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Light regulation of the RNA-binding activity of 46 kDa protein in chloroplast of *Chlamydomonas reinhardtii*

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ABSTRACT

Light regulation of the RNA-binding activity of 46 kDa protein in chloroplast of *Chlamydomonas reinhardtii*

Shengwu Wang

In this thesis project, I undertook a study of the light regulation of a 46 kDa RNA binding protein associated with chloroplast membranes in *C. reinhardtii*. The optimal conditions for binding of this protein were characterized. Initial purification steps using $(NH_4)_2SO4$ precipitation and affinity chromatography revealed that this protein is present at extremely low abundance. This activity is low in the dark and increases drastically within 1-10 minute following a shift to light. This activation does not require protein synthesis in either the chloroplast or the cytosol. Finally activation is abolished by a proton ionophore and an inhibitor of the cytochrome b_6/f complex, and partially reduced by an inhibitor of photosystem II. With a previous finding that ADP inhibits the RNA-binding activity of the protein *in vitro*, these results support a role of ADP in repression of this binding activity.

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ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine monophosphate
bp	base pairs
cpm	counts per minute
DBMIB	2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone
DCMU	3-(3', 4'-dichloro-phenyl)-1,1-dimethylurea
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiolthreitol
EDTA	ethylenediaminetetraacetic acid
FCCP	carbonylcyanide *p*-(trifluoro-methoxy)phenylhydrazone
FPLC	fast protein liquid chromatography
HPLC	high pressure liquid chromatography
L	liter
LDM	low density membrane
kbp	kilo base pairs
kDa	kilo Daltons
KOAc	potassium acetate
HA	hydroxylapatite
LHC	light harvesting complex
μE	micro-Einstein

mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
PAGE	polyacrylamide gel electrophoresis
PEG	polyethyleneglycol
PMSF	phenylmethylsulfonlyfluoride
PQ(Q)	plastoquinone
QH2	plastoquinol
PS	photosystem
RNA	ribonucleic acid
RBP	RNA-binding protein
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
UTR	untranslated region
VLDM	very low density membrane
X g	times gravity

I. Introduction

1, Photosynthesis

Oxygenic photosynthesis originated 1-2 billion of years ago in an ancestor of cyanobacteria. The light-dependent reactions of photosynthesis use light energy absorbed by chlorophyll and other pigments and electrons from oxidation of water to produce a pH gradient across thylakoid membranes (ΔpH) and reducing potential in the form of NADPH. The ΔpH is used by the ATP synthase complex to convert ADP and inorganic phosphate to ATP. Light-independent reactions use ATP and NADPH to drive the formation of organic molecules from atmospheric carbon dioxide. Almost all the life on the earth depends on photosynthesis as energy source, whether directly or indirectly.

1.1, photosynthetic organelles-chloroplasts have evolved from a prokaryote.

Cells of plant and eukaryotic algae express genomes in three intracellular compartments: the nucleus, mitochondria and plastids. Chloroplasts are the developmental plastid fate that specializes in photosynthesis. The prokaryotic nature of genetic systems and division systems of plastids and mitochondria has led to the widely held belief that these organelles evolved from bacteria that took up residence in ancestral eukaryotic cells and established a mutually beneficial "endosymbiotic" relationship with them (Gray, 1999). A photosynthetic cyanobacterium moved into the cells of the common ancestor of plants and green algae. Since giving up life as free-living organisms, plastids and mitochondria have developed severe codependences upon the eukaryotic cells they inhabit. These

organelles have relinquished most control over their metabolism, division, development, and gene expression and become tightly integrated into the physiology of their resident cells (Gillham, 1978; Gray, 1999; Gray et al., 1999; Gillham, 1994). Signal transduction pathways relay information between them and other intracellular compartments and the environment (Jarvis, 2001). Plastid and mitochondrial genomes have been reduced by deletions and translocations to the nuclear genome. Most organelle proteins are now encoded by nuclear genes, synthesized in the cytosol by 80S ribosomes, and then directed to a specific organellar subcompartment by N-terminal transit peptide signals (Caliebe, 1999). Minor subsets of the genes from the original endosymbionts have been retained in organelles as well as complete gene expression systems which is account for nearly half of the genes of plastid genomes (Gillham, 1994).

1. 2, Chloroplast membranes

There are three kinds of membranes in chloroplasts: the outer and inner membranes of the envelope, and the internal network of thylakoid membranes. The outer and inner envelope membranes neither contain chlorophyll nor participate directly in photosynthesis. The thylakoid membrane consist of a network of flattened vesicles which are arranged in stacks termed grana or extend into the stroma as "stroma lamella". Thylakoid membranes contain many multi-subunit complexes such as light harvesting complexes (LHCs), photosystem I (PSI), photosystem II (PSII), the cytochrome b_6/f complex, and the ATP synthase.

1. 3, Photosystems

There are two photosystems in chloroplast, PSI and PSII. PSI is driven by light of wavelength 700 nm or less. PSII absorbs optimally light of shorter wavelength of 680 nm or less. PSI is located primarily in non-oppressed "stromal lamella" region of thylakoid membranes and PSII is primarily located in oppressed membranes of the grana (Lodish, et al. 1995a).

PSII is a large multisubunit complex. Six reaction center polypeptides (D1, D2, CP43, CP47 and the α and β subunits of cytochrome b-559) are encoded by chloroplast genes (*psbA*, *psbD*, *psbC*, *psbB*, *psbE*, and *psbF*)(de Vitry, 1991), and many peripheral subunits are encoded by nuclear genes.

1. 4, Linear and cyclic electron flow: In the linear electron flow, both PSI and PSII are involved, but with different functions. The light harvesting complex LHCII absorbs light energy and transfers it to P680, a special pair of chlorophylls in the PSII reaction center. P680 is oxidized and the electrons go through the complex via a series of electron carrying cofactors to Qb, a plastoquinone bound to the stromal side of the complex. The oxidized chlorophyll $P680^+$ is a strong oxidant and, in a reaction catalyzed by the oxygen-evolving complex on the luminal face of PSII, removes an electron from H_2O, forming O_2 and protons. The protons from oxidation of water remain in the thylakoid lumen and contribute to the proton motive force of the trans-thylakoid membrane pH gradient (ΔpH). The electrons freed from P680 move to the quinone Qb on the stromal face of the thylakoid membrane. Reduced plastoquinonol ($PQ-H_2$) is formed after Qb accepts two electrons from two sequential charge separation reactions by $P680^+$ and two protons from the stroma. Reduced plastoquinol diffuses within the lipid bilayer of the

thylakoid membrane to the cytochrome b6/f complex where it binds to a site on this complex. It releases its electrons at the cytochrome b/f complex, and its two protons into the thylakoid lumen. This shuttling of protons from stroma to thylakoid lumen by plastoquinol contributes to the ΔpH. Redox reactions within the cytochrome b6/f complex are coupled to additional transport of protons from the stroma to the lumen. The mobile electron carrier protein plastocyanin accepts the electrons from the cytochrome b/f complex, and carries them to photosystem I. The oxidation of plastocyanin releases the electrons to P700+ of PSI. $P700^{+}$ is formed when P700 is oxidized following absorption of excitation energy from the LHCI antenna. The electron released from P700 is transported, by a series of electron carriers in the reaction center, to the stromal surface, where soluble ferredoxin transfers the electron to FAD and finally to NADP+ through NADP-FAD reductase. The synthesis of NADPH from NADP and proton from the stroma further contributes to the ΔpH. One ATP is synthesized from ADP and Pi when 3 protons move down their concentration gradient through Fo of the ATP synthase complex in the thylakoid membrane. NADPH and ATP synthesized by the reaction of the photosynthetic electron transport chain are used in CO_2 fixation (Lodish, et. al., 1995a).

In cyclic electron flow, PSI and the cytochrome b6/f complex are involved. Electrons from PSI can be directed back to the plastoquinol pool. In linear electron flow, PSII removes electrons from H_2O, and electrons from ferredoxin are transferred to NADP+, forming NADPH. Alternatively, ferredoxin can reduce a PQ and form reduced PQ-H_2. The PQ-H_2 then diffuses through the thylakoid membrane to its binding site on the luminal side of the cytochrome b_6/f complex, where it releases two electrons to the b cytochromes and two protons to the thylakoid lumen, generating a proton-motive force.

As in linear electron flow, these electrons return to PSI via plastocyanin. This process is called "cyclic electron flow". In this pathway, the ΔpH gradient generated permits the synthesis of additional ATP, but not NADPH. PSII is not involved and no O_2 or NADPH are generated (Lodish, et. al., 1995a).

2, *Chlamydomonas reinhardtii*

The unicellular eukaryotic green alga *Chlamydomonas reinhardtii* is used as an experimental system for studies of photosynthesis and chloroplast physiology for several reasons. First, much is known about its genome and cytology. Methods for culturing and manipulating this alga have been developed over the past century (Harris, 1989a). *C. reinhardii* is viable in the absence of photosynthesis, unlike vascular plants. Therefore, mutants lacking photosynthesis can be isolated and studied. *C. reinhardtii* can also be subjected to genetic analyses. Many studies have used mutant selections and screens to identify functions involved in a wide variety of processes, including photosynthesis and chloroplast biogenesis. Most relevant to this study, chloroplasts and chloroplast membranes can be isolated in large quantities with relative ease. One can also easily expose this organism in liquid culture to specific inhibitors of a variety of processes (Harris, 1998, Harris, 2001).

3, Genome of the chloroplast

3. 1, chloroplast proteins are encoded by the chloroplast genome: The 196-kb chloroplast genome of *C. reinhardtii* encodes many components of the photosynthetic apparatus and the organellar gene expression system.(Harris, 1987, Chloroplast genome

map at *Chlamaydomonas* genetics center of Duke University from internet at http://www.biology.duke.edu/chlamy_genome/chloro.html). Chloroplast genes encode components of photosystem II, b6/fcomplex, photosystem I, ATP synthase, and the chloroplast gene expression system (Gray, 1999). The principle thylakoid membrane proteins encoded by the chloroplast genome include 51-kDa ch1 a apoprotein (PsbB); p700 chl a apoprotein 1a, 1b; 44-kDa chl a apoprotein (PsaC); 32-kDa herbicide-binding protein D1 (encoded by *psbA*); 32-kDa protein D2(encoded by *psbD*); the α and β subunits of cytochrome b-559 (encoded by *psbE and psbF*); cytochrome f (*petA*); cytochrome b6 (*petB*); the CF_0 and CF_1 subunits of the ATP synthase (α (*atpA*), β (*atpB*), ε (*atpE*), I (*atpF*), III (*atpH*), IV (*atpI*) subunits).The large subunit of ribulose bisphosphate carboxylase/oxygenase. Components of the gene expression system encoded by the chloroplast genome include: EF_{tu}; Ribosomal proteins (14 proteins of the small subunit, 5 or 6 proteins of the large subunit); at least 12 tRNAs; 23S, 16S, 7S, 5S, and 3S rRNAs. .

3. 2, Many chloroplast proteins are encoded by the nuclear genome:

Due to the loss of chloroplast genes to nuclear genome, chloroplast can not undergo photosynthesis without the products of some nuclear genes which are synthesized on cytosolic 80S ribosomes and subsequently imported into appropriate compartments in chloroplast (Caliebe and Soill, 1999). The following chloroplast components are coded by nuclear genes: 24 thylakoid membrane proteins including proteins of light-harvesting LHC and oxygen evolving complexes, ch1 a/b binding protein and a 34-kDa protein; ferredoxin; plastocyanin; cytochrome c_{552}; CF_0CF_1ATPase (γ, δ, II); the ribosomal

proteins (17 proteins of small subunit, and 27 or 26 proteins of large subunit); phosphoribulokinase; and the RUBISCO small subunit (Harris, 1989b, Nuclear genome map at Chlamaydomonas genetics center of Duke University from internet at http://www.biology.duke.edu/chlamy_genome/nuclear_maps.html).

Allen proposed that oxygen free radicals generated by the electron transport reactions are so mutagenic that they exert a strong selective pressure for translocation of chloroplast genes to the nucleus (Allen, 1996). First, most genes of the bacterial ancestors of plastids and mitochondria have moved to the nucleus where they are less likely to acquire free radical-induced mutations. Second, the genes encoding the proteins that position the electron carrying cofactors within the oxidoreductase complexes (e.g. photosystems I and II and the cytochrome b6/f complex) have been retained within the organelle because their expression would be directly and rapidly regulated in response to changes in the redox states of specific electron carriers within the system called "redox sensors".

4, Regulation of chloroplast transcription:

We discussed the chloroplast genome and its cooperation with the nuclear genome. The next question is how the chloroplast gene expression is regulated. Gene expression is regulated at different levels, including transcription (Stern et al. 1997, Christopher et al., 1997, Mullet, 1993), translation (Rochaix, 1996, Sugita and Sugiura, 1996, Stern et al. 1997, Danon, 1997, Hauser, et al. 1998, Zerges, 2000), and post-translational event, such as protein modification and complex assembly (Adam, 2000, Choquet and Vallon, 2000).

4. 1, Structure and promoters of the chloroplast genome:

Chloroplast genes of vascular plants are usually organized into polycistronic transcription units, but algal chloroplast genes tend to be more dispersed and monocistronic (Stern et al. 1997). These genes usually have prokaryotic-like promoters, which are recognized by plastid-encoded RNA polymerase (PEP). For example, the light-responsive promoter of *psbD-psbC* operon in vascular plants is activated by blue light or ultraviolet light (Harris, Boynton, Gillham, 1994, Christopher and Mullet, 1994). Alternative promoters also were reported, for example, the 16S rRNA genes are preceded by PEP promoters and a NEP promoter which is recognized by a nuclear-encoded RNA polymerase (Stern et al. 1997). Correspondent to these promoters, both prokaryotic and eukaryotic RNA polymerases exist in chloroplasts (Gruissem, et al. 1983, Hess, et al 1993, Allison,, Simon, and Maliga, 1996, Lerbs-Mache, 1993).

4. 2, Transcription order of NEP and PEP promoters:

Housekeeping genes and genes encoding components of the chloroplast gene expression system are transcribed from NEP promoters from an RNA-polymerase similar to that of the T7 bacteriophage early in chloroplast development. Later, upon illumination. transcription of photosynthetic genes with PEP promoters by the chloroplast genome-encoded RNA-polymerase increases. While regulation of chloroplast transcription plays an important role in the differential expression of chloroplast genes, this is not the case for mature chloroplasts where post-transcriptional control predominates (Stern et al. 1997).

4. 3, Translatability of transcripts:

Not all newly synthesized chloroplast transcripts are translatable. A transcript may have to undergo post-transcriptional modifications, such as editing, splicing, and processing (Bock, 2000, Hirose, 1997, Reinbothe, 1993), and reversible modifications, such as association with specific proteins, to be competent for translation (Danon, 1997). Both types of modifications in the chloroplast have been reported. The translatability of *psbL* mRNA requires editing of an ACG codon to the initiator codon AUG in tobacco (Chaudhuri, 1995) .

5, Translational regulation of chloroplast gene expression:

mRNA stability and translational efficiency are now recognized to be major control points in the regulation of gene expression in chloroplasts. The half-lives of most mRNA species are determined by a complex set of interactions that depend on the primary and secondary structure of the mRNA, ribonuclease activity, RNA binding proteins, and translational rate (Thomson, et. al, 1999). While expression levels of specific proteins in the chloroplast are enhanced 50 to 100-fold after illumination of plant and algae cells, the absence (in many cases) of corresponding changes in the amount of the mRNAs encoding these proteins indicated that gene regulation occurs mainly at posttranscriptional levels (Kirk, 1985, Klein, 1988, Malnoe, 1988). An accumulating body of evidence indicates that translational control plays a key role in the regulation of chloroplast gene expression in mature chloroplasts (Gillham et. al., 1994; Mayfield et. al., 1995; Rochaix, 1996). Because light intensity fluctuates, a quick and dynamic adjustment of gene expression is required and the expression of many chloroplast genes is modulated in response to light

intensity (Bruick, 1999, Danon, 1997). Transcriptional regulation is not as rapid as translational regulation for gene expression, which can rapidly modulate the rate of protein synthesis from an existing pool of transcripts. That may be the reason that chloroplasts use predominantly translational regulation to control rates of gene expression.

5. 1, Shine-Delgarno sequences: are they present ?

In eubacteria, such as *E. coli*, the ribosomal small subunit is recruited to the initiation codon by a base-pairing interaction between the 3' end of the 16S ribosomal RNA and a complementary sequence positioned 5-9 bases upstream of the initiation codon in the mRNA. This sequence is called the Shine-Delgarno (SD) sequence. About 40% of the chloroplast mRNAs contain a potential SD sequence which is typically located five to nine nucleotides upstream of the initiation codon suggesting that other mechanisms are involved in recruiting the chloroplast small ribosomal subunit to initiation codons (Harris et al., 1994).

5. 2, Possible ribosome binding mechanism:

Many chloroplast mRNAs do not have SD-RBS. How these mRNAs are initiated in translation attracts the attention of many scientists (Fargo, 1998, Stern, 1997). Because chloroplast mRNAs lack the 5'-m^7G cap in the 5' untranslated leader regions (UTRs) and poly-A tails at the 3' termini of these mRNAs, which are involved in translation initiation in the eukaryotic cytosol, the mechanism of translation initiation on chloroplast mRNAs should be different from translation in the eukaryotic cytosol. There are two possible

mechanisms for the initiation in chloroplasts. First, there could be special binding sites other than SD-RBS for the small ribosome subunit in the 5'-UTRs of these chloroplast mRNA. The binding does not require a m^7G-cap in the mRNA and the special binding is due to the specific interactions between the small ribosome subunit, possibly ribosome-associated factors, and *cis*-elements in the mRNA. Second, there are some cis-elements in 5'-UTR which are structured and required for translation initiation (see below). With the help of *trans*-acting factors, e.g., 5'-UTR binding proteins, the small ribosome subunit could bind the mRNA and be directed to the initiation codon. There is some limited evidence for scanning of the small ribosomal subunit to the initiation codon (Danon, 1997). In such cases, the sequence complementarity between the 5'-UTR or the initiation codon and a small ribosomal subunit would not be required for the initiation (Danon, 1997).

5. 3, *Cis*-acting sequence elements control translation of chloroplast mRNAs:

Some translational *cis*-acting sequences have been identified within the 5'-UTRs of chloroplast mRNA by site-directed and random mutagenesis (Zerges and Rochaix, 1994, Sakamoto et al., 1994, Hirose and Sugiura, 1996, Zerges et al., 1997, Stampacchia et al., 1997, Nickelsen, 1999). Furthermore, some reports show that a secondary structure in 5'-UTR is also required for translation initiation (Koo and Spremulli, 1994, Betts and Spremulli, 1994). A predicted stem-loop structure in the 5'-UTR of the *C. reinhardtii psbA* mRNA has been proposed to interact with translational activation factors and regulate translation of the *psbA* mRNA in response to light (Mayfield et al.1994). The *cis*-element in the 5'-UTR of *Chlamydomonas psbC* mRNA also has the potential to form

a stem-loop secondary structure with a loop of 47 nt. There are two "bulges" in one strand of the predicted stem, which result from sites of non-complementarity with the other strand. A mutation that eliminates the bulges completely abolishes translation of *psb*C mRNA *in vivo* (Rochaix et al. 1989, Zerges et al. 1997). Therefore, these stem-loop secondary structure with the bulges seems to play a very important role in translation initiation. Some factors may control alternate secondary structures in the RNA which either promote or repress translation initiation. Alternatively, binding of these factors may promote or repress translation, by recruiting or blocking the small subunit.

5. 4, Trans-acting factors that control translation of chloroplast mRNAs:

Many mRNA binding proteins also have been detected to bind to 5'-UTR of chloroplast mRNA either by genetic analysis or by in vitro experiments including Gel mobility shift assays and UV-cross linking techniques.

