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Identification of a Sequence-Specific DNA Binding Factor Required for Transcription of the Barley Chloroplast Blue Light-Responsive *psbD-psbC* Promoter

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The plastid gene *psbD* encodes the photosystem II reaction center chlorophyll protein D2. *psbD* is located in a complex operon that includes *psbC*, *psbK*, *psbI*, *orf62*, and *trnG*. The operon is transcribed from at least three different promoters. One of the *psbD* promoters is differentially activated when plants are exposed to blue light. In this study, the *psbD* blue light-responsive promoter was accurately transcribed in vitro in high-salt extracts of barley plastids. Transcription required supercoiled templates and was inhibited by tagetitoxin, an inhibitor of plastid transcription. *Escherichia coli* RNA polymerase did not recognize the *psbD* light-responsive promoter with the same specificity as plastid RNA polymerase. Deletion analyses demonstrated that sequences between −39 and −68, upstream of the transcription initiation site, were required for transcription of the *psbD* blue light-responsive promoter. This DNA region is highly conserved among plant species and contains multiple AAG sequences. Gel shift assays and DNase I footprinting experiments demonstrated that the AAG-rich DNA sequence interacts with a sequence-specific DNA binding factor termed AGF. Point mutations in the AAG *cis* element decreased binding of AGF and inhibited transcription from the *psbD* light-responsive promoter. We concluded that AGF is an essential factor required for transcription of the *psbD* light-responsive promoter.

INTRODUCTION

The chloroplast genome in most higher plants is circular and ranges in length from 120 to 217 kb (for review, see Palmer, 1990; Sugiura, 1992). The genome encodes ~135 genes, including genes for rRNAs, tRNAs, subunits of the plastid 70S ribosome, subunits of an RNA polymerase (*rpoA*, *rpoB*, *rpoC1*, and *rpoC2*), and proteins that comprise the photosynthetic apparatus (for review, see Mullet, 1993). Transcription of different plastid genes varies as much as 300-fold (Rapp et al., 1992). Genes such as 16S rDNA, *rps16*, and *rpoB* are transcribed at higher rates early in chloroplast development relative to plastid genes encoding photosynthetic proteins, such as *rbcL*, which encodes the ribulose bisphosphate carboxylase large subunit. Genes encoding proteins of the photosynthetic apparatus are transcribed at higher rates later in chloroplast development (Baumgartner et al., 1993). Differences in the relative rates of plastid gene transcription and modulation of transcription as a function of chloroplast development have been attributed to differences in promoter structure, the presence of multiple plastid RNA polymerases (Greenberg et al., 1984; Morden et al., 1991; Hess et al., 1993; Lerbs-Mache, 1993), and proteins that modulate transcription activity (Tiller and Link, 1993a; Iratni et al., 1994). Many plastid promoters contain −10/−35 sequences (TATAAT/TTGACA) that function in bacteria to promote transcription. The plastid-encoded RNA polymerase, which is homologous with bacterial RNA polymerases, utilizes these promoter elements. In contrast, the *rps16* promoter lacks −35 sequence elements but retains a −10-like domain (Neuhaus et al., 1989). Moreover, transcription of *trnR1* and *trnS1* does not require any upstream sequences (Gruissem et al., 1986). These promoters may be recognized by a nuclear-encoded plastid RNA polymerase, (Morden et al., 1991; Hess et al., 1993). Alternatively, plastid sigma-like factors (Tiller and Link, 1993a) may alter the specificity of the plastid-encoded RNA polymerase, allowing promoters that lack −35 elements to be utilized.

Light stimulates chloroplast development and modulates plastid gene expression (Link, 1988; Schrubar et al., 1990; Chory, 1993). In some plants, light absorbed by the photoreceptor phytochrome stimulates leaf and chloroplast development and plastid gene expression. Analysis of *cop* (for constitutive photomorphogenic) and *det* (for deetiolated) mutants indicates the presence of a complex regulatory network that connects phytochrome activation, leaf development, and selective activation of photosynthetic gene expression (for review, see Chory, 1993; Deng, 1994). Light also activates chlorophyll biosynthesis, which induces the accumulation of chlorophyll binding proteins and assembly of the photosynthetic electron transport complexes photosystem I and photosystem II (Kim et al., 1994). Light-induced activation of plastid transcription that occurs during leaf development in sorghum is correlated with an increase in the level of the plastid-encoded RNA polymerase (Schrubar et al., 1990). In mustard, light-induced changes in

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overall plastid transcription activity appear modulated through phosphorylation of sigma-like factors (Tiller and Link, 1993b). In addition to the regulation of overall plastid transcription activity by light, plastid genes, such as *psbA* (Klein and Mullet, 1990), *petE* (Haley and Bogorad, 1990), and *psbD* (Sexton et al., 1990b), are differentially activated by plant illumination. The biochemical mechanisms that mediate differential transcription of specific plastid genes in response to light have not been elucidated.

