day of injection. The vehicle was a bicarbonate-buffered physiological saline of 122 mM NaCl, 3.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 1.2 mM $CaCl_2$. After data collection, we verified the location of the injection site histologically by examining 75-μm-thick vibratome sections cut through the injection site.
23. S. Shibata, A. Watanabe, T. Hamada, M. Ono, S. Watanabe, *Am. J. Physiol.* **267**, R360 (1994); A. Watanabe, T. Hamada, S. Shibata, S. Watanabe, *Brain Res.* **646**, 161 (1994).
24. S. Amir, *Brain Res.* **586**, 336 (1992).
25. D. Chen and M. U. Gillette, unpublished observations.
26. R. A. Prosser, A. J. McArthur, M. U. Gillette, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6912 (1989).
27. D. S. Bredt *et al.*, *Neuron* **7**, 615 (1991); S. R. Vincent and H. Kimura, *Neuroscience* **46**, 755 (1992).
28. P. L. Huang, T. M. Dawson, D. S. Bredt, S. J. Snyder, M. C. Fishman, *Cell* **75**, 1273 (1993).
29. B. Wendland *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 2151 (1994); T. J. O'Dell *et al.*, *Science* **265**, 542 (1994).
30. E. Southam, R. Morris, J. Garthwaite, *Neurosci. Lett.* **137**, 241 (1992); Y. Kamisaki *et al.*, *Brain Res.* **644**, 128 (1994).
31. H.-C. Pape and R. Mager, *Neuron* **9**, 441 (1992); A. Gelperin, *Nature* **369**, 61 (1994).
32. S. Shibata, A. Tsuneyoshi, T. Hamada, K. Tominaga, S. Watanabe, *Brain Res.* **597**, 257 (1992); T. Hamada, S. Shibata, A. Tsuneyoshi, K. Rominaga, S. Watanabe, *Am. J. Physiol.* **265**, R1199 (1994).
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Light-Regulated Translation of Chloroplast Messenger RNAs Through Redox Potential

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Translation of key proteins in the chloroplast is regulated by light. Genetic and biochemical studies in the unicellular alga *Chlamydomonas reinhardtii* suggest that light may regulate translation by modulating the binding of activator proteins to the 5′ untranslated region of chloroplast messenger RNAs. In vitro binding of the activator proteins to *psbA* messenger RNA and in vivo translation of *psbA* messenger RNA is regulated by the redox state of these proteins, suggesting that the light stimulus is transduced by the photosynthesis-generated redox potential.

Synthesis of specific proteins in the chloroplast is enhanced about 50- to 100-fold after illumination of plant and algae cells (*1–4*). This marked increase in translation occurs in the absence of a corresponding change in the amount of the mRNA (*1, 3, 5, 6*), indicating that translation of chloroplast mRNAs is light-regulated. Translation of these chloroplast mRNAs requires nuclear-encoded translational activators that interact with the 5′ untranslated region (5′-UTR) of the mRNAs (*7–10*). We have identified mRNA-binding proteins that bind to a stem-loop RNA structure, which contains the ribosome-binding site, in the 5′-UTR of *psbA* mRNA (*11, 12*) (encoding the D1 protein of photosystem II). The amount of *psbA* mRNA–protein complex (RNP) correlates with the amount of translation of *psbA* mRNA in light- and dark-grown *Chlamydomonas reinhardtii* cells, suggesting that modulation of RNP complex formation may regulate the translation of this mRNA (*11*). We therefore set out to identify the mechanism by which the binding of these proteins to the mRNA—and consequently the translation of the mRNA—is regulated in response to light. Adenosine diphosphate (ADP)–dependent phosphorylation inactivates *psbA* mRNA binding after transfer of cells into the dark (*13*). Here, we show that binding of the protein complex to *psbA* mRNA and translation of *psbA* mRNA are modulated by redox potential, perhaps through ferredoxin and thioredoxin. Utilizing the reducing power generated by photosynthesis to regulate translation provides a dynamic and direct link between the amount of light absorbed by photosynthesis and the synthesis of the photosystem II (PS II) reaction center proteins.