5. 4. 1, trans-elements found by genetic analysis:

Genetic analysis show that three nuclear gene products (*Tbc1*, *Tbc2*, *Tbc3*) interact with *cis*-elements in the 5'-UTR of the *psbC* mRNA to activate its translation initiation (Rochaix et al. 1989, Zerges and Rochaix, 1994, Zerges et al. 1997). While mutations of the nuclear *TBC1* gene is suppressed by point mutations within the 5'-UTRs of *psbC* mRNA, a dominant suppressor mutation of *TBC3* can suppress a mutation of the *psbC* 5'-UTR both in the endogenous *psbC* gene and a *psbC* 5'-UTR fused reporter gene in chloroplast genome transformants (Zerges, 1997). The nuclear gene AC115 is required for the initiation of *psbD* mRNA translation (Wu, 1995). In maize, nuclear Atp1 gene is

required for the translation of atpB mRNA, which encodes the β subunit of the chloroplast ATP synthase (McCormac, 1999). The nuclear Crp1 gene is required for the translation of the chloroplast *petA* and *petD* mRNA which encode the polypeptides in the cytochrome b6/f complex (Barkan, 1994). Other nuclear gene products that are required for mRNA translation include F35 for the *psbA* mRNA, *Nac1* for the *psbD* mRNA, *Sim30* and Mcd1 for the *petD* mRNA, *Tab1* for the *psaA* mRNA, *F54* and *Atp1* for the *atpA* (Zerges W., 2000, Goldschmidt-Clermont, 1998, Barkan, 2000, Zerges, 1994, Stampacchia, 1997, Zerges, 1997, Rochaix, 1989, Yohn, 1998b, Girard-Bascou, 1992) From these results, we can see that each chloroplast mRNA requires its own translational factor, which gives the full control of chloroplast photosynthesis system expression to nuclear genes. *A priori* one would expect that a simpler global regulatory system controls expression of all subunits of a particular complex. The unexpected complexity of the mRNA-specific translational regulators suggests that they could control expression of a particular subunit in ways that are required for its assembly into the complex in which it functions. Retention of genes encoding subunits of electron transport complexes and the existence of analogous (Fisk, 1999) gene-specific translational regulators suggest the existence of common translational requirements for mRNAs encoding polytopic membrane proteins of macromolecular complexes in mitochondria, chloroplasts, and possibly other systems where these requirements have not been studied. While there exists no direct evidence to support the functions of 5' leaders and mRNA-specific translational regulators in regulating translation in assembly pathways in chloroplasts, in yeast mitochondria the 5' leaders of the *COX2* and *COX3* mRNAs are required for assembly of Cox2p and Cox3p into the cytochrome c oxidase complex (Sanchirico,

1998). These leaders interact with nucleus-encoded factors that specifically control their ability to promote translation of the coding sequence linked to them. Thus, it is tempting to speculate that gene-specific translational activators and 5' leaders function together to target organelle mRNAs to the correct organellar membrane and control the assembly of their polypeptide products into an integral membrane complex (Zerges, 2000).

5. 4. 2, Chloroplast mRNA binding proteins discovered by affinity chromatography and gel mobility shift assay: Using affinity chromatography with immobilized in vitro synthesized *psbA* 5'-UTR to gel beads, a complex of proteins that binds to this 5'-UTR have been purified from *C. reinhardtii* (Danon and Mayfield, 1991). RNA gel-shift experiments were used to identify and follow the activity of this complex. The complex contains four proteins, RB38, RB47, RB55, RB60, and binds the 5'-UTR of *psbA* mRNA through an RNA binding protein called RB47 (Trebitsh, 2000). Cloning of the genes for RB47 and RB60 and determination of their DNA sequence revealed that RB47 is a homologue of poly-A binding protein and RB60 is a homologue of protein disulfide isomerase-like protein (Kim and Mayfield, 1997, Yohn, Cohen, Danon and Mayfield, 1998). Poly-A binding proteins are a family of eukaryotic, cytoplasmic proteins which bind to 3'-poly A tails of mRNA and stimulate translation through synergistic interactions with other factors bound at 5'-end (Sachs, 1997). Some authors think that this nucleus-encoded eukaryotic protein has been exploited for use in the chloroplast. This is consistent with a general trend of bi-directional exchange of genetic information between the chloroplast and the nucleus (Morden, 1992)

Using UV cross-linking technique, Dr. Zerges found a set of proteins (30-32, 46, 47, 60, and 80 kDa proteins) which can bind to the 5'-UTR of the chloroplast *psbC* mRNA of *C. reinhardtii* (Zerges and Rochaix, 1998). The 47 kDa RNA binding protein could be RB47, which has been reported to be a specific activator of *psbA* mRNA translation (Danon, 1991). Other evidence suggests that RB47 may not be specific for *psbA* (Fargo, 2001). These proteins are associated with membranes isolated from broken chloroplasts by sucrose density gradients (Zerges and Rochaix, 1998). The buoyant density and analyses of acyl-lipids and pigments show the similarity with the inner chloroplast envelope membrane (Zerges and Rochaix, 1998).

5. 5, Activity regulation of chloroplast mRNA binding proteins:

Although many mRNA-binding proteins have been identified, cloned and sequenced, the molecular mechanisms by which the activity of these RNA binding proteins are regulated is still unresolved (Fong, 2000). An intensive study of RB47 shows that light enhances the RNA-binding activity of the complex containing this protein. Reduction by dithiothriotol activates the RNA binding activity of this complex, and oxidation inhibits the activity. ADP inactivates the RNA-binding activity *in vitro* (Danon, 1994). The protein complex contains a kinase activity which specifically uses the β-phosphate of ADP to phosphorylate RB60. The 60 kDa protein of the *psbA* mRNA-binding protein complex is phosphorylated during inactivation of RNA-binding, and contains a redox-active regulatory site responsive to thioredoxin (Danon and Mayfield, 1994, Kim and Mayfield, 1997, Trebitsh, et. al., 2000). These discoveries lead to a complicated model in which phosphorylation, dephosphorylation prime the complex to bind in response to

light-induced changes in ADP level, and the redox state of thioredoxin modulates the binding activity of the primed complex in response to relative light intensity (Trebitsh, et al., 2000).

5. 6, Control of translation initiation and elongation on chloroplast mRNA:

While many studies have shown that mRNA translation is regulated through the translation initiation (Danon, 1994, Zerges, 2000), some reports show evidence that light regulation of the mRNA translation is through the translation elongation, the phase when the ribosome translocates along the coding region and synthesizes a protein (Muhlbauer, 1998, Inagaki, 1992, Taniguchi, 1993, van Wijk, 1996). Accumulation of D1 translation intermediates in the dark was observed in pea chloroplasts (Taniguchi, Kuroda, and Satoh, 1993) and in spinach (Inagaki, and Satoh, 1992, Van Wijk, and Eichacker, 1996). About the components involved in translation regulation, some researchers attributed reduced protein expression in the dark to the reduced level of ATP in the chloroplast (Michaels, 1990). Some researchers have proposed that ΔpH is involved in light regulation of translation elongation (Muhlbauer, 1998).

5. 6. 1, Evidence for translational control by ATP/Energy supply:

One study using *C. reinhardtii* showed evidence for translational control by ATP supply (Michaels and Herrin, 1990). Chloroplast mRNA translation can be induced during the normal dark period by light or by acetate, and the induction is blocked by an inhibitor of ATP synthesis, the proton ionophore CCCP. ATP levels in synchronous cells were found to be 2-5-fold lower during the dark than in the light period. The administration of acetate or light at the mid-dark period increased the ATP level 2-3-fold. consistent with a

regulatory role of this molecule. In their experiments, total cellular ATP instead of chloroplast ATP was measured, possibly because purification of chloroplast would take time and make real-time measurement impossible. In this way, we do not know exactly what changes in ATP level occurred in the chloroplast and cytoplasm. The synthesis of many proteins in the cytoplasm, excluding those destined to the chloroplast, occurs during the dark period. If we could measure the ATP level in cytoplasm and chloroplast, we could determine whether or not ATP level affects mRNA translation both in chloroplast and cytoplasm. In addition, they did not show to what extent ATP level affects mRNA translation. This would require strictly controlled *in vitro* and *in vivo* experiments to show if 2-5-fold ATP level change is sufficient to explain the change of mRNA translation. Hence, to measure the ATP levels both in chloroplast and cytoplasm, and to show to what extent ATP level affects mRNA translation needs to be done to confirm the ATP/energy supply hypothesis for translational control.

5. 6. 2, Evidence for translational control by trans-thylakoid membrane proton gradient:

Muhlbauer and Eichacker showed evidence for translational control by the proton gradient ΔpH (Muhlbauer and Eichacker, 1998). In darkness, translation elongation is retarded even in the presence of exogenously added ATP and dithiothreitol; In the light, addition of the electron transport inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethyl urea inhibits translation elongation even in the presence of ATP. This inhibition can be overcome by the addition of artificial electron donors in the presence of light, but not in darkness; Electron flow between photosystem II and I induced by far red light of 730 nm

is sufficient for the activation of translation elongation. This activation can also be obtained by electron donors to photosystem I, which can contribute to the ΔpH by cyclic electron flow. Release of the proton gradient by uncouplers prevents the light-dependent activation of translation elongation; The induction of translation activation is switched off rapidly upon transfer from light to darkness. However, because of the highly correlations among the formation of proton gradient, ATP synthesis, NADPH synthesis, reduction of electron transport chain, more experiments need to be done to confirm the hypothesis that the ΔpH regulates translation in reponse to light. In a cell free translation system, one should determine whether ATP, NADPH, NADH and other proton gradient affecting components can alter the level of protein synthesis. The proton gradient should be monitored with H^+-electrode to make sure that there is a correspondence between the proton gradient and protein synthesis. This will provide direct evidence that specific molecules are, or are not, involved in this light regulation. Second, one could put lysed chloroplasts in solutions with various pH and determine the rates of protein synthesis to determine whether or not an artificially changed proton gradient will affect the rate of protein synthesis.

6, Importance of studying the regulation of photosynthesis:

C. reinhardtii, a widely used model organism for genetic studies, is believed to have potential to power 21th-century automobiles by making hydrogen gas when they cut off the culture's supply of oxygen and sulfur (Melis *et al*, 2000). Currently, hydrogen for fuel purposes is extracted from natural gas. But as fossil-fuel resources dwindle and environmental concerns rise, these renewable technologies are likely to become more

attractive. Beside, the exhaust from a hydrogen-fueled car would be simple H_2O, in the form of water vapor. Researchers are trying to increase the hydrogen gas yield. To understand the regulation of metabolism and photosynthesis in *C. reinhardtii* will be a key to achieve this goal because hydrogen production requires both PSI and PSII .

In this thesis project, I undertook a study of the light regulation of a 46 kDa RNA binding protein (RBP) associated with chloroplast membranes in *C. reinhardtii* (Zerges and Rochaix, 1998). The optimal conditions for binding of this protein were characterized. Initial purification steps using $(NH_4)_2SO4$ precipitation and affinity chromatography revealed that this protein is present at extremely low abundance. This activity is low in the dark and increases drastically within 1-10 minute following a shift to light. This activation does not require protein synthesis in either the chloroplast or the cytosol. Finally activation is abolished by a proton ionophore and an inhibitor of the cytochrome b_6/f complex, and partially reduced by an inhibitor of photosystem II. With a previous finding that ADP inhibits the RNA-binding activity of the protein *in vitro*, these results support a role of ADP in repression of this binding activity.

II. Materials and Methods

1, Media and growth conditions.

Cultures were grown under mixotrophic conditions in Tris-acetate-phosphate (TAP) medium (Gorman, 1965) to a cell density of 2-5 x 10^6 cells/ml. Light-grown cultures were exposed to a light intensity of 100 $\mu E\ m^{-2}\ sec^{-1}$. Dark-grown cultures were grown in the absence of light by wrapping the flask in at least two layers of aluminum foil and cells were harvested in darkness or, when pouring of the culture into centrifuge bottles was required, with indirect illumination with a 25W green light bulb. Chloroplasts were isolated from strains carrying the *CW15* mutation, which affects the cell wall (Davies, 1971), to allow breakage of cells, but not chloroplasts. In experiments of light regulation, CC503 (*CW15*, Chlamydomonas Stock Center, Duke University) was cultured in the dark in 500 ml TAP medium with agitation. At the specified times before isolation of membranes, cultures were exposed to approximately 250 $\mu E\ m^{-2}\ sec^{-1}$ of white light (from two fluorescent bulbs located 20 cm on either side of the flasks). To the specified cultures and 15 min prior to light exposure, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU), 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB), carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP),were added to a final concentration of 10^{-5} M, cycloheximide to 10 μg/ml and chloramphenicol was added to 100 μg/ml.

2, Chloroplast isolation and subfractionation

Cells were pelleted for 10 min at 4,000 X g, resuspended in 40 ml of isotonic solution (0.3 M sorbitol, 20 mM Tricine-HCl pH 7.8, 5 mM $MgCl_2$) per L of original culture. Chloroplasts were obtained from cells carrying the *CW15* mutation by passage through a Yeda press at 4 bars or broken with 0.25% (wt/vol) saponin, which disrupts the plasma membrane but not the chloroplast envelope (Detmers, 1983), in the isotonic solution. Samples were maintained at 0-4°C thereafter. Chloroplasts were pelleted by centrifugation at 3,000 X g for 1.0 min and then gently resuspended in the same isotonic solution. Samples were loaded on to a discontinuous (45/75%) Percoll gradient (with 0.3 M sorbitol, 10 mM Tricine-HCl pH 7.8, 5 mM $MgCl_2$), centrifuged at 6,000 X g for 20 min, and collected at the interface of the Percoll solutions. Two volumes of the isotonic buffered solution were added to dilute the Percoll so that the chloroplasts could be pelleted by centrifugation for 5 min at 4,000 X g. Chloroplasts were osmotically lysed in 2 ml of a hypotonic buffered solution (10 mM Tricine pH 7.8, 10 mM EDTA, 5 mM β-mercaptoethanol) by repeatedly pipetting at least 20 times with a Pipetman p1000 (Gilson) or a Pasteur pipette. To prepare the low-density membranes (LDM), this chloroplast lysate was fractionated on a discontinuous sucrose gradient, which had 0.3 and 1.0 M sucrose phases in10 mM Tricine pH 7.8, 10 mM EDTA, and 5 mM β-mercaptoethanol, and was centrifuged for 16 hr at 10^5 X g in an SW40 rotor (Beckman). Membranes were collected from the interphase and resuspended in 10 mM Tricine pH 7.8, 0.2 mM EDTA, 60 mM KCl, 5 mM β-mercaptoethanol, and 20% glycerol. Each extract was standardized for protein concentration using the assay of Smith (1985) and stored at -80°C.

3, RNA-Binding activity assays.

The RNA probe corresponding to the *psb*C 5' leader was transcribed in vitro from pDH245 (Zerges, 1994) in a 10 µl reaction containing 0.5 µg of linear DNA template, 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, 25 U porcine RNase inhibitor (Biofinex), 60 µCi of [α–^{32}P]UTP (800 mCi/mmole, Amersham), 12 µM non-radiolabelled UTP, 0.3 mM each of ATP, CTP, and GTP, and 8 U T7 RNA polymerase (New England Biolabs) for 45 minutes at 37°C. 1 U of RNase-free DNase was added and the reaction was incubated for an additional 10 minutes at 37°C. The reactions were extracted once with phenol-chloroform. The RNA probes were separated from the non-incorporated nucleotide-triphosphates on G-50 "spin-columns" (Sambrook, J. et al., 1989). Each probe had the expected length as determined by agarose RNA-gel electrophoresis. Binding reactions contained 30 mM Tris-HCl (pH 7.0), 5.0 mM $MgCl_2$, 5 mM dithiothreitol, 50 mM KCl, and were performed at 22-25°C. Each reaction contained approximately 2-10 x10^5 cpm of [^{32}P]RNA probe. Cross-linking reactions were performed with a 254 nm UV irradiation of 1.0 J/cm^2 for approximately seven minutes duration using a Stratalinker UV Cross-linker (Stratagene). After irradiation, the probe was digested by treatment with 10 µg RNase A (Sigma) for 10 minutes at 37°C, leaving only small [^{32}P]RNA fragments covalently cross-linked to the proteins. Samples were incubated at 85°C for 5 min in protein loading buffer (10% glycerol, 1% SDS, 100 mM DTT, 30 mM Tris-HCl (pH 6.8), 0.01% bromophenol blue), fractionated by SDS-PAGE (11% acrylamide), and analyzed by autoradiography or phosphorimaging. In each experiment, coomassie -blue staining of protein in the acrylamide gels following SDS-PAGE confirmed that equal amounts of the low-density membranes were used.

4, Precipitation of 46 kDa RNA binding protein with ammonium sulfate at different pH.

Ammonium sulfate was gradually added to combined LDM fractions (from sucrose gradient) to reach a saturation of 85%. Samples were maintained at 4°C for at least 1 hour and with slow stirring. Centrifugation was carried out at 5000 X g for 15 minutes to pellet the protein. Pelleted proteins were resuspended in storage buffer and stored at –80°C. The pH of supernatant was adjusted from 7.5 to 9.0 by adding slowly 0.1 M NaOH with slow stiring. This step was followed by centrifugation again at 5000 X g for 15 minutes to further pellet the protein at pH 9.0. Pelleted proteins were resuspended in storage buffer and stored at –80°C.

5, Purification of 46 kDa RBP

5. 1, Purification with DEAE-sepharose CL-6B

DEAE-sepharose CL-6B (Sigma) was washed with buffer A [20 mM Tris-HCl (pH 7.5), 3 mM $MgCl_2$, 0.1 mM EDTA, 5 mM 2-mercaptoethanol] containing 2 M KOAc and equilibrated in buffer A before loading the sample. The sample was applied at 0.5 ml / min using a peristaltic pump. The column was washed at the same flow rate with 4 column bed volumes of buffer A containing 0.5 M KOAC. 1 ml fractions were collected, dialyzed against dialysis buffer [20 mM HEPES (pH 7.8), 60 mM KCl, 0.2 mM EDTA, 20% glycerol, 5 mM 2-mercaptoethanol] and stored at - 80°C.

5. 2, Purification of 46 kDa RBP with Q-sepharose

In the batch test, 100 µl Q-sepharose gel (Sigma) was mixed with 100 µl LDM protein extract for 15 minutes in a 1.5 ml tube with shaking, and then centrifuged for 1 minute at 3000 rpm. The non-bound fraction in the supernatant was kept for detection of the RNA binding activity. Three washing steps were done each with 100 µl washing solution (buffer A) the same way as above. Then, the bound proteins were eluted successively with 100 µl Buffer A containing 0.3 M, 0.6 M, 1.0 M, and 1.6 M KOAc.

5. 3, Purification of 46 kDa RBP with Superdex-75 using HPLC

The Superdex-75 column (Amersham Pharmacia Biotech, Inc. Canada) was washed and equilibrated with buffer A before loading the sample. The sample was applied at 0.5 ml/ min using HPLC. The column was washed at the same flow rate with 3 column bed volumes of buffer A and 0.5 ml fractions were collected.

5. 4, Purification of 46 kDa RBP with HA chromatography

In this batch test, 100 µl HA matrix (Sigma) was mixed with 100 µl LDM protein extract for 15 minutes in 1.5 ml tube with shaking, and then centrifuged for 1 minute at 3000 rpm. The supernatant was kept for detection of the RNA binding activity. Three gel washing were done each with 100 µl washing solution (buffer A) the same way as above. Then, the bound proteins were eluted with 100 µl buffer A containing 0.03 M, 0.06 M, 0.125 M, 0.25 M, and 0.5 M phosphate buffer.

5. 5, Purification of 46 kDa RBP with Poly (A)- sepharose 4B

Preparation of the gel: Poly (A)- sepharose 4B (Pharmacia Biotech) was swelled in 1.0 M NaCI for 5 min, and washed with 0.1 M NaCI (25 ml/ml gel). Then, the gel was washed with a formamide buffer e.g. 0.01 M phosphate, 0.01M EDTA in 90% formamide pH 7.5(25ml/ml gel) followed by equilibration with at least 5 bed volumes of the buffer A.

For the batch procedure, 100 µl Poly (A)- sepharose 4B was mixed with 100 µl LDM protein extract for 15 minutes in 1.5 ml tube with shaking, and then centrifuged for 1 minute at 3000 rpm. The supernatant was kept for detection of the RNA binding activity. Three gel washing were done each with 100 µl of buffer A the same way as above. Then, the bound proteins were eluted with 100 µl of buffer A containing 0.3 M, 0.6 M, 1.0 M, 1.6 M, and 2.0 M KOAc.

5. 6, Purification of the 46kDa RBP using heparin-agarose batch procedure.

100 µl heparin-agarose gel (GIBCO BRL Life Technologies, Inc. USA) was mixed with 100 µl LDM protein extract for 15 minutes in 1.5 ml tube with shaking, and then centrifuged for 1 minute at 3000 rpm. The supernatant was kept for detection of the RNA binding activity. Three gel washing were done each with 100 µl of buffer A as described above. Then, the bound proteins were eluted with 100 µl buffer A containing 0.3 M, 0.6 M, 1.0 M, and 1.6 M KOAc.

5. 7, Purification of the 46kDa RBP by heparin-agarose using FPLC.

A heparin agarose (GIBCO BRL Life Technologies, Inc. USA) column with 2 ml bed volume was washed with 3 column volume of buffer A containing 2 M KOAc and equilibrated with four column of buffer A before loading the sample. A 6 ml sample was

applied to the column at 0.5 ml/ min using the FPLC. The column was washed at the same flow rate with three column bed volumes of buffer A containing 0.3 M KOAc. RBPs were eluted with a 0.3 to 1.0 M KOAc gradient over four column bed volumes and collected in 0.5 ml fractions. Finally, the column was washed with three bed volumes of buffer A containing 2 M KOAc. Fractions were tested for RBP activity immediately.