psbD encodes the photosystem II reaction center chlorophyll protein D2. Transcription of this gene is differentially stimulated when plants are illuminated by blue light but not by red or far-red illumination (Gamble and Mullet, 1989). Because D2 is degraded when plants are exposed to high light, it has been proposed that blue light activation of *psbD* transcription helps to maintain D2 levels under these conditions (Christopher and Mullet, 1994). The mechanism that activates *psbD* transcription was difficult initially to delineate because this gene is located in a complex operon that also encodes *psbC*, *psbK*, *psbI*, *orf62*, and *trnG* (Sexton et al., 1990a). Two *trnS* genes are encoded within this DNA region on the opposite strand. The *psbD* operon is transcribed from at least three different promoters (Sexton et al., 1990b). One of the *psbD* promoters is activated when plants are illuminated, and transcripts arising from this promoter become the most abundant *psbD* transcripts in chloroplasts of mature barley leaves (Christopher et al., 1992). Sequences surrounding the *psbD* blue light–responsive promoter (LRP) are conserved among cereals and dicots (Christopher et al., 1992), and light-induced accumulation of *psbD* transcripts has been observed in a wide variety of plants (Christopher et al., 1992; Wada et al., 1994).

In this article, we elucidate some of the factors required for transcription of the *psbD* LRP. In vitro transcription, gel shift, and DNase I footprinting assays identified a sequence-specific DNA binding factor that is required for transcription of the *psbD* LRP.

RESULTS

Organization of the *psbD* LRP

The barley chloroplast *psbD* LRP is shown in Figure 1. This promoter is located 572 bp upstream of the *psbD* open reading frame. The 5′ termini of transcripts generated from the light-responsive promoter in vivo span ~25 nucleotides (Christopher et al., 1992). This region is shown as a solid box in Figure 1, with +1 corresponding to the most abundant transcript generated in vivo and in vitro (see later discussion). The 100-bp DNA region upstream of the *psbD* light–induced transcript 5′ terminus is highly conserved among higher plants (~50% at the nucleotide level; Christopher et al., 1992). Sequences between the *psbD* LRP and the *psbD* open reading frame are not highly conserved (<10%). Alignment of the DNA sequence upstream of the *psbD* LRP from several plants revealed two blocks of conserved sequence. The first is located between −36 and −64 in the case of barley (AAAGTAAGTAGACCTGACT). This sequence was previously designated as containing aa′ and bb′ motifs (underlined in Figure 1; Christopher et al., 1992). The aa′ motif contains two AAG sequences. Additional AAG repeats were observed immediately upstream of the aa′ motif in cereals and somewhat farther upstream in black pine (Wakasugi et al., 1994). For convenience, this DNA region is referred to as the AAG box. The second conserved sequence is located between −71 and −100 in the case of barley. This region is referred to as the PGT (plastid GT) box because it is rich in GT sequences (Figure 1).

In Vitro Transcription from the *psbD* LRP

In a previous study, pea chloroplast transcription extracts were able to transcribe the barley *psbD* light-responsive promoter (Sexton et al., 1990b). To test whether barley chloroplast high-salt extracts (Orozco et al., 1985) could initiate transcription from the *psbD* LRP, a recombinant plasmid, pLRP185, that

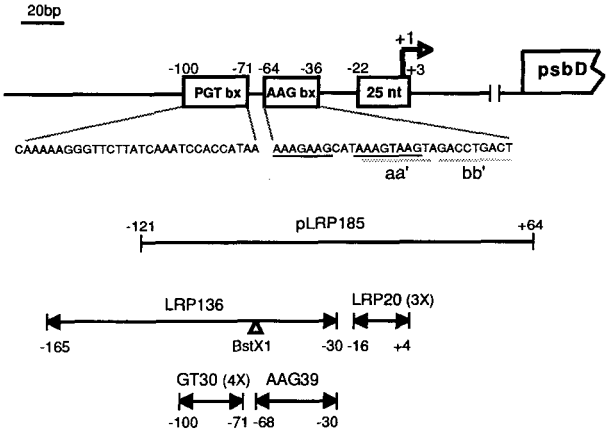

Figure 1. Schematic Representation of the Barley Chloroplast *psbD* LRP.

