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A Light-Regulated DNA-Binding Activity Interacts with a Conserved Region of a *Lemna gibba rbcS* Promoter

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We have characterized a DNA-binding activity, designated light-regulated nuclear factor (LRF-1), which interacted with a specific sequence located 150 nucleotides upstream from the transcription start site of a phytochrome-regulated *Lemna gibba rbcS* gene (*SSU5B*). There was a higher level of LRF-1 activity recovered from nuclei of light-grown plants than from dark-treated plants. In light-grown plants given a 1-day dark treatment, either white light or a single 2-min red illumination caused a rapid twofold to threefold increase in this activity, suggesting that the phytochrome system is probably involved in its regulation. The nuclear extracts also contained an activity that bound specifically to Box II sequences from a pea *rbcS* gene [Green, P.J., Yong, M.H., Cuozzo, M., Kano-Murakami, Y., Silverstein, P., and Chua, N.-H. (1988). EMBO J. 7, 4035–4044], but this activity was not higher in the light-grown compared with the dark-treated plants. Comparison of about 700 base pairs upstream from the *SSU5B* transcription start site with the upstream sequences of two other *Lemna rbcS* genes revealed several conserved regions. One of these regions is found upstream of *rbcS* genes in other species and is contained in the sequence which was shown to interact with LRF-1.

INTRODUCTION

Light regulates many fundamental processes in plant growth and development. Phytochrome is the best characterized of the several different pigments which mediate the responses of plants to light (Quail, 1983). In dark-grown plants, photomorphogenesis can be initiated by the photoreversible conversion of phytochrome from its inactive state (P_r) to the active form (P_{fr}) upon absorption of light. One of the most interesting and important effects of P_{fr} on plant growth and development involves the transcriptional regulation of specific nuclear genes, including *rbcS*, encoding the small subunit of ribulose-1,5-bisphosphate carboxylase (Rubisco) (Tobin and Silverthorne, 1985). The details of the signal transduction pathway by which phytochrome controls gene expression are unknown.

Analysis of the terminal connection of this pathway to the activated genes provides a convenient starting point for understanding the phytochrome response. In dicotyledonous plants, published reports from several laboratories have identified DNA-binding proteins that interact with upstream genetic elements known to influence light-regulated expression of the Rubisco small subunit gene (Green et al., 1988; Datta and Cashmore, 1989; Donald and Cashmore, 1990), chalcone synthase gene (Schulze-Lefert et al., 1989), and phenylalanine ammonia-lyase gene (Lois et al., 1989). Binding activity has been shown to be influenced by ultraviolet light in the cases of the chalcone synthase and phenylalanine ammonia-lyase genes, but the activities of the *rbcS* binding factors, GT-1 (Green et al., 1987) and GBF (Giuliano et al., 1988b), are unaffected by light.

The duckweed *Lemna gibba* G-3 is an aquatic monocot in which the Rubisco small subunit is encoded by a 12- to 14-member gene family (Wimpee et al., 1983). The transcription of this gene family (Silverthorne and Tobin, 1984) and the mRNA level from at least six of the genes (Silverthorne et al., 1990) have been shown to be regulated by phytochrome. Genomic clones for six members of the family and a cDNA clone for a seventh have been isolated and characterized (Stiekema et al., 1983; Silverthorne et al., 1990).

We have now sequenced and compared the upstream regions from three of these genes that show significant differences in their mRNA abundance (Silverthorne et al., 1990). The most abundantly expressed of these, *SSU5B*, has been clearly demonstrated to be regulated by phytochrome at the transcriptional level using a gene-specific probe (P.A. Okubara, unpublished results). We selected the promoter region from this gene to identify DNA-binding proteins that may be involved in the phytochrome regulation of transcription of *rbcS* genes.

In this paper, we identify a light-regulated DNA-binding protein, designated light-regulated nuclear factor (LRF-1),

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that interacts with a conserved DNA sequence motif present in the 5′-region of several *rbcS* genes. We conclude from our results that LRF-1 may be part of the signal transduction pathway mediating transcriptional control of plant genes by light.

RESULTS

Identification of a DNA-Binding Activity in Nuclear Extracts

Sequence comparison of the upstream regions from the *Lemna SSU5B*, *SSU5A*, and *SSU13* genes is shown in Figure 1. These sequences are at least 60% conserved between nucleotides −300 and +1, and contain three shorter stretches of much higher similarity, designated as Boxes X, Y, and Z. There are no obvious similarities to the previously characterized Box II motif (Green et al., 1987) or the G-box motif (Giuliano et al., 1988b), which have been suggested to play a role in reducing transcription in dark-treated plants. Within Box X there is a GATAAG motif that is present in a number of other *rbcS* genes (cf. Manzara and Gruissem, 1988). A DNA restriction fragment containing the conserved region from +20 to −255 of the highly transcribed, phytochrome-regulated *SSU5B* gene, shown at the bottom of Figure 1 (B1 + B2 region), was chosen to assay for DNA-binding proteins in nuclear extracts from light-grown *Lemna*. Electrophoretic mobility shift gels revealed the presence of a diffuse, slightly shifted, ^{32}P-labeled band. The resolution of this bound complex was improved by optimizing the binding reaction conditions and reducing the size of the probe. Initially, fragment B2, a 175-bp fragment shown in Figure 1, was found to form a complex that was sensitive to pretreatment with proteinase K, but not to RNase A, indicating that a protein component is involved in the binding activity (data not shown). We have designated the activity bound in this complex LRF-1.