5. 8, Separation of 46 kDa RBP with Hitrap heparin HP column using HPLC.

A Hitrap heparin HP column (5 ml) (Amersham Pharmacia Biotech, Inc. Canada) was washed with buffer A containing 2 M KOAc and equilibrated in buffer A before loading the sample. The sample was applied at 0.5 ml/ min using the HPLC. The column was washed at the same flow rate with 4 column bed volumes of buffer A. Protein were eluted with a 0 to 1.6 M KOAc gradient over 3 column bed volumes and collected in 0.5 ml fractions. Fractions were dialyzed against dialysis buffer and stored at - 80°C.

6, Silver staining of protein bands in the polyacrylamide gel.

The Silver Stain Plus Kit from Bio-Rad was used for protein staining in polyacrylamide gels as recommended by the manufacturer.

7, Isolation of LDM by Aqueous Polymer Two-Phase Partitioning (Norling, 1994, Norling, 1998)

125ml of *C. reinhardtii* culture CC503 (1 x 10^6 /ml) was pelletted and suspended in breaking buffer. The cells were broken with 0.25% saponin, and loaded on 40% percoll, 0.3M Sorbitol, 20 mM Tricine, pH 7.5, 1 mM EDTA and then chloroplast were pelletted

by centrifugation at 5000 X g for 5 minutes. The pellet fraction was resuspended in 7 ml hypotonic lysis buffer (20 mM Tricine, 1 mM EDTA, and 20 µl cocktail of protease inhibitors) to lyse the chloroplast, or using sonication to break chloroplast (2 x 30 seconds at intensity level 3 or 6). Membranes from broken chloroplasts were pelleted by centrifugation at 100,000 X g for 1 hour, and then resuspended in 0.25 M sucrose, 5 mM Potassium phosphate buffer (pH 7.8). Equal volume of 2 x 0.25 M sucrose, 5 mM potassium phosphate buffer pH 7.8, and 5.4% to 6.4% (W/W) of Dextran T-500 (Sigma) and PEG 3350 (Sigma) was added. Partitions were performed at 4° C from 1 hour to overnight.

III. Results

1, Optimization of the yield of chloroplast membranes with the 46 kDa RBP.

The assay used involves incubation of a uniformly ^{32}P-labelled RNA probe with chloroplast subfractions, containing membranes and proteins (Leibold, 1988). These reactions are then exposed to UV light, which induces covalent bonds between the RNA probe and proteins bound to it. Non-bound probe is digested with RNase A, leaving small fragments of labeled RNA covalently linked to the proteins of interest. Proteins are then fractionated by SDS-PAGE and the ^{32}p-labeled proteins are revealed by autoradioagraphy.

1. 1, Comparisons of methods to break cells: The initial goal of this project was to purify and characterize the 46 and 47 kDa RBPs. Towards this end, optimal conditions and procedures were established for isolation of LDM fractions with these activities. Higher levels of the binding activity of the 46 kDa RNA-binding protein (RBP) were detected by using chloroplasts isolated from cells broken with either a yeda press at 4 bar or the detergent saponin (Figure 1 and Figure 16). Saponin solubilizes the plasma membrane but not the chloroplast envelope (Detmers and Goodenough, 1983). Homogenization of cells using sonification or a polytron yielded less RBP binding signals (data not shown).

1. 2, Separation of LDM from chloroplast by Aqueous Two-phase Partitioning: Aqueous two-phase partitioning has been used to separate plasma membranes from thylakoid membranes in cyanobacteria with considerable success (Norling, 1994,

Norling, 1998). A substance partitions between the two liquid phases according to its surface properties, such as electrical charge and hydrophilic-hydrophobic character. If electrolytes are included in the two-phase system, an unequal distribution of cations and anions across the interface creates a potential difference between the phases. This interfacial potential depends on the salt used and directs the partition of other charged substance present in deficit as compared with the salt. Certain salts eliminate the interfacial potential and the partition of a substance is, in this case, independent of its net charge. (Hjalmar et. al., 1976).

To determine whether or not this technique can separate LDMs from thylakoid membranes of chloroplasts, I used aqueous two phase partitioning of chloroplast membranes using 5.4% to 6.4% Dextran T-500/(W/W) and PEG 3500. Cells were broken and chloroplasts isolated on percoll gradients. Chloroplasts were broken by hypotonic lysis or sonification. KOAc or NaCI were used to change the electrical status of the two phase (see also Material and Methods and Discussion sections). Envelope membranes and LDM can be easily detected on the basis of their orange color, due to enrichement in beta carotene (Joyard et al. 1991, Zerges and Rochaix, 1998). Thylakoid membranes are dark green due to enrichement in chlorophlyll. After 1 hour of equilibration in the two-phase mixture, the phases had separated and most of the green thylakoid membranes were observed in the top phase. Some yellow floating materials still remained in the bottom layer with some green thylakoid membranes. However, after overnight equilibration, almost all the membranes went to the top phase. In order to decrease the density of the two-phase to keep the LDM in the bottom layer, I tried two-phase partitioning without sucrose. Still under these conditions almost all of membranes moved to the top layer by

overnight equilibration. The same results were obtained from changing electrostatic status of two-phase by using KOAc or NaCI. Therefore, I concluded that two-phase partitioning could not be used to fractionate chloroplast membranes.

2, Optimization of the RNA-binding conditions: To optimize the RNA-binding conditions, different amounts of RNA probe, and LDM proteins were used in RNA-binding reactions, prior to UV cross-linking (Figure 1). Strong RNA binding signals were detected using 1 x 10^5 cpm probe (*psbC* 5'-UTR) from the T3 template (300 bp) and pDH-245 template (210 bp), and using 20 μg of proteins from purified LDM (Figure 1). Effects of varying pH, KOAc concentration, time of UV cross-linking, and distance from UV-bulbs were also determined to optimize the RNA-binding signals (Figure 3 for effect of pH, other data not shown). The following conditions were found for the maximum RNA-binding activity: pH> 7.5, 20μg LDM protein/ 10^5cpm probe, salt< 0.5M, 254 nm UV irradiation for at least 1.0 J/cm^2 using a Stratalinker UV Cross-linker (Stratagene), and 10 to 15 cm distance from UV-bulbs.

3, Stability studies of 46 kDa RNA-binding Protein (RBP):

Strong RNA-binding signals were detected following storage of the LDM preparations at 4°C for four weeks or at -80°C for three months in storage buffer (50 mM Hepes, 0.2 mM EDTA, 60 mM KCI, 5 mM β-mercaptoethanol, 15% glycerol). Weaker RNA-binding signals or no binding signal were detected from LDM extracts which were frozen and thawed more than four times (data not shown).

Template	T3	T3	T3	PDH	PDH	PDH
Probe ($x10^4$cpm)	10	5	2	10	5	2
Proteins (µg)	20	5	5	20	5	5

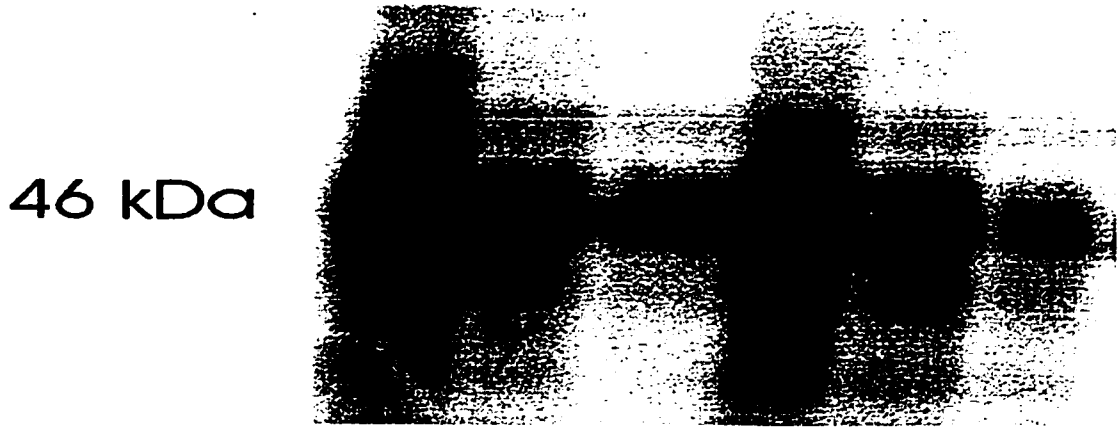

Figure 1, Determination of optimal RNA-binding conditions. Cells were broken with a yeda press at 4 bar.

4, Properties of the 46 kDa RBP

4. 1, Biochemical properties of 46 and 47 kDa RNA-binding proteins.

In order to ensure that the bands observed were derived from proteins, extracts were treated with proteinase K for 45 minutes or SDS and then tested for the RBP activities. RNA structures are expected to be resistant to these treatments, while most proteins are degraded or denatured. As seen in Figure 2, lanes 10, 2 and 3, these treatments eliminated the RBP activities. Therefore, these bands are generated by protein. To determine whether or not the activities of these proteins are sensitive to heat treatment, a property that would provide an easy purification step, LDM extract was incubated at 100°C for 3 minutes and then tested for activity. In Figure 2, no 46 kDa RBP binding signal was detected from LDM protein extracts which had been treated at 100°C for 3 minutes (lane 8). This result further confirmed the protein property of the 46 kDa RNA-binding signal. Loss of the 46 kDa RNA-binding signal after treatment of LDM protein extract with 0.1M DTT (lane 11), or 0.1M β-mercaptoethanol (lane 12) at 37 °C for 45 minutes indicate the requirement of disulfide bonds for RNA-binding. DNase treatment (lane 9) of LDM protein extracts did not affect the signal intensity of 46 and 47 kDa RBPs (Figure 2) reducing the possibility that DNA in the extracts or remaining from the probe template somehow generates these signals. No difference in the 46 and 47 kDa RNA-binding signal is observed between EDTA (lane 7) and $MgCl_2$ treatments (Lane 6) of LDM protein extract suggesting that magnesium ions do not play an important role in the binding (Figure 2). In protein stained gel (corresponding to lane 10 of figure 2; data not shown), only one protein band (proteinase K) was seen. This shows that treatment of LDM proteins with proteinase K for 45 min at 37°C completely hydrolyzed proteins,

except proteinase K itself, and at the same time we lost the RNA-binding activity. It confirmed that this mRNA-binding signal requires the presence of proteins.

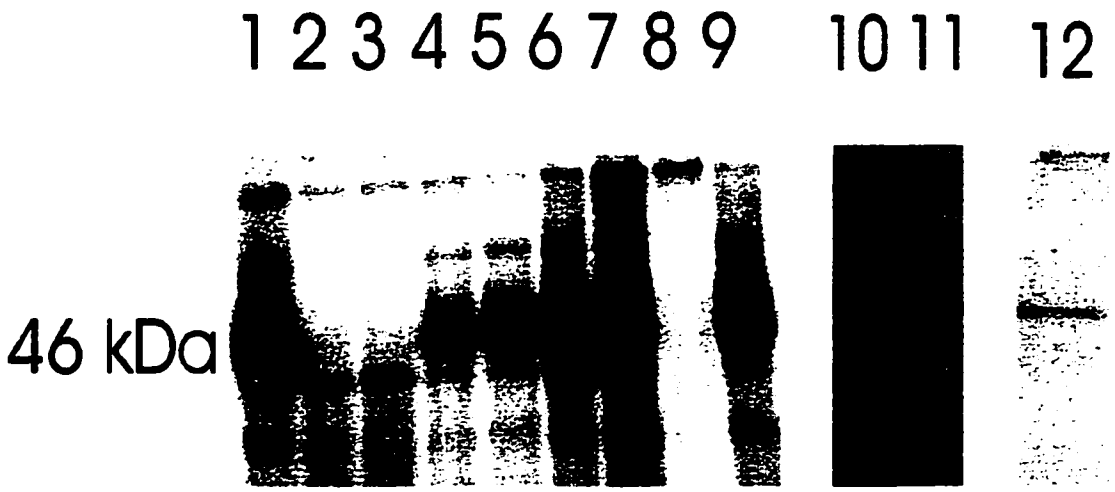

Figure 2, The signals obtained by the UV-cross linking assays are generated by proteins. Prior to UV-cross-linking analysis, LDM fractions containing the RNA-binding activities were not treated as a control (lane 1), or treated with SDS (2% wt/vol, lanes 2 and 3), Triton X-100 (lanes 4 and 5), Mg^{2+} (lane 6), or EDTA (lane 7), incubated at 100° C for 3minutes (lane 8), DNase (0.5 U/μl, lane 9), proteinase K (100 μg/ml, lane 10), DTT (0.1 M, lane 11), or β-mercaptoethanol (0.1 M, lane 12).

4. 2, Effect of pH on the activity of the 46 kDa RNA-binding protein: In order to determine the optimal pH for the RBP activities, LDM extracts were incubated in solutions buffered at various pH values, and then assayed for binding using the UV cross-linking assay. Strong RNA-binding signals were detected when assayed at pH from 7.0 to 9.0, and weaker banding signals were detected when assayed at pH 6.5 and 6.0 (Figure 3, A). Coomassie blue staining of proteins in this gel shows that comparable amount of proteins were loaded in the lanes (Figure 3, B). The results show that higher pH values enhance the 46 kDa RNA-binding activity, and lower pH inhibits the binding with a maximum between 7.5 and 8.5.

4. 3, Effects of salt on RNA-binding activity: It is known that the presence of salt can change the electrostatic properties of proteins and, thereby, alter their ability to bind to other macromoleucles, including RNA. To guarantee the detection of the 46 kDa RBP binding signal which could be disturbed by salt and control the salt concentration in RNA-binding assays, the effect of KOAc on the 46 kDa RNA-binding has been studied. Weaker or no RNA-binding signal were detected when concentrations of KOAc over 0.5 M were added to RNA-binding reactions (data not shown). Therefore, KOAc concentration was maintained under 0.5 M in all RNA-binding assays in my experiments.

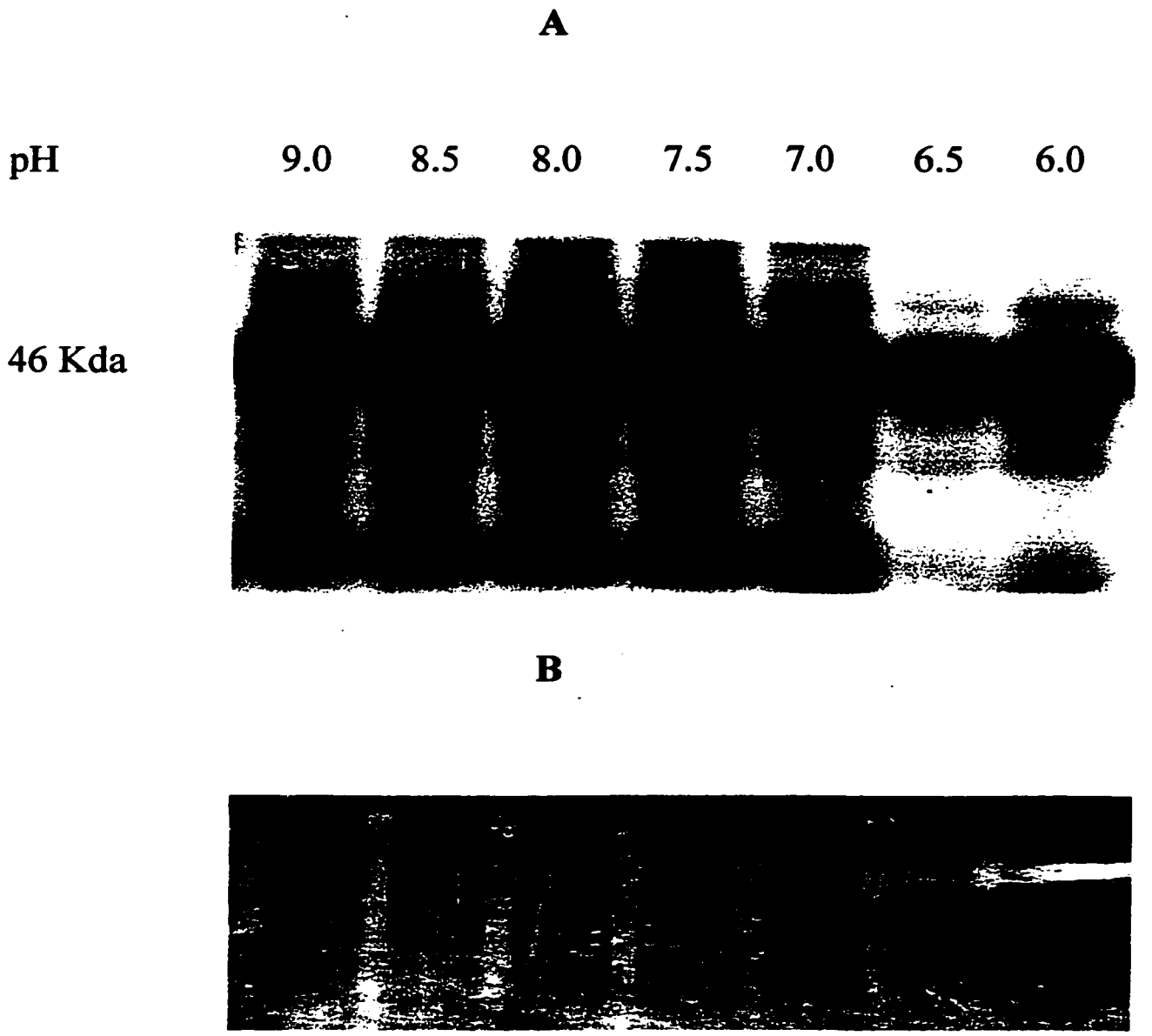

Figure 3, UV cross-linking analysis of the RNA-binding activity revealed highest 46 kDa RBP activity at pH 8.5. A: Samples of LDM with RNA-binding activity were incubated in solutions buffered at various pHs and used for the UV cross-linking assays. A: Autoradiograph shows the level of labeling, a measure of RNA-binding activity. B: Proteins in the gel were stained with coomassie blue.

5, Partial purification of the 46 kDa RBP

5. 1, Precipitation of 46 kDa RNA-binding protein; Step-wise precipitation with increasing [$(NH_4)_2SO_4$]or changing pH is an easy initial method of protein purification (Englard and Seifter, 1990). Therefore, the pH and concentration of $(NH_4)_2SO_4$ required to efficiently precipitate the RBPs were determined. Compared with that of 85% ammonium sulfate precipitation at pH 7.5 (lane 1 and 2, Figure 4), stronger 46 kDa RBP binding signals (lane 3 and 4, A) and less proteins (lane 3 and 4, B) were detected in further precipitation after adjusting pH from 7.5 to 9.0 (Figure 4). Therefore, the maximum RBPs precipitation can be achieved at pH 9.0 with 85% $(NH_4)_2SO_4$.