The top of the figure shows conserved sequence regions (boxed) that are present upstream of the *psbD* transcription initiation site induced by blue light. Transcription occurs from left to right. The *psbD* open reading frame is shown at the far right. The most abundant light-induced *psbD* transcript generated in vivo and in vitro is marked by an arrow. This position is designated +1. The boxes (bx) PGT and AAG represent two blocks of sequences that are conserved in several plants (Christopher et al., 1992). The sequence containing a repeated AAG sequence and the previously designated aa′ and bb′ motifs are underscored. LRP185 is a 185-bp DNA fragment from the *psbD* LRP (−121 to +64). This DNA was used in construction of the transcription plasmid pLRP185. The double-headed arrows represent DNA fragments from the *psbD* light-responsive promoter used for gel retardation, competition binding, and DNase I footprinting assays. GT30 and LRP20 represent four (4X) and three (3X) tandem copies of each region indicated in the figure. The open triangle indicates a BstXI restriction site. nt, nucleotide.

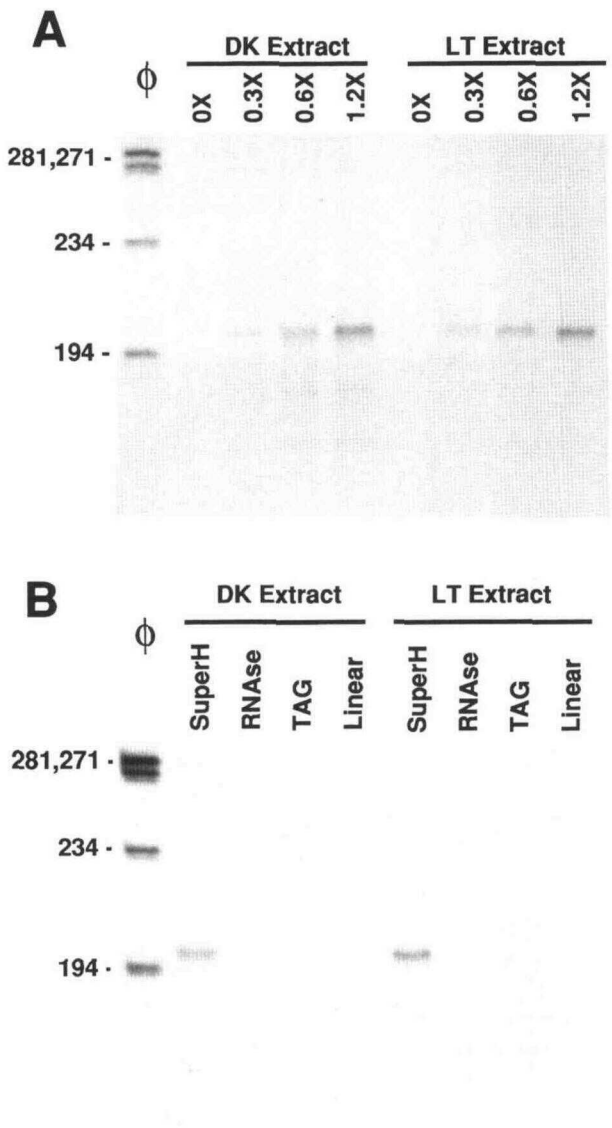

Figure 2. In Vitro Transcription of the *psbD* LRP in Barley Plastid Extracts.

(A) Transcription of the *psbD* LRP in plastid extracts. Transcription of the *psbD* LRP constructs was carried out in extracts of plastids isolated from barley plants grown 4.5 days in darkness (DK Extract) or similar plants illuminated for 4 hr (LT Extract). Primer extension analysis revealed the accumulation of transcripts with increasing concentrations of extract (1X represents a high-salt extract obtained from 10^8 plastids). Recombinant plasmid pLRP185 was used as the exogenous DNA template. Lane φ shows some of the φX174-HaeIII restriction fragments used as length markers. The lengths of these markers are indicated at left in base pairs.

(B) Transcription of the *psbD* LRP is inhibited by tagetitoxin and requires supercoiled templates. In vitro transcription of pLRP185 was performed as described in Figure 2A. *psbD* transcripts were produced when the plasmid was supercoiled (SuperH) but not when the template was linearized by digestion with XbaI (Linear). The products of transcription were sensitive to RNase (lanes marked RNAse). The addition of 10 μM tagetitoxin (TAG) inhibited transcription from the *psbD* promoter. DNA length markers are indicated at left in base pairs.

contains the *psbD* LRP, was added to barley chloroplast in vitro transcription extracts. As shown in Figure 1, pLRP185 contains 121 bp of barley *psbD* DNA upstream of the light-inducible transcription initiation site. Transcription initiated from the *psbD* LRP would extend into the plasmid vector sequences located in pLRP185. To avoid background from plastid RNA in the extracts, primer extension assays were carried out using a primer specific for vector sequences.

