 25 μl reaction final concentration of 0.5 mM (ATP, CTP & GTP) each and cold UTP 40 μM along with 100 μCi of ^{32}P-UTP were used to make the radiolabeled RNAs using 20 Units of T3 or T7 RNA polymerase at 30^{o} C in the presence of 1 unit of RNase inhibitase (5'-3' Inc.). The reaction was terminated

by adding 1 unit of RNase free DNase (Promega). One µl from the reaction was loaded on a urea PAGE and exposed to X-ray film to confirm the complete transcript. Quantification of the transcript was done by TCA precipitation method. One µl was removed from the reaction and added to 9 µl of sterile water. For TCA precipitation, 5 µl was taken while rest 5 µl was used for total count. The radioactivity was estimated by liquid scintillation counting. Percentage of radioactive UTP incorporation was calculated from the total and TCA precipitable count. From this, total NTP incorporation into RNA synthesis was extrapolated and total ng RNA synthesized was estimated. *In vitro* transcribed RNA, after phenol and chloroform extractions followed by ethanol precipitation the resultant pellet was dissolved in sterile water to give 20 fmole/µl or 100 fmole/µl depending on the experiment.

In a reaction containing 20 mM HEPES, 100 mM KCl, 1 mM DTT, 10% glycerol, 4 µg of yeast tRNA, 1 unit of RNase inhibitase (5'-3' Inc.), 20 or 100 fmole of radiolabeled RNA were incubated with 500 ng of purified protein. The reaction was incubated at 4^{o} C for 10 min and transferred onto a parafilm sheet and exposed to UV light in a UV crosslinker for 7 mins (the distance between the UV source and the sample was about 5 cm). SDS-PAGE sample buffer was added and subjected to electrophoresis. After completion of electrophoresis the gel was dried and exposed to X-ray (Kodak Omart) film for 12 to 48 hours depending on the signal.

Subcellular fractionation:

Sucrose step gradient (50 to 64%) was used for fractionation. One ml of each sucrose solution was overlaid on one above the other in an ultra centrifuge tube and left in the cold room for 1 to 2 h before loading the seed extract. About 3 g of dehulled rice seeds were gently ground in either cytoskeleton stabilizing buffer (as described above) or high salt PB-extraction buffer (2 ml) containing 20 mM HEPES-NaOH (pH 7.5), 50 mM MgCl2, 100 mM KCl, 200 mM Sucrose, 1 mM DTT and 1 mM PMSF. The slurry was passed through miracloth and then spun at 500 RPM twice. As a control, 10 µl of extract was saved from both high and low salt extract before loading onto the sucrose gradient (this is to compare the levels of Rp120 in 10 µl volume extract before and after the

centrifugation of the extract at different salt concentrations). The resulted supernatant was loaded onto the sucrose gradient. The tubes were loaded in a SW50 rotor and spun at 32 K RPM for 90 minutes. Different layers from the gradient were collected and subjected to SDS-PAGE for further immuno blot analysis.

Immuno blot analysis and immuno screening:

Protein samples were subjected to SDS-PAGE and transferred to nitrocellulose membrane according to the manufacturer's protocol (Biorad). Membranes were blocked with 5% milk powder in TBS and incubated with primary antibodies in TBS containing 2% milk powder. After 3 washes in TBS the blots were incubated in the secondary antibodies containing horseradish peroxidase (HRP). After three washes with TBS, HRP substrate (Pierce) was spread on the blots and the chemiluminescence was detected by exposing the blot to the X-ray film.

Immuno screening was mainly performed according to the manufacturer's protocol (Stratagene) with slight modification. About a million plaques of lambda ZAP II rice developing seed cDNA library were spread on solid LB medium. Nitrocellulose membranes presoaked in 10 mM IPTG were spread on the plaques grown for about 6 hours and the plate containing membranes were left in the incubator overnight. Membranes removed from the plates were washed with TBST and the antibodies incubation was followed as stated before for immuno blots. Two positive clones were identified and *in vivo* excision was performed as per the company protocol. Plasmid DNAs were prepared using Promega Wizard kit. DNA sequencing of these clones was carried out which revealed that they were identical.

Immuno localization:

Rice developing seeds (about 12 DAF) were harvested and fixed in solution containing 1.25% formaldehyde and 2% paraformaldehyde. Dehydration was carried out by gradually increasing percentage of ethanol. Embedding was done by gradually increasing the concentration of LR white to ethanol as 1:3 followed by 1:2, 1:1, 2:1, 3:1 and then incubated in LR white solution. The embedded tissue was cured at 50^0 C

overnight. Thin sections were taken using a glass knife in a microtome. Sections were taken onto a grid and then incubated in blocking solution containing 10 mM Tris pH 7.4, 500 mM NaCl and 1% BSA. Primary antibody was added to this blocking solution and incubated for 2 to 6 hours. Grids were washed in washing solution and then incubated in blocking solution containing secondary antibody conjugate. Grids were washed and observed under TEM and photographed.

RESULTS

Purification of Rp120 from cytoskeleton enriched fraction:

A cytoskeleton-PB enriched fraction from developing rice seed was prepared by sucrose density gradient centrifugation as described in the previous chapter. After extraction of proteins by high ionic strength buffer and clarification by high speed centrifugation followed by dialysis, the protein extract was subjected to Poly-(U) Sepharose chromatography. Protein fractions were collected at 300 mM and 1.0 M KCl and analyzed by RNA-protein UV-crosslinking assay. A prominent 120 kD RNA binding acivity was observed in the 300 mM KCl fraction. Such an activity was not observed in the crude extract. Minor binding activities were also evident at 100 kD, 75 kD and 40 kD. The 1 M KCl fraction showed a strong binding activity at 40 kD which was characterized in the previous chapter. The 300 mM KCl fraction was further subjected to heparin column chromatography. The 120 kD binding activity together with a few weak activities at the 100 and 75 kD regions eluted off the heparin column at 300 mM KCl.

Rp120 binds to several different mRNAs but shows specificity to certain regions:

When 20 fmole of ^{32}P radiolabeled prolamine RNA was used in the crosslinking reaction, a strong binding activity at 40 kD was readily detected whereas a weak reaction was observed for the 120 kD protein. Analysis by SDS-PAGE, however, revealed that the 120 kD binding activity was associated with a prominent polypeptide band stained by Coomassie blue whereas the 40 kD binding activity was associated with very faint polypeptide band. When the amount of ^{32}P-radiolabeled RNA probe was increased to 100 fmole of radiolabeled RNA a prominent binding activity by the 120 kD polypeptide

was evident. These results indicate that the 120 kD RNA binding activity had a much lower affinity as compared to the 40 kD RNA binding activity.

The substrate specificity of the Rp120 was studied using three different mRNAs. Radiolabeled prolamine, glutelin and EF1 alpha RNAs were incubated with heparin 300 mM KCl fraction (Fig 1a). UV crosslinking results showed that Rp120 not only bound to prolamine RNA but to glutelin and EF1 alpha as well. Since Rp120 bound to several RNAs, probably it is a general RNA binding protein expressed in the rice seeds.

Radiolabeled prolamine mRNA containing the complete sequence or excluding the 3'UTR were used in the UV crosslinking reaction. The full length mRNA sequence at 50 and 100 molar excess competed the binding efficiently whereas even 100 molar excess prolamine RNA without 3'UTR (along with 111 bases 3'UTR, 80 bases of coding region was removed for *in vitro* transcription reaction) had no effect on binding to the radiolabeled probe (Fig.1b). These results indicate that Rp120 binds to prolamine RNA 3'UTR containing poly(A). In order to ensure that the Rp120 binding activity is specific to 3'UTR, ^{32}P radiolabeled 3'UTR lacking the poly(A) was used in the RNA binding UV crosslinking assays. An interaction of the 120 kD binding activity was observed with the 3'UTR (Fig. 1c), indicating the presence of unique binding sequences.

Rp120 is associated with the cytoskeleton:

Association of 120 kD RNA binding protein with the cytoskeleton was studied by subcellular fractionation using sucrose density gradient followed by immunoblot analysis. Cytoskeleton is made up of actin and tubulin and is sensitive to high salt treatment. Based on this principle, rice seed extracts were prepared in low and high salt buffers and the association of 120 kD protein with cytoskeleton was established by immunoblot experiment. A cytoskeleton-PB enriched fraction was isolated in the presence of low salt buffer with Triton X-100 and subjected to 60-80% sucrose density gradient. The rice seed extract prepared in high salt without Triton X-100 was subjected to 50-64% sucrose density gradient. An aliquot of protein from different layers including the supernatant of

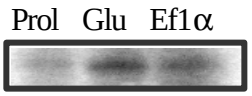

Fig. 1a

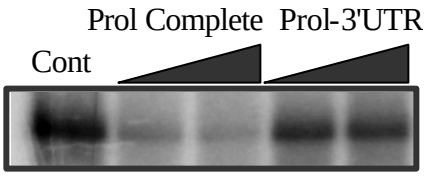

Fig. 1b

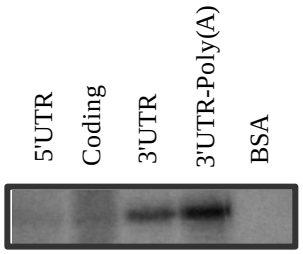

Fig. 1c

Fig. 1 RNA-protein UV crosslinking showing the binding of Rp120 to 3'UTR of prolamine RNA and to different RNAs.

(a) Radiolabeled prolamine (Prol) (20 fmole), glutelin (Glu) and EF1 alpha (Ef1α) RNA were incubated with equal quantity of heparin 300 mM KCl fraction proteins.