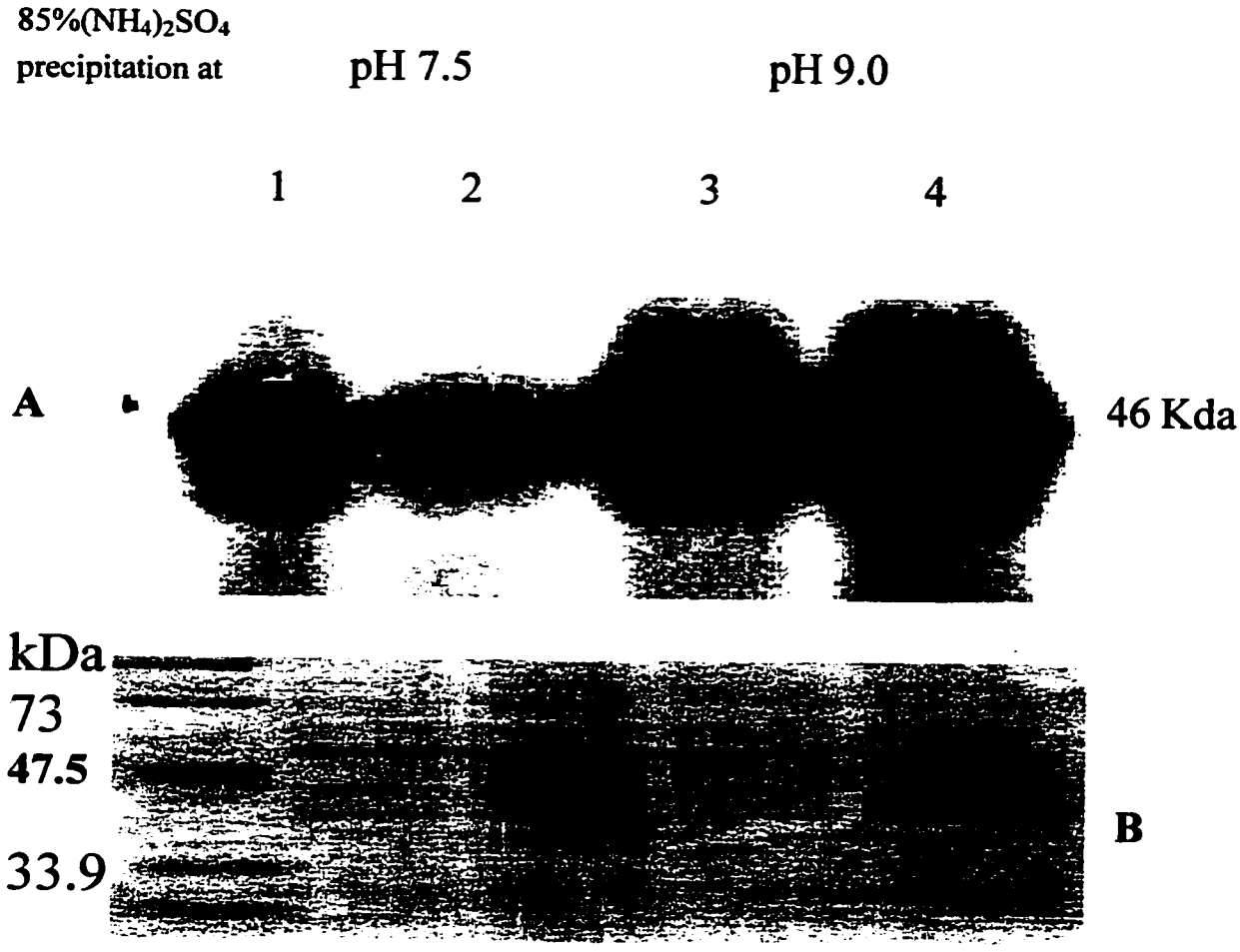

Figure 4, 46 kDa RNA-binding protein was precipitated at pH 9.0 and 85% $(NH_4)_2SO_4$.

A: autoradiographs of the UV cross-linking assay. B: Correspondent gel stained with coomassie blue. Lane 1, 85% $(NH_4)_2SO_4$ precipitation at pH7.5. Lane 2, Dialyzed protein sample from 85% $(NH_4)_2SO_4$ precipitation at pH 7.5. Lane 3, pH 9.0 precipitation after $(NH_4)_2SO_4$ precipitation at pH 7.5. Lane 4, Dialyzed protein sample from pH 9.0 precipitation after $(NH_4)_2SO_4$ precipitation at pH 7.5.

5. 2, Attempts of purification of the 46 kDa RBP by chromatography:

5. 2. 1, DEAE-sepharose CL-6B: To eliminate the RNA and DNA which have strong affinity to DEAE-sepharose and may exist in the LDM protein extracts, a step of DEAE-sepharose chromatography was used. LDM membranes were solubilized with 0.1 % Triton X-100 and passed over a column of DEAE-sepharose CL-6B (Sigma) by elution with 0.5 M KOAc in buffer A. This step was designed to retain RNA and DNA in the column while the proteins pass through the column because of the 0.5 M KOAc in the elution buffer (Gruissem, *et al.*, 1986). However, after one pass through of the column by LDM protein extracts with 0.5 M KOAc, no 46 and 47 kDa RBP binding signals were detected in the flow-through. Relatively strong binding signals of 30, 40, 60 kDa were detected in elution solution (Figure 5, lane 2, 3, and 4).

5. 2. 2, Q-sepharose: Proteins from Triton X-100 solubilized LDMs were mixed with Q-sepharose (Sigma), which separates proteins on the basis of their negative charge). LDM protein extract was incubated with Q-sepharose in low salt buffer. The non-bound fraction and successive washing steps with solutions of increasing concentrations of potassium acetate were carried out. These fractions were tested for RNA-binding activities using the UV cross-linking assay. As seen in Figure 6, no 46 kDa RBP binding signal was detected in the fraction of second washing, or in elutions up to the highest concentration of salt used; 2 M KOAc (Figure 6, A). However, strong 40 kDa and 44 kDa RBP binding signals appeared in 1.0 M KOAc elution (Figure 6, A). The coomassie blue stained gel shows that most of proteins were eluted from the Q-sepharose with 1.0 M KOAc in buffer A (B, Figure 6).

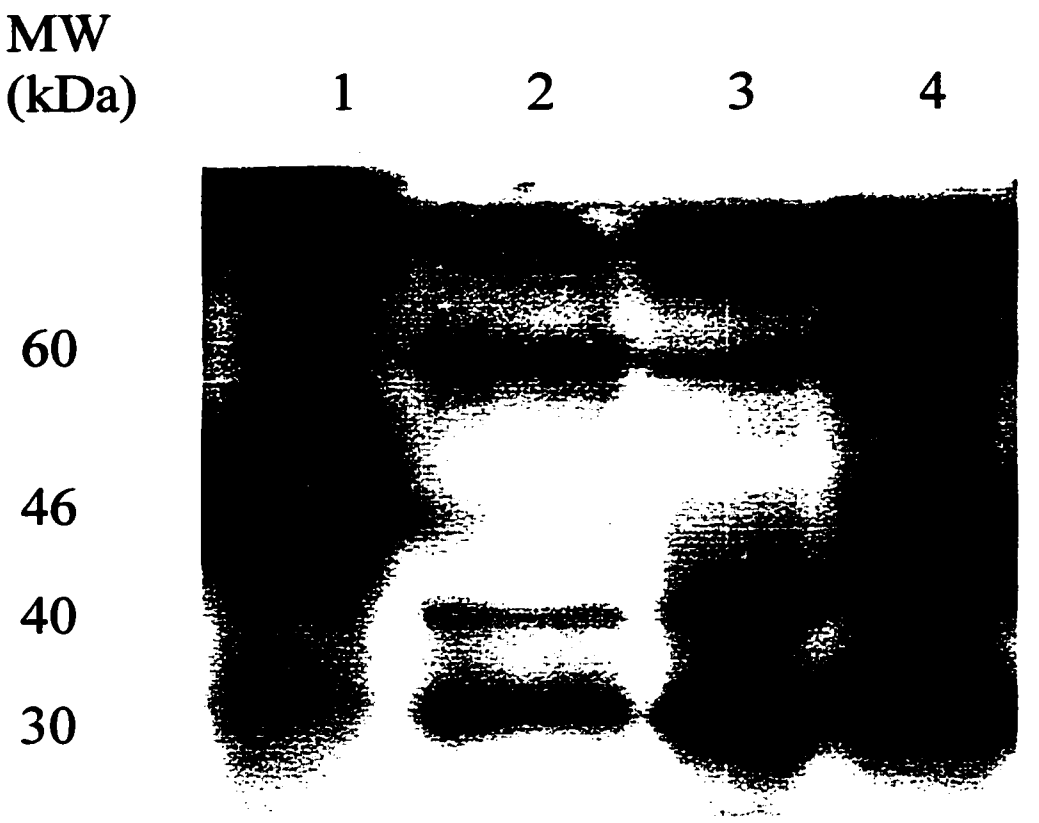

Figure 5, Analysis of fractions from DEAE-sepharose CL-6B and DEAE sephadex A-75 using the UV cross-linking assay: Lane1, Protein extract from LDM. Lane 2, dialyzed elution solution after DEAE sephadex A-25. Lane 3, non-dialyzed elution solution after DEAE sephadex A-25. Lane 4, non-dialyzed elution solution after DEAE sepharose CL-6B.

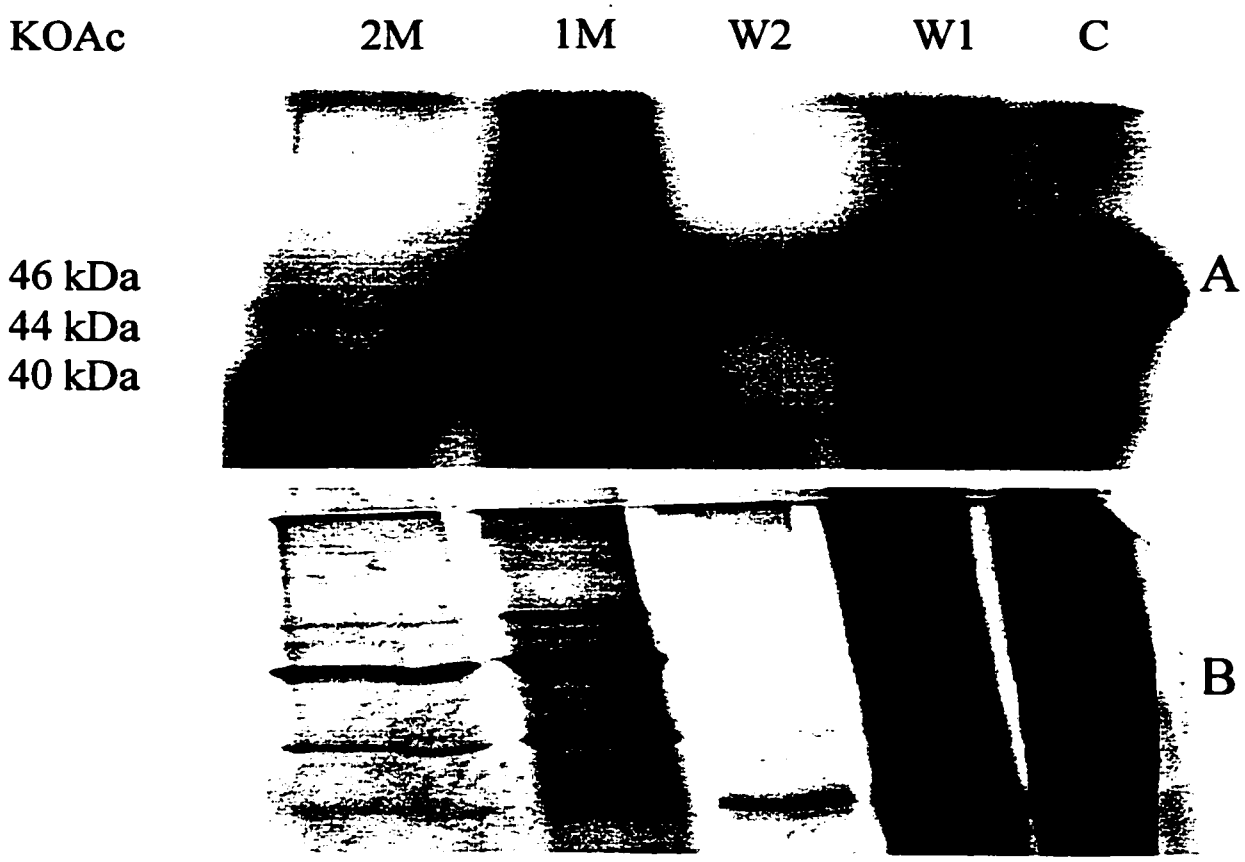

Figure 6, Analysis of fractions from Q-sepharose chromatography using the UV cross-linking assay.

A: autoradiographs of the UV cross-linking assay. B: Correspondent silver stained gel (SDS-PAGE). Lane C: Control of LDM protein extracts. Lane W1: first washing. Lane W2: second washing. Lanes 1M and 2M: 1M and 2 M KOAc elution.

5. 2. 3, Superdex-75 using HPLC: In order to separate the 46 kDa RBP from proteins with different molecular weights in LDM proteins extract, Superdex-75 was used with HPLC to maximize the separation of proteins according to their molecular weight. Activities of RBPs were tested for fractions with UV cross-linking assay. Very few and weak protein bands was detected even using silver staining (data not shown). The binding signal of a 30 kDa RBP was detected, but no 46 kDa RBP binding signal was detected (data not shown). The dilution in size-exclusion chromatography as shown in the silver staining may leads to the disappearance of the 46 kDa RBP binding signal.

5. 2. 4, Hydroxyapatite chromatography: Although the exact mechanism of the separation is unknown, hydroxyapatite (HA) has been used widely and efficiently for separation of different proteins. Proteins from Triton X-100 solubilized LDM were mixed with hydroxyapatite. After 0.03 to 0.5 M phosphate buffer washing, the 46 and 47 kDa RBPs were detected from 0.125 to 0.5 M phosphate buffer elution, and partially separated from 33, 40, 60, and 80 kDa RBPs (Figure 7). In 0.5 M elution, although there is a strong 46 kDa RBP binding signal (Figure 7), there is no apparent corresponding protein band in the coomassie blue stained gel (Figure 8). These results show that HA column works well in separating the 46 kDa RBP, and can be used together with other steps to finally purify the 46 kDa RBP, if a large quantity of algae cells is available.

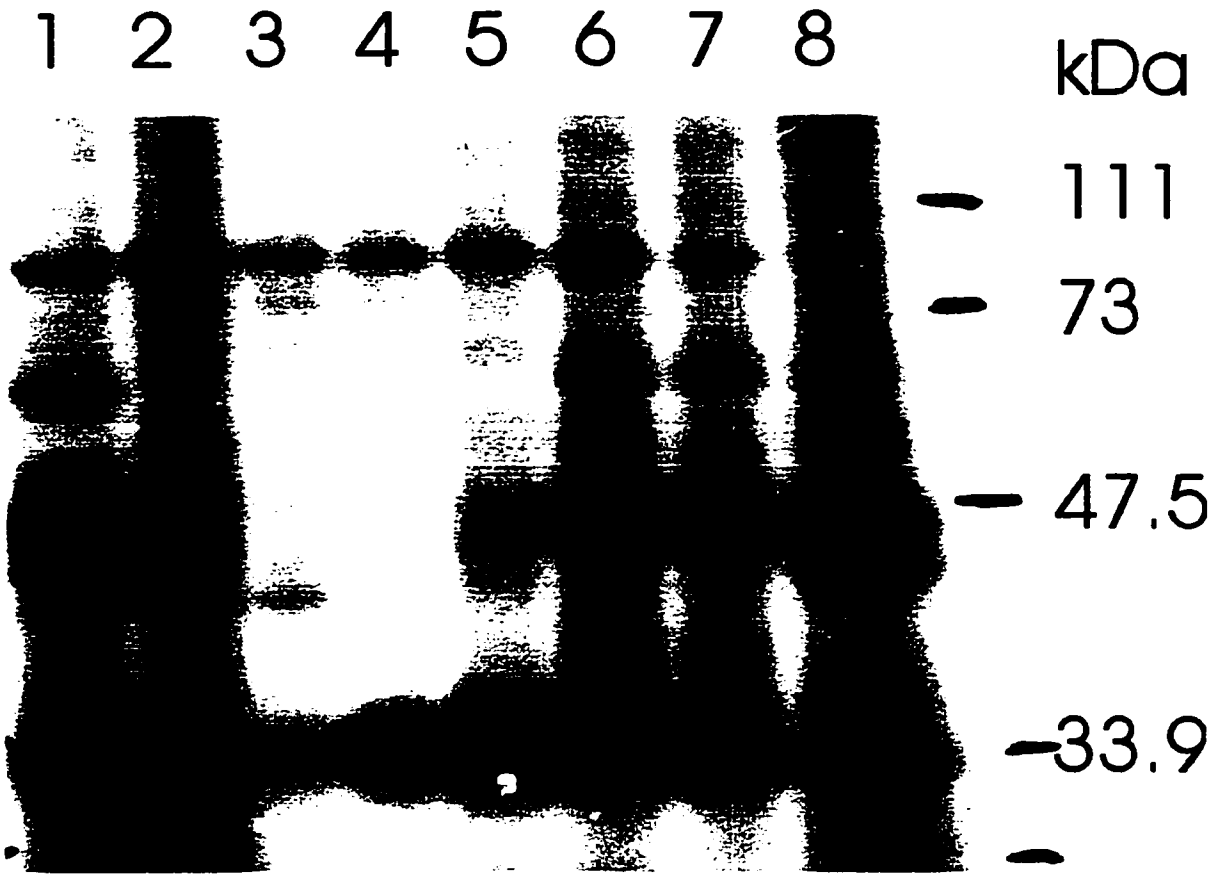

Figure 7, Purification of 46 kDa RNA-binding protein using Hydroxyapatite chromatography (autoradiographs). Lane 1, LDM protein extract; Lane 2, Non-bound fraction; Lane 3, Washing solution. Lanes 4 to 8, Eluted fractions with 0.03M (Lane 4), 0.06M (Lane 5), 0.125M (Lane 6), 0.25M (Lane 7), 0.5M (Lane 8) phosphate buffer.

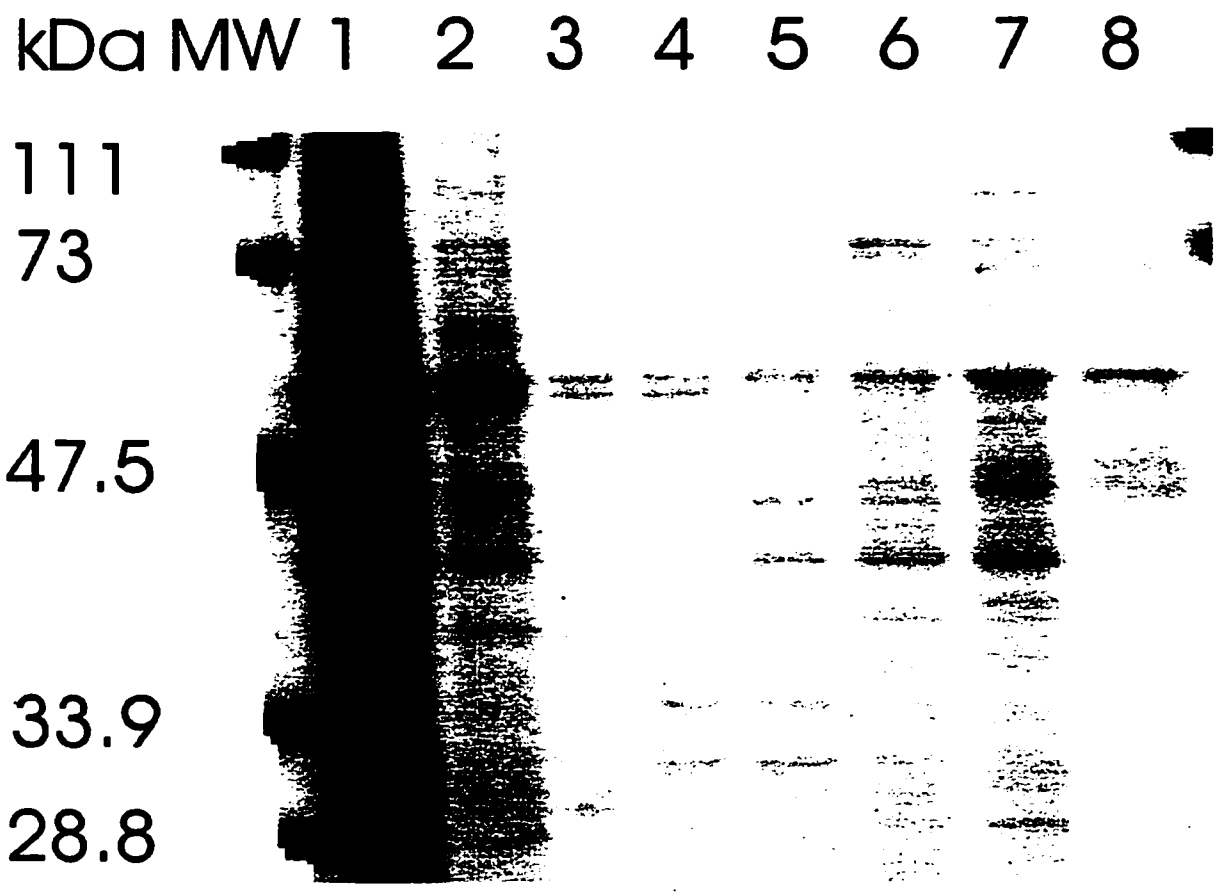

Figure 8, Purification of 46 kDa RNA-binding protein using HA chromatography (coomassie blue stained proteins in the gel shown in Figure 7). Lane 1, LDM protein extract. Lane 2, non-bound fraction. Lane 3, washing solution. Lanes 4 to 8, Eluted fractions with 0.03M (Lane 4), 0.06M (Lane 5), 0.125M (Lane 6), 0.25M (Lane 7), 0.5M (Lane 8), phosphate buffer.