Transcription extracts were prepared from an equal number of plastids isolated from 4.5-day-old dark-grown barley plants or similar plants that had been illuminated for 4 hr. Figure 2A shows that both extracts synthesized similar amounts of RNA from pLRP185 as a function of extract concentration (per plastid or per microgram of protein). The in vitro–synthesized RNA identified by the most intense primer extension signal corresponds to the most abundant RNA synthesized from the *psbD* LRP in vivo (Christopher et al., 1992; see later discussion). The amount of transcript synthesized in vitro increased with an increasing amount of both etioplast and chloroplast extracts and then declined (data not shown). Figure 2B shows that transcripts were produced when supercoiled templates were added to extracts but not when templates were linearized. Transcription was sensitive to tagetitoxin, and the products of transcription were sensitive to RNase (Figure 2B). The concentration of tagetitoxin (10 μM) that inhibited in vitro transcription from the *psbD* LRP was previously found to completely inhibit transcription of *trnM* in pea chloroplast extracts (Mathews and Durbin, 1990). Tagetitoxin also prevents light-induced accumulation of *psbD* RNAs in vivo (Sexton et al., 1990b).

The *psbD* LRP Is Not Recognized by *Escherichia coli* RNA Polymerase with the Same Specificity as Plastid RNA Polymerase

The ability of *E. coli* RNA polymerase to recognize the *psbD* LRP was tested, and the results are shown in Figure 3. Transcripts produced from the *psbD* LRP by plastid extracts (lanes 4 and 7) were compared with transcripts synthesized from this same DNA in the presence of *E. coli* RNA polymerase (lane 10). Primer extension assays showed that the major transcript synthesized by *E. coli* RNA polymerase had its 5′ terminus upstream from the transcripts produced by plastid extracts. Furthermore, the level of transcription from the *psbD* LRP by *E. coli* RNA polymerase was much lower than that from the *psbA* promoter. In contrast, the *psbA* promoter, containing −10/−35 promoter elements, was transcribed similarly by plastid transcription extracts (lanes 13 and 16) or *E. coli* RNA polymerase (lane 19), and transcripts having the same 5′ termini were produced by both sources of RNA polymerase.

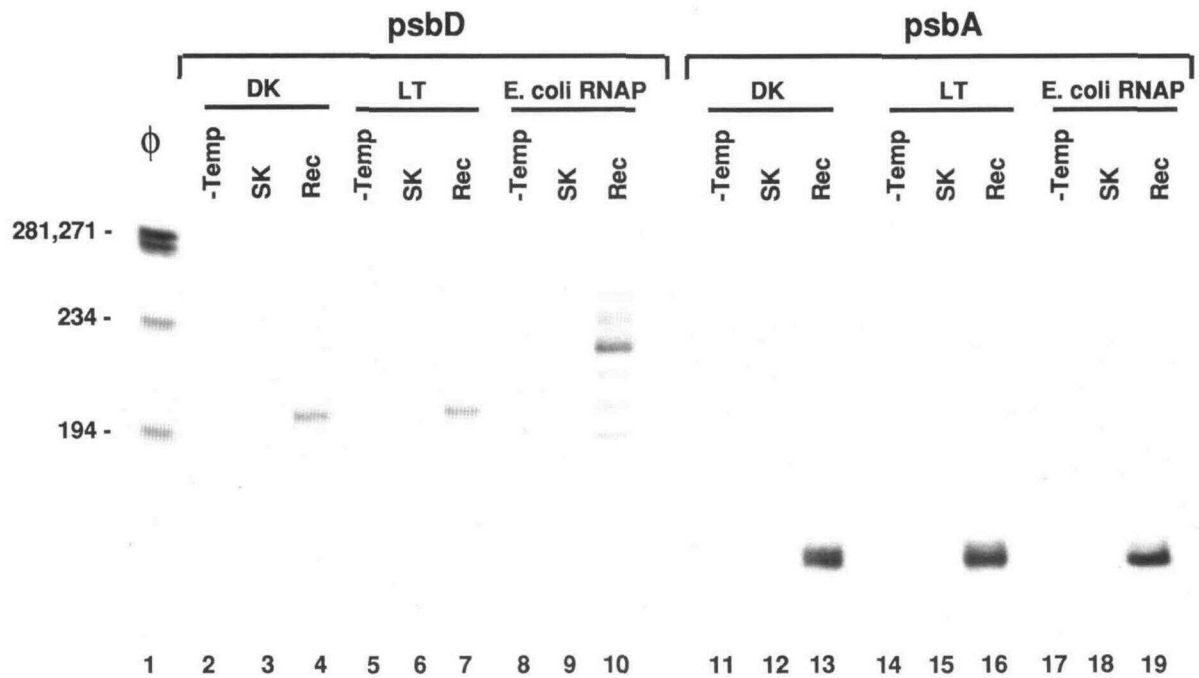

Figure 3. In Vitro Transcription of *psbD* LRP by the Plastid High-Salt Extracts and the *E. coli* RNA Polymerase Holoenzyme.