Specificity and Localization of LRF-1 Binding

The ability of various unlabeled DNA fragments to compete for binding on the electrophoretic mobility shift gels was employed to test the specificity of LRF-1 for the B2 fragment. A series of experiments demonstrated that the amount of bound complex was reduced by 50% at a 2:1 molar ratio of unlabeled B2 and by 90% at a 10:1 molar ratio. Subdividing this fragment into two smaller restriction fragments, B2b, including Box X, and B2a, including Box Y (Figure 1), allowed further localization of the binding activity. Figure 2, which shows the results of competition assays with a 4:1 molar ratio of the competitor to ^{32}P-labeled B2b, demonstrates that the B2b fragment includes the strongest binding site. At a higher molar ratio of 10:1, competition with either the B2b fragment or the complete B2 fragment reduced the amount of binding by 90%, whereas the B2a and B1 fragments reduced the binding by about 50%, suggesting the possibility of weaker binding sites in these regions. The slightly higher competition seen in lane B, in which the competitor contains the entire region from +20 to −255 (B2 + B1, Figure 1), was probably due to these additional, weaker sites of interaction. Competitor C, a cloned fragment from downstream of a *Lemna cab* gene, shows no competition at the 4:1 ratio shown in Figure 2, and did not compete for binding up to an 80:1 molar ratio, the highest tested. At a 20:1 mass ratio, poly(dA-dT)·poly (dA-dT), poly(dG-dC)·poly(dG-dC), and *Escherichia coli* genomic DNA cut with Sau3A showed no competition (data not shown). These experiments identified the B2b fragment as the region within B1 + B2 with the strongest binding to LRF-1.

Footprint Analysis of the Binding Activity

DNase I protection was employed to map the binding sequence more precisely within the B2b region. As shown in Figure 3 (solid lines), footprints of 21 and 11 nucleotides were observed on the coding and noncoding strands, respectively, of the bound probes. Two additional nucleotides on the noncoding strand were protected in a separate experiment (dashed line). These footprints overlapped by 8 nucleotides, including the GATAAG sequence at −149, within the highly conserved region referred to here as Box X (Figure 1). A similar GATA sequence is present at −163 in the upstream half of the split footprint seen on the coding strand. There are also many hypersensitive sites, particularly apparent on the noncoding strand (Figure 3). This analysis enabled us to localize a specific region within the B2b fragment that interacts with LRF-1.

LRF-1 Activity Decreases after Dark Treatment

We initially tested whether LRF-1 activity might be correlated with light regulation in *Lemna* by comparing the amount of binding activity in nuclei from light-grown plants with that present after dark treatment for 5 to 7 days. Figure 4A shows that there was a substantially decreased binding activity in the dark-treated plants. In this experiment, the activity was at least 10-fold higher in the light-grown nuclear extract than in that from dark-treated plants, based on comparing the relative activity in dilutions of each extract. The range of differences seen over a total of 14 additional experiments was threefold to 10-fold. The differences could not be attributed to the presence of an inhibitor in extracts from dark-treated plants because mixing of extracts from dark-treated and light-grown plants produced an additive effect (data not shown).