5. 2. 5, Poly-A sepharose : The 46 kDa RBP is known to bind A-U rich RNA because Poly-A and Poly-U compete for its binding to the RNA probe derived from the *psbC* 5' UTR (Wang et al, submitted). It is reasonable to think that the 46 kDa RBP should have affinity for poly-A and that this can be used for affinity purification. Proteins from Triton X-100 solubilized LDM were mixed with Poly-A, and then eluted with solutions of increasing concentrations of KOAc. In a batch test, the 46 and 47 kDa RBP were eluted with 0.3 M KOAc. In the elution containing 0.3 M KOAc, weaker 46 and 47 kDa RBP binding signals and strong 30 kDa RBP (which was not seen in the control) may be caused by the degradation of the 46 and 47 kDa RBPs (Figure 9, A). In any case, the 46 and 47 kDa proteins were not detected in any of the fractions eluted with salt. Therefore, poly-A affinity chromatography is probably not an efficient purification step for this study. Strong binding signal for the 40 kDa RBP was also detected in 0.3 M KOAc elution as in control. Staining of the proteins in the gel with coomassie blue revealed that the RBP activities were separated from most proteins, which did not have affinity to Poly-A and were present in the supernatant and washing solution. Most bound proteins and the RBPs were eluted from the Poly-A with 0.3 M KOAc (Figure 9, B).

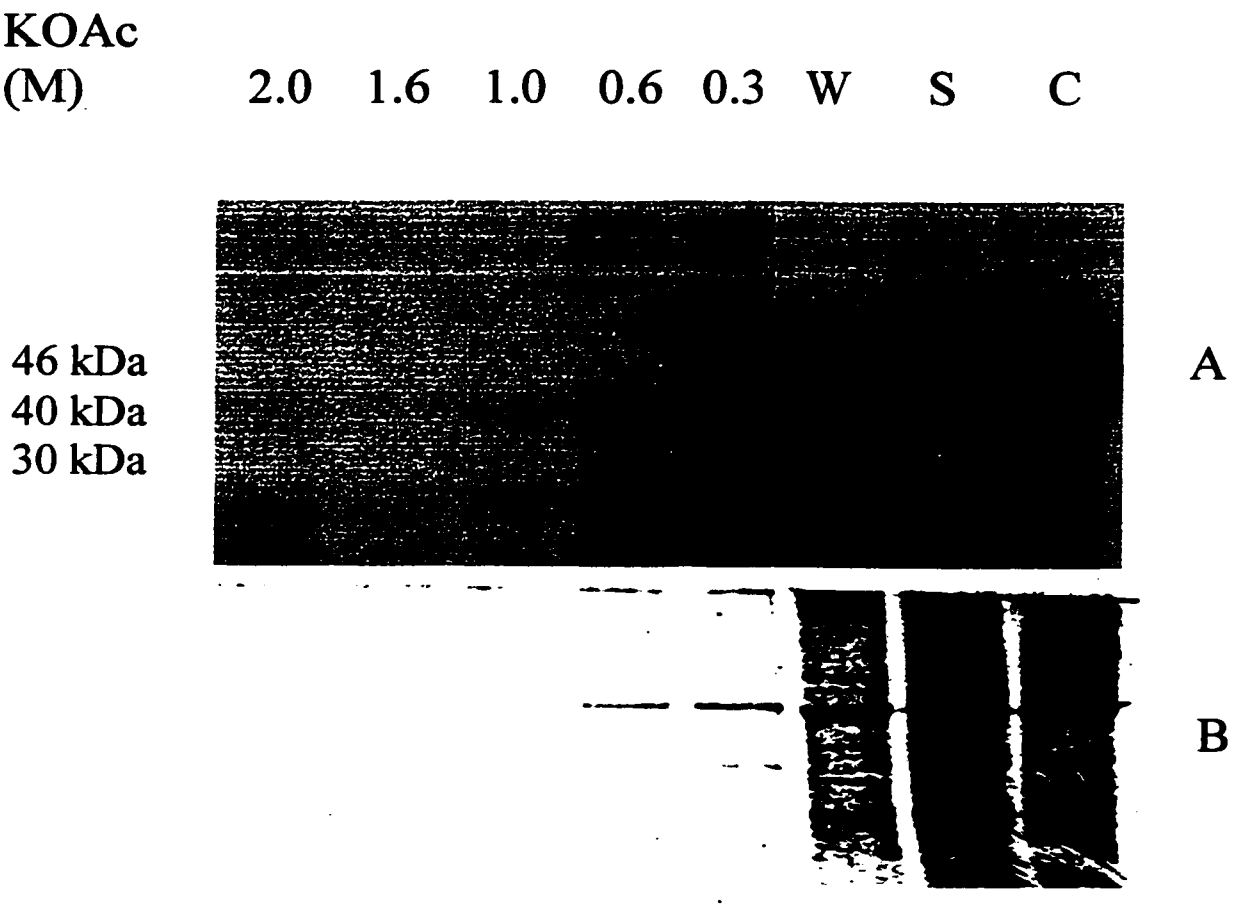

Figure 9, Purification of 46 kDa RBP with affinity chromatography using Poly-A sepharose: A: autoradiographs of the SDS-PAGE B: Correspondent SDS-PAGE stained with coomassie blue. C: LDM protein extract. S: Supernatant after mixing gel and RBP extracts. W: Wash.

5. 2. 6, Heparin-agarose batch tests: To determine whether the RBPs bind to heparin linked to agarose, a batch test was done first. Heparin is commonly used for affinity purification of nucleic acid binding proteins (Steven Ostrove, 1990). In these "batch tests", LDM protein extracts (with membranes solubilized with Triton X-100) and heparin-agarose gel suspensions were mixed and incubated with agitation in a 1.5 ml tube for 15 minutes. Centrifugation was used to pellet the gel beads. The non-bound fraction and elution fractions obtained by washing the matrix with solutions of progressively higher KOAc concentration were analyzed by the UV cross-linking assay. In the supernatant and first washing solution, the 30 and 40 kDa RBP binding signals were detected (Figure 10). Comparison of the binding activities following elution with increasing concentration of potassium acetate revealed that the 46 kDa RBP is retained by the heparin-agarose until 0.6 M KOAc elution. In lane 5 (Figure 10, A), we can see that the 46 kDa RNA-binding protein has been successfully separated from other RNA-binding protein by step-wise elution with 0.6 M KOAc from heparin-agarose. Staining of proteins in the gel with coomasie blue revealed two strong bands (Figure 10, B). Relative to the 46-kDa protein, one is larger and the other smaller, and we can not detect a protein that corresponds in mobility to the 46-kDa RNA-binding signal on the autoradiographs (Figure 10). The stained gel also showed that the majority of proteins were separated from the 46 kDa RBP.

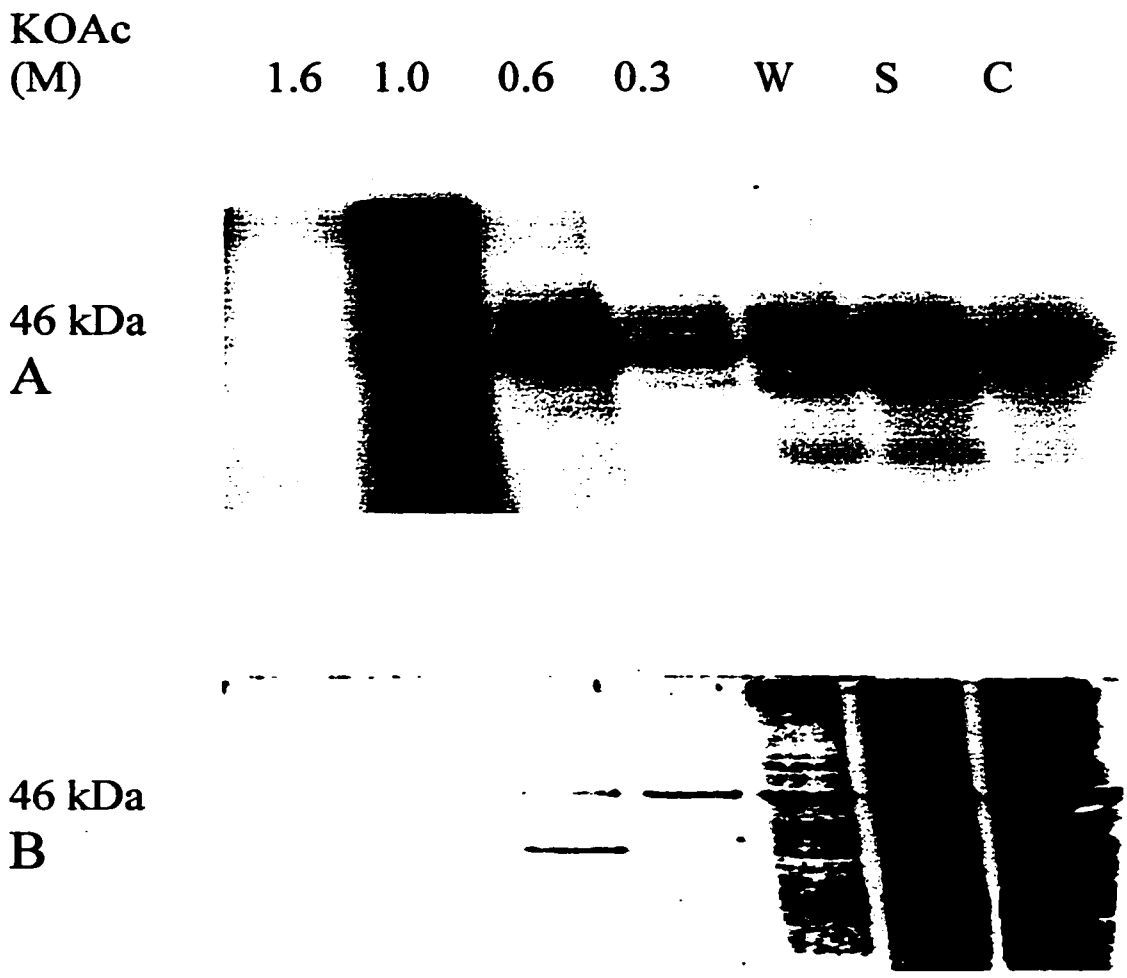

Figure 10, Purification of the 46 kDa RBP with heparin-agarose gel -batch test. A: autoradiographs of the SDS-PAGE. B: Correspondent SDS-PAGE stained with coomassie blue. C: LDM protein extract. S: supernatant after mixing and equilibrating gel with RBP extracts. W: buffer A washing solution.

5. 2. 7, Heparin-agarose using fast protein liquid chromatography (FPLC): Because we observed retention of the RBP activities by heparin agarose in the batch fractionations, column chromatography using the same matrix was carried out on a much larger scale. Most of proteins were eluted from the column in 0.3 M KOAc washing step, and no binding signal was detected in this washing solution. During the application of 0.3 to 1.0 M KOAc gradient to elute proteins bound to the heparin, the 46 kDa RBP binding signal was detected in a minor protein peak on the FPLC elution profile (Figure 11) and which begins at 0.6 M KOAc.

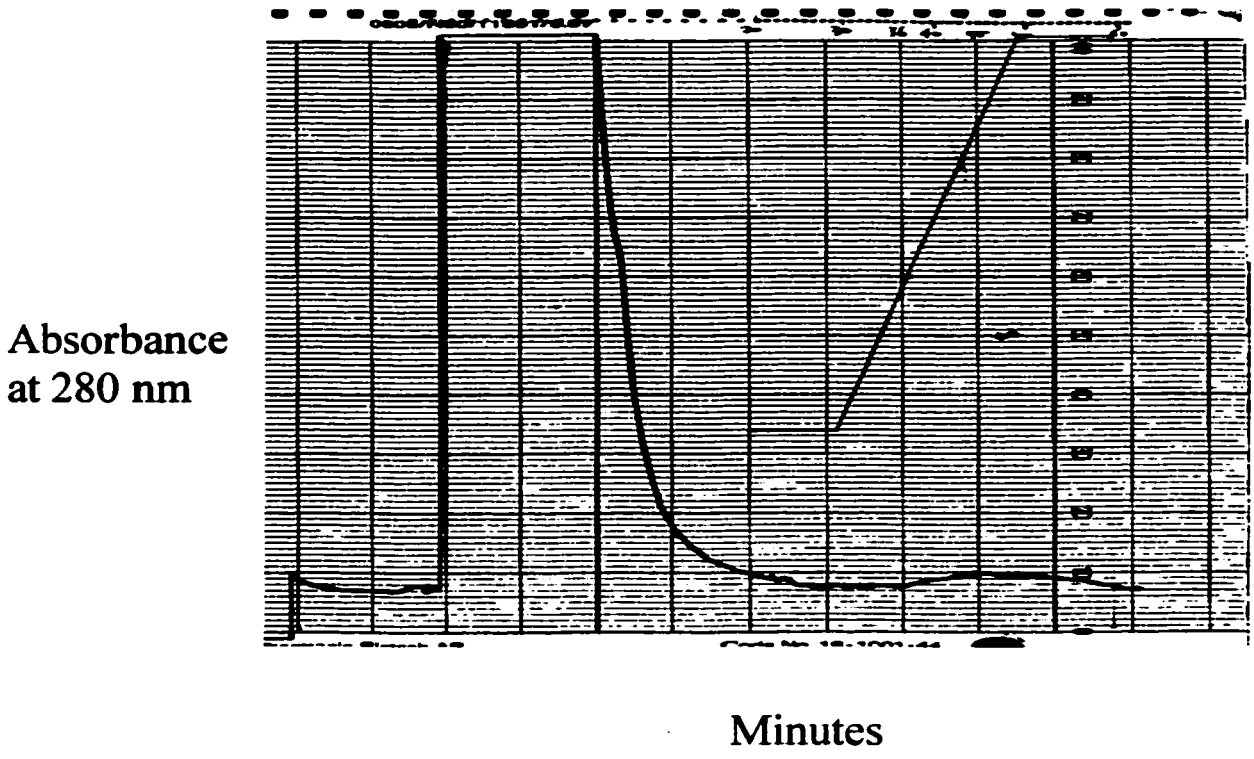

Figure 11, FPLC-plot using heparin-agarose gel

5. 2. 8, Heparin-agarose affinity chromatography, using HPLC.

LDM preparations were solubilized with 0.1% Triton X-100 and then loaded onto a Hitrap Heparin HP Column (Amersham Pharmacia Biotech). Following washing with 4 column bed volumes of buffer A, a linear gradient of 0 to 1.6 M potassium acetate was applied over 3 column bed volumes (see material and methods). Fractions were collected and tested for RBP activity using the UV cross-linking assay. (The samples were diluted at least 6 fold in the RNA-binding reactions to minimize effects of variable salt concentration in the fractions.) Four main peaks of protein were eluted from the column in the plot of absorbance at 280 nm, a measure of protein concentration, (Figure 12). Proteins in the fractions were separated by SDS-PAGE and revealed by silver staining (Figure 13, B) and autoradiography (Figure13, A). The 46 kDa RBP binding signal was detected in fractions 25 to 29 which correspond to Peak 4 (P4) at 52.783 minutes in the elution profile (Figure 12). The protein in these fractions account for about 2% of total protein, calculated from the area of each peak. The 46 kDa RBP was eluted out of the column at approximately 1.0 M KOAc (Figure 12 and 13). Despite a purification and concentration of the 46 and 47 kDa RBPs by 50 fold in fractions 25 to 29, no silver-stained proteins bands aligned with the ^{32}P labeled RBPs in the autoradiograph of RNA-binding signals (Figure 12 and 13).

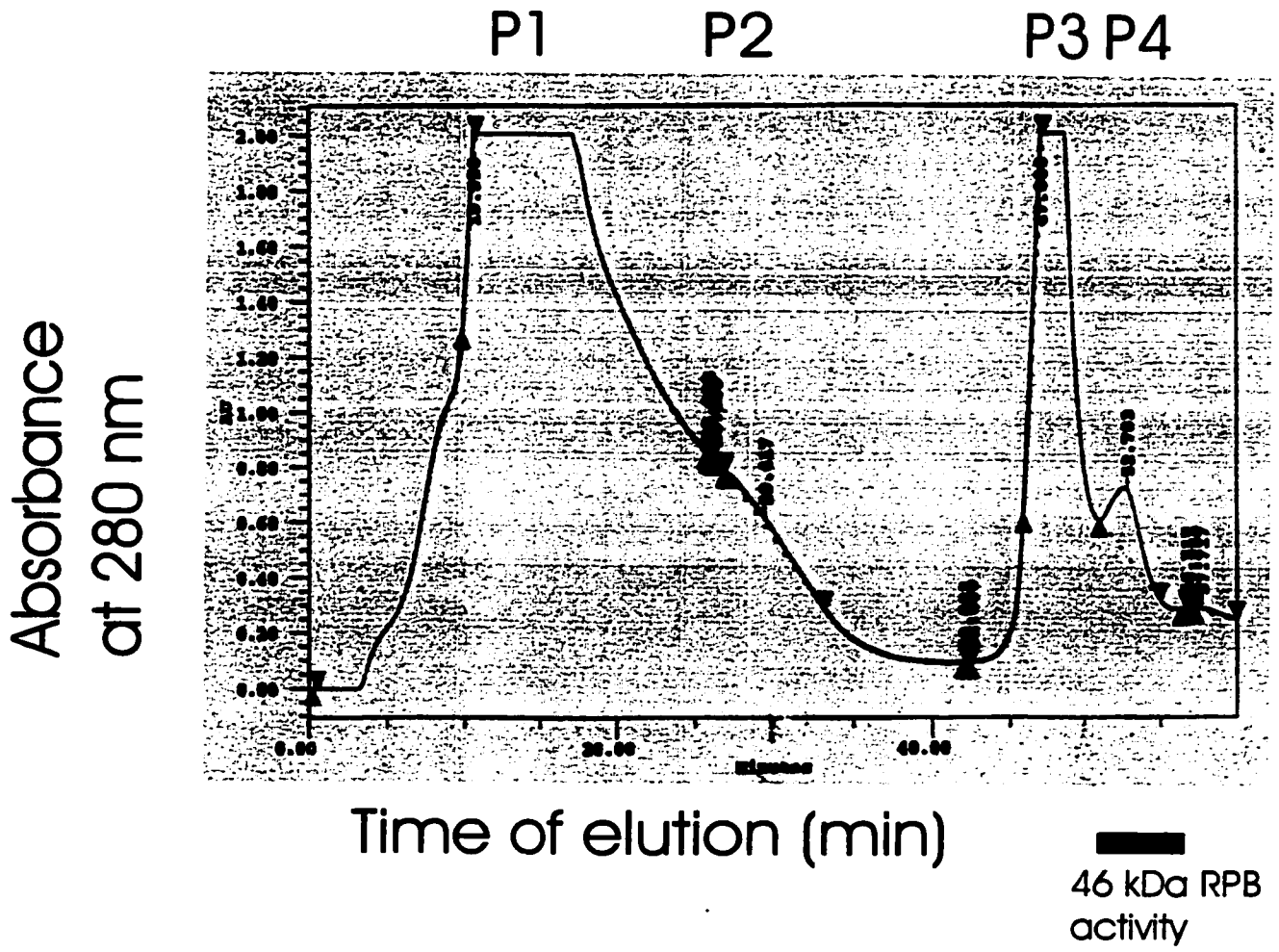

Figure 12, Heparin-Agarose HPLC. The elution profile of proteins from the heparin-agarose column was revealed spectrophotometrically by the absorbance at 280 nm (vertical axis). Four peaks of proteins were eluted and are labeled P1-P4.