(b) In all the reactions, 100 fmoles of radiolabeled prolamine RNA and equal quantity of protein (Cont. lane) were incubated with cold complete prolamine RNA 50X and 100X excess (lanes 2, 3) or cold truncated prolamine RNA (without 3'UTR) 50X and 100X excess (lanes 4, 5, respectively)

(c) Direct biding of Rp120 to different parts of prolamine RNA namely 5'UTR (lane 1), coding region (lane 2), 3'UTR (lane 3), 3'UTR-poly(A) (lane 4) and the last lane prolamine RNA (20 fmoles) allowed to crosslink to BSA

these sucrose gradients and an aliquot of the extract before centrifugation were subjected to SDS-PAGE and proteins were transferred onto nitrocellulose membranes. These blots were probed with antibodies raised against Rp120, actin and ER membrane marker calnexin. Low salt immunoblot results indicated that supernatant of the sucrose gradient (after the centrifugation) contained very less quantity of Rp120 as compared to control (an equal volume of crude cytoskeleton-PB extract before loading onto the gradient) (Fig.2a). This indicates that majority of Rp120 was associated with subcellular fraction which was heavier, and thus appeared down the gradient upon centrifugation. However the quantity of calnexin in sucrose gradient supernatant (after centrifugation) and the control lane (an equal volume of crude cytoskeleton-PB fraction before loading on to the sucrose gradient) was nearly equal, since the extract was made in the presence of membrane dissolving agent, non ionic detergent Triton X-100. In the interface between the supernatant and sucrose there were two membrane layers which appeared close to each other. A significant amount of Rp120 and a small quantity of actin were observed in the heavier membrane fraction compared to the lighter membrane layer. As expected Rp120 and actin were detected in the heavy (cytoskeleton) fraction (Fig.2a) at about 65% density region and calnexin was not detected there. In case of high salt extract, five layers of different densities and the supernatant were recovered for immunoblot analysis. As expected a significant amount of Rp120 detected in the supernatant (after the spin) was readily comparable to the control (an equal volume of crude extract of PB fraction before the spin) (Fig.2b). This observation supported our low salt immunoblot results that Rp120 was associated with cytoskeleton and its interaction was sensitive to high salt. The first layer, which was at the bottom of the supernatant, also contained significant amount of Rp120, calnexin and actin. While in the subsequent layers, down the gradient, quantity of calnexin was reduced whereas actin and Rp120 could not be detected. Even at high salt condition significant quantity of Rp120 was associated with the ER membrane and the cytoskeleton in the first layer was interesting to note (Fig.2b). These results clearly indicated that at low salt Rp120 was associated with the heavy cell matrix (cytoskeleton) and appeared at denser region while at high salt condition Rp120

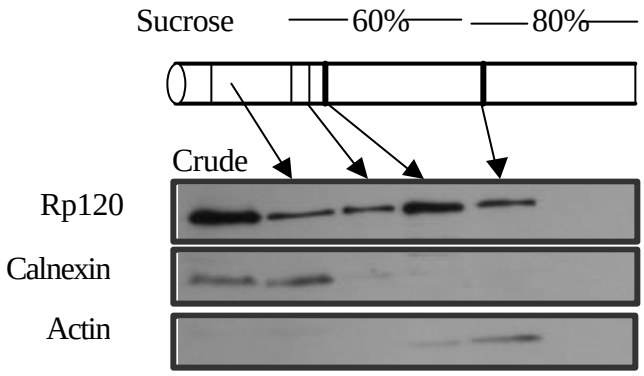

Fig. 2a

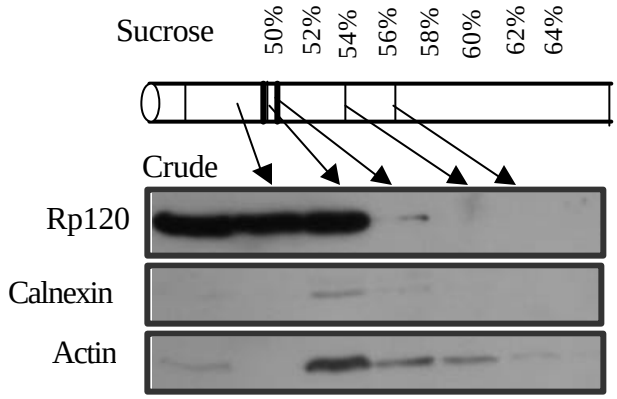

Fig. 2b

Fig. 2 Cytoskeleton-Rp120 interaction studies by sucrose density gradient (SDG) followed by immuno blot analysis using anti-Rp120, anti-calnexin and anti-actin antibodies.

(a) Developing rice seed extract prepared in low salt buffer were subjected to SDG and different layers obtained that include crude low salt extract (lane 1) supernatant of SDG (lane 2) down the gradient layer 1 (lane 3) layer 2 (lane 4) layer 3 (lane 5).

(b) Developing rice seed extract prepared in the high salt buffer were subjected to SDG and different layers obtained that include crude high salt extract (lane 1) SDG supernatant (lane 2) layer 1 (lane 3) layer 2 (lane 4) layer 3 (lane 5) layer 4 (lane 5) were analyzed by immuno blot.

association with cytoskeleton was destabilized and appeared in the supernatant. Even at high salt condition a significant portion was associated with ER membrane-cytoskeleton complex.

Immunolocalization of Rp120 in rice endosperm cells:

About 12 DAF (days after flowering) rice seeds were embedded in LR white. Thin sections obtained from the embedded tissue were incubated with primary antibodies followed by secondary antibodies conjugated with gold colloid. The sections were visualized using a transmission electron microscope. Control antibodies did not show any localized signal rather they were randomly seen (Fig. 3a). Signals were seen around the ER membrane especially around the PBs and cisternal ER membrane (Fig. 3b). Signals were seen strongly on cell wall region as well. Possible reason could be that antibodies tend to bind to the sugar residues on the cell wall or may be this protein could be localized in the intercellular regions as well. Although subcellular fraction experiment indicate that 120 kD protein appear in the layer containing ER membrane-cytoskeleton complex (Fig. 2b lane 3), immunolocalization experiment has to be repeated to confirm the localization of 120 kD protein to ER membrane.

Tissue specific and temporal expression of Rp120:

In order to study the tissue specific expression of Rp120, total protein from the roots, leaves of seedlings, flowers and developing seeds were subjected to SDS-PAGE and blotted onto nitrocellulose membrane. When this blot was probed with Rp120 antibodies, the Rp120 expression was observed only in the developing seeds and not in roots, leaves and flowers (Fig.4a). The temporal expression of Rp120 with respect to prolamine and glutelin expression was studied by assessing the amount of the Rp120 in developing seeds of varying stage (4 to 12 days after flowering (DAF)). The immuno blot analysis showed that the Rp120 was expressed as early as 4 DAF and attained a maximum steady state level between 7 to 10 day old developing seeds (Fig. 4b).

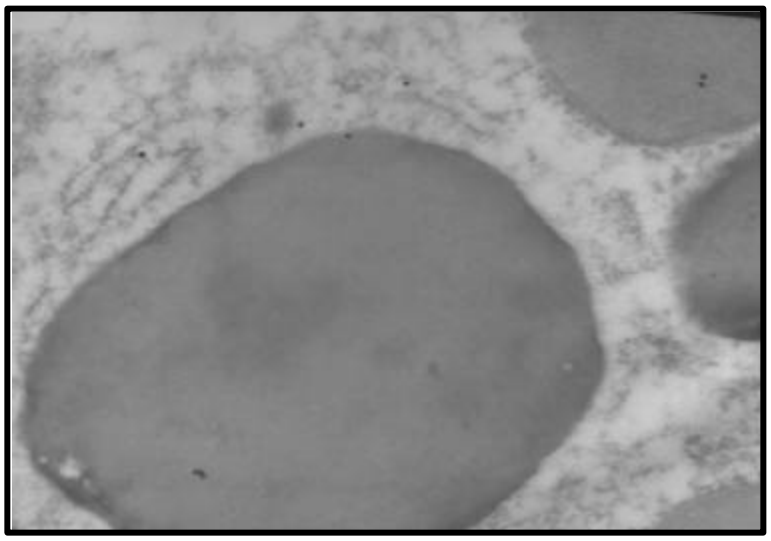

Fig. 3a

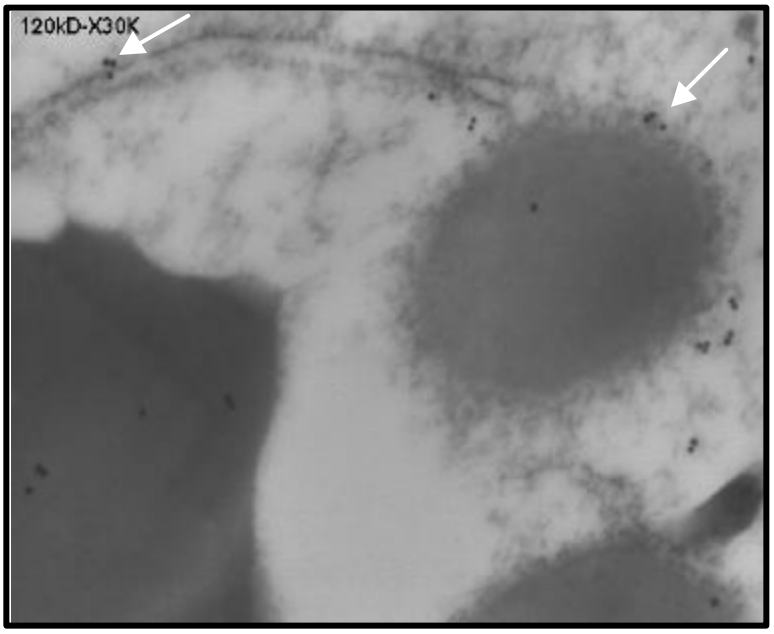

Fig. 3b

Fig 3 Immuno localization of Rp120 at transmission electron microscope level.

(a) Rice section incubated with pre immune serum.

(b) Rice section incubated with Rp120 antibodies. Arrows indicate Rp120 localization on ER membrane.

Isolation of Rp120 cDNA:

About one million plaques from a developing rice seed cDNA library were screened with Rp120 antibodies. After three rounds of screening two positive clones were purified, which were identical as determined by DNA sequence analysis. The isolated cDNA was about 3.0 kb in length and lacked the initial ATG codon. Since Northern analysis indicated that the Rp120 transcript is about 4 kb in length (data not shown), a considerable length of the 5' end of the cDNA was missing in these clones.

The translated peptide contained 875 amino acids (Fig.5). Simple BLAST search indicated that Rp120 shared overall about 30% identity with human nuclear protein, transcriptional coactivator p100 class protein which is conserved from yeast to mammals. Interestingly, the Rp120 amino acids 600-750 shared about 26 to 40% sequence identity with multiple region of the Drosophila tudor protein (Fig.6b) (for details refer the discussion). Two regions of the Rp120, amino acid positions 213-254 and 562-600, shared 25-33% sequence identity with 52-140 amino acid region in the SN fold motif of staphylococcus nuclease (Fig.6a). Other than the SN-like sequences, the Rp120 lacked the usual RNA recognition and ribonucleo protein motifs. MOTIF (http://www.genome.ad.jp/htbin/) search results indicate a putative P-loop (GTP-binding) like motif at 67-74 amino acid region of Rp120 (Fig.5). Rp120 might also contain a putative transmembrane motif in between the region 90-106 amino acids (http://www.isrec.isb-sib.ch/software/TMPRED) towards the N-terminal region of the polypeptide.