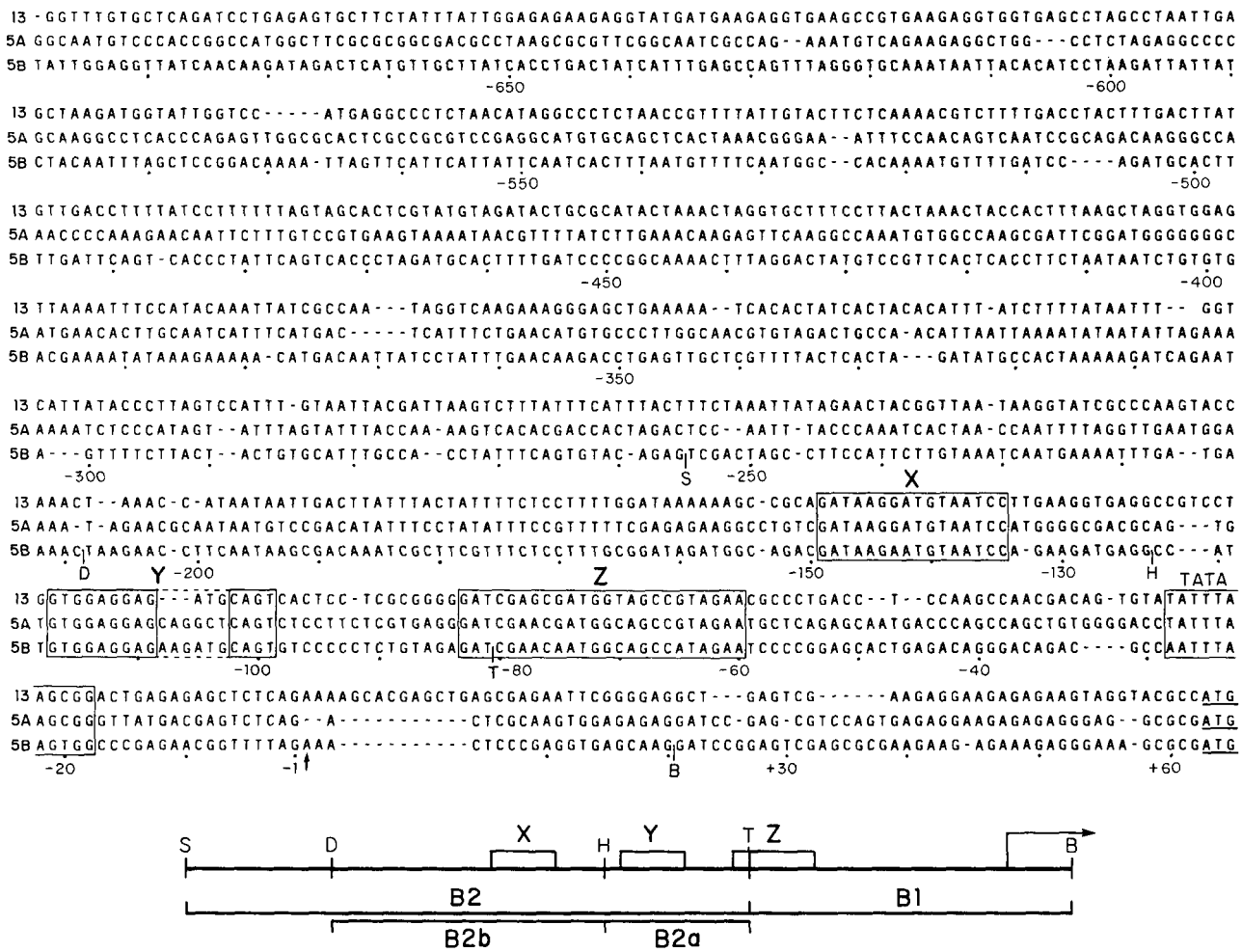


Figure 1. Upstream Sequences of *L. gibba rbcS* Genes.

Sequences of about 700 nucleotides upstream from transcription start sites (arrow) of the three genes determined in this study were compared and aligned using Pustell Sequence Analysis Programs (Pustell and Kafatos, 1982). Boxes indicate regions of ≥80% conservation (X, Y, and Z) and TATA sequences. Numbering is based on distance from transcription start site for *SSU5B*. Diagram below represents a 275-bp restriction fragment, referred to as fragment B, containing the highly conserved regions upstream of *SSU5B*. Restriction fragments of B are shown at bottom. S, SalI site; D, DdeI site; H, HaeIII site; T, TaqI site; B, BamHI site.

All of the above experiments were performed using equivalent amounts of total nuclear protein in each reaction. To determine whether the amount of nuclear protein recovered varied with the light/dark treatments, protein/DNA ratios were determined for samples of both types of tissue. All samples were found to contain 30 ± 4 μg of protein/μg of DNA in the final extracts and 3.8 ± 0.8 μg of protein/μg of DNA before ammonium sulfate precipitation. Although not as reliable as a standard, the yields of nuclear protein per gram of plant tissue were also comparable, as were the polypeptide profiles observed with SDS-PAGE (data not shown). Because there were no detectable changes in total nuclear protein content, even after the longest period of dark treatment, dilution of LRF-1 could not account for the decrease in binding activity.

As a positive control for the light/dark changes in LRF-1 activity, we utilized another sequence, originally found upstream of a pea *rbcS* gene and designated Box II (Nagy et al., 1986). This sequence binds a factor, GT-1, which has been shown to be present at comparable levels in nuclear extracts from both light-grown and dark-treated pea plants (Green et al., 1987). Figure 4B (Box II lanes) shows that *Lemna* also contains a GT-1-like activity that formed a slow-moving complex with the probe consisting

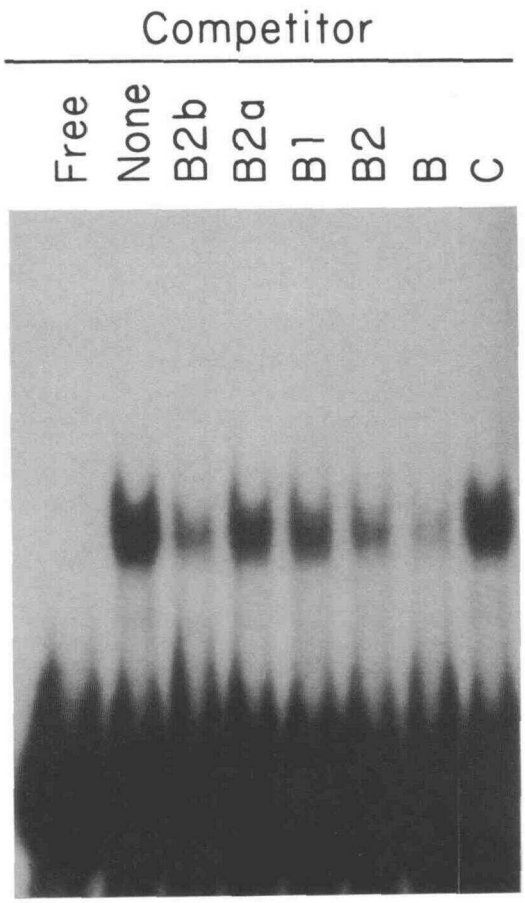
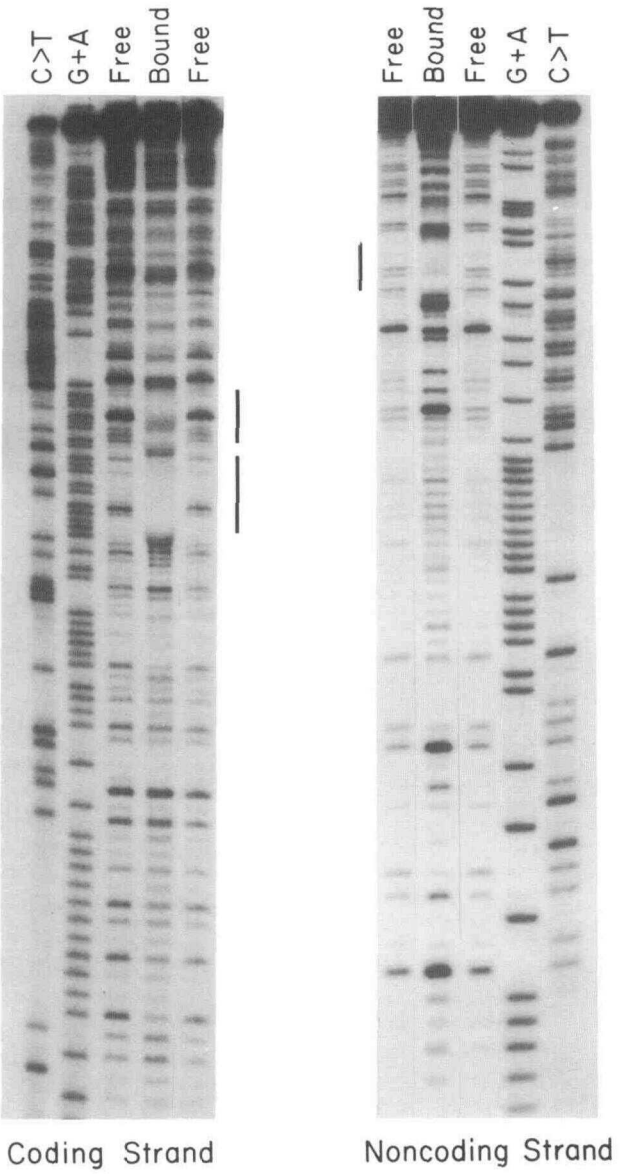