In vitro transcription of pLRP185 was used to assess transcription from the *psbD* LRP (lanes marked *psbD*), whereas transcription from the *psbA* promoter was assessed using the plasmid p*psbA*138 (lanes marked *psbA*). Transcription was performed in extracts of plastids from 4.5-day-old dark-grown plants (DK) and plastid extracts from similar plants that had been illuminated for 4 hr (LT) or using 0.1 units of *E. coli* RNA polymerase (lanes labeled E. coli RNAP). –Temp, SK, and Rec indicate transcription reactions with no DNA template, with the pBluescript SK+ plasmid, or with the recombinant plasmid template, respectively. For lanes 10 and 19, one-tenth volume of the in vitro transcription reaction was loaded onto the gel. DNA length markers in base pairs are indicated at left.

The 5′ termini of the *psbD* transcript produced by plastid extracts and *E. coli* RNA polymerase were fine mapped using primer extension analysis in Figure 4. The 5′ termini of RNAs synthesized by plastid extracts were mapped in the middle of the sequence GATATATAA(A), which represents the greatest concentration of signals observed when in vivo RNA is analyzed (Christopher et al., 1992). In contrast, the 5′ end of the major transcript produced by *E. coli* RNA polymerase was mapped 23 bp upstream of the RNA transcripts synthesized by plastid RNA polymerase in vitro.

The AAG Box Domain Is Required for Transcription from the *psbD* LRP

The requirement for sequences upstream of the *psbD* transcription initiation site was investigated using the barley plastid in vitro transcription extracts. The diagram in Figure 5A shows the plastid DNAs tested for transcription activity. As shown earlier, pLRP185 was transcribed accurately in plastid extracts (Figure 5B, lane 1). DNA constructs that lacked 5′ sequences containing the PGT box were also able to support transcription in vitro (Figure 5B, lane 2). Similarly, insertion of 175 bp between the AAG box and the PGT box had little influence on transcription (lane 3). In contrast, deletion of sequences containing the AAG box (−68 to −39) completely eliminated transcription from the *psbD* LRP (Figure 5B, lane 4). Similar results were obtained with plastid extracts from 4.5- or 7.5-day-old barley seedlings grown in darkness or illuminated (data not shown).

Identification of Proteins in Plastid Transcription Extracts That Interact with the *psbD* LRP

The conservation of sequences upstream of the *psbD* light-responsive transcription start site and results from in vitro transcription assays prompted us to search for sequence-specific DNA binding proteins that associate with the *psbD* promoter. As a starting point, gel retardation assays were carried out by mixing DNA probes containing 136 bp (LRP136, Figure 1) of the *psbD* LRP region with plastid high-salt transcription extracts obtained from 4.5-day-old dark-grown barley plants. Figure 6A shows that LRP136 binds to proteins to form multiple complexes and that complex composition was altered with increasing levels of poly(dI-dC). The major complexes are

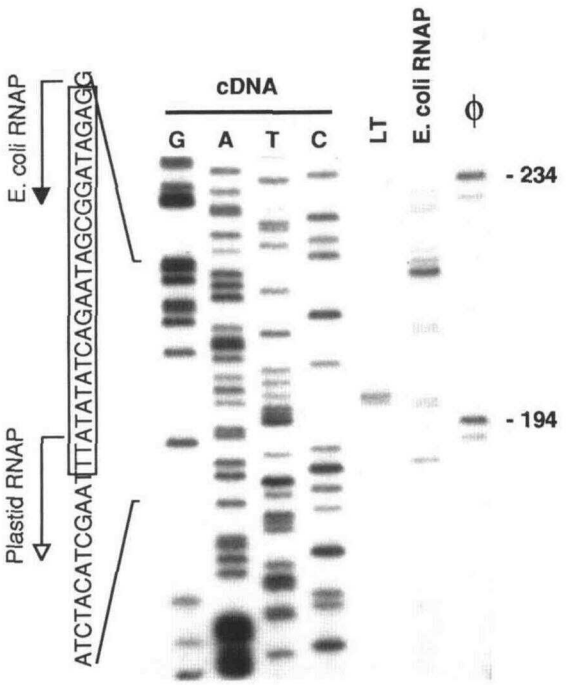

Figure 4. Fine Map of the 5′ Ends of Transcripts Synthesized from the *psbD* LRP in Chloroplast High-Salt Extracts and by the *E. coli* RNA Polymerase Holoenzyme.

Lanes designated cDNA (G, A, T, and C) refer to dideoxy nucleotide sequencing reactions performed on pLRP185 using the same primer as that used for primer extension analysis of transcripts. The cDNA sequence in the region of transcription initiation is indicated at left. Transcript 5′ termini generated by plastid RNA polymerase present in the high-salt extract (lane LT) or by *E. coli* RNA polymerase (lane E. coli RNAP) are indicated by an open or solid arrow. The boxed region is the location where multiple *psbD* RNA 5′ termini have been observed. DNA length markers are shown at right in base pairs.