DISCUSSION

Non random distribution of mRNA in the cytoplasm has been reported in a wide range of eukaryotic systems (Hesketh 1996, Mohr 1999, St Johnston 1995). In rice endosperm cells, segregation of prolamine and glutelin RNAs to different subdomains of the ER membrane has been documented (Li et al., 1993). Although molecular mechanism of

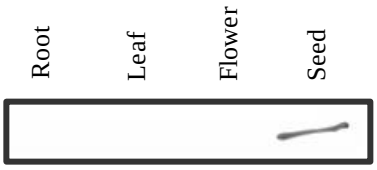

Fig. 4a

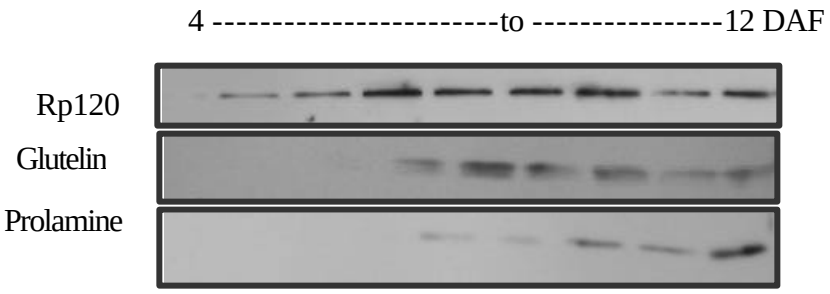

Fig 4b

Fig. 4 Tissue specific and temporal expression of Rp120.

(a) Total protein from root, leaf, flower and developing seed were analyzed by immuno blot using Rp120 antibodies.

(b) An immuno blot prepared out of total protein from different stages of rice seed development ranging from 4 to 12 days after flowering and was probed with Rp120, glutelin and prolamine antibodies individually.

1 ARGVKEQGPKGGEPSPYLTELLRLEEVAKQQGLGRWSKEPGAAEESIRDLPPSAIGEASG 60

61 FDAKGFAVANKGKSLEAIVEQVRDGSTV**RVYLLPSFQFVQIYVAG**VQSPSMGRRPPNPTV 120

121 VAAAESTADGATNGGDSEEAPAPLTTAQRLAAAAVSTEIPPDRFGIEAKHFTETHVLNRD 180

181 VRIVVEGTDSFSNIIGSVYYSDGDTLKDLALELVENGLAKYVEWSANMMDVDAKIKLKNA 240

241 ELQAKKDQLRIWTGFKPPVTNSKPIHDQKFTGKVVEVVSGDCIIVADDAAPYGSPSAERR 300

301 VNLSSIRAPKMGNPRRDEKPDNFAREAKEFLRTRLIGKQVTVEMEYSRRISTVDGQPTTN 360

361 TADARVLDYGSVFLGSPSQADGDDVSSIPSSGNQPGINIAETLLSRGFAKTSKHRDYEKR 420

421 SHYFDLLLAAESRAEKAKKGVHSAKKSPVMHITDLTTVSAKKARDFLPFLQRNRRHSAIV 480

481 EYVFSGHRFKLTIPKETCSIAFSFSGVRCPGKDEPYSNEAIALMRRRILQRDVEIEVEAV 540

541 DRTGTFLGSLWESKTNMASVLLEAGLAKLSSFGLDRIPDANVLMRAEQSAKQQKLKIWEN 600

601 YVEGEEVSNGSASESKQKEILKVVVTEVLGGGKFYVQTVGDHRVASIQQQLASLKLKDAP 660

661 VIGAFNPVKGEIVLAQFSADNSWNRAMIVNGPRGAVSSQDDKFEVFYIDYGNQEVVPYSR 720

721 IRPADPSISSSPALAQLCSLAFIKVPNLEDDFGHEAAVYLNDCLLNSQKQYRAMIEERDT 780

781 SGGKSKGQGTGTILIVTLVDAKTETSINATMLEEGLARLERSKRWDTRERKAALQNLEQF 840

841 QEKAKKERLQIWQYGDVESDEEEQAPAARRTGGRR 875

Fig. 5

Fig. 5 Predicted amino acid sequence of Rp120 cDNA. Putative ATP/GTP binding motif obtained by MOTIF search is high-lighted (amino acids 67-74). Predicted transmembrane motif is in bold letters (amino acids 90-106). Two SN-fold motifs are underlined (amino acids 213-254 & 561-600). Drosophila tudor like domain is shown by open box (amino acid 632-715).

```
                  helix                        helix       helix
Rp120 domainI  LVEN GLAK ..YVEWSANMMDVDAKIK LKNA ELQ AK KDQ LR IW TG
Rp120 domainII LLEA GLAK LSSFGLDRIPDANV....L MRA EQS AK QQK LK IW EN
TNase          LVRQ GLAK VAYVYKPNNTHEQH....L RKS EAQ AK KEK LN IW SE
```

Fig. 6a

```
                 X
Rp120      632-GK FYV QTVGDHRVASIQQQLASLKLKDAPVIGAFNPVKGEIVLA
Human p100 669-LH FYV QDVETGTQFQKLMENMRNDIASHPPVEGSYAPRRGEFCI
tudor I    424-YL FWV HLKSSDHDLSTMMGQIER....TKLKALAQAPELGTACV
tudor II   608-SN FYV QKVANIGKFEQLMDEMFS..YYNATREFPDQLILGAPCI
tudor III 1322-YR VYV QPQAIVPSMQTLLDNMYE..HYKAKGDSLKKFDVGQICA
tudor IV  1630-AQ FYV HPIDQLSKLNQLHENLQI...VSPSLPQLMNVVNGADCV
tudor V   1808-CD FFI QLERDSKALELIELYLRK....KDTLKPLEGFEKGLIVA
tudor VI  1992-SR IYL QFSEKDSLMDIICEKLNG....SKLQPKTEKAAVDDMCV
tudor VII 2176-KS FYV QMKHNSADLDLIVKTLQS..LKKEKLKKLIDPTTNSNGV
tudor VIII2360-MS FYV QMESDVPALEQMTDKLLD...AEQDLPAFSDLKEGALCV
Consensus
```

```
                    y                    z
Rp120      QFSADNSWNRAMIVNGPRGAVSSQDDKFEVFYIDYGNQEV-715
human p100     AKF.VDGEWYRARVEKVESPAK.IHVFYIDYGNREV-746
tudor I        ARFSEDGHLYRAMVCAVYAQR..YRVVYVDYGNSEL-497
tudor II       VKC..DQEWYRAEILRVDDS...VIVRHVDFGYEQN-680
tudor III      VRSS.DGNWYRARISGKDSNAACFEVFYIDYGNTEE-1398
tudor IV       SMYSVDKCWYRAKIIDAEL....MVLLFIDYGNTDC-1702
tudor V        ALFEDDELWYRAQLQKELPDSR.YEVLFIDYGNTST-1882
tudor VI       VQFADDLEFYRSRILEVLEDDQ.YKVILIDYGNTTV-2066
tudor VII      CYSQEDACYYRCSIKSVLDPSQGFEVFLLDYGNTLV-2253
tudor VIII     AQFPEDEVFYRAQIRKVLDDGK.CEVHFIGDFNNAV-2435
Consensus               **               ********
```

Fig. 6b

Fig. 6 (a) Amino acid sequence alignment of Rp120 with Staphylococcal nuclease SN-fold motif.

(b) Sequence alignment of Rp120 with HCA output of Drosophila tudor domain.

prolamine RNA localization is not understood fully, available evidence suggests that the prolamine RNAs are localized to the PB-ER by an RNA-based mechanism. Since this infers the existence of RNA binding activity associated with cytoskeleton that recognizes the prolamine RNAs, we decided to identify and purify RNA-binding proteins associated with the cytoskeleton-PB enriched fraction. Cytoskeleton-enriched fraction was obtained from the sucrose density gradient and subjected to poly(U) Sepharose and heparin column chromatography. Different protein fractions eluted from the column were subjected to RNA-protein UV crosslinking assay. A major protein of about 120 kD (Rp120) along with few weak signals were detected on the autoradiogram with molecular weights ranging from 35 to 100 kD. A strong signal at about 40 kD was observed in 1 M KCl fraction. The binding of 40 kD protein to 3'UTR of prolamine mRNA and to poly(A) has been described in the previous chapter. Although the signal obtained in 300 mM KCl heparin column was weak for 120 kD protein, SDS-PAGE profile indicates the abundance of 120 kD (data not shown). Moreover, two-dimensional gel electrophoresis of 300 mM heparin fraction indicated Rp120 as a single major polypeptide at 120 kD region. Since this protein was abundant in the cytoskeleton-enriched fraction and bound to RNA, we decided to isolate the gene and characterize its RNA binding activity and association with cytoskeleton.

Immuno screening of a rice developing seed expression library using Rp120 antibodies resulted in identification of a positive clone sharing homology with a wide range of proteins (Fig.5). Overall, Rp120 protein shows 35% identity with human nuclear protein p100 (Tong et al., 1995). Hydrophobic cluster analysis (HCA) of human p100 revealed repetitive domains similar to the subdomains appearing in Staphylococcal nuclease [or thermonuclease (TNase)] (Callebaut & Mornon 1997). Staphylococcal nucleases are calcium-dependent enzymes that hydrolyze both DNA and RNA to yield 3'-mono nucleotides and polynucleotides and the crystal structure of the staphylococcal nuclease has been solved. There were two motifs in Rp120 that shared 30% identity with 3 helices which appeared in SN-fold motif of TNase(Fig.6a). In the first helix most of the amino acids were conserved in both the domains of Rp120 except the hyrdophilic

glutamine residue was replaced by a hydrophobic alanine residue in domain II whereas in domain I glutamine is replaced by asparagine residue. In second and third helices most of the hydrophobic residues are conserved. Conservation of these helices in Rp120 is interesting to note, since these three helices appear near the vicinity of the active site of TNase and are likely to be involved in nucleic acid binding. SN-fold proteins belong to OB-fold super family, which recognize wide range of nucleic acids (double or single stranded DNA, tRNA, mRNA). Therefore, the Rp120 regions sharing homology with TNase OB-fold might possibly be involved in RNA-binding. Besides, Rp120 share significant similarity with a 80 amino acid region called tudor domain of Drosohpila tudor protein (Fig.6b). The maternally encoded tudor protein is required for posterior pole formation during oogenesis in Drospophila embryo. HCA analysis indicates three motifs x, y and z present in the tudor and human p100 proteins. The invariant amino acids of x and z motifs are also conserved in Rp120 (Callebaut & Mornon 1997) while in y motif a basic invariant arginine residue is replaced by a polar aspargine and acidic aspartate is replaced by a basic arginine (Fig. 6b). Moreover, the Drosophila *homeless* (*hls*) gene product required for RNA localization contains a single tudor domain (Ponting 1997). Mutation in *hls* gene resulted in disruption of localization of specific mRNAs during oogenesis. A transmembrane helix like motif, between amino acids 90 and 106 may be present in Rp120 (Fig.1) however we do not know the significance of this motif in Rp120.