Figure 2. *Relative Affinity of LRF-1 for Sites within SSU5B −255 to +20 Region.*

Electrophoretic mobility shift assays shown were performed as described in Methods. Unlabeled competitor DNA was included in binding reactions at a 4:1 molar ratio to ^{32}P-labeled B2b. Competitors: B2b, homologous B2b DNA (−210 to −125); B2a, B2a DNA (−125 to −80); B1, B1 DNA (−80 to +20); B2, B2 DNA (−255 to −80); B, B DNA (−255 to +20); C, 100-bp fragment from downstream of the *Lemna AB30 gene*.



of four tandem copies of Box II (Green et al., 1988). This complex did not form with a mutant form of the site that does not bind the pea GT-1 factor (Box II^m, Green et al., 1988), and was competed completely by a 25:1 molar ratio of unlabeled Box II. By contrast, Box II^m did not compete for this GT-1-like binding activity. Unlike the results from pea, in *Lemna* the GT-1-like activity was present at a higher level in the extracts from dark-treated plants, whereas LRF-1 activity was reduced by a factor of three (B2b lanes: F,L,D). This result argues against the possibility of a general loss of chromatin-associated proteins in the extracts from the dark-treated plants. Together, the experiments presented in Figure 4 clearly demonstrate that dark treatment of *Lemna* reduces LRF-1 activity.

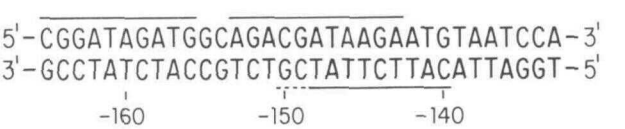


Figure 3. Mapping of the LRF-1 Binding Site.

Fractions of each probe both shifted (Bound) and unshifted (Free) by LRF-1 binding were prepared as described in Methods. C > T, pyrimidine sequencing ladder; G + A, purine sequencing ladder. Coding Strand is from 3′-end-labeled B2 fragment and Noncoding Strand (transcribed strand) is from 3′-end-labeled B2b fragment. Solid bars alongside each set of lanes designate regions protected from DNase I digestion and correspond to bars on the sequence shown below. The dashed bar on the sequence below indicates additional bases protected in a separate experiment (not shown).

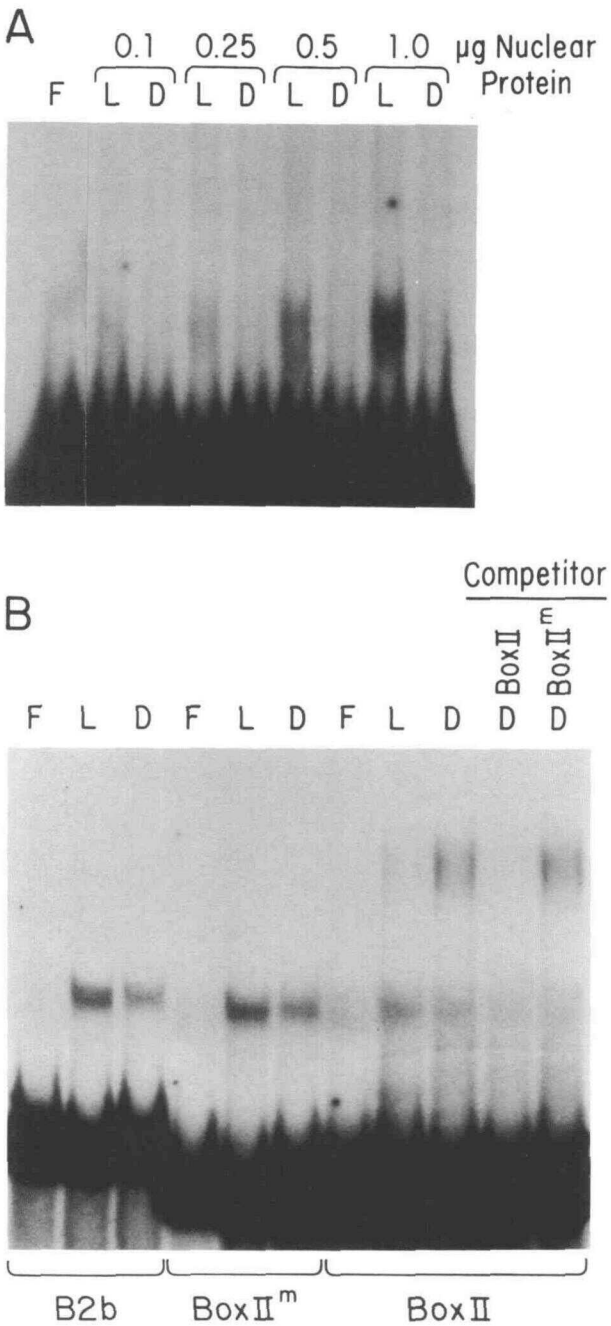


Figure 4. Light Dependence of DNA-Binding Activity.