Light may affect chloroplast metabolism by its effect on a photoreceptor, such as phytochrome, which in turn affects nuclear gene transcription (*14*). Light also generates the products of photosynthesis, such as redox potential, adenosine triphosphate, or carbohydrates, which can subsequently affect activity of chloroplast enzymes (*15*). For example, chloroplast enzymes can be activated by the ferredoxin-thioredoxin system (*16*). Reducing power, generated by the light-reactions of photosynthesis, is used to reduce thioredoxin, which in turn activates enzymes of the reductive pentose phosphate cycle to regulate carbon assimilation.

To investigate whether redox potential affects the assembly of the *psbA* mRNA–binding protein complex, we subjected purified *psbA* RNP to either reducing or oxidizing reagents and analyzed binding activity by T1-ribonuclease (RNase) gel mobility–shift assay (T1-GMS) (*11*). Incubation of the protein complex with the reductants β-mercaptoethanol (β-Me) or dithiothreitol (DTT), prior to T1-GMS, did not alter the RNA-binding capacity of the RNP (Fig. 1, lanes 2 to 4). However, addition of the oxidant dithionitrobenzoic acid (DTNB) (*17*) completely abolished the RNA-binding capacity of the protein complex (Fig. 1, lane 5). This inhibition of RNP formation persisted after removal of the oxidant by gel permeation chromatography (*18*). To assess whether inactivation of RNA binding by DTNB could be reversed by reduction of the protein complex, we incubated the DTNB-oxidized proteins with either β-Me or DTT and subjected the proteins to T1-GMS. Addition of up to 50 mM β-Me did not restore the RNA-binding activity (Fig. 1, lanes 6 and 7), in-

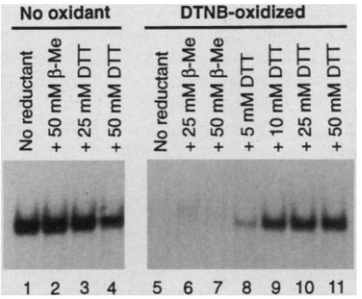

Fig. 1. Redox control of *psbA* mRNA binding in vitro. Autoradiograph of gel mobility–shift assay, displaying the labeled 5′-UTR of *psbA* mRNA complexed with *psbA* mRNA–affinity chromatography–purified proteins (*psbA*-RACPs) (*11*). RNA-binding assays contained *psbA*-RACPs that were treated for 10 min with the reductants β-mercaptoethanol (β-Me, lane 2) or dithiothreitol (DTT, lanes 3 and 4) at concentrations indicated above each lane, or the oxidant dithionitrobenzoic acid (DTNB, lane 5) before the addition of labeled RNA. To examine whether the RNA-binding capacity of the oxidized *psbA*-RACPs could be restored by reduction, we treated DTNB-oxidized *psbA*-RACPs (lane 5) with either β-Me (lanes 6 to 7) or DTT (lanes 8 to11) for 10 min before incubation with labeled RNA and compared RNA binding of these proteins to that of untreated proteins (lane 1). Proteins were then incubated with 0.25 U of the RNase inhibitor Inhibit-Ace (5 Prime→3 Prime) in the presence of 3 mM $MgCl_2$ for 15 min before the addition of 0.065 pmol of in vitro–synthesized ^{32}P-labeled 5′-UTR of *psbA* mRNA and 20 μg of wheat-germ tRNA. The binding reactions were then digested with 0.25 U of RNase T1 and fractionated with a nondenaturing polyacrylamide gel electrophoresis. The presence of bound RNA-protein complex was detected by autoradiography. Only the portions of the gel containing the RNA-protein complexes are shown.