Besides radiolabeled prolamine, glutelin and EF1 alpha RNAs were used as substrate in the RNA binding experiments (Fig. 1a). The results indicate that Rp120 not only binds to prolamine RNA but glutelin and EF1 alpha RNAs as well. Therefore, we believe that Rp120, a general RNA binding protein, may bind to a wide range of mRNAs expressed during the seed development. Vera RNA-binding protein not only binds to Vg1 but to Xcat-2 as well. Rp120 might play a role in localizing several mRNA molecules during seed development. Recently, in cultured PC12 cells, a 160 kD general RNA binding protein associated with cytoskeleton has been identified by *in vivo* UV crosslinking experiments which binds to RNA in a sequence independent but length

dependent manner (DeFranco et al., 1998). This protein is identified as a part of ~1600 kD complex with the microtubule associated protein MAP 1A.

Using RNA-protein UV crosslinking technique RNA binding activity of Rp120 was studied. In order to map the region being bound by Rp120 on prolamine RNA, radiolabeled prolamine RNA was competed with excess unlabeled intact prolamine RNA and truncated prolamine RNA (without 3'UTR). Unlabeled intact RNA efficiently competed the binding activity while truncated RNA was unable to compete the binding even at higher concentration indicating that Rp120 binds to 3'end of the labeled RNA (Fig 1b). Direct binding assay results supported these observations that Rp120 binds to 3'UTR of prolamine RNA (Fig 1c).

Rp120 interaction with the cytoskeleton was evaluated by subcellular fractionation of rice endosperm cells on a sucrose cushion followed by immunoblot analysis. It is known that protein-protein interaction is sensitive to high salt treatment and this property was exploited to probe the interaction of Rp120 with the cytoskeleton. When cytoskeletal extract was prepared in the presence of low salt buffer (cytoskeleton stabilizing) and nonionic detergent Triton X-100, Rp120 co-sedimented with the actin in the sucrose gradient. Most of the calnexin, an ER membrane marker, appeared in the supernatant of the gradient and the cytoskeleton fraction was free of the ER membrane due to the nonionic detergent (Fig 2a). This clearly indicates that the association of Rp120 with cytoskeleton is independent of ER membrane.

A drastic change in distribution pattern of Rp120 was observed in high salt extract fractionation. Significant quantity of Rp120, which is equivalent to the same volume of crude extract (before spin), was detected in the supernatant of the gradient (Fig.2b) due to dissociation of Rp120 from cytoskeleton. It was also interesting to note that a substantial portion of Rp120 was also associated with the ER membrane-cytoskeleton complex even at high salt concentration (Fig. 2b lane 3). In maize, cytological study using confocal microscopy indicated that PBs are enmeshed with a network of cytoskeleton (Clore et al., 1996). Moreover, in Xenopus oocytes, RNA binding protein Vera is co-localized with

the ER membrane marker TRAP alpha as shown by subcellular fractionation study (Deshler et al., 1997). Since Rp120 binds to prolamine RNA and its interaction with cytoskeleton is sensitive to high salt treatment, it is possible that Rp120 associates the prolamine mRNA to cytoskeleton. Moreover, Rp120 polypeptide sequence indicates the presence of a putative P-loop (ATP/GTP binding motif) (Fig.5) which is found in kinesin like motor proteins and kinases (Saraste et al., 1990). It is not known, if this P-loop motif is functional in Rp120. Although the function of Rp120 is not clearly understood, the density gradient experiment data suggest that Rp120 is associated with cytoskeleton and also cytoskeleton-ER membrane complex. A close association of cytoskeleton with ER membrane is understood very recently (Clore et al., 1996). Although Rp120 shows a putative transmembrane like motif, appearance of Rp120 in ER fraction can be due to cytoskeleton rather than a direct interaction with ER membrane (Fig.5). Immuno localization at electron microscope level indicates that patches of Rp120 signals are seen around the PBs and cisternal ER membranes (Fig 3b). Recently, a Staufen like protein identified in human kidney was mainly seen around the ER membrane and was associated with the polysomes as revealed by confocal microscopic study (Marion et al., 1999).

Rp120 is expressed only in seeds (Fig 4a) and its expression is detected earlier than prolamine and glutelin during seed development. Circumstantial evidences of this study suggest that Rp120 may be directly or indirectly involved in prolamine mRNA localization process. Moreover, since Rp120 binds to glutelin and EF1 alpha as well, it may be speculated that Rp120 is involved in localization of wide range of mRNAs during seed development.

Chapter IV

Message specific RNA binding proteins associated with ER membrane in rice endosperm cells

ABSTRACT

Systematic distribution of prolamine and glutelin transcripts on different domains of endoplasmic reticulum (RE) has been well documented. To address the molecular mechanisms of nonrandom distribution of mRNAs on ER membrane, different domains of ER membranes were fractionated by ultracentrifugation. Prolamine and glutelin message specific RNA binding proteins associated with different domains of the ER membranes were analyzed by RNA protein UV crosslinking method. Prolamine specific, about 25 kD and 32 kD RNA binding proteins seem to be uniformly distributed on both protein body-ER (PB-ER) and cisternal-ER domains (c-ER). Interestingly, about 36 kD prolamine RNA specific binding protein is detected only in PB-ER but not in c-ER. Possible role of this 36 kD in enriching or translational activation of prolamine mRNA on PB-ER is discussed. A 28 kD, glutelin specific RNA binding protein is detected relatively more on c-ER than PB-ER. Message specific RNA binding proteins associated with different domains of ER membrane have been identified and their possible role in prolamine and glutelin RNA localization are discussed.

INTRODUCTION

The endoplasmic reticulum (ER), the largest endomembrane system of eukaryotic cells, performs a wide range of functions including synthesis, processing and secretion of proteins. Earlier it was classified into three compartments as rough ER, smooth ER and nuclear envelope. In rice endosperm cells, rough ER was further classified into two subdomains based on their morphology and RNA distribution (Okita et al., 1994). Synthesis and aggregation of prolamine seed storage proteins on rough ER membrane have demarcated the endomembrane into two domains that include single layer of cisternal ER membrane (c-ER) and a spherical compact prolamine protein body ER (PB-ER). Microsomal fraction, which is rich in c-ER, contains glutelin transcripts twice the amount of prolamine (Kim et al., 1993) while transcripts obtained from PB-ER enriched fraction could yield only prolamine polypeptide in an *in vitro* translation system (Yamagata et al., 1986). By *in situ* localization using fine resolution electron microscopy and subcellular fractionation, it has been shown that prolamine transcripts are mainly associated with PB-ER while glutelin transcripts are seen on c-ER (Li et al., 1993). In case of UT-1 cells, it was shown that HMG-CoA reductase is translated mainly on nuclear envelope and is absent on extensive rough ER membrane of the cells (Pathak et al., 1986). These findings demonstrate that rough ER membrane is divided into several subdomains and specific ER domain is enriched with a particular type of transcript.

Like animal viruses, synthesis of several plant viral RNAs takes place in a compartmentalized manner in association with endomembrane system (Restrepo-Hartwing & Carrington 1994, Restrepo-Hartwing & Ahlquist 1996, Schaad et al., 1997). Using florescence microcopy, the impact of TMV on ER membrane has been studied in live cells. TMV (Tobacco Mosaic Virus) movement protein (MP) modified to incorporate green fluorescent protein (GFP) has been used to trace the viral protein on ER membrane and a drastic morphological change of ER membrane has been noted upon TMV infection (Reichel & Beachy 1998). Microsomal fractionation analysis results indicate that TMV MP behaves as an integral membrane protein on ER membrane (Reichel & Beachy 1998). Using immuno staining and *in situ* localization studies of protoplasts infected with TMV genomic RNA, it has been shown that viral RNAs are

colocalized with viral proteins. Moreover, TMV viral RNA associated with ER membrane has been confirmed by simultaneously immuno staining the ER luminal protein Bip (binding protein) (Mas & Beachy 1999). These studies have brought out a clear-cut evidence that ER membrane plays an important role in viral RNA and protein synthesis.

Although Blobbel (1980) has proposed that targeting of a polypeptide to ER membrane is directed by signal sequences, growing number of evidences strongly support the view that mRNAs are localized at discrete locations in the cytoplasm and the localization is due to the cognate sequences within the RNA itself. Perhaps, studies from the polarized egg cells, somatic, neuronal and plant cells have shown that mRNAs are localized at discrete regions of the cytoplasm rather than having a random distribution (Wharton & Struhl 1989, Gavis & Lehman 1992, Melton 1987, Li et al., 1993, Bouget et al., 1996). In fibroblast cells, mutations in the 3'UTR and microinjection has indicated that a sequence motif in c-*myc* 3'UTR is required for localization of reporter gene at the perinuclear cytoplasm. In case of β-actin mRNA localization, 54-nucleotide region called "zipcode" at 3'UTR can direct the localization of entire transcript to periphery (Kislauskis et al., 1993, Kislauskis et al., 1994). A 68 kD protein which binds to this zipcode, thought to play a role in β-actin mRNA localization has been reported (Ross et al., 1997). In a recent study, by microinjecting *in vitro* synthesized RNA, it has been shown that two distinct cis elements located at 3'UTR are responsible for transport and localization of MBP mRNA (Ainger et al., 1997). These findings demonstrate that localization of RNA is directed by a signal (sequence motif/secondary structure) which is recognized by a specific RNA binding activity during localization process.