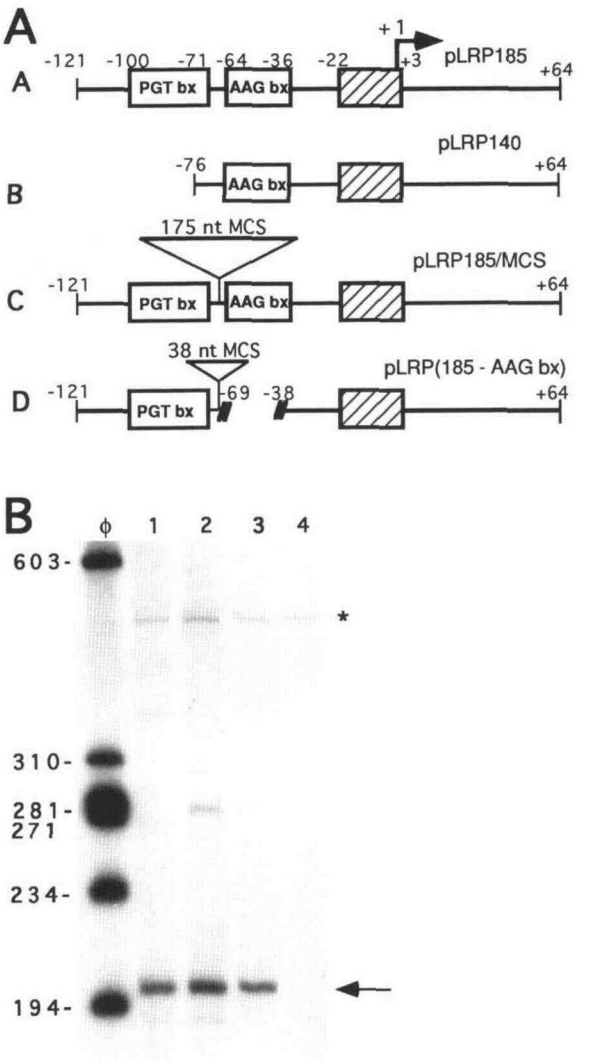

Figure 5. In Vitro Transcription of DNAs Containing Modified *psbD* LRP Domains.

(A) Schematic representation of the recombinant plasmids used for in vitro transcription experiments shown in **(B).** Construct A is the original pLRP185 described in Figure 1. Construct B (pLRP140) contains a 5′ deletion that removes most of the PGT box sequences. Construct C is pLRP185, with an insertion containing the 175 bp of the multiple cloning site (MCS) from the KS vector (pLRP185/MCS). Construct D (pLRP[185-AAG bx]) was derived from construct C by deletion of most of the MCS (38 bp remain) and deletion of the AAG box (AAG bx) region (−38 to −69).

(B) Transcription of modified *psbD* LRPs. The constructs described in **(A)** were transcribed in vitro in barley plastid extracts and transcripts analyzed by primer extension analysis. Lanes 1 to 4 represent the results obtained with constructs A to D, respectively. The plastid high-salt extract (5.2×10^8 plastids per reaction) used for the experiments was obtained from 7.5-day-old dark-grown barley plants. Similar results were obtained using extracts of 4.5-day-old plants. The arrow indicates the primary transcript synthesized from the *psbD* LRP in vitro. An asterisk marks the position of a signal produced from extracts in the absence of template. DNA length markers are indicated at left in base pairs.

designated A to E for convenience. All of the retarded bands were removed by protease treatment, indicating that they are formed by protein–DNA interactions (data not shown).

Proteins interacting with LRP136 were investigated by incubating radiolabeled LRP136 with protein extracts in the presence of various competitor DNAs prior to gel shift assay (Figure 6B). In the presence of poly(dI-dC), the addition of unlabeled LRP136 reduced the abundance of complexes A, C, and D dramatically. In contrast, only complexes A and C showed reduced abundance in the presence of unlabeled GT30 (tetramer of PGT box sequences). LRP20 (see Figure 1) did not compete with any of the complexes (Figure 6B, lanes 6 and 7). These results indicate that complexes A and C contain proteins that bind to LRP136 within the GT30 region, whereas complex D is composed of protein(s) that binds outside the GT30 and LRP20 sequences. The abundances of complexes B and E decreased gradually in the competition experiments, suggesting that these complexes are formed by