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dicating that the *psbA* mRNA–binding activity is not responsive to monothiol reductants. However, the inclusion of 5, 10, 25, or 50 mM DTT reactivated the RNP formation (Fig. 1, lanes 8 to 11), demonstrating that the *psbA* mRNA–binding activity is sensitive to redox state and suggesting that a redox-responsive regulatory site might be present within the protein complex. We suggest, therefore, that reduction of the regulatory site activates the RNA-binding activity and that oxidation inhibits binding to RNA. In vertebrate systems, binding of a translational repressor protein (IRE-BP) to the cytoplasmic ferritin mRNA is responsive to redox potential and can be modulated by reduction with either β-Me or DTT (*19*). Unlike the IRE-BP, activation of binding of *psbA* RNP is sensitive to reduction by DTT but not by β-Me, indicating that in contrast to the IRE-BP, reactivation of binding of *psbA* RNP requires vicinal dithiol groups. This requirement for vicinal dithiol groups is reminiscent of the light-activation of chloroplast stromal enzymes by thioredoxin (*15*).

To assess the potential role of thioredoxin in the redox-sensitive regulation of *psbA* mRNA binding, we analyzed the effect of inclusion of thioredoxin in the in vitro binding assay (Fig. 2A). Incubation of the DTNB-oxidized proteins (Fig. 2A, lane 2) with thioredoxin reduced by DTT (Fig. 2A, lane 6) restored RNA-binding activity more effectively than did DTT alone (Fig. 2A, lane 3). Oxidized thioredoxin (Fig. 2A, lane 5), or thioredoxin reduced by β-Me (Fig. 2A, lane 7), did not reactivate RNA binding. These results suggest that thioredoxin may be the in vivo factor that reduces a regulatory disulfide bond in the *psbA* mRNA–binding complex, resulting in reactivation of binding to the mRNA.

To determine whether reactivation of RNA binding resulted from a switch from a disulfide bond to dithiol groups, we treated the RNA-binding complex with *N*-ethylmaleimide (NEM), a nonreversible modifier of thiols (Fig. 2B, lane 3). As expected, inactivation by NEM could not be reversed by either β-Me or DTT (Fig. 2B, lanes 4 and 5). Because NEM does not modify disulfide bonds, and oxidation by DTNB converts only vicinal dithiol groups to disulfides, DTNB oxidation followed by NEM treatment should protect vicinal (regulatory) dithiols from nonreversible modification by NEM. As expected, the RNA-binding protein complex that was first oxidized by DTNB and then treated with NEM was reactivated by reduction with DTT but not with β-Me (Fig. 2B, lanes 6 and 7), verifying the formation of disulfide bonds during oxidative inactivation of RNA binding. In addition, the restoration of RNA binding by coincubation with DTT and thioredoxin was more efficient than reactivation with DTT alone (Fig. 2B, lane 8), again demonstrating the enhancement with thioredoxin in the reduction of the regulatory disulfides.

To assay the in vivo effect of chloroplast redox potential as a modulator of *psbA* mRNA binding and translation, we analyzed the synthesis of the D1 protein in a strain of *C. reinhardtii* (*cc703*), containing a mutation in the *psaB* gene that results in the absence of the photosystem I (PS I) reaction center (*20*). PS I is the primary reducer of ferredoxin and hence of thioredoxin. If reduced thioredoxin is required for the activation of *psbA* mRNA binding and translation in vivo, then the *cc703* mutant should have less than normal RNP-complexed *psbA* and a decreased amount of translation of the *psbA* mRNA. Proteins were isolated from both *cc703* and wild-type (wt) cells grown in the light and assayed for binding to *psbA* mRNA by gel mobility–shift assay. Less RNP complex was formed by proteins isolated from *cc703* cells than by an equal amount of proteins isolated from wt cells (Fig. 3). To determine whether the diminished binding ca-

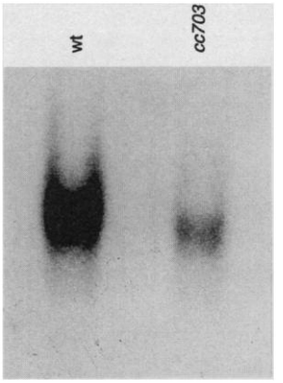

Fig. 3. Decreased *psbA* mRNA–binding activity in a mutant lacking photosystem I (PS I, the primary reducer of ferredoxin and thioredoxin). Autoradiograph of gel mobility–shift assay, displaying the labeled 5′-UTR of *psbA* mRNA complexed with proteins, isolated from light-grown wt cells or from a light-grown PS I–deficient mutant cells (*cc703*) lacking a PS I reaction center. Conditions for gel mobility–shift assay are as in Fig. 1. Only the portions of the gel containing the RNA-protein complexes are shown.