Recently, localization dependent translation hypothesis has been put forth (Gavis & Lehmann 1994, Curtis et al., 1995). In Drosophila oocytes, it has been shown that unlocalized *nos* mRNAs are translationally suppressed due to the 3'UTR (Gavis & Lehmann 1994). *Oscar*, another posterior pole determinant, is translationally repressed prior to localization and the repression is mediated by an ovarian RNA binding protein Bruno, which binds to the multiple cis-elements located at the 3'UTR of *oskar* (Kim-Ha

et al., 1995). In Xenopus embryo, a 75 kD polypeptide (Vera) which binds to Vg1 localization element (VgLE) has been identified in cytoplasmic extract. Moreover, Vera binding activity co-sediments with an ER membrane marker as has been shown in a sucrose density gradient experiment (Mowry & Melton 1991, Deshler et al., 1997). Recently, *staufen* homolog has been isolated from human brain cDNA library. Using confocal microscopic technique, it is shown that hStaufen (human Staufen) is localized onto rough ER membrane. Although hStaufen is not an integral membrane protein, the subcellular fractionation and *in vitro* studies demonstrate that hStaufen is a cytoskeleton associated protein (Wickham et al., 1999). However, a close interaction of ER membrane with cytoskeleton has been documented recently in plants and animals (Clore et al., 1996, Klopfenstein et al., 1998). Therefore, possibly the association of hStaufen could be mediated through cytoskeleton, which surrounds the ER network. Using a yeast two-hybrid system, with influenza virus RNA binding protein as bait, *staufen* like transcript has been identified from a human kidney cDNA library. This hStaufen-like protein is co-localized with ER membrane marker and associated with polysomes in transfected cells (Marion et al., 1999). These studies have indicated that RNAs are transported in a translationally suppressed manner and RNA binding proteins seem to be associated with ER membrane.

In rice endosperm cells, blot hybridization and *in situ* localization demonstrated orderly distribution of storage protein mRNAs on different domains of ER membrane (Li et al., 1993). Although biochemical evidences indicate the possible role of cytoskeleton and associated RNA binding protein in prolamine mRNA localization (Muench et al., 1998, Sami-Subbu et al., 2000), the exact mechanism of prolamine mRNA localization is obscure. In this part of work, a direct approach of studying RNA binding proteins associated with different domains of ER membrane of rice endosperm cells was adopted to study their distribution pattern. Message specific RNA binding proteins have been directly obtained from ER membrane fraction and their differential distribution on different domains of ER membrane is shown.

MATERIALS AND METHODS

ER membrane fractionation:

Developing rice seeds of Basmati-370 were frozen in liquid N_2. Using a coffee grinder, seeds were ground to fine powder and suspended in homogenizing buffer consisting of 20 mM Tris-HCl pH 8.5, 50 mM MgCl2, 100 mM KCl, 1 mM DTT, 1 mM PMSF and 200 mM sucrose. The protocol followed was as described by Li et al., (1993) with slight modifications. Seed extract was passed through a nylon cloth and centrifuged at 100 g for 5 min using SS34 rotor in a Sorval centrifuge to remove the nuclei and starch grains. The homogenate was then spun at 2000 g using SS34 rotor of Sorval centrifuge and the pellet containing PB-ER was saved. The supernatant was collected and spun at 30,000 g for 45 min and the resulted pellet containing c-ER was saved. Both PB-ER and c-ER pellets were suspended in high salt buffer containing 1 M KCl, 1% Triton X-100, 10 mM $MgCl_2$, 200 mM Tris HCl pH 8.5, 1 mM DTT and 1 mM PMSF to dissolve the ER membrane and to release RNA binding proteins associated with ER membranes. High salt extracts of ER membrane fractions were spun at 30,000 g for 40 min to remove the polysomes and storage proteins. Supernatant was dialyzed overnight in a buffer containing 20 mM Tris HCl pH 8.5, 5 mM $MgCl_2$, 10 mM KCl and 10 % glycerol. Dialyzed proteins were stored at freezer compartment for RNA protein crosslinking assay.

***In vitro* transcription:**

Plasmids were digested with *Bam*HI or *Xba*I and processed as described in the earlier chapter. *In vitro* transcription was carried out in a 25 µl reaction containing 1 µg of template DNA, 100 µCi of ^{32}P-UTP, 40 µM cold UTP (incase of glutelin RNA synthesis 80 µM cold UTP was used), 400 µM each of CTP, GTP and ATP (Ambion), 40 units of RNase Inhibitor (Promega or Boheringer) and 20 units T3 or T7 RNA polymerase (Ambion). The reaction was incubated at 30^{o}C for 1.5 h and terminated by adding 1 unit of RNase free DNase (Boheringer). An aliquot of the reaction was loaded on a urea-PAGE and exposed to X-ray film to ensure complete RNA synthesis in

transcription reaction. The reaction volume was made up to 100 µl with sterile water and 20 µg of yeast tRNA was added and extracted with equal volume of chloroform. Precipitation was carried out by adding 1/10 volume of 3 M NaOAc, two volumes of ice cold ethanol and left in the freezer for 2-3 h. It was spun at 10,000 RPM at 4°C for 10 min in a microcentrifuge. Pellet was washed with 70% ethanol and dried and suspended in sterile water. Radioactivity in one µl of this RNA solution was counted using scintillation counter and the quantity of RNA in the solution was calculated from the counts.

RNA protein UV crosslinking

A 25 µl reaction containing 20 mM Tris HCl pH 7.5, 0.2 mM EDTA, 1 mM $MgCl_2$, 10% glycerol, 10 µg of yeast tRNA (Sigma), 1 mM DTT, 100 fmole of radiolabeled RNA and 40 µg of ER protein or 50 µg of crude seed protein were incubated at 8°C for 10 min. Reaction was transferred onto a parafilm and exposed to UV in a crosslinker for 7 min (the distance between the parafilm and the UV tubes was about 3 inches). The reaction was transferred back to the tubes and treated with 100 units of RNase T1 (Boheringer) and 30 µg of RNase A and incubated at 30°C for 30 min. Rest of the steps were as described in the previous chapter.

RESULTS

Message specific RNA binding proteins associated with ER membranes:

To address the molecular mechanisms of differential RNA distribution on ER membrane of rice endosperm cells, initially specific RNA binding proteins for prolamine and glutelin RNAs in the crude seed extract were analyzed and their distribution on different domains of ER membrane were studied. Low speed centrifugation of rice seed extract yielded PB-ER fraction and the supernatant containing light microsomal fraction (c-ER) was collected by high-speed ultracentrifugation.

RNA-protein UV crosslinking is a powerful technique used in this study to identify proteins that bind to prolamine and glutelin RNAs. In this technique, protein fraction was incubated with radiolabeled RNA and protein-RNA complex was exposed to

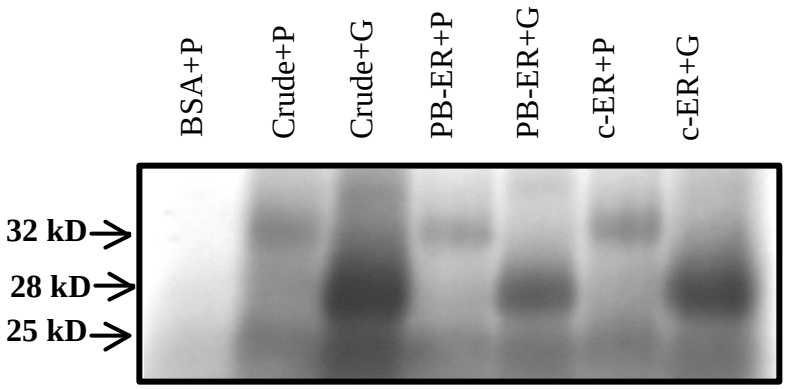

Fig. 1 Message specific RNA binding proteins associated with PB and c-ER membranes: In all the reactions either radiolabeled prolamine (P) or glutelin (G) RNAs were crosslinked to BSA (lane 1) or crude seed protein (lanes 2 & 3) or PB-ER fraction (40 µg) (lanes 4 & 5) or c-ER fraction (lanes 6 & 7).

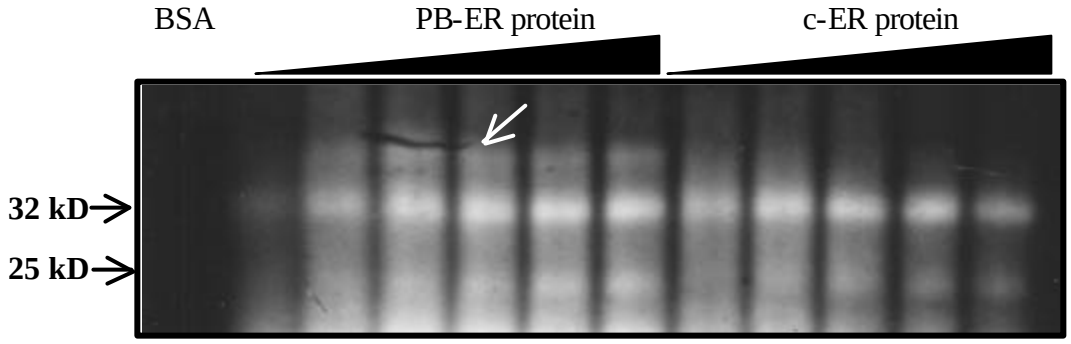

Fig. 2 Localization of prolamine RNA to PB-ER is associated with polypeptide of about 36 kD: In all the reactions 100 fmole radiolabeled prolamine RNA was incubated with BSA (lane 1) or 10 to 60 µg of PB-ER protein (lanes 2 to 7) or 10 to 50 µg c-ER protein (lanes 8 to 12). An arrow in the image indicates the position of 36 kD protein. The autoradiogram was used to print the image directly on a photographic paper.

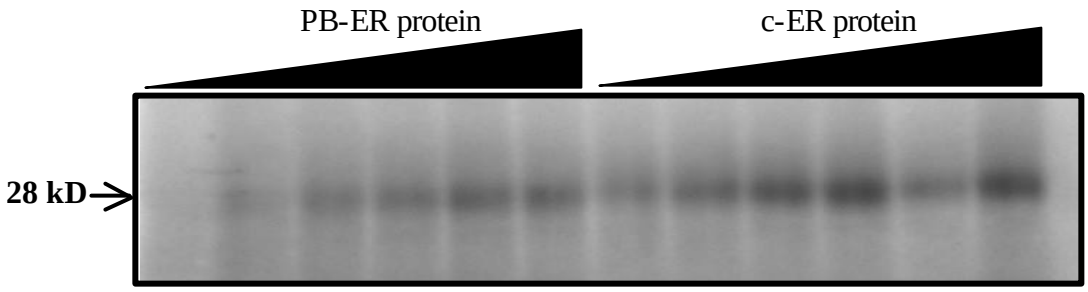

Fig. 3 Differential distribution of glutelin specific RNA binding protein on PB-ER and c-ER membranes: All the reactions contained 100 fmole of radiolabeled glutelin RNA and were incubated with 10 to 60 µg PB-ER protein (lanes 1 to 6) and 10 to 60 µg of c-ER protein (lanes 7 to 12).