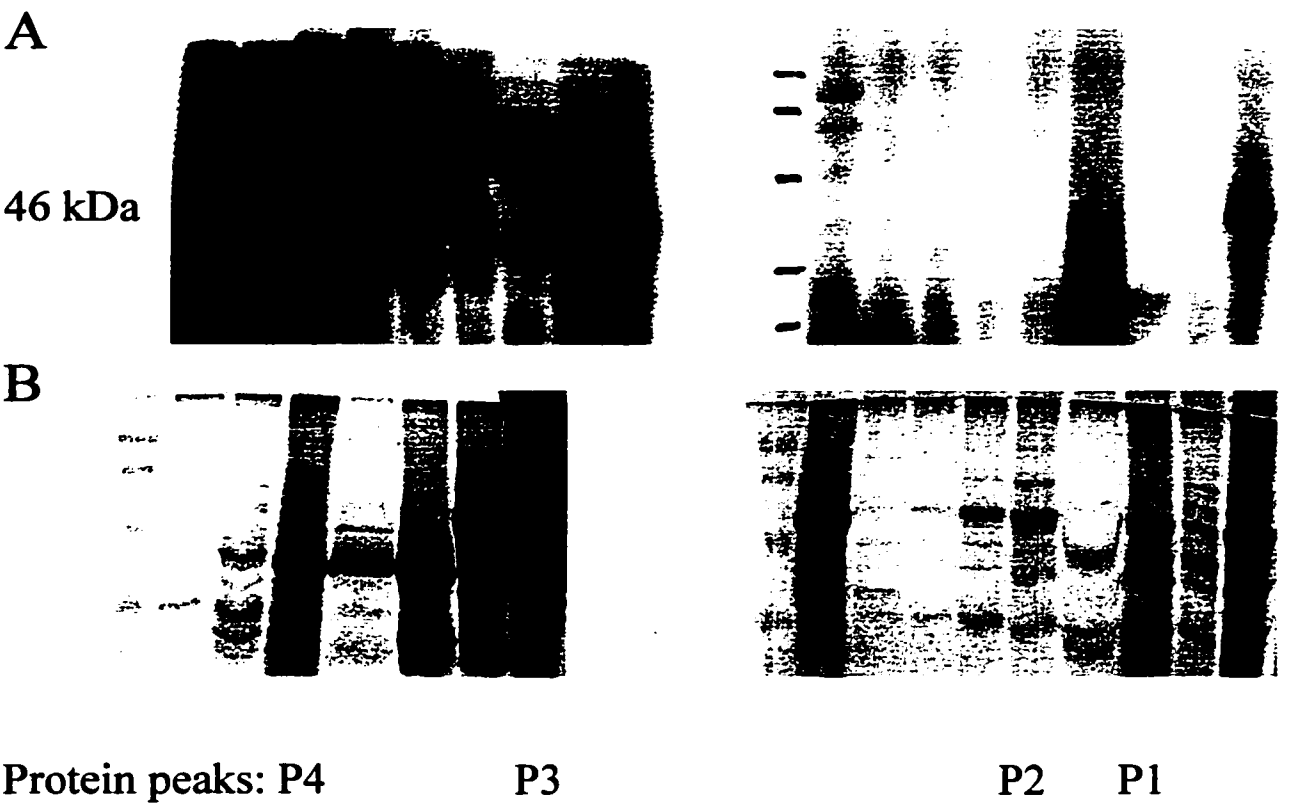

Figure 13, Purification of the 46 kDa RBP with HPLC using heparin-agarose and silver staining. A: autoradiographs of the SDS-PAGE. B: Correspondent silver stained gel (SDS-PAGE). The lane labeled "c" contains a control with non-fractionated LDM protein extract.

5. 3, Detection of the 46 kDa protein band with concentrated RBP fractions and silver-staining: Fractions 26 to 29 from the HPLC purification with heparin-agarose (Hitrap, Amersham) were concentrated from 0.5 ml to 10 μl with Microcon YM-30 (Millipore) whose nominal molecular weight limit is 30 kDa. Parallel experiments were done for each fraction with equal or not equal amount of proteins. The 46 and 47 kDa RBP signals were detected on autoradiographs, but no clear matched protein bands were found on silver staining gel (Figure 14). Because the 46 kDa RBP are very rare protein, its purification will require a large quantity of cells, and several different purification steps. Therefore, I took my project in a new direction as described below.

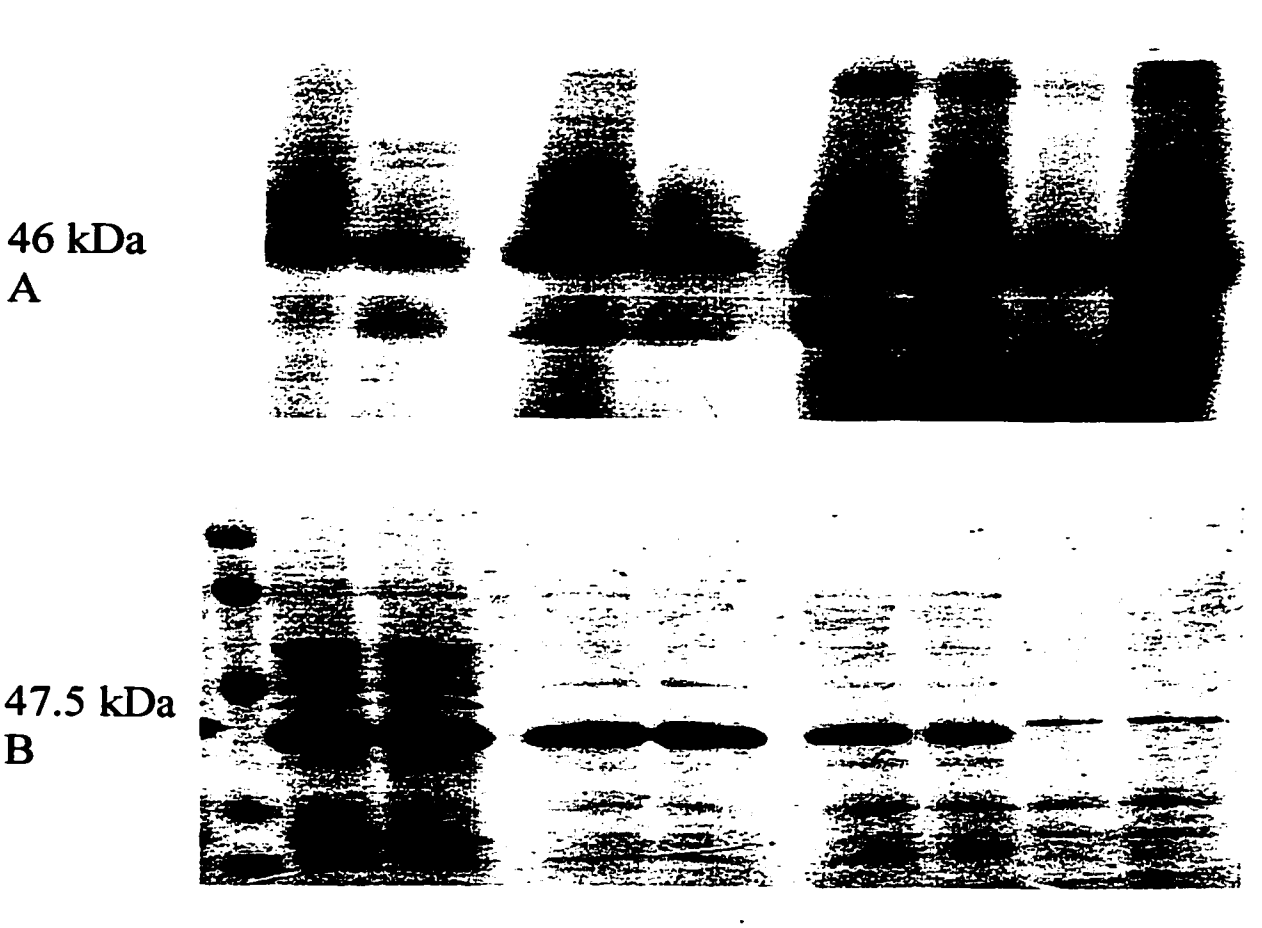

Figure 14, I was unable to detect a protein band corresponding to the 46 kDa RBP in the concentrated RBP fractions using silver staining; The UV cross-linking assay was used to determine the level of the RBP activities in the fractions eluted from the heparin agarose column shown in Figure 12. Panel A: RNA-binding signals were revealed by autoradiography. Panel B: Proteins in the gel were revealed by silver staining.

6, Regulation of the activity of the 46 kDa RBP by light.

6. 1, Light regulation of VLDM RNA-binding activity:

Previous experiments had shown that the levels of the RBP activities or the 46 and 47 kDa proteins were low in LDM preparations from cells grown in the dark, and drastically higher in cells grown in the light. As this difference reflected the steady-state conditions, i.e., following extended grown in the presence or absence of light, it was of interest to determine how rapidly this difference is established following a shift of a dark-grown culture to light conditions. It should be noted that the light intensities used in these experiments (250 $\mu Em^{-2}s^{-1}$) are sufficient to drive photosynthesis but not to cause extensive damage to the photosynthetic apparatus caused by higher intensities. Our assumption was that rapid light induction, i.e. induction within a few minutes, would suggest that these RBPs have regulatory or direct roles in gene expression. If the induction requires longer time, these RBPs may have roles that are indirectly related to the effects of light and perhaps are regulated by general changes in the physiology of the cells due to light and photosynthesis.

In order to determine how rapidly the RBP activities are induced by light, cultures were grown in the dark and then exposed to light for varying amounts of time. A control culture was maintained in constant darkness. Cells were broken rapidly with saponin (see above). Chloroplasts were pelleted by a brief (1 minute) centrifugation and then hypotonically lysed. Membranes were fractionated by discontinuous sucrose gradient centrifugation. A "very low density membrane" (VLDM) fraction was collected from the top of the 0.3 M sucrose, and LDM was collected at the interphase of the 0.3 and 1.0 M sucrose phases. Membranes in these fractions were then solubilized with 0.1% Triton

X-100. The levels of the RBP activities were determined with same amount of proteins, using the UV cross-linking assay. The dark grown culture was harvested under dim green light to avoid inducing the response.

Autoradiography in Figure 15 shows very weak or no binding signals of 46, 47, 60 kDa VLDM RBPs prepared from continuous light (lane 1) or continuous dark (lane 2) cultures. Strongest binding signals of 46, 47, 60 kDa VLDM RBPs were achieved from culture with 10 minutes light exposition before membrane separation (lane 6), and the strength of binding signals decrease with the time of light exposition from 10 minutes to 6 hours (lane 6 to lane 3). The strength of the 30 kDa RBP binding signal was not affected by light. The protein gel stained with coomassie blue in Figure 16 showed that equal amount of proteins were loaded in the lanes. The 46 kDa RBP activity in VLDM increased during the first 10 minutes of light exposure and decreased thereafter for 6 hours. The results show the strong positive regulation of the 46 kDa RBP activity by light.

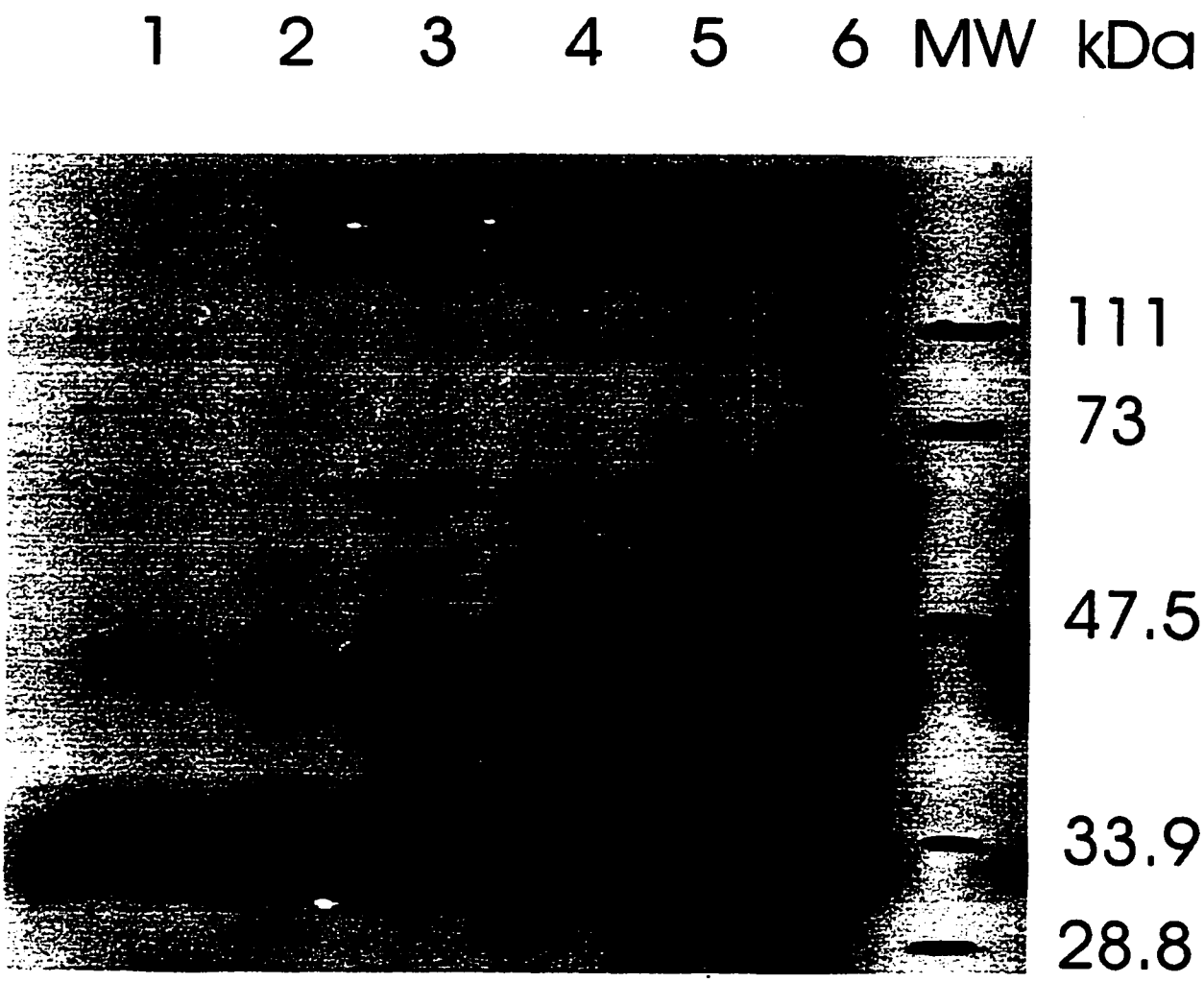

Figure 15, Light regulation of VLDM RNA-binding activity (autoradiography). LDM samples were prepared from cells grown in constant light (lane 1), constant darkness (lane 2), or constant darkness followed by illumination for various times: 6 hours (lane 3), 2 hours (lane 4), 30 min (lane 5), or 10 min (lane 6).

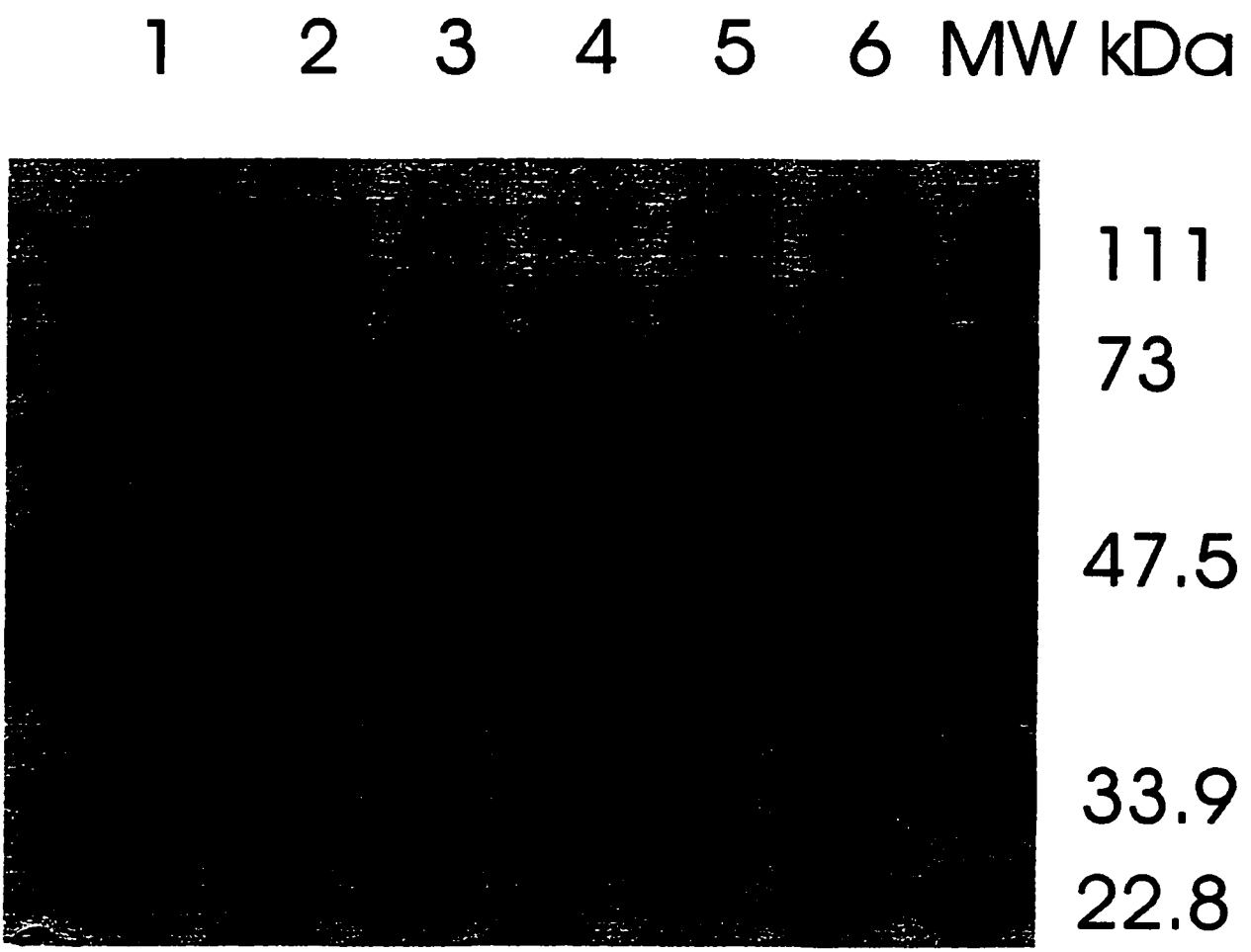

Figure 16, Coomassie blue stained gel shows equal amount of VLDM proteins loaded.

LDM samples were prepared from cells grown in constant light (lane 1), constant darkness (lane 2), or constant darkness followed by illumination for 6 hours (lane 3), 2 hours (lane 4), 30 min (lane 5), or 10 min (lane 6).

6. 2, Light regulation of LDM RNA-binding activity:

LDM RBPs were obtained and their activities were determined as described in 6.1 and in Materials and Methods. The autoradiograph in Figure 17 shows weak binding signals of 40, 46, 47, 60 kDa LDM RBPs prepared from continuous light (lane 1) or continuous dark (lane 2) cultures. Strong binding signals of 46, 47, kDa LDM RBPs were achieved and maintained at least for 6 hours in culture exposed to light. The protein gel stained with coomassie blue showed that equal amount of proteins were loaded (Figure 18). In lane 1, it seems that more protein was loaded. But this does not affect the explanation of my results because it still shows low binding signals in LDM fractions from this continuous light culture. The results show again here the activity regulation of the 46 kDa RBP by light.

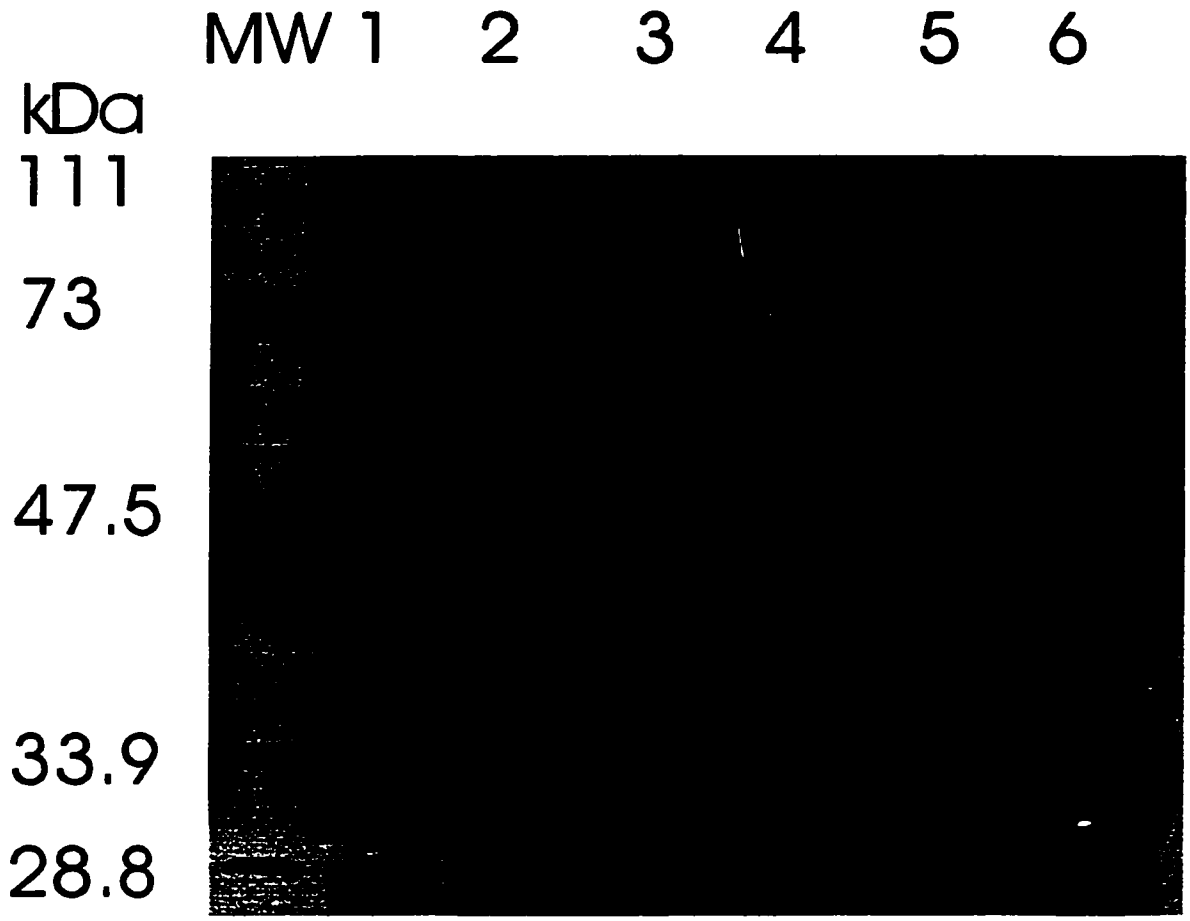

Figure 17, Light regulation of LDM 46 kDa RNA-binding activity.