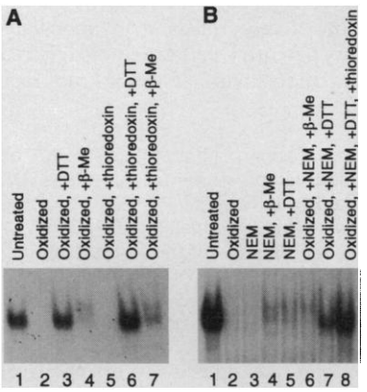

Fig. 2. Thioredoxin enhances the reactivation of *psbA* mRNA binding by the reduction of a regulatory disulfide bond. (**A**) Reactivation of *psbA* mRNA binding by thioredoxin. Autoradiograph of gel mobility–shift assay, displaying the labeled 5′-UTR of *psbA* mRNA complexed with *psbA*-RACPs. RNA-binding reactions that contained *psbA*-RACPs were first oxidized for 5 min with DTNB (Oxidized, lane 2) and then treated for 10 min with the reductants DTT (lane 3), β-Me (lane 4), with oxidized thioredoxin (lane 5), with thioredoxin reduced with DTT (lane 6) or thioredoxin reduced with β-Me (lane 7), before the addition of labeled RNA and compared to RNA binding of untreated proteins (lane 1). Conditions for the gel mobility–shift assay are as in Fig. 1. Only the portions of the gel containing the RNA-protein complexes are shown. (**B**) Oxidation with DTNB transforms the regulatory dithiols of *psbA* RNP to disulfide. Autoradiograph of gel mobility–shift assay, displaying the labeled 5′-UTR of *psbA* mRNA complexed with *psbA*-RACPs. RNA-binding reactions contained *psbA*-RACPs that were either oxidized for 5 min with DTNB (Oxidized, lane 2) or modified with *n*-ethylmaleimide (NEM, lane 3). The RNA-binding activity of NEM-treated proteins cannot be recovered after 10 min of incubation with either β-Me (lane 4) or DTT (lane 5). However, the RNA-binding activity of *psbA*-RACPs that were first oxidized for 5 min with DTNB, reduced with β-Me for 5 min, and then treated for 5 min with NEM could be partially recovered by 10-min reduction with DTT (lane 7) or fully recovered by 10-min incubation with DTT-reduced thioredoxin, but not with β-Me (lane 6). Conditions for the gel mobility–shift assay are as in Fig. 1. Only the portions of the gel containing the RNA-protein complexes are shown.

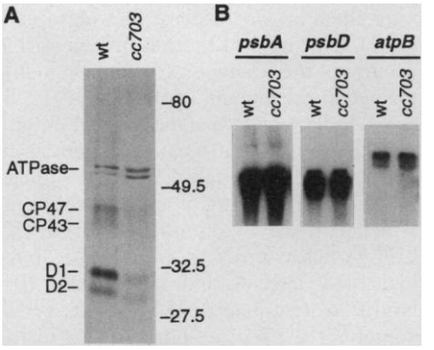

Fig. 4. Decreased translation of *psbA* mRNA in a mutant lacking PS I (the primary reducer of ferredoxin and thioredoxin). (**A**) Autoradiograph of pulse-labeled proteins isolated from wt and *cc703* cells. Light-grown cells were labeled for 10 min with [^{14}C]acetate in the presence of cycloheximide (8 mg/liter). Thylakoid proteins were isolated (*3*) and fractionated by denaturing polyacrylamide gel electrophoresis. The positions of specific proteins are identified at left. Molecular sizes are indicated on the right (in kilodaltons). (**B**) Autoradiograph of RNA-blot analysis of RNA isolated from light-grown wt and *cc703* cells. Duplicate blots were hybridized with labeled DNA fragments of *psbA*, *psbD*, and *atpB* genes (*3*) to assay the steady-state level of each of these mRNAs in wt and *cc703* cells.