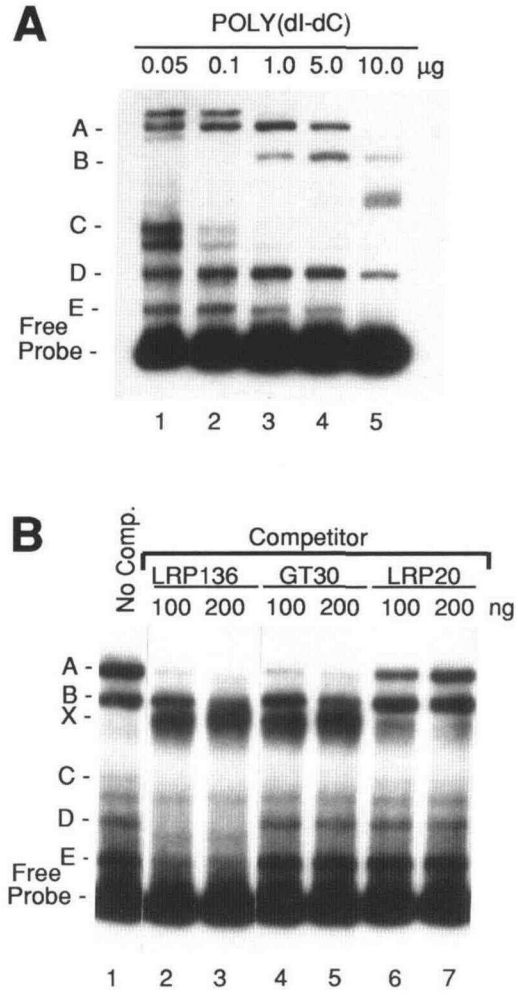

Figure 6. Gel Retardation and Competition Binding Experiments with the Radiolabeled LRP136 DNA Fragment and Plastid Transcription Extracts.

(A) Gel retardation experiment in the presence of various amounts of the nonspecific competitor DNA poly(dI-dC)·(dI-dC). Plastid high-salt extracts obtained from 4.5-day-old dark-grown barley plants were used to obtain data shown here and in **(B)**. The amount of poly(dI-dC)·(dI-dC) in each reaction is indicated above the lanes.

(B) Competition binding experiments in the presence of nonradiolabeled DNA fragments from the *psbD* LRP region. Binding reactions were performed in the presence of either 100 or 200 ng of the nonradiolabeled competitor DNA fragment indicated above the lanes (LRP136, GT30, and LRP20; see Figure 1). All binding reactions contained 1.0 µg of poly(dI-dC)·(dI-dC). A control reaction (lane marked No Comp.) was performed in the absence of any competitor DNA. The new complex observed in the presence of competitor LRP136 and GT30 is labeled X.

The major complexes observed in **(A)** and **(B)** are labeled A, B, C, D, and E, and the migration of the free probe is indicated.

either abundant, nonspecific DNA binding proteins or proteins that have a low affinity for competing DNA. A complex marked X appears below complex B whenever complexes A and C were removed by competitive binding (Figure 6B, lanes 2 to 5). Complex X or another complex with slightly faster migration can also be observed in the presence of 10 µg of poly(dI-dC) (see Figure 6A, lane 5). Competition assays indicated that complex X arises from non-sequence-specific binding (data not shown).

Specific Protein Binding to the AAG Box Region

Complex D was formed by proteins interacting with LRP136 (Figure 6). The abundance of complex D was not reduced by the addition of GT30 or LRP20 to protein extracts. Therefore, complex D could be formed by the association of protein(s) with the AAG box sequences in LRP136 that are located downstream of the GT30 domain. To test this possibility, the ability of AAG39 (a DNA fragment that contains the AAG sequences; see Figure 1) was used in competition binding assays. Figure 7 shows that the addition of AAG39 to protein extracts

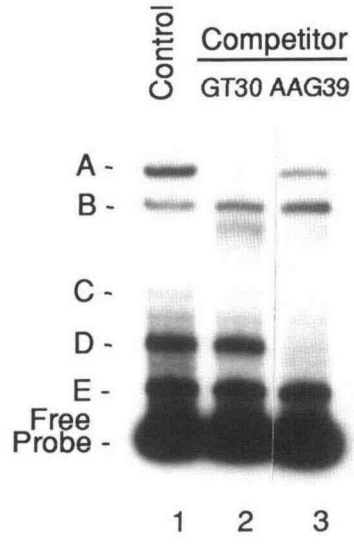

Figure 7. *Gel Retardation Competition Experiment Showing That the* Factor Forming Complex D Binds to the AAG-Rich Domain of the *psbD* LRP.

The radiolabeled probe LRP136 DNA fragment and extracts obtained from 4.5-day-old dark-grown barley plants were used for binding reactions. Binding reactions were performed in the presence of 100 ng of the nonradiolabeled competitor DNA fragment indicated above the lanes (GT30 and AAG39). All binding reactions contained 1.0 µg of poly(dI-dC)·(dI-dC). The major complexes observed in the absence of specific competitor DNA (control reaction, lane 1) are labeled A, B, C, D, and E. Migration of the free probe is indicated.