LDM samples were prepared from cells grown in constant light (lane 1), constant darkness (lane 2), or constant darkness followed by illumination for 6 hours (lane 3), 2 hours (lane 4), 30 min (lane 5), or 10 min (lane 6).

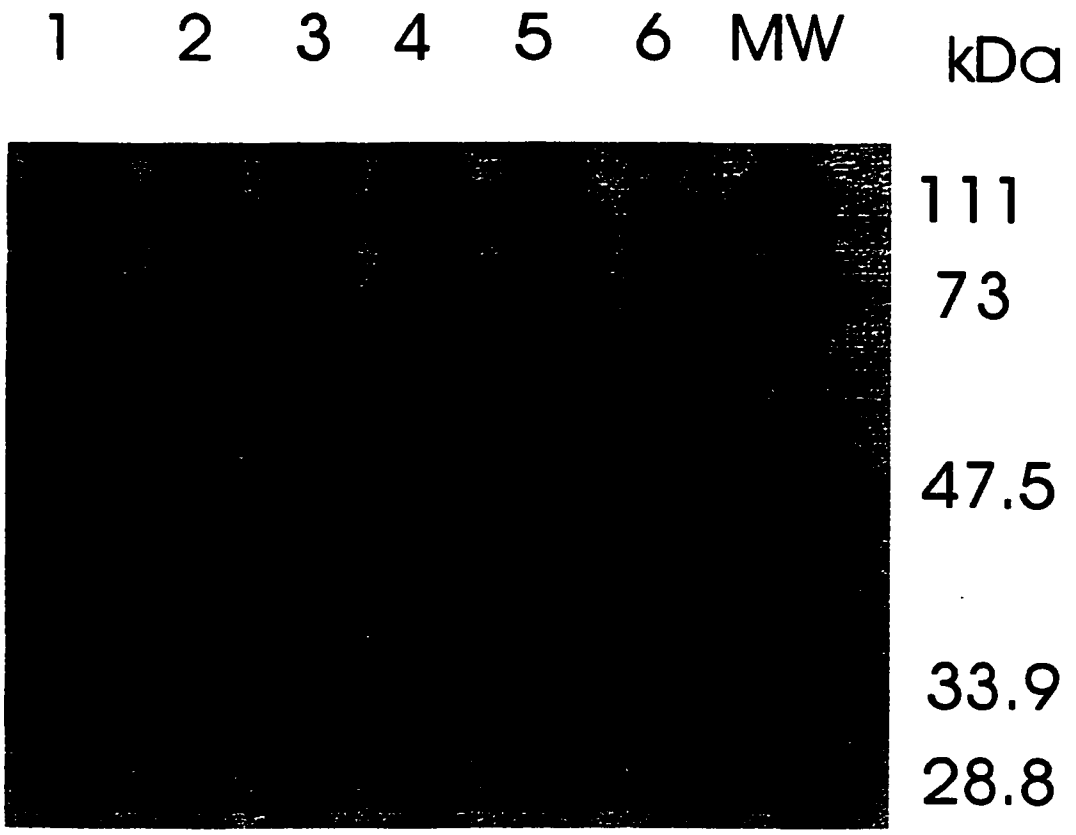

Figure 18, Equal amounts of protein were analyzed in the RNA-binding reactions shown in Figure 17. Proteins in the gel, for which the autoradiograph is shown in Figure 17, were revealed by staining with coomassie blue. LDM samples were prepared from cells grown in constant light (lane 1), constant darkness (lane 2), or constant darkness followed by illumination for 6 hours (lane 3), 2 hours (lane 4), 30 min (lane 5), or 10 min (lane 6).

7, Mechanism of the light regulation of the 46 kDa RBP activity:

7. 1, Light-induction of RNA-binding activity of the 46 kDa RBP is rapid and results from the activation of RNA-binding activities of preexisting proteins. The RBP binding activities are clearly dependent upon some effects of light (Figure 15 to 18). Next, I did an experiment to determine whether light stimulates the synthesis of the RBPs or activates existing RBPs with the assumptions that light activation of existing RBPs should be insensitive, while enhanced protein synthesis would be sensitive, to inhibitors of protein synthesis. By comparing the levels of RBP activities in membranes isolated from cells grown in the dark or shifted to light (250 $\mu Em^{-2}s^{-1}$) for 1 or 10 minutes, we concluded that the increase in the 46 kDa RBP activity occurs within 10 minutes (Figure 19, panel A). The RNA-binding activities of the 30 and 46 kDa protein in the LDM preparation from the culture that was maintained in darkness were low (Figure 19, Panel A, lane 1) and increased progressively following light exposure for 1 min (lane 2) and 10 min (lane 3). It should be noted that in most trials of the experiments shown in Figure 19 only binding of the 30 and 46 kDa proteins to the *psbC* RNA probe was detected (see Discussion). We suspect that the 30 kDa protein and the 40 kDa protein seen in other experiments are degradation products of the 47 kDa RBP because one or both of these smaller proteins were detected when the 47 kDa RBP was undetectable. We do not know what variable(s) affect the disappearance of the 47 kDa RBP binding signal, or the appearance of the smaller binding proteins. Binding of the 46 kDa RBP was detected in low-density membranes in all experiments.

To determine whether the increases in the levels of the binding activities of the 30 and 46 kDa RBPs following light exposure results from an increase in the accumulation

of these proteins (e.g., due to an increased rate of synthesis or a reduced rate of degradation) or activation of their RNA-binding activities, we determined whether inhibitors of protein synthesis in the cytosol (cycloheximide) or chloroplast (chloramphenicol) affect the induction of these activities by light. Cultures were grown in constant darkness and inhibitors were added to them 15 min prior to exposure of white light at 250 $\mu Em^{-2}s^{-1}$ for 10 min. The binding of the 30 and 46 kDa proteins detected in low-density membranes was not affected to a significant extent by pre-treatments with the inhibitors (Figure 19 B, lanes 2-4). Therefore, we conclude that light activates the RNA-binding activities of proteins that are synthesized but inactive in the dark.

7. 2, Light-induction of the 46 kDa RBP (and that of a 30 kDa protein) requires ATP synthesis and/or the trans-thylakoid membrane pH gradient. Cultures grown in the presence of an inhibitor of photosystem II (DCMU) showed partially reduced levels of the binding activities of the 46 and 47 kDa RBPs (Zerges, unpublished results). This indicates that linear electron flow and net synthesis of NADPH can only partially account for the induction of the 46 and 47 kDa RBPs, because this pathway is completely inhibited by DCMU. Six hours treatments with inhibitors of the cytochrome b_6/f complex (DBMIB) and a proton ionophore (FCCP) drastically reduced the yield of low-density membranes for unknown reasons (data not shown). To determine whether DBMIB or FCCP affect the induction of the 46 kDa protein, dark grown cultures were treated with DBMIB, FCCP, or DCMU at 10^{-5} M for 15 min prior to light exposure. Following exposure to 250 $\mu Em^{-2}s^{-1}$ white light for 10 min, LDMs were isolated and the levels of RBP activity determined with the UV cross-linking assay. As seen in lane 4 of

Figure 19A, the RNA-binding activity of the 46 kDa protein, and a 30 kDa protein, are reduced by the DCMU treatment, but a significant light stimulation is still observed. DBMIB, the inhibitor of the cytochrome b_6/f complex, completely eliminates the binding of the 30 and 46 kDa RBPs to the RNA probe (Figure 19A, lane 5). In the presence of DBMIB and light, the plastoquinol pool is reduced by electrons from photosystem II. Moreover, DBMIB abolishes both linear and cyclic electron flows, thereby eliminating all contribution of the photosynthetic electron transport chain to the trans-thylakoid membrane pH gradient. In order to determine whether the trans-thylakoid electrochemical gradient or ATP synthesis is required for light induction of the RBP activities a potent proton ionophore and uncoupler of the electrochemical proton gradient and ATP synthesis (FCCP) was added 15 min prior to exposure of a culture to light. FCCP also drastically inhibited the induction of the 30 and 46 kDa RBPs associated with the LDMs (Figure 19A, lane 6), indicating that the electrochemical trans-thylakoid membrane proton gradient or ATP synthesis is required for the light regulation.

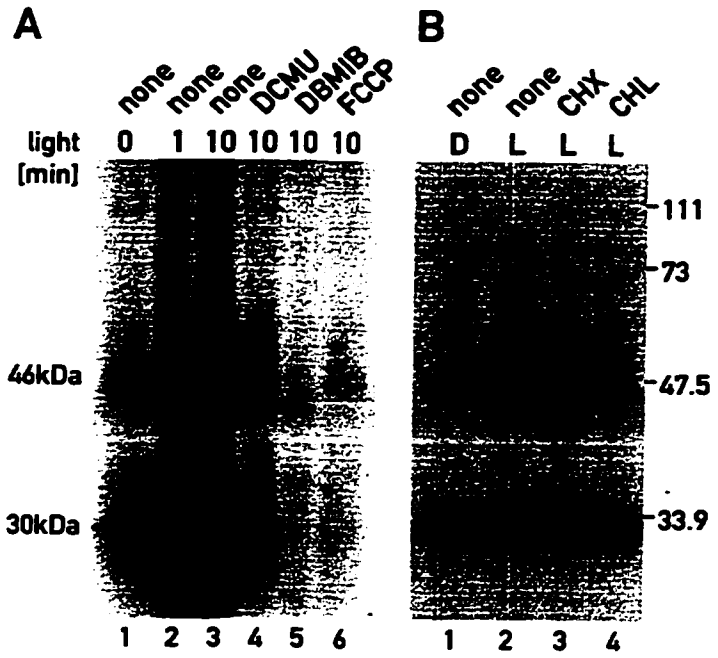

Figure 19, Mechanism of the light regulation of the 46 kDa RBP activity.

Panels A; Cells were cultured for 72 hours in darkness and then maintained in darkness (lane 1) or exposed to 250 μE m^2 sec^{-1} of white light for 1 minute (lane 2) or 10 minutes (lanes 3-6). The following inhibitors were added at 10^{-5} M final concentration 15 minutes prior to light exposure; lane 4, DCMU; lane 5, DBMIB and lane 6, FCCP.

Panel B; UV cross-linking assays were performed with LDM fraction prepared from cells grown in the dark and either maintained in darkness (lane 1) or exposed to 250 μE m^2 sec^{-1} of white light for 10 min (lanes 2-4). Cells were treated for 15 min prior to light exposure with inhibitors of protein synthesis in the cytosol (lane 3, cycloheximide) or chloroplast (lane 4, chloramphenicol).

7. 3, Effect of 10 mM DCMU, DBMIB, and FCCP on LDM RNA-binding activity of continuous light culture: In order to determine whether the redox status of the PQ pool or the proton gradient play an important role in light regulation of the 46 kDa RBP activity, DCMU, DBMIB, and FCCP were used to change the redox status of the PQ pool or to alter the proton gradient. DCMU can block the reduction of the PQ pool, therefore the PQ pool will be in oxidized state. DBMIB can block the oxidization of the PQ pool, therefore the PQ pool will be in reduced state. FCCP is a proton ionophore that can abolish the proton gradient directly. In this experiment, the strain was cultured in continuous light for three days to reach a cell density of 2×10^6/ml, and then electron transport inhibitors (DCMU, DBMIB) or FCCP were added. To ensure that the electron transport is more efficiently blocked, the cultures with these inhibitors or FCCP were kept in dark thereafter by covering the flasks with foils. In Figure 20, control experiments show no apparent difference in signal intensities of 30, 40, 46, and 60 kDa RBP bands between continuous light culture (lane 1) and continuous light culture with methanol for 6 hours in dark (lane 2). (Methanol was added to control for effects of the solvent used to solubilize the inhibitors.) While the signal intensities of the 46 and 30 kDa RBPs were not affected by the combination of dark treatment and treatment with methanol (lane 3), no 40 kDa RBP was detected in LDM fractions prepared from cells incubated in the dark and treated with DCMU (lane 4). Almost no LDM were obtained from cultures treated with DBMIB (lane 5) or FCCP (lane 6) in dark for six hours, and therefore no RBP binding signals were observed.

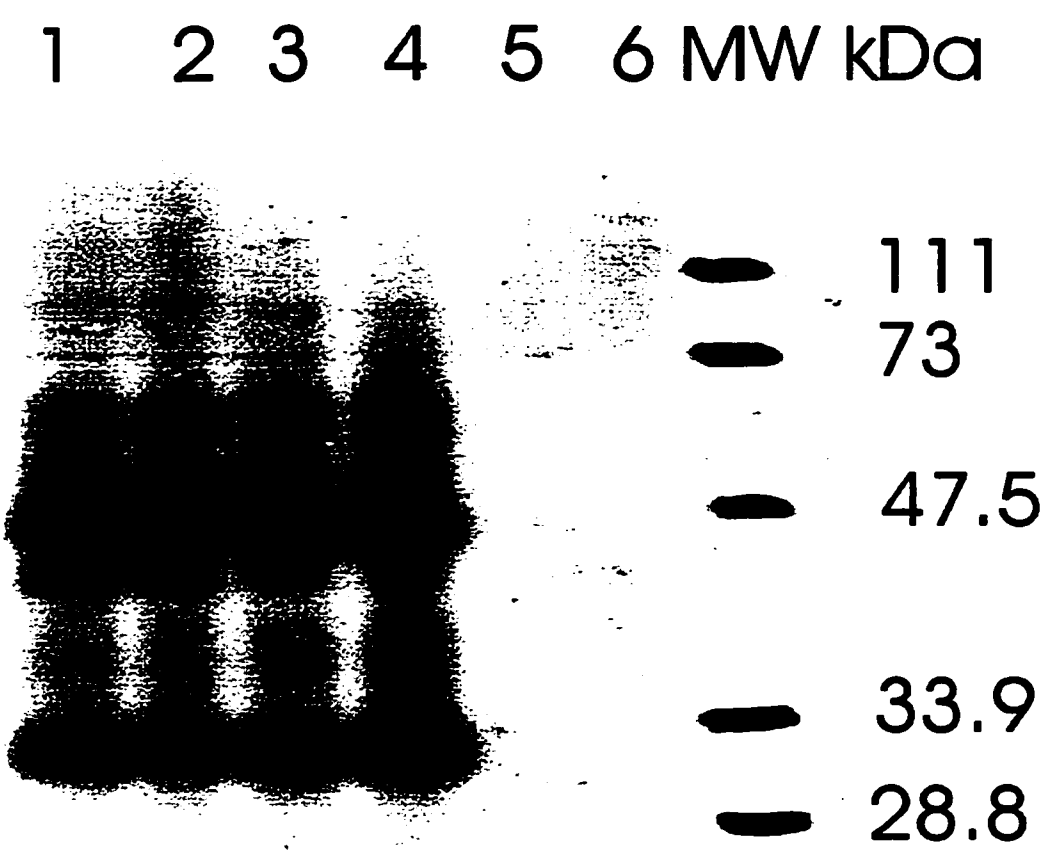

Figure 20, Effect of 10 mM DCMU, DBMIB, and FCCP on LDM RNA-binding activity of continuous light culture.

UV cross-linking assays revealed RBP activities in LDM fractions obtained from cells cultured in the light and maintained in light (lane 1), maintained in light and with methanol for 6 hours (lane 2), shifted to darkness and with methanol for 6 hours (lane 3), or shifted to darkness for 6 hours in the presence of DCMU (lane 4), DBMIB (lane 5), or FCCP (lane 6) which are dissolved in methanol first and then added to the cultures. 20 μg protein was used for each RNA binding reaction.

7. 4, Effect of 10 mM DBMIB and FCCP on light induction of VLDM 46 kDa RNA-binding activity: For light induction experiments of the 46 kDa RBP activity, cells were cultured in darkness for at least 7 days. In Figure 21, compared with binding signal in from continuous dark culture (lane 1), a much stronger 46 kDa binding signal observed in lane 2 in which RBP was extracted from a culture exposed to light for 10 minutes. Induction of the 46 kDa RNA-binding signal during the same period of 10 minutes was drastically diminished in LDM preparations from cells treated with DBMIB or FCCP (lanes 3 and 4, respectively). For unknown reasons, extremely low yields of VLDM and LDM were obtained from cells cultured continuously in the presence of DBMIB and FCCP. DCMU did not affect these yields under similar growth conditions.

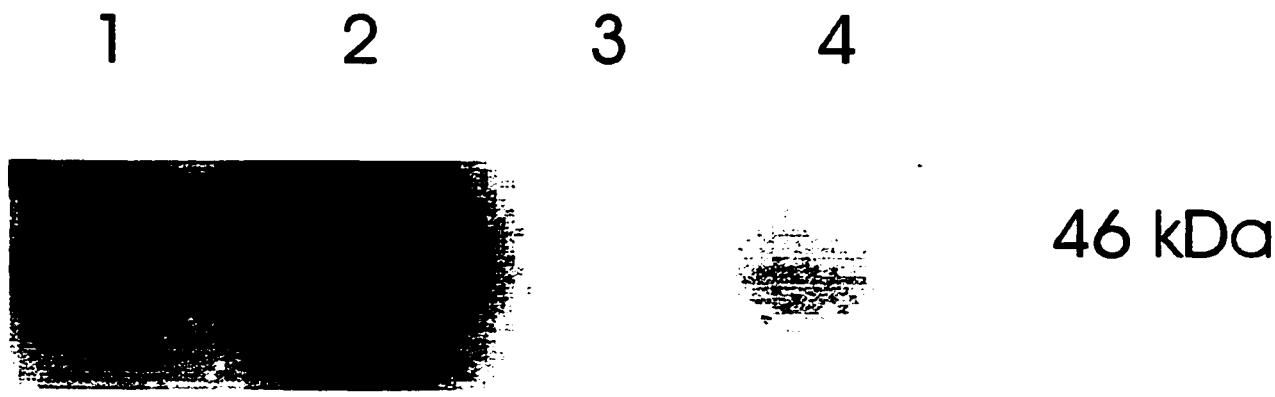

Figure 21, Effect of 10 mM DBMIB and FCCP on the 46 kDa VLDM RNA-binding activity (continuous dark culture).

UV cross-linking assays revealed RBP activities in VLDM fractions obtained from cells cultured in the dark and maintained in darkness (lane 1), shifted to light for 10 min (lane 2), or shifted to light for 10 min in the presence of DBMIB (lane 3), or FCCP (lane 4).

7. 5,The RPBs are associated with membranes which rise during sucrose gradient centrifugation: To ensure that the 46 kDa RBP in VLDM and LDM fractions is associated with membranes, the VLDM and LDM were prepared using the bouyant density centrifugation (Figure 22). In these experiments, broken chloroplasts were mixed with sucrose to a final concentration ofl M. This suspension was placed at the bottom of an ultra-centrifuge tube, and then 0.3 M sucrose layered gently on top. On top of that layer, a third phase of buffer without sucrose was layered. During a 16 hours centrifugation at 100,000 X g, membranes float and soluble protein complexes pellet. If the RBP activities float with the membranes, this result would indicate that these proteins are not present in complexes that co-sediment with, but are not associated with, membranes.

As seen in Figure 22, lane 1, the 46 kDa RBP activity was detected in membranes that were floated during this centrifugation. Therefore, this supports that this RBP is indeed associated with membranes and that its behavior in sucrose gradient sedimentation used in the other experiments is not due to cofractionation of a large protein complex with membranes.