Electrophoretic mobility shift assays shown were performed as described in Methods.

(A) Effect of dark treatment on binding of LRF-1 with B2. Lane F, no nuclear protein; lanes L, increasing amounts of nuclear protein from light-grown plants; lanes D, increasing amounts of nuclear protein from dark-treated plants (5 days).

(B) Effects of dark treatment on binding of nuclear factors with B2b and Box II. Each ^{32}P-labeled probe is indicated below its respective lanes. Reactions containing Competitor included unlabeled DNA at a 25:1 molar ratio to ^{32}P-labeled Box II. Lanes F, no nuclear protein; lanes L, equivalent amounts of nuclear protein from the same light-grown plant extract; lanes D, equivalent amounts of nuclear protein from the same dark-treated plant extract. A complex with a mobility close to the LRF-1 complex with B2b was seen with both Box II and Box II^m.

Light Reinduces LRF-1 Activity after Dark Treatment

We next tested the effects of light treatments after LRF-1 activity had been reduced by a dark treatment. We determined that a 1-day dark-treatment period was sufficient to produce a reduction in LRF-1 activity that could be reversed by subsequent light treatments. The shorter period of dark treatment was found to produce a slightly higher level of dark activity than generally observed after longer periods, as in Figure 4A. Such a short dark treatment has been shown to decrease the transcription of a *Lemna cab* gene by about fivefold (P.A. Okubara and E.M. Tobin, unpublished results). After dark treatment for 22 hr from the end of the 8-hr light period of the growth cycle, one set of plants was harvested, whereas another was transferred into white light for 2 hr before harvesting. Figure 5A shows that the level of LRF-1 activity increased after 2 hr of white light (lane L2), compared with the relatively low level present before light treatment (lane D). Thus, 2 hr of white light was sufficient to reinduce LRF-1 activity.

Figure 5A also shows the result of another experiment in which two sets of plants were placed in the dark for 22 hr as before, whereas a third set received its regular 16 hr of darkness, followed by 6 hr of light before harvesting. LRF-1 activity was much higher after the longer period in white light (lane L6) than the dark level (lane D′). If a circadian rhythm were responsible for the increase in LRF-1 activity seen after 6 hr of white light, then this increase would also be expected in plants that remained in the dark during this same time period (e.g., Giuliano et al., 1988a). The dark-treated plants did not show such an increase, suggesting a distinct light response.

The results of a time-course experiment from a series of nuclear extracts from white light-treated plants are presented in Figure 5B. There was a peak in white light-induced activity after 4 hr, which was about the time in the growth cycle when most of the light-grown plants had been harvested previous to this result.

Brief Red Illumination Can Induce LRF-1 Activity

Figure 5A (lane R) shows the results of treating plants with 2 min of red light 2 hr before harvesting after a 22-hr dark treatment. This brief red illumination increased LRF-1 activity by about twofold, which is comparable with phytochrome-induced transcription increases previously observed for *Lemna rbcS* genes (Silverthorne and Tobin, 1984). LRF-1 activity was not consistently increased by brief red light treatment after depletion by long periods of dark treatment (5 to 7 days). In a time-course experiment

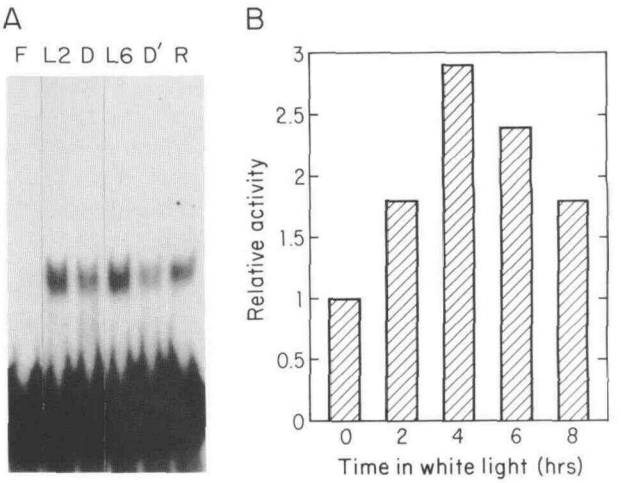


Figure 5. Effects of Light on LRF-1 Activity after Dark Treatment.

Electrophoretic mobility shift assays were performed as described in Methods, using ^{32}P-labeled B2b.

(A) Effect of white or red light on LRF-1 activity. Each reaction contained 1 μg of nuclear protein from plants subjected to light-treatment regimes described in text, except F, which contained no nuclear protein.

(B) LRF-1 activity as a function of white light exposure time. Activity was measured by liquid scintillation counting of excised, radioactive bands shifted by LRF-1 binding. Values shown are relative to average, basal activity of companion dark-treated extracts.

with plants given 2 min of red light after a 22-hr dark treatment, then returned to the dark, the binding activity was found to show a transient increase of about twofold with a peak at 2 hr after the light treatment (data not shown). These experiments strongly suggest that phytochrome action can affect the activity of LRF-1.