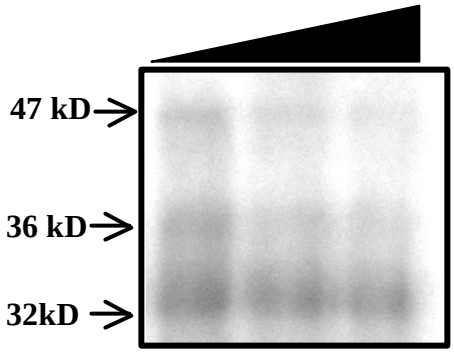

Fig. 4 The 36 kD protein binds to the 5'UTR/coding region of prolamine mRNA. All the reactions containing 100 fmole of radiolabeled prolamine RNA and excess of cold RNA that lacks the 3'UTR 25, 50 &100 fmole were incubated with equal quantity of PB-ER protein.

the UV light. RNA protein complex was then treated with RNase and subjected to SDS PAGE followed by autoradiography.

When BSA was incubated with prolamine RNA in a crosslinking reaction, it was unable to bind to prolamine RNA, indicating that it did not possess the RNA binding activity. Apart from specific binding activities (25 kD and 32 kD polypeptide crosslinked to prolamine RNA while a 28 kD polypeptide bound to glutelin RNA) in crude seed extract, several polypeptides crosslinking to prolamine and glutelin, with different intensities of signals were observed. Polypeptides that bind to both prolamine and glutelin RNAs are possibly general RNA binding proteins. Among these general RNA binding proteins, a polypeptide of about 120 kD is likely to be Rp120 described in the earlier chapter. Heparin, a poly anion, often used in RNA-protein UV crosslinking as a nonspecific competitor, when added to the RNA binding reaction did not have much effect on binding activity, although there was a slight increase in intensity of signal upon adding heparin.

As seen in Fig 1., crude seed extract, PB-ER, and c-ER protein fractions were allowed to crosslink to radiolabeled prolamine and glutelin RNAs. In crude seed extract, as described in the previous para, apart from general RNA binding proteins, specific RNA binding proteins for prolamine and glutelin were detected. In case of PB-ER extract, there were two polypeptides of about 25 kD and about 32 kD which crosslinked specifically to prolamine RNA. When the same extract was used to crosslink to glutelin RNA, a polypeptide of about 28 kD specifically bound to glutlein RNA was noted. Similarly, when c-ER fraction was used to crosslink to labeled RNAs, 25 kD and 32 kD polypeptides were specifically crosslinked to prolamine RNA while 28 kD protein bound to glutelin RNA (Fig. 1). When concentrated ER membrane fractions were used to see if there were more binding activities, there was not any significant difference in the RNA binding activities in concentrated fraction and ER membrane fractions that was used directly in the crosslinking. The general RNA binding proteins, which appeared in crude seed extract, were not observed in the ER membrane fractions.

Differential distribution of message specific RNA binding protein on ER domains:

It has been shown that prolamine transcripts dominate over glutelin on PB-ER membrane (Li et al., 1993). To uncover the molecular mechanisms of domination of prolamine transcripts over glutelin on PB-ER membrane, the relative levels of prolamine specific RNA binding proteins associated with PB-ER and c-ER membrane fractions were analyzed. As seen in Fig. 2, different amounts of PB-ER protein ranging from 10 to 60 µg, and c-ER ranging from 10 to 50 µg were allowed to crosslink to 100 fmoles of radio labeled prolamine RNA. Interestingly, in addition to 25 and 32 kD proteins, another polypeptide of about 36 kD crosslinking to prolamine RNA in PB-ER extract was observed but it was absent in the c-ER extract. Polypeptides, 32 kD and 25 kD were seen in both ER membrane indicating that these two polypeptides were possibly distributed uniformly throughout the rough ER membrane (i.e. in both PB-ER and c-ER).

It has been documented that glutelin transcripts are seen more than prolamine transcripts on c-ER (Li et al., 1993). Therefore, to unwind the molecular cues of glutelin RNA localization to c-ER, relative levels of glutelin specific RNA binding protein was analyzed in PB-ER and c-ER extracts. As seen in Fig 3, PB-ER protein extracts in the range 10 to 60 µg and c-ER extracts in the range 10 to 60 µg were allowed to crosslink to 100 fmole of radio labeled glutelin RNA in each reaction. The results indicated that the intensity of band obtained for 28 kD polypeptide in 30 µg PB-ER protein was equivalent to the intensity of band obtained for 10 µg of c-ER extract (Fig. 3). From c-ER extract, 40 µg of protein could saturate 100 fmole glutelin RNA probe used in the study (based on the intensity). Therefore, in 40 µg of c-ER protein, approximately 100 fmole of glutelin specific RNA binding proteins are present, assuming that one RNA binding protein binds to one glutelin RNA molecule, and hence in 10 µg of c-ER extract, 25 fmole glutelin RNA binding proteins can be extrapolated. Since, the intensity of 10 µg c-ER was equivalent to the intensity obtained with 30 µg of BP-ER for glutelin RNA, 30 µg of PB-ER would have 25 fmole of glutelin RNA binding proteins, which was three folds less than what was seen in 30 µg of c-ER extract. These results are in agreement with the

previous finding that glutelin transcripts dominate the prolamine in the c-ER (Li et al., 1993).

Mapping of binding sites for prolamine RNA binding proteins:

Prolamine specific RNA binding activities were detected by using complete radiolabeled RNAs, however, exact region of RNA (whether 5'UTR or coding region or 3'UTR) being bound by these proteins needs to be determined. Therefore, to map the region of prolamine RNA that was bound by a specific protein, radiolabeled complete prolamine RNA was competed with excess of cold truncated prolamine RNA lacking 3'UTR. As seen in Fig. 4., 32 kD protein, binding to radio labeled RNA was not competed by cold truncated RNA, indicating their biding specificity to 3'UTR of prolamine. Interestingly, a 47 kD protein that binds to prolamine RNA was also detected in the competition experiment. In case of 36 kD and 47 kD polypeptides, truncated, excess cold prolamine RNA could chase their binding to radiolabeled prolamine RNA. This indicates that possibly these proteins bind to either 5'UTR or the coding region. The chances of these proteins binding to coding region are remote, since several RNA binding proteins bind to either 3'UTR or 5'UTR of a mRNA. Therefore, it is likely that 36 kD and 47 kD prolamine RNA binding proteins bind to 5'UTR of prolamine RNA.

DISCUSSION

In rice endomembrane system, differential distribution of storage protein mRNAs has been well documented (Li et al., 1993, Kim et al., 1993, Yamagata et al., 1986). Biochemical studies have indicated the possible role of cytoskeleton in RNA localization in endosperm cells (Muench et al., 1998, Sami-Subbu et al., 2000). However, molecular mechanisms underlying these storage protein mRNAs are still not known. Moreover, several cellular and viral mRNAs are translated on the ER membrane but their mode of transport and anchoring to ER membrane is not explored fully. To get more insight into the molecular mechanisms of prolamine and glutelin mRNA localization, RNA binding proteins associated with different domains of ER membranes were studied and their implications in mRNA localization were assessed.

The presence of PB as a distinct subdomain of ER membrane makes it possible to fractionate the endomembrane system in rice endosperm cells into low speed sedimenting PB-ER and high-speed sedimenting microsomal c-ER fraction. These two fractions were obtained by differential ultracentrifugation and the associated RNA binding proteins were studied by UV crosslinking method. When developing rice seed extract was subjected to the UV crosslinking, several polypeptides were observed crosslinking to prolamine and glutelin RNAs with different densities along with few message specific RNA binding proteins. One possible reason for different intensities of signals by different polypeptides may be they protect variable length of labeled RNA or their binding regions differ in terms of number of uridine residues (since *in vitro* transcription was carried out in presence of ^{32}P-UTP). When PB-ER was allowed to crosslink to prolamine and glutelin RNAs, there were two polypeptides (about 25 kD and 32 kD) specifically bound to prolamine RNA but did not crosslink to glutelin RNA. There was a polypeptide of about 28 kD bound to glutelin transcripts in PB-ER fraction that did not crosslink to prolamine RNA. These three polypeptides were also detected in the c-ER protein extract. Detecting glutelin RNA binding activity in PB-ER might be due to contamination of c-ER fraction during low speed centrifugation. However, *in vivo*, we do not know if all these specific RNA binding proteins are bound to respective RNAs or some are present in free form to receive the newly coming RNA to ER. Moreover, *in situ* localization study has brought out the ratio of prolamine to glutelin on PB-ER membrane (Li et al., 1993) and hence glutelin RNA binding protein is expected to be associated with PB-ER apart from prolamine specific RNA binding protein.

Protein fractions from PB-ER and c-ER were analyzed for differential distribution of prolamine and glutelin message specific RNA binding proteins. The 32 kD protein from 40 µg of PB-ER extract could saturate the 100 fmole of prolamine RNA, whereas 25 kD protein intensity was found increasing even with 60 µg of protein. This indicates that 32 kD protein quantity is more than that of 25 kD protein and these two proteins bind to different regions (specific sequence/secondary structure) of the prolamine RNA. Similar pattern was observed for 25 kD and 32 kD proteins in different quantities of c-ER extract when used in the UV crosslinking study. However, most interesting pattern was

observed with respect to prolamine RNA specific 36 kD protein distribution in PB-ER and c-ER. The 36 kD protein was detected only in PB-ER extract that was as low as 30 µg, which was absent in c-ER extract even at 50 µg protein used in the crosslinking. We speculate that 36 kD protein is possibly involved in associating the prolamine RNAs onto PB-ER membrane. On the other hand, we believe that 32 and 25 kD proteins are probably involved in anchoring of prolamine RNAs to ER membrane. However, it is not clear, if this 36 kD protein is involved in either enriching the prolamine transcripts on PB-ER or making the prolamine transcripts translationally competent upon reaching the surface of PB-ER. *In situ* localization study by Li et al., (1993) has brought out the association of prolamine transcripts with PB-ER and c-ER, however, we do not know if the prolamine transcripts associated with c-ER are translationally competent. It has been shown that PB-ER contains prolamine polypeptides (Krishnan et al., 1986) and hence 36 kD protein association with PB-ER membrane can be interpreted as a translational activator of prolamine mRNA. Localization dependent translation concept put forth by Gavis & Lehmann (1994) supports this speculation that 36 kD prolamine specific RNA binding protein presumably activates the translation of prolamine RNA on the surface of PB-ER.

When glutelin transcript was allowed to crosslink to different concentration of protein from PB-ER and c-ER, substantial difference in distribution of glutelin specific 28 kD RNA binding protein was observed. The band intensity obtained with 60 µg of PB-ER protein for glutelin RNA was equivalent to the signal obtained with 30 µg of c-ER protein. This supports the previous finding that glutelin transcripts dominate the c-ER membrane (Kim et al., 1993, Li et al., 1993). Although glutelin specific RNA binding proteins are detected more on c-ER compared to PB-ER, it is not clear the way glutelin transcripts dominate the c-ER membrane as we could not detect any additional polypeptide other than 28 kD in c-ER fraction. It can be speculated that glutelin RNA binding protein possibly interacts with another protein and enriches the glutelin transcript on c-ER.