7. 6 Effect of O_2, N_2, and 2 μM FCCP on the activity of 46 kDa RBP of VLDM prepared from continuous dark culture. In this set of experiments, I addressed whether the redox status of the PQ pool regulates the 46 kDa RBP activity. O_2, N_2, and 2 μM FCCP treatments have been used. Flushing nitrogen into the culture will deplete its supply of oxygen, which will block the oxidization of the PQ pool and lead to the accumulation of reduced PQ. In contrast, flushing air with oxygen will lead to the

oxidization of the PQ pool, and oxidized PQ will accumulate. Treatment with FCCP in the dark for more than 21 minutes is another way to obtain a fully reduced PQ pool. As no ATP is synthesized under these conditions, the glycolytic pathway is accelerated (the Pasteur effect) and this leads to the reduction of the PQ pool by the chlororespiratory chain. As seen in Figure 22, reduction of the PQ pool by flushing nitrogen (lane 3) or adding FCCP in the dark (lane 4) does not induce 46 kDa RBP binding activity. Similar results were obtained when the plastoquinol pool was oxidized by flushing the culture with air. Therefore, it would appear that the redox potential of PQ pool can not account for the light activation of the 46 kDa RBP

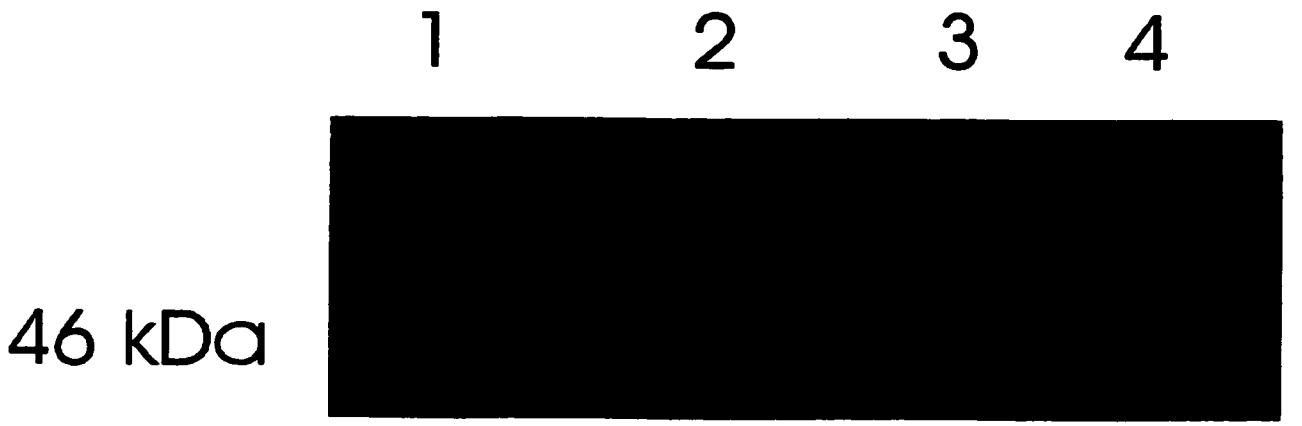

Figure 22, Effect of O_2, N_2, and 2 μM FCCP on the activity of the 46 kDa RBP of VLDM prepared from continuous dark culture by floating method.

UV cross-linking assays measured binding activity of the 46 kDa RBP in VLDM extracts prepared from cells cultured in darkness and then exposed to light for 10 min (lane 1), or maintained in darkness in the presence of air (lane 2) or nitrogen (lane 3) for 1 hour, or 2 μM FCCP for 10 min (lane 4).

IV. Discussion

1, LDM appears to be similar to thylakoid membrane in chemical and electrical properties: Aqueous polymer two-phase partitioning has been used successfully to separate plasma membranes from thylakoid membranes in cyanobacteria and plants (Norling, 1994, Rochester, 1987). A substance partitions between the two liquid phases according to its surface properties, such as electrical charge and hydrophilic-hydrophobic character.

I have used a PEG/Dextran two-phase system in an attempt to separate VLDM and LDM from thylakoid membrane. However, under conditions that efficiently separate plasma membrane from thylakoids of cyanobacteria (Norling, 1994), I was unable to separate LDM or envelope membranes from thylakoids of *Chlamydomonas*. In attempts to optimize the fractionation, I varied the concentrations of the two polymers which contain either KOAc or NaCl. The inability of two-phase partitioning to separate LDM from thylakoid membrane suggests that these membranes have similar surface properties, further supporting the hypothesis that these membranes are related by a developmental pathway (Zerges, 2000).

2, The 46 kDa RBP is a rare protein in cells and even in LDM fractions: Although we observed an approximately a 50-fold enrichment in binding activity of the 46 kDa RBP in fractions eluted in peak 4 during the heparin-agarose chromatography (Figures 12-14), with silver staining we were unable to detect any protein which comigrated with the 46 kDa RBP detected by autoradiography (Figure 13 and 14). Therefore, this 46 kDa

protein accounts for a very small percentage of total proteins present in LDM preparations. This protein must be of low abundance in chloroplasts.

It should be noted that one usually determines the degree to which the desired protein and its activity are enriched with each step. These data are tabulated to show the efficacy of each step and the overall degree of purification relative to the starting-material. However, with the UV cross-linking assays used in this study, only relative amounts of the RBP activity in samples analyzed in a single experiment can be determined and no activity unit has been defined for RNA binding activity. First, specific activity of the probe varied due to varying age of it or the [^{32}P]UTP used in its preparation. Second, I used autoradiography to measure the signals. These signals do not have an absolute value and their intensity varies with exposure time and the quality of development reagents. Third, as two days is required to reveal the RNA binding activity for each purification step, the RNA binding proteins usually gradually loss their activity in storage, especially by freezing and thawing. Fourth, as I was unable to detect the 46 kDa protein with staining methods, only the activity could be revealed. This activity appeared to be sensitive to the protein concentration of the sample, and other factors which are variable in the eluted fractions. Therefore, activity may not be an accurate measure of the absolute degree of enrichment. All of variance in above multiple processes makes the comparing of two test of RNA binding activity not practical. In addition, I was unable to detect the RBP activities in extracts of whole cells, or even isolated chloroplasts. For these reasons, I was only able to determine the degree of enrichment of the activity of the 46 kDa RNA-binding protein relative to the total amount

of protein used as starting-material. These values are expressed in the relevant figure legends and the text.

3, Binding properties of the 46 kDa RBP: Mg^{2+} ions are often required in interaction between proteins and RNA. For example, ribosome binding to tRNAs requires 10 mM $MgCl_2$ (Rheinberger, 1987). However, my observation that the presence of EDTA or $MgCl_2$ had no effect on the 46 and 47 kDa protein RNA binding activities excludes the possibility of requirement of Mg^{2+} in RNA binding by this protein (Figure 2). Maximal binding activity was achieved at pH 8.5 and lower pH (< 6.0) inhibits binding (Figure 2).

4, Purification of the 46 kDa RBP from LDM: Besides the 46 kDa RBP, some other proteins also bind to the probe from *psbC* mRNA 5'-UTR. This makes it impossible for us to use crude protein extract to study the role of this 46 kDa protein in chloroplast mRNA translation. Separation of this 46 kDa protein from other RNA binding protein will enable us to do *in vitro* translation inhibition experiments to determine whether this protein plays a positive or a negative role in chloroplast mRNA translation. The first *in vitro* translation system for chloroplasts was developed recently (Hirose, 1996). The molecular weight of the 46 kDa protein was determined by SDS-PAGE, in which all the peptides are separated and we do not know if this protein is associated with other proteins or not. The separation will also enable us to determine the molecular weight of this protein in non-denaturing conditions by native PAGE. If this protein is associated with other proteins, we would observe the RNA binding signal from much higher molecular weight than 46 kDa in native PAGE.

4. 1, Reasons that could cause the change of pattern of RNA binding signal: The changes of RNA binding signals were observed after different chromatography including DEAE-sephadex, Q-sepharose, Poly-A and heparin-agarose chromatography. On the batch test using heparin-agarose gel (Figure 10), we can see that two new bands lower than 46 kDa appeared. I obtained the similar pattern in the first 100 μl washing solution. The change of the pattern of RNA binding signals may result from several possible factors. First, a specific protease and its inhibitor could exist in the LDM protein extract. Separation of this protease and its inhibitor in different chromatography leaded to the hydrolysis of the 46 and 47 kDa RNA binding protein and produced lower bands. Second, some RNA binding proteins and their binding inhibitors could exist in LDM protein extract. Separation of these RNA binding proteins and their inhibitors leaded to the release of this inhibition and we saw some new binding signals. Third, the 46 kDa RBP could have been separated from another factor(s) which is required for its RNA-binding activity.

4. 2, Other methods could be used to purify the 46 kDa RBP: The 46 kDa RNA binding protein has been successfully separated from the majority of proteins and other RNA binding proteins by step elution with 0.6 M KOAc from heparin-agarose gel (Figure 10). However, I was unable to separate this protein from some non-RNA binding protein only by heparin-agarose affinity chromatography. Separation of 46 kDa RNA binding protein from non-RNA binding proteins could be achieved by specific affinity chromatography- immobilization of psbC mRNA 5'-UTR to some solid gel beads.

With these RNA transcripts immobilized columns, one could load the partially purified 46 kDa RBP fraction onto column, wash out non-RNA binding proteins, and then elute the 46 kDa RBP with an KOAc gradient buffer. However, even if such techniques are successful, a very large amount of starting material would be required because of the rareness of the 46 kDa RBP.

5, Light regulation of the activity of the 46 kDa RNA binding protein.

5. 1, The activity and not the synthesis of 46 kDa RBP from LDM and VLDM induced by light: Following growth in the dark, the level of the 46 kDa RBP activity detected in VLDM fractions increased within 10 minutes of light exposure and decreased thereafter at least until 6 hours light exposure (Figures 15, 16). The 46 kDa RBP activity from LDM increased in one minute and ten minutes of light exposure and maintain the high relative level of activity throughout 6 hours (Figure 17,18). This study focuses on the RNA-binding activity of the 46 kDa RBP because its activity was reproducibly detected in all trials of the experiments presented here. Nevertheless, some conclusions could also be reached regarding the light regulation and binding specificity of the other RBPs.

Previous studies have shown that 100μg/ml chloramphenical completely blocks protein synthesis in the chloroplast and 8μg/ml cycloheximide completely blocks protein synthesis in the cytoplasm (Bulte and Wollman, 1992). I have provided strong evidence that light activates these RBPs, rather than induces their synthesis, because these inhibitors of protein synthesis did not affect induction of the RBP activities (Figure 19).

Previous studies showed that LDM fractions prepared from gametes have very low levels of the 46 and 47 kDa RBP activities (Zerges, unpublished). The correspondence of these RBP activities and requirements for photosynthesis (i.e., in vegetative cells and with light) suggests that these proteins function in the metabolism or translation of mRNAs encoding polypeptides of the photosynthetic apparatus.

The light intensities used in this study (150-250 $\mu E\ m^2\ sec^{-1}$) are sufficient to drive photosynthesis, without causing damage to the photosynthetic apparatus associated with photoinhibition. Moreover, the strains used in this study synthesize chlorophyll in the dark and, consequently, do not undergo the drastic changes in gene expression and membrane organization that occur when etioplasts differentiate to chloroplasts in the photosynthetic tissues of angiosperms or *C. reinhardtii* yellow-in-the-dark mutants following a shift from dark to light. Therefore, the light regulation of these RBP activities probably.

5. 2, Light-induced activation of the 46 kDa RBP could be caused by reduced concentration of ADP. There are different reports about how the activity of the RBPs are regulated (Michaels and Herrin, 1990, Muhlbauer and Eichacker, 1998, Danon and Mayfield,1991. Danon and Mayfield, 1994). ADP, phosphorylation and dephorsphorylation of the RB60 protein in the *psbA* UTR binding complex, redox state of the plastoquinol pool, NADPH, thioredoxin, the trans-thylakoid membrane proton gradient, and intermediates in the chlorophyll biosynthetic pathway all have the potential to regulate the RBP activity.

We have used inhibitors of photosystem II (DCMU) and the cytochrome b_6/f complex (DBMIB) and a proton ionophore (FCCP) to identify mediators of the light regulation of the RBP activities. The photosynthetic electron transport chain converts light energy into reducing power (i.e., reduced ferredoxin, thioredoxin, NADPH and glutathione) and an electrochemical proton gradient across thylakoid membranes. The ATP synthase complex uses this electrochemical proton gradient to synthesize ATP from ADP and inorganic phosphate. In the presence of DCMU, cells lack photosystem II activity and light energy can only be used to cycle electrons through photosystem I and the cytochrome b_6/f complex, thereby generating an electrochemical proton gradient across the thylakoid membrane. Cyclic electron transport does not contribute to net reduction of electron carriers of the chloroplast stroma because the electrons from photosystem I are directed back into the transport chain.

My data suggest that activation by light of the 46 and 47 kDa RBP results primarily from the lowered stromal ADP concentration caused by ATP synthesis. First, their activation is prevented by inhibitors that eliminate the conversion of ADP and inorganic phosphate to ATP, either by preventing all light-driven electron flow through the photosynthetic electron transport chain (DBMIB, Figure 19A, lane 5) or by dissipation of the electrochemical proton gradient across thylakoid membranes (FCCP, Figure 19A, lane 6). In these experiments the exposure of the cells to the inhibitors was limited to 25 min to minimize secondary effects. Second, ADP inhibits the RNA-binding activity of the 46 kDa protein *in vitro*, while other nucleotide tri- or di-phosphates have little or no effect (Zerges, unpublished data). In addition, full activation of the 46 kDa RBP appears to require linear electron flow through the photosynthetic electron transport chain because

DCMU partially inhibits its light activation. As linear electron flow generates both reduced products and an electrochemical proton gradient, it is difficult to distinguish between effects of DCMU on the redox states of substrates or products of linear electron flow, the electrochemical proton gradient, or ATP synthesis. However, treatment of the cells with DBMIB, an inhibitor of the cytochrome b6/f complex, which leads to the reduction of the PQ pool, inhibited the RNA binding activity of the 30 and 46 kDa RBPs (Figure 19, Panel A). Thus these results suggest that the reduction of the plastoquinone pool cannot account for the light activation of the 46 RBP.

In the dark, both reduction of the PQ pool by flushing nitrogen or adding FCCP to the culture, and oxidization of PQ pool by flushing oxygen to the culture failed to induce the 46 kDa RBP activity. It further confirmed the above deduction. Treatment of algae with FCCP in the dark more than 30 minutes is another way to obtain a fully reduced PQ pool. As no ATP is synthesized under these conditions, the glycolytic pathway is accelerated (Pasteur effect) and this leads to reduction of the PQ pool by the chlororespiratory chain. It is likely that this activation depends on a complex interplay between light and the redox state of several components of the photosynthetic electron transfer chain (Zhang L. et al., 2000, Trebitsh, T. et al. 2000). My results are consistent with the a model for the activation of translation of the chloroplast *psbA* mRNA (encoding the D1 protein of the photosystem II reaction center core) by light through two distinct mechanisms in chloroplasts of *C. reinhardtii* (Trebitsh, 2001) and spinach (Muhlbauer, 1998, Zhang, 2000). First, stimulation of *psbA* translation requires the electrochemical proton gradient across thylakoid membranes or low ADP levels resulting from ATP synthesis using this gradient. A secondary level of redox control modulates

translation rate in response to reducing potential generated by the photosynthetic electron transport chain. This secondary redox regulation is consistent with the effect of DCMU upon the level of the 46 kDa RBP described here.

5. 3, Transient cascade and programming inhibition: Rapid induction of the 46 kDa RBP activity and slow decrease of the activity after 10 minutes of light exposing (Figure.15) cannot be explained only by ADP level. A transient change caused signal amplification may be involved to explain the continuous decrease of RBP activity after 10 minutes of light induction. The transient cascade probably is through phosphorylation and dephosphorylation (Danon and Mayfield, 1994), and I call this the programming inhibition of the RBP, which can explain why after 10 minutes of light exposing, the 46 kDa RBP activity decrease continuously when ADP level is still low. An alternative explanation is that the change of VLDM to LDM by incorporation of more proteins made with time reduce the amount of the 46 kDa RBP available for new vesicle formation from chloroplast inner membrane (see also discussion in sections 6, and 7).

5. 4, Function of the 46 kDa RBP. The correspondence of the 46 kDa RBP activity and light exposure, which leads to photosynthesis, suggests that the 46 kDa RBP plays some role(s) in the stability or translation of mRNAs encoding polypeptides of the photosynthetic apparatus (PSI, PSII, b6/f complex, or ATP synthesis complex). Further experiments are required to test this hypothesis.

Together with the phenomenon of inhibition of ADP on the RNA binding, I think that this 46 kDa RBP probably plays a negative role in chloroplast mRNA translation by

binding and anchoring chloroplast mRNA in membrane when enough CP43 or PS II and more ATP (less ADP) are available. Previous observations that the level of the *psbC* mRNA in *C. reinhardtii* is not altered by the presence or absence of light during growth support roles of these proteins at steps following transcription and mRNA stability (Kirk, 1985, Klein, 1988, Malnoe, 1988). In the dark, the mRNA could be released due to the inhibition of the 46kDa RBP by high level of ADP, but lack of ATP make the protein synthesis in a very low level. This is consistent with the a previous result showing that the level of ADP or ATP affects translation rate in the chloroplast of *C. reinhardtii.* (Michaels and Herrin, 1990). In contrast, most ADP is converted to ATP by photosynthesis in light (Michaels and Herrin, 1990), the inhibition of RBP binding activity will be released, and more and more chloroplast mRNA will be restricted in low density membrane to prevent them from translation. It is worth to mention that protein synthesis may occur in association the membranes of the envelope and thylakoids (Zerges, 1998, Zerges, 2000, Sato, 1999).

6, Additional evidences that the 46 kDa RBP is associated with chloroplast membranes: Continuous and step sucrose gradients were used to fractionate the chloroplast stroma, and various membranes. Similarity between LDM and inner membrane in phospholipid and pigment composition has been shown by Dr. Zerges (Zerges and. Rochaix., 1998). To further support the membrane association of the 46 kDa RBP, I also did floating experiments in which membranes are loaded at the bottom of the sucrose gradient and float to their position instead of sedimenting to their position during centrifugation. Large protein complexes would not float under these conditions, but would remain at the bottom of the tube. That I obtained VLDM and LDM with

associaed RBP activities from floating experiments further confirmed the membrane association of the 46 kDa RBP.

7, Where do the LDM and VLDM come from? In the chloroplast, there are several kinds of membranes, the outer and inner membranes of the envelope and thylakoid membranes. Other uncharacterized compartments may exist. The following evidences suggest that the VLDM and LDM are derived from the chloroplast inner membrane. First, the VLDM and LDM are different from thylakoid membrane in chlorophyll content. VLDM and LDM are yellow and the thylakoid membranes are green. Second, phospholipid analysis showed similarity of the LDM with chloroplast inner membrane and thylakoids (Zerges and Rochaix 1998). Third, for most organelles, the inner envelope membrane usually is rich in proteins and outer membrane is not, such as mitochondrion (Lodish, Harvey et. al., 1995b). Fourth, the optimum pH for the RNA binding of the 46 kDa RBP is above 7.5 in vitro, while the pH of the thylakoid lumen is 5. Therefore, this protein is unlikely to function in the thylakoid lumen. Fifth, according to the hypothesis proposed by Kenith Hoober (Hoober, 1991, Hoober, 1998), thylakoid membranes arise from vesicles originating from the inner envelope membrane. Thylakoid may be formed from vesicles derived from inner membrane. Thylakoid proteins may be inserted into these vesicles during their synthesis and while the vesicles are migrating to and fusing with thylakoid membrane (Hoober et. al 1991, Hoober et. al 1998, Zerges, 2000). I speculate that light stimulates the formation of new vesicles, which incorporate newly synthesized protein and become heavier and heavier. This could be how the VLDM and LDM are formed. The 46 kDa RBP specific activity

decreases and density increases in the order of VLDM, LDM, and thylakoid membrane. These observations agree with the Hoober's hypothesis because while thylakoid proteins are inserted into the membrane, the RBP specific activity decreases in there. Lastly, that two aqueous phase partitioning (PEG/Dextran) failed to separate VLDM and LDM from thylakoid membrane shows that VLDM, LDM, and the thylakoid membrane have similar chemical and electrical properties, which further suggests that they are related, either by colocalization or that LDMs correspond to a thylakoid biogenesis pathway.

In addition, vesicle formation, protein synthesis, and their incorporation into the vesicles need energy from ATP. Completely blocking ATP synthesis with DBMIB and FCCP should block the vesicle formation, protein synthesis and protein insertion into membranes. This may explain my observation that less or no VLDM and LDM were obtained from cultures treated with 10 mM DBMIB and FCCP for several hours. Once DBMIB and FCCP are added to the culture, the energy supply will be almost completely blocked.

V. References

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