Competition for LRF-1 Binding Correlates with *rbcS* mRNA Induction

We tested whether there is any correlation between the binding of LRF-1 and the expression of the three *Lemna rbcS* genes characterized in this paper. We previously used gene-specific probes to compare the phytochrome regulation of mRNAs from individual *Lemna rbcS* genes (Tobin et al., 1985; Silverthorne et al., 1990). The red light-induced increases in mRNA levels of *SSU5B*, *SSU5A*, and *SSU13* genes varied, as shown in Figure 6A (Silverthorne et al., 1990). A transcriptional increase in response to phytochrome action has been demonstrated for the *SSU5B* gene using the gene-specific probe and "run-on" assays with isolated nuclei (P.A. Okubara, unpublished data). The other two genes were expressed at too low a level to detect new transcription in response to brief red illumination in this way. The binding of LRF-1 to the B2b fragment was used to test the ability of the corresponding DNA fragments from upstream of *SSU5A* and *SSU13* to compete for this binding on the mobility shift gel shown in Figure 6B. At a 4:1 molar ratio of unlabeled competitor DNA, the +20 to −255 region of *SSU5B* (B2, Figure 1) competed very strongly, +20 to −345 from *SSU5A* competed more weakly, and −15 to −295 from *SSU13* did not

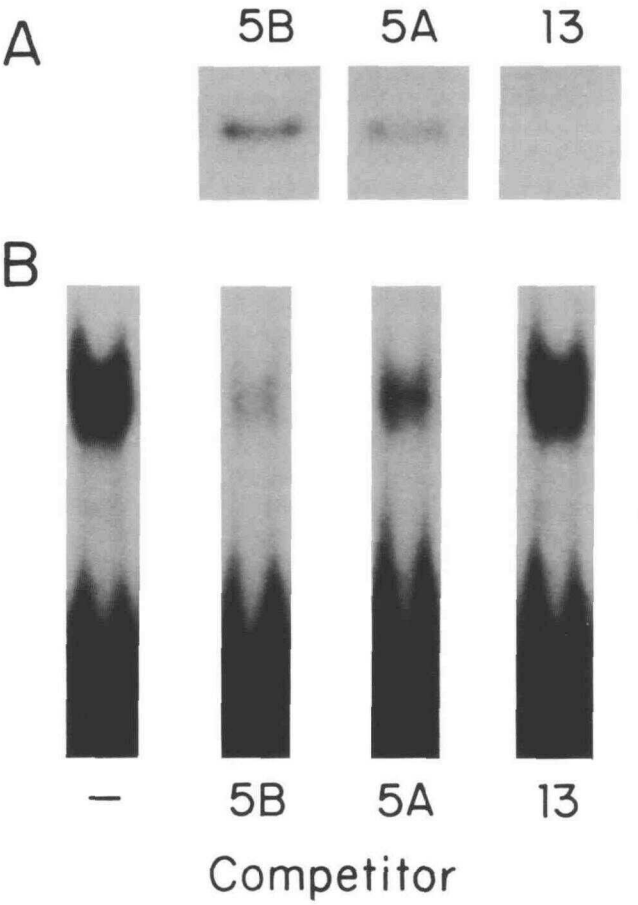


Figure 6. Comparison of *rbcS* mRNA Levels and Competition for LRF-1 Binding.

(A) Relative *rbcS* mRNA levels. Total RNA was isolated from red light-treated plants, gel fractionated, blotted onto nitrocellulose, and hybridized with ^{32}P-labeled gene-specific probes from the 3′-untranslated regions of *SSU5B*, *5A*, and *13*, as described (Silverthorne et al., 1990).

(B) Relative affinity of LRF-1 for the upstream region of each *rbcS* gene. Electrophoretic mobility shift assay was performed as described in Methods. Unlabeled competitor DNA was included in binding reactions at a 4:1 molar ratio to ^{32}P-labeled B2b probe (−210 to −125). Competitors: 5B, B DNA (−255 to +20); 5A, −345 to +20 of *SSU5A*; 13, −295 to −15 of *SSU13*.

compete for LRF-1 binding. This fragment from *SSU13* also did not form any detectable complexes when used as a probe for mobility shift gels (data not shown). We conclude that the *SSU5B* gene, which showed the highest transcriptional response to phytochrome action and whose mRNA accumulated to the highest level of the three *rbcS* genes, also showed the strongest binding of LRF-1. By contrast, the *SSU13* gene, whose expression could not be detected in total RNA, showed no evidence for binding LRF-1. All of the three promoter regions used as competitors contained the conserved GATAAG motif. Therefore, our results suggest that sequences in addition to GATAAG are important in determining the response of these genes to phytochrome action.

DISCUSSION

In this study, we describe a *Lemna* DNA-binding activity, LRF-1, that recognizes and binds a distinct, highly conserved sequence upstream of a *Lemna rbcS* gene, *SSU5B*. The binding activity was greater in extracts from light-grown plants than from those that had been dark treated, and it could be rapidly increased by light after short periods of dark treatment. The increase in activity could also be induced transiently by brief red illumination. LRF-1 had a greater affinity for the promoter region of *SSU5B* than for those of the two other *rbcS* genes whose mRNAs are less abundant. Our results suggest that LRF-1 plays a role in the phytochrome signal transduction chain.

The binding of LRF-1 protected two adjacent stretches of the coding DNA strand, one of which was also protected on the noncoding strand, approximately −150 bases from the transcription start site. The sequences protected on both strands include nucleotides lying within the highly conserved Box X region (Figure 1) and containing a GATAAG motif. The additional sequence that was protected only on the coding strand also contains a similar motif, GATAGA. The GATAAG motif has been recognized as a common feature in the region within 300 bp upstream from the transcription start site of most dicotyledonous *rbcS* genes thus far characterized (Dean et al., 1985; Giuliano et al., 1988b; Manzara and Gruissem, 1988). It is also present, more proximal to the TATA box, in many dicotyledonous *cab* genes (Castresana et al., 1987; Gidoni et al., 1989), and has been uncovered independently in a computer search of the promoter regions of numerous phytochrome-regulated genes (Grob and Stuber, 1987). Mutations in two GATAAG sequences upstream of the *Arabidopsis thaliana rbcS-1A* gene have recently been shown to significantly reduce expression of reporter genes in light-grown transgenic tobacco plants (Donald and Cashmore, 1990). A factor in tobacco leaf nuclear extracts (ASF-2) has been reported to bind a GATA motif upstream from a petunia *cab* gene, but no evidence of light dependence was presented (Lam and Chua, 1989).