To map the region bound by prolamine specific RNA binding proteins on prolamine RNA, radiolabeled prolamine RNA was competed with cold prolamine RNA that lacks the 3'UTR. Results indicate that 32 kD protein binds to 3'UTR of prolamine RNA, since the binding activity was not chased by adding excess of cold RNA that lacks 3'UTR. However, 36 kD and a 47 kD proteins were competed by adding cold prolamine RNA which lacks 3'UTR. Therefore, the binding site for 36 kD and 47 kD could be located either in the 5'UTR or in the coding region. Although in the present study, the region bound by these two proteins has not been shown clearly, available evidences indicate that several RNA binding proteins bind to either 3'UTR or to the 5'UTR of a mRNA. Further work needs to be carried out to confirm the binding sites of 36 kD and 47 kD proteins on prolamine RNA. Moreover, the glutelin specific RNA binding protein (28 kD) binding site on glutelin RNA is yet to be determined. Specific sequence motif at 3'UTR responsible for localization of several RNAs has been reported (Macdonald & Struhl 1988, Ephrussi & Lehmann 1992, Kim-Ha et al., 1993, Macdonald et al., 1993). Recently, it has been shown that 5'UTR is necessary for the localization in oocytes and regulates the translation of viral RNAs (Saunders & Cohen 1999, Park et al., 1999). These reports tempt us to speculate that 36 kD prolamine specific RNA binding protein which is found in PB-ER extract is possibly involved in translational activation of prolamine RNAs or in enriching the prolamine RNAs on PB-ER.

Localization of mRNA involves several steps that include formation of RNA protein complex, translational repression, translocation (cytoskeleton mediated), localization/anchoring and translational activation. In rice endosperm cells, the possible role of cytoskeleton in prolamine RNA localization has emerged from the biochemical studies (Muench et al., 1998, Sami-Subbu et al., 2000, Sami-Subbu & Okita unpublished). In Xenopus oocytes, RNA binding protein Vera, binds to 3'UTR of Vg1 RNA and is associated with ER membrane (Deshler et al., 1997). Moreover, a mammalian Staufen (double stand RNA binding protein) like protein isolated by two different groups seems to be associated with ER membrane (Wickham et al., 1999, Marion et al., 1999). Therefore, it is suggested that these message specific RNA binding proteins associated with ER membrane play a vital role in associating prolamine and

glutelin RNAs on ER membrane. Most interestingly, 36 kD prolamine specific RNA binding protein is found only on PB-ER and not c-ER. Localization and further study on 36 kD and other prolamine RNA binding proteins will lead to a better understanding of segregation of prolamine mRNA to PB-ER domain of the ER membrane. Since several viral and cellular proteins are translated on ER membrane, our data will provide an insight to understand more about segregation of RNAs to different regions on ER membrane of eukaryotic cells.

Chapter V

General discussion

Eukaryotic cells have developed a compartmental system along with an extensive endomembrane system to carry out complex cellular functions in an efficient manner. Protein synthesis takes place at different regions of the cytoplasm depending on the nature of the protein. Earlier it was thought that the nascent polypeptide which emerges from the translation machinery directs the protein to an appropriate organelle of the cell. However, growing number of evidences have now indicated that the RNA itself contains sequences responsible for its localization at a specific region of cytoplasm and hence, the translation is localized. Moreover, it would be economically viable for cells to localize a RNA at a specific region of the cytoplasm and to make many copies of a protein than translating a RNA at several regions of cytoplasm and transporting the protein to a specific region. Initially, RNA localization was mainly documented in polarized egg cells but it was evident that RNA localization is rather a general mechanism found in a wide range of somatic, neuronal and plant cells.

Rice endosperm cells contain two distinct domains of endomembrane system that include prolamine transcript enriched PB-ER and glutelin transcript dominated c-ER. Biochemical characterization and cytological studies of rice endosperm have indicated a possible role of cytoskeleton in prolamine RNA localization to PB-ER. However, the molecular mechanisms of prolamine RNA localization are still not understood fully and hence attempts have been made to unravel these details in the present study.

Till today, there have been several reports stating that polysomes are associated with cytoskeleton which plays a vital role in transporting RNAs to the site of localization. However, in rice endosperm cells, role of cytoskeleton in localization of prolamine RNA is not understood well. In this work, a few RNA binding proteins associated with cytoskeleton that includes 40 kD and a 120 kD (Rp120) proteins have been identified. The 40 kD protein shares properties with poly(A) binding protein and interestingly, binds not only to the poly(A) region but to prolamine 5'UTR as well. Recently, it has been reported that 3'end and 5'end of a mRNA come closer during translation and hence it can be assumed that 40 kD binds to poly(A) region and 5'UTR of prolamine RNA and bring

them closer during translation. However, it is not clear if this 40 kD protein binds to other RNAs and associate them on cytoskeleton.

Rp120, a cytoskeleton associated RNA binding protein, has been purified and cDNA clone is isolated. Rp120 binds to the 3'UTR of prolamine mRNA. Although we do not know if the localization signal for prolamine RNA is located at the 3'UTR, available evidences in other systems suggest that several RNAs contain localization signal at the 3'UTR. Predicted polypeptide sequence reveals that Rp120 contains a SN-fold like sequence motif, which is found in a wide range of nucleic acid binding proteins. Unlike DNA, RNA has a more complex structure and hence, the proteins that bind to RNA contain a wide range of domains. Based on these observations, we believe that Rp120 belongs to SN-fold type RNA binding protein. Interestingly, a tudor like domain is found in Rp120 polypeptide sequence. In Drosophila, *homeless* gene product that is required for RNA localization during oogenesis, contains a single tudor domain. Since Rp120 binds to prolamine, glutelin and Ef1 alpha mRNAs, it is most likely that Rp120 binds to several RNAs expressed in rice seed. Moreover, Rp120-cytoskeleton interaction studies have shown that Rp120 is a cytoskeleton associated protein and the interaction is sensitive to high ionic condition. It is particularly interesting to note the co-localization of Rp120 with a ER membrane marker in the subcellular fractionation study. Since the ER membrane is surrounded by a cytoskeletal network and interacts with it, the co-localization could be due to the association of Rp120 with cytoskeleton rather than a direct interaction with ER membrane. Rp120 polypeptide sequence shows a P-loop like sequence motif, which is found in majority of the motor proteins. This tempts us to speculate that Rp120 is possibly a motor protein and transports RNAs in an energy dependant manner on cytoskeletal track. In that case, we may not expect a concentrated signal around the ER membrane in immunolocalization study. In conclusion, these results indicate that Rp120, a general RNA binding protein, is presumably involved in transport of prolamine RNA from perinuclear region to PB-ER.

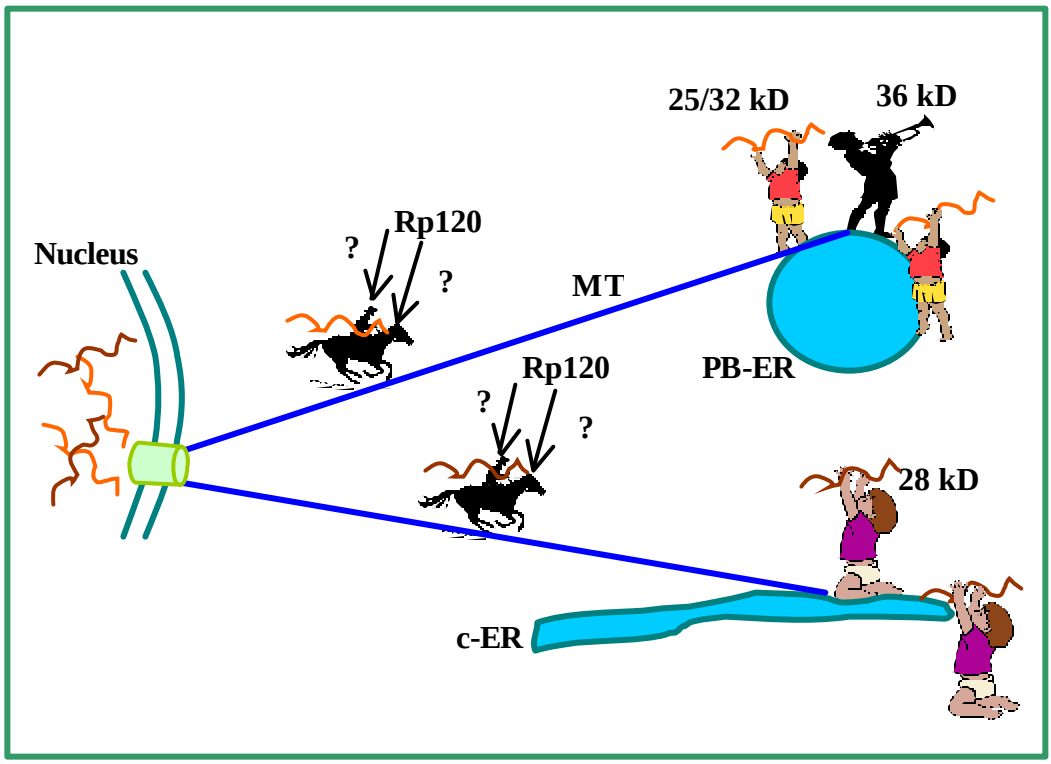

Fig. 1. A diagram showing the proposed model of RNA localization in rice endosperm cells. Rp120 transports the RNAs from perinuclear to ER membrane on cytoskeleton track (MT), message specific RNA binding proteins associated with different domains of ER membranes anchor the RNAs on ER membrane. The 36 kD protein found on PB-ER is presumably involved in enriching the prolamine RNA on PB-ER.