pacity of these proteins resulted in a decreased amount of translation of the *psbA* mRNA, we analyzed the synthesis of D1 protein in vivo by pulse labeling proteins with [^{14}C]acetate. Synthesis of the D1 protein, as well as of other light-regulated proteins (D2, CP47, and CP43), was less in *cc703* cells than in wt cells (Fig. 4). In contrast, the synthesis of the α and β subunits of adenosine triphosphatase (ATPase), which do not show light-regulated translation (*3*), was not affected in *cc703* cells (Fig. 4A). The synthesis of proteins in light-grown *cc703* cells (Fig. 4A) is similar to the synthesis of proteins in dark-grown wt cells (*3*) and is in agreement with the hypothesis that reducing power, generated by PS I, is responsible for the activation of translation of the light-regulated chloroplast mRNAs. Analysis of amounts of *psbA*, *psbD*, and *atpB* mRNAs by RNA-blot analysis (Fig. 4B) showed no difference between wt and *cc703* cells. Thus, differences between wt and *cc703* cells in the synthesis of D1 and other proteins were due to differences in the rate of translation and not to altered amounts of mRNAs.

Thioredoxin serves as a transducer of redox potential generated by the "light-reactions" of photosynthesis (*15*), providing the chloroplast with a mechanism to coordinate the activity of various components of photosynthesis to light. Our results show that the formation of *psbA* RNP is also modulated by redox in vitro and in vivo and that translation of the core proteins of PS II is regulated by redox in vivo. These data suggest that thioredoxin may act as a link between the "light-reactions" of photosynthesis and the amount of *psbA* mRNA binding and hence *psbA* mRNA translation in vivo. According to this model, translation of light-regulated proteins is activated in response to changes in redox potential generated by photosynthesis. Following illumination, the reducing power generated by photosynthesis is used to reduce thioredoxin through a series of oxidation-reduction reactions involving ferredoxin (Fd), ferredoxin-thioredoxin reductase, and thioredoxin. Reduced thioredoxin drives reduction of the *psbA* mRNA–binding proteins, increasing their capacity to bind the 5′-UTR of *psbA* mRNA. Binding of this protein complex to the *psbA* mRNA allows for recruitment of *psbA* mRNAs onto polysomes and subsequent translation of the D1 protein. This direct link between light and the translation of the D1 protein, which is part of the PS II reaction center, provides the cell with a capacity to respond to fluctuating light levels, replacing photooxidized reaction center proteins (*21*, *22*) at a rate that is appropriate to the rate of photosynthesis.