The region protected by LRF-1 is also flanked by 10 to 11 bp of homopurine·homopyrimidine tracts, of opposite polarity, within about 25 bp on either side. The tract downstream of the binding site lies within the highly conserved Box Y, and the upstream tract is also well conserved (Figure 1). Such tracts are common in eucaryotic promoter regions and are capable of forming unusual (see Wells, 1988, for review) S1 nuclease-sensitive structures (Pulleyblank et al., 1985; Evans and Efstratiadis, 1986). They have been shown to influence the recognition of DNA by proteins (Drew and Travers, 1984) and may play a role in the regulation of transcription (Wells, 1988). In this context, it is noteworthy that a footprint was not observed on the coding strand of the B2b fragment, in which the Box Y homopurine·homopyrimidine tract downstream of the binding site is missing.

Despite the high sequence conservation of Box X in the three differentially expressed *Lemna rbcS* genes, the larger DNA fragments containing this region from these genes competed quite differently for LRF-1 binding (Figure 6). Additionally, a 16-bp synthetic oligonucleotide covering part of the footprinted region including the GATAAG motif (−156 to −141) did not compete as well for binding as did the B2b fragment (J.S. Buzby and E.M. Tobin, unpublished data). These findings emphasize the importance of regions in addition to the GATAAG motif for interaction with LRF-1.

Although binding was reasonably specific to the Box X region (Figure 2), LRF-1 also had low affinity for sequences on the B1 and B2a fragments that have no obvious similarities (Figure 1) to the footprinted sequences on B2b (Figure 3). Likewise, both the Box II^m and Box II probes formed complexes that resembled the B2b complexes with LRF-1 (Figure 4B), even though these two boxes have no obvious similarities to the footprinted sequence. In fact, there are no sequences with evident homology to Box II within −2500 of the *SSU5B* and *SSU5A* transcription start sites (T. Yamada and E.M. Tobin, unpublished data). We do not yet know the significance, if any, of these interactions.

The fact that LRF-1 activity was not found to be consistently reinduced by red light after 5- to 7-day dark-treatment periods may be indicative of its degradation during that time. The activity did not decrease as much with a 1-day dark treatment. The shorter, 1-day dark treatment may, then, permit a more rapid reinduction of observable activity either by resynthesis or modification of the LRF-1 protein(s) in response to white or red light.

The correlation of the relative affinity of LRF-1 for each of the three *Lemna rbcS* genes tested with their relative mRNA levels after red light treatment (Figure 6) lends support to the argument for the involvement of LRF-1 in their normal regulation. Although the sequence bound by LRF-1 has not yet been shown to directly influence light-regulated transcription in *Lemna*, this correlation, along

with the high degree of binding site conservation and the response of LRF-1 to light, suggests a functional relationship. In any case, the influence of the surrounding DNA sequences and the possible interactions of other protein factors [see Gilmartin et al. (1990) for review] will also need to be considered to understand fully how phytochrome can act to affect gene expression.

METHODS

Growth of Plants

Lemna gibba G-3 was cultured aseptically in E medium supplemented with 1% sucrose (Tobin, 1981), and with 30 μM kinetin added 1 to 2 days before harvest (Tobin et al., 1985). Plants were routinely grown in an 8-hr light/16-hr dark cycle at 25°C ± 2°C for about 4 weeks to a density such that the surface of the medium was covered by a thick monolayer of plants. Dark-treated plants were grown to this stage in the light and then transferred to total darkness for up to 7 days before harvest, as indicated in Results.

Isolation of Nuclei and Nuclear Proteins

Nuclei were prepared by modification of a method described previously (Flores and Tobin, 1988). Mortar and pestle were used to grind the harvested plants and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) was included in all buffers. Dark-treated plants were harvested and nuclei prepared under a dim green safelight.

The isolated nuclei were gently lysed with 0.4 M NaCl in 5 mM spermidine for 30 min. After centrifugation for 30 min at 24,000*g*, the supernatant was precipitated with 60% ammonium sulfate. The precipitate was resuspended in 5 μL of storage buffer (10 mM Tris·HCl, pH = 7.5, 0.5 mM PMSF, 10 mM 2-mercaptoethanol, 10% glycerol) per gram of plant tissue and dialyzed. Optimum yields were about 10 μg of nuclear protein/g of tissue. Protein concentrations were determined using the Bradford assay method (Bio-Rad). The binding activity of this nuclear extract was stable for at least 4 weeks when stored at −70°C.

Enrichment for LRF-1 was accomplished by Affi-Gel-heparin (Bio-Rad) affinity chromatography (Farooqui, 1980). From 300 to 700 μg of total nuclear protein was loaded onto a 200-μL packed column in storage buffer supplemented with 0.15 M KCl and 5 mM $MgCl_2$. The LRF-1 binding activity was eluted by 0.3 M KCl, separating it from some nonspecific DNA-binding factors and DNase impurities. The active fraction pool was concentrated and desalted in a Centricon-30 microconcentrator (Amicon), which retains proteins of >30 kD.

Isolation of *rbcS* Genomic Clones

The genomic clone λSSU5, which contains both the *SSU5B* and *SSU5A* gene family members, was isolated as described previously (Wimpee et al., 1983). A clone containing the *SSU13* gene, λSSU13, was isolated from a Charon 35 phage library. The library was constructed by partial BamHI digestion of *Lemna* genomic DNA, size fractionation, and ligation with the BamHI-digested vector arms (Loenen and Blattner, 1983). In vitro packaged phage particles were transfected into *Escherichia coli* K802, and the library was screened by plaque hybridization (Maniatis et al., 1982), using a probe from the 3′-untranslated region of the pLgSSU1 cDNA clone (Stiekema et al., 1983) that is specific for the *SSU1* and *SSU13* gene family members (Silverthorne et al., 1990).