Apart from the above two proteins, specific RNA binding proteins associated with ER membrane that bind to prolamine and glutelin RNAs have also been identified. The 25 kD and 32 kD polypeptides bind to prolamine mRNA and are distributed almost uniformly on both PB-ER and c-ER membranes. It is likely that these polypeptides are involved in anchoring of prolamine mRNA onto ER membrane. However, it is not clear whether the prolamine RNAs that are anchored to c-ER are translationally competent. Interestingly, a polypeptide of about 36 kD which binds to prolamine RNA is found only on PB-ER but not on c-ER. It is well known that PB-ER is mainly packed with prolamine polypeptide. This tempts us to speculate that 36 kD protein is possibly a translational activator located on PB-ER membrane. However, the possible involvement of 36 kD protein in enriching the prolamine RNA on PB-ER can not be ignored. A model of RNA transport and localization is shown in a diagrammatic illustration in Fig. 1. A polypeptide of about 28 kD specifically binds to glutelin RNA and a differential distribution pattern of glutelin RNA binding protein on PB-ER and c-ER is observed. In 10 µg of c-ER protein extract, glutelin RNA binding proteins are about three folds more than equivalent amount of protein from PB-ER. This indicates that glutelin RNA binding proteins are enriched on c-ER domain of endomembrane system. Unlike a specific polypeptide (36 kD) associated with PB-ER, no additional polypeptide associated with c-ER binds to glutelin mRNA and hence, it is not clear the way glutelin transcripts are enriched on c-ER.

The basic findings emerging from this thesis are:

1. **RNA binding proteins are associated with cytoskeleton.**
2. **Rp120 is possibly involved in transport of prolamine mRNA to PB-ER on cytoskeleton track.**
3. **Message specific RNA binding proteins associated with ER membrane are presumably involved in anchoring of mRNAs on ER membrane.**
4. **The 36 kD prolamine specific RNA binding protein is possibly involved in enriching prolamine RNA onto PB-ER or could be a translational activator.**

Summary

The role of cytoskeleton-associated RNA-binding proteins in mRNA sorting during seed development in rice (*Oryza sativa L.*)

Background:

Recent findings from animal and plant systems suggest that mRNAs are localized at discrete sites in the cytoplasm instead of random distribution. For instance, *bicoid* mRNA is localized at the anterior pole of Drosophila oocytes whereas *oscar* and *nanos* mRNAs are localized at the posterior pole. Beta-actin mRNAs are localized at the peripheral cytoplasm while the onco-gene transcript c-myc is localized in the perinuclear cytoplasm. The best example of mRNA localization in plants comes from rice endosperm cells where prolamine and glutelin mRNAs are differentially localized on different domains of the ER membrane. *In situ* localization at electron microscope level reveals that transcript levels of prolamine are seven folds higher than those of glutelin on PB-ER while glutelin transcripts are two folds higher than those of prolamine on c-ER membrane. Further dot blot analysis using poly(A) RNA from PB-ER and c-ER supports the *in situ* localization observation.

Localization of a transcript at a specific site in the cytoplasm leads us to postulate binding of a protein at specific secondary structure of RNA leading to localization. Indeed, evidences suggest that specific regions of the 3'UTR in MBP mRNA are responsible for its localization at the myelin region of the mouse brain cells. Truncated Xcat-2 RNA when microinjected in Xenopus oocytes resulted in identification of localization element located in 3'UTR. Similarily c-*myc* RNA localization in fibroblast cell was studied by transfecting with appropriate construct followed by hybridization with labeled anti-sense RNA. The 3'UTR of c-*myc* when fused with β-globin ORF resulted in detection of localization at the perinuclear cytoplasm. These findings clearly indicate the presence of a distinct region of mRNA that contains the signals responsible for localization.

Cytoskeletal elements mediate a number of functions including transport of organelles and macromolecules, cell division, cell motility and cell enlargement. Treatment of Xenopus oocytes with microfilament destabilizing drug cytochalasin and microtubule destabilizing drug colchicin indicates that microtubules are necessary for the transport of the *Vg1* RNA to the vegetal cortex while microfilaments are important for the anchoring process. In Drosophila, treatment of embryos with cytochalasin B, actin microtubule depolymerizing agent did not affect the localization of staufen particle to the extremities of the mitotic spindle. In contrast to this finding, embryos treated with microtubule destabilizing drug colcemid resulted in appearance of *bicoid* 3'UTR-staufen in a random fashion. It thus appears that microtubules play vital role in localization of *bicoid* 3'UTR-staufen complex to the anterior pole and are independent of microfilaments.

It has been shown that RNA binding proteins directly or indirectly associated with the cytoskeleton bind to specific sequences of mRNA and are involved in the RNA localization process. A classical example is Vrea, thst binds to 366 nucleotide region at the 3'UTR of Vg1 named as Vg1 localization element (VgLE). Staufen, a double stranded RNA binding protein, associates with *bicoid* 3'UTR and moves in a microtubule dependent manner. These evidences demonstrate that RNA binding proteins associated with cytoskeleton are involved in RNA localization process.

Biochemical and sub-cellular fractionation analysis have brought out some information regarding prolamine RNA localization. Non-ionic detergent and salt treatment of isolated protein body indicate that association of prolamine polysomes with protein body (PB) is detergent resistant but salt sensitive. This association could be either through mRNA or ribosome but not due to nascent polypeptide. NaF treatment of rice seeds, followed by membrane-stripped PB isolation and sucrose density gradient analysis, reveal that ribosome free prolamine RNAs remain associated with membrane-stripped PB. Thus the biochemical evidences have indicated the presence of RNA binding activity associated with cytoskeleton possibly involved in prolamine RNA

localization onto PBs. To understand the molecular mechanism of prolamine and glutelin mRNA localization, present work was undertaken with the following objectives:

1. **To purify RNA binding proteins directly associated with cytoskeleton.**
2. **Assess cytoskeleton-RNA binding protein interaction.**
3. **Identification of genes that encode cytoskeleton associated RNA-binding proteins.**
4. **Identification of specific RNA-binding proteins for prolamine and glutelin RNAs that are associated with ER-membrane**

A summary of important findings is as follows:

A cytoskeleton associated RNA-binding protein binds to untranslated regions of prolamine mRNA and to poly(A)

Systematic distribution of prolamine mRNAs on different domains of ER membrane in rice endosperm cells has been documented earlier. Recent findings suggest that prolamine polysomes are not only attached to the surface of the prolamine PBs but also to the cytoskeleton which is associated with this organelle. To understand the prolamine mRNA localization to PB-ER, cytoskeleton-PB enriched fraction was obtained and RNA binding activities were fractionated on poly(U)-Sepharose and studied. Using RNA-protein UV cross-linking assay, a dominant 40 kD RNA-binding protein was identified. The 40 kD protein bound to the 3' and 5' untranslated regions of prolamine mRNA and 3' untranslated region of glutelin mRNA but not to their coding sequences. The binding activity was readily competed by polyadenylic acid indicating that the binding site is A-rich.

Rp120 : A cytoskeleton associated tudor domain protein binds to mRNA

Cytological studies from animal systems clearly indicate that cytoskeleton plays vital role in localization of mRNAs in the cytoplasm. Biochemical evidences reveal the possible role of cytoskeleton in RNA localization process in rice endosperm. In order to look for direct evidence, Rp120 was purified from the cytoskeleton-enriched fraction by

column choromatography. Rp120 bound to the 3'UTR of prolamine RNA and to glutelin and elongation factor mRNAs. A partial cDNA which encodes 875 amino acids of 120 kD protein was isolated using Rp120 antibodies. Predicted amino acid sequence of Rp120 reveal that it contains Drosophila tudor domain and nucleic acid binding motifs. Rp120 was associated with the cytoskeleton at low salt conditions while at high salt condition, Rp120 dissociated from the cytoskeleton. A significant portion of Rp120 was also associated with endoplasmic reticulum as indicated by subcellular fractionation analysis. Transmission electron microscopic observations showed the association of Rp120 with endoplasmic reticulum. Rp120 was expressed only in seeds and the protein was detected much early in the seed development. A possible role of Rp120 in transport of RNA is discussed.

Message specific RNA binding proteins associated with ER membrane in rice endosperm cells

Previous studies have shown that prolamine transcripts are enriched on PB-ER whereas glutelin transcripts are mostly seen on c-ER membrane. To understand the molecular mechanisms of prolamine and glutelin RNA localization, message specific RNA binding proteins associated with PB-ER and c-ER were obtained using differential ultracentrifugation. RNA protein interactions of these proteins were studied by UV crosslinking method. The polypeptides, about 25 kD and 32 kD which bind to prolamine RNA were detected in both PB-ER and c-ER extracts. Interestingly, about 36 kD protein which binds to prolamine RNA (5'UTR/coding region) was seen only in PB-ER extract but was absent in c-ER extract. About 28 kD protein that binds to glutelin RNA was seen more in c-ER than PB-ER. The 36 kD prolamine specific RNA binding protein is presumably involved in segregation of prolamine RNA to PB-ER.

Conclusion

Nonrandom distribution of prolamine and glutelin transcripts are seen in rice endosperm cells. Prolamine transcripts are enriched on PB-ER membrane, whereas glutelin transcripts are mainly associated with c-ER. Hence, making it an attractive model system to study the transport and localization of RNAs in rice endosperm cells.

The 40 kD protein obtained from the cytoskeleton fraction shared functional similarities with poly(A) binding protein and bound to 3'UTR and 5'UTR of prolamine and 3'UTR of glutelin transcripts. Yet another protein called Rp120 was purified from cytoskeleton enriched fraction and showed binding specificity to 3'UTR of prolamine RNA and glutelin and prolamine RNAs. Predicted amino acid sequence of Rp120 indicates the presence of Drosophila tudor domain, nucleic acid binding motifs and a P-loop like motif. Rp120 was associated with cytoskeleton in a salt sensitive manner. Message specific RNA binding proteins obtained from ER membrane fractions demonstrate that possibly these proteins are involved in anchoring of prolamine and glutelin transcripts onto ER membrane. The 36 kD prolamine specific RNA binding protein is possibly involved in enriching the prolamine transcripts on PB-ER or it could be a translational activator. In summary, Rp120 is possibly a RNA cargo protein involved in transportation of RNA from the nucleus to the ER membrane and message specific RNA binding proteins anchor the prolamine and glutelin transcripts on different domains of ER membrane.

Publication

1. Sami-Subbu R., D. G. Muench & T. W. Okita (2000) A cytoskeleton associated RNA-binding protein binds to the untranslated regions of prolamine mRNA and to poly(A). Plant Science 152, 115-122

2. Sami-Subbu R., S. B. Choi, Y. Wu, Wang, M. Ogawa, Takemoto & Okita T. W. Rp120: A cytoskeleton associated tudor domain protein binds to mRNA (to be communicated)

3. Sami-Subbu R., M. V. Katti, A. P. Aspatwar, P. K. Ranjekar, T. W. Okita & V. S. Gupta. Segregation of mRNAs to different domains of ER membrane is associated with message specific RNA binding proteins in rice endosperm cells (under preparation)

Conference

Sami-Subbu R., D. G. Muench & T. W. Okita. Purification of cytoskeleton associated RNA binding proteins. Plant Biology '97, Vancouver, Canada. Supplement to Plant Physiology 114:3 page 89

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