REFERENCES AND NOTES

1. H. Fromm, M. Devic, R. Fluhr, M. Edelman, *EMBO J.* **4**, 291 (1985).
2. R. R. Klein, H. S. Mason, J. E. Mullet, *J. Cell Biol.* **106**, 289 (1988).
3. P. Malnoë, S. P. Mayfield, J. D. Rochaix, *ibid.*, p. 609.
4. K. Krupinska and K. Apel, *Mol. Gen. Genet.* **219**, 467 (1989).
5. X.-W. Deng and W. Gruissem, *EMBO J.* **7**, 3301 (1988).
6. J. Mullet, *Annu. Rev. Plant Physiol.* **39**, 475 (1988).
7. K. H. Jensen, D. L. Herrin, F. G. Plumley, G. W. Schmidt, *J. Cell Biol.* **103**, 1315 (1986).
8. M. R. Kuchka, S. P. Mayfield, J.-D. Rochaix, *EMBO J.* **7**, 319 (1988).
9. J.-D. Rochaix *et al.*, *ibid.* **8**, 1013 (1989).
10. J. Girard-Bascou, Y. Pierre, D. A. Drapier, *Curr. Genet.* **22**, 47 (1992).
11. A. Danon and S. P. Mayfield, *EMBO J.* **10**, 3993 (1991).
12. S. P. Mayfield, A. Cohen, A. Danon, C. B. Yohn, *J. Cell Biol.* **127**, 6 (1994).
13. A. Danon and S. P. Mayfield, *EMBO J.* **13**, 2227 (1994).
14. E. M. Tobin and J. Silverthorne, *Annu. Rev. Plant Physiol.* **36**, 569 (1985).
15. R. A. Wolosiuk, M. A. Ballicora, K. Hagelin, *FASEB J.* **7**, 622 (1993).
16. B. B. Buchanan, *Arch. Biochem. Biophys.* **288**, 1 (1991).
17. G. L. Ellman *et al.*, *Biochem. Pharmacol.* **7**, 88 (1961).
18. A. Danon and S. P. Mayfield, unpublished data.
19. R. D. Klausner, T. A. Rouault, J. B. Harford *Cell* **72**, 19 (1993).
20. D. M. Grant, N. W. Gillham, J. E. Boynton, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6067 (1980); J. Girard-Bascou, *Curr. Genet.* **12**, 483 (1987).
21. A. K. Mattoo, U. Pick, H. Hoffman-Falk, M. Edelman, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1572 (1981).
22. M. Wettern and I. Ohad, *Isr. J. Bot.* **33**, 253 (1984).

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Differential Activation of ERK and JNK Mitogen-Activated Protein Kinases by Raf-1 and MEKK

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Growth factors activate mitogen-activated protein kinases (MAPKs), including extracellular signal–regulated kinases (ERKs) and Jun kinases (JNKs). Although the signaling cascade from growth factor receptors to ERKs is relatively well understood, the pathway leading to JNK activation is more obscure. Activation of JNK by epidermal growth factor (EGF) or nerve growth factor (NGF) was dependent on H-Ras activation, whereas JNK activation by tumor necrosis factor α (TNF-α) was Ras-independent. Ras activates two protein kinases, Raf-1 and MEK (MAPK, or ERK, kinase) kinase (MEKK). Raf-1 contributes directly to ERK activation but not to JNK activation, whereas MEKK participated in JNK activation but caused ERK activation only after overexpression. These results demonstrate the existence of two distinct Ras-dependent MAPK cascades—one initiated by Raf-1 leading to ERK activation, and the other initiated by MEKK leading to JNK activation.

The transactivation function of c-Jun, a component of AP-1, is stimulated by phosphorylation at serines 73 and 63 (*1*, *2*) in response to H-Ras activation (*1*). Phosphorylation at Ser^{63} and Ser^{73} is also stimulated by other oncoproteins, including v-Sis and v-Raf (*3*), or by exposure of cells to ultraviolet radiation (*4*), TNF-α (*5*), growth factors (*6*), or factors that activate T cells (*7*). Both Ser^{63} and Ser^{73} of c-Jun are efficiently phosphorylated by a single type of protein kinase termed JNK, which exists in several isoforms (*8*–*10*). The JNKs are members of the MAPK family (*9*, *10*) and, like ERK1 (pp44) and ERK2 (pp42) (*11*), are activated through phosphorylation at conserved Thr and Tyr residues (*9*). Both the ERKs (*12*) and JNKs (*8*, *9*) are activated by EGF and oncogenic H-Ras (*6*–*8*). The two forms of JNK expressed by human cells, JNK1 and JNK2, are also efficiently activated in cells exposed to ultraviolet radiation or TNF-α, which are weak ERK activators (*6*). In contrast, 12-O-tetradecanoyl phorbol-13-acetate (TPA), a potent ERK activator (*12*), has little effect on JNK activity in fibroblasts and epithelial cells (*6*). These differences suggest that the JNKs are activated by a different signaling cascade than that which

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