DNA Sequencing and S1 Mapping

DNA fragments containing the *rbcS* upstream regions were subcloned into phage M13mp18/19 or pUC18/19 (Yanisch-Perron et al., 1985). DNA sequencing was performed using the dideoxynucleotide chain-termination method and ^{35}S-dATP (Biggin et al., 1983). Both DNA strands were sequenced and overlaps obtained at each restriction site used for cloning.

S1 nuclease mapping was used to determine the transcriptional start sites. The noncoding strands were prepared for hybridization with the mRNAs essentially as described (Karlin-Neumann et al., 1985). Recombinant M13 clones containing the coding strands of the respective *rbcS* genes were used as templates for primer extension by Klenow fragment in the presence of α-^{32}P-dCTP, dATP, dGTP, and dTTP. The labeled probes were hybridized with total *Lemna* RNA (20 to 40 μg) at 50°C for 6 hr, diluted in S1 buffer (Yamada and Shimaji, 1987), and digested with S1 nuclease (500 to 1000 units/mL) at 37°C for 30 min. The protected fragments were analyzed on 8% (1:19 bis:acrylamide) polyacrylamide, 8 M urea sequencing gels.

DNA Fragment Preparation

DNA restriction fragments, for use as ^{32}P-labeled probes and unlabeled competitors, were isolated from polyacrylamide gels either by electroelution onto NA45 DEAE-membrane (Schleicher & Schuell) or by solubilization of *N,N′*-bis-acrylylcystamine (BAC; Bio-Rad) cross-linked gels (Hansen, 1981). A Hoefer DNA fluorometer (model TKO-100) was used with the DNA-specific Hoechst 33258 dye for quantitation of DNA (Labarca and Paigen, 1980). Probes were 3′-end-labeled using Klenow fragment of DNA PolI. Single-strand ^{32}P-labeled probes for DNase I protection were prepared by removing about 10 bp of either labeled 3′-end with a restriction endonuclease.

Fragment C is a 100-bp AluI restriction fragment cloned from downstream of the *Lemna AB30* gene (Kohorn et al., 1986). The Box II and Box II^m fragments, containing four tandem copies of the sequences 5′-TGTGTGGTTAATATG and 5′-TGTGTCCTTAATATG, respectively (Green et al., 1988), were generously provided by Steve Kay from the laboratory of Nam-Hai Chua. All other DNA fragments are described in Results.

Electrophoretic Mobility Shifts

Nuclear protein-DNA binding reactions were routinely performed in 25-μL binding buffer (10 mM Tris·HCl, pH = 7, 150 mM KCl, 5 mM $MgCl_2$, 0 to 2 mM EDTA, 0.5 mM PMSF, 10 mM 2-mercap-

toethanol, 15% glycerol) with 0.5 to 5 μg of poly(dI-dC)·poly(dI-dC) (Pharmacia LKB Biotechnology Inc.) nonspecific, bulk competitor DNA, 1 to 2 μg of nuclear protein, and 0.5 ng of a ^{32}P-labeled DNA probe. After incubation at room temperature for 5 to 10 min, the samples were electrophoresed in Tris-acetate buffer (Maniatis et al., 1982) on a 4.2% (1:29 bis:acrylamide) polyacrylamide gel, which was then dried and autoradiographed.

DNase I Protection

Preparative scale binding reactions were scaled up to a final volume of 50 μL containing 0 to 5 μg of poly(dI-dC)·poly(dI-dC), 10 μg of nuclear protein extract or the amount of Affi-Gel-heparin enriched nuclear protein obtained from about 250 μg of extract, and 5 to 10 ng of a single-strand ^{32}P-labeled DNA probe. Affi-Gel-heparin enrichment was found to greatly improve the reproducibility and quality of the resulting footprint. The conditions for the reactions were also modified such that the binding buffer contained 10 mM EDTA and no $MgCl_2$, and the incubation was carried out on ice for 0.5 to 10 min. The reactions were then treated with 10 μg/mL DNase I (Worthington) in the presence of 20 mM $MgCl_2$ for 30 sec at room temperature, after which EDTA was added to a final concentration of 50 mM. A preparative electrophoretic mobility shift gel, composed of 5% (1:21 BAC:acrylamide) solubilizable polyacrylamide, was used to separate the DNase I-treated bound and free ^{32}P-labeled probes (Singh et al., 1986; Jofuku et al., 1987). After autoradiography of the wet gel, the radioactive bands were isolated essentially as described (Hansen, 1981), using Elutip-d columns (Schleicher & Schuell) for purification. Equivalent counts per minute of bound and free fractions recovered from the gel were electrophoresed on a 10% (1:19 bis:acrylamide) polyacrylamide, 8 M urea sequencing gel, along with Maxam and Gilbert purine and pyrimidine marker ladders (Bencini et al., 1984).

Techniques and Reagents

Routine procedures involving DNA manipulation were performed essentially as described (Maniatis et al., 1982). Except where noted, chemicals were obtained from Sigma Chemical Co. and enzymes from Bethesda Research Laboratories, following the manufacturers' recommendations for their usage.

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NOTE ADDED IN PROOF

Although the nuclear extract apparently formed an LRF-1-like complex with both ^{32}P-BoxII and ^{32}P-BoxII^m (Figure 4B), the affinity for these probes is relatively low if this binding is due to LRF-1. In competition experiments using the ^{32}P-B2b probe, a 10:1 molar ratio of unlabeled BoxII^m showed no significant competition for LRF-1 binding, whereas this molar ratio of unlabeled B2b showed almost complete competition for binding, as described in Results.

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