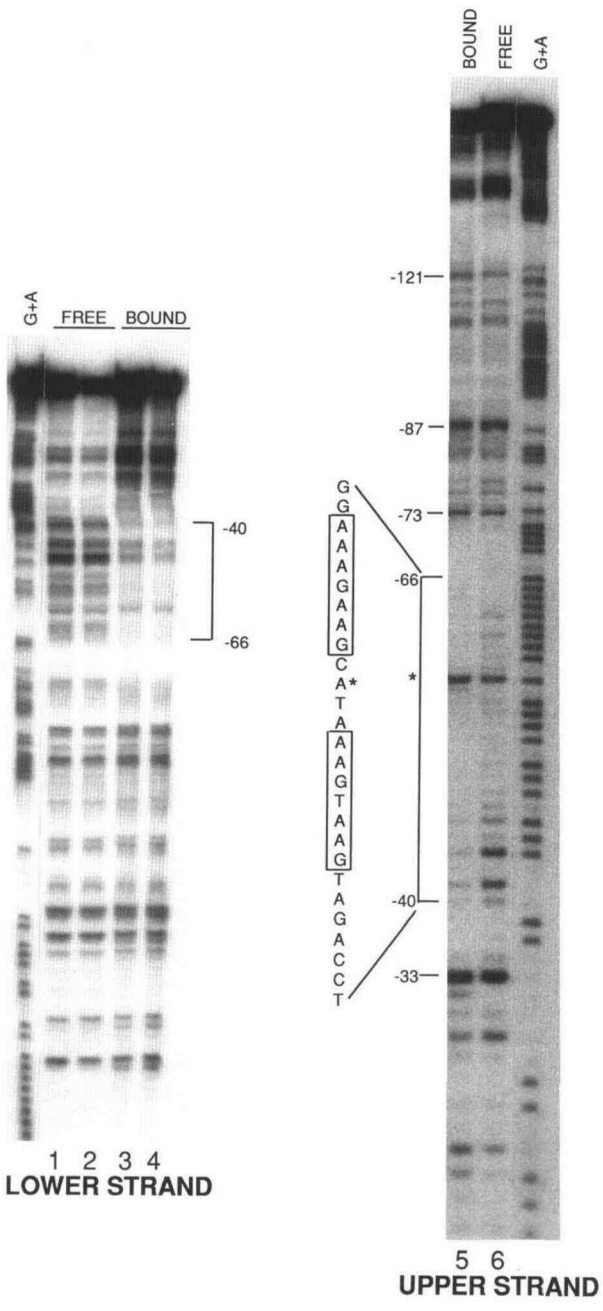

Figure 8. DNase I Footprint Analysis of the *psbD* LRP in the Presence of Proteins That Form Complex D.

Lower Strand and Upper Strand indicate LRP136 DNA fragments radiolabeled on the lower or upper strand. Bound indicates DNase I digestion of the mixture of DNA probe and 0.3 to 0.4 M KCl fraction from heparin-agarose chromatography. Free refers to DNase I digestions carried out in the absence of protein extract. DNase I digestion was performed for either 1 min (lanes 1, 3, 5, and 6) or 2 min (lanes 2 and 4). The brackets indicate the regions protected from partial DNase I digestion. The sequence corresponding to the protected region of the upper strand is indicated based on (A+G) chemical sequencing (Maxam and Gilbert, 1990), with the AAG repeat sequences in the protected region boxed. The asterisks indicate the nucleotide in the upper strand that was not protected from DNase I digestion.

significantly reduced the abundance of complexes D and C (Figure 7; compare lanes 1 and 3). In contrast, the addition of AAG39 reduced the abundance of complex A to only a small extent. This result indicates that the protein(s) forming complex D is interacting specifically with the AAG39 DNA fragment. In contrast, the protein(s) forming complex C either binds with less specificity or is modified by other protein interactions because its binding is reduced by the addition of either GT30 or AAG39 to protein extracts (Figure 7, lanes 2 and 3).

Additional fine mapping of the protein binding sites in the AAG39 fragment was accomplished by DNase I footprinting experiments. Proteins that form complex D were enriched by separation of plastid high-salt transcription extracts on heparin-agarose columns. Fractions eluting between 0.3 and 0.4 M KCl were enriched in a protein(s) that forms complex D and lacked most proteins forming complexes A, B, C, E, and X (data shown below). This separation helped to delineate the protein binding sites for complex D by DNAse I footprinting experiments, because LRP136 interacts with multiple proteins at different binding sites. For footprint analysis, proteins from the 0.3 to 0.4 M KCl fraction were mixed with radiolabeled LRP136 and then incubated for various times with DNase I. The resulting digests of protein-free LRP136 and LRP136 complexed with protein were phenol extracted and separated on sequencing gels. Figure 8 shows that the sequence from −40 to −66 was protected from digestion by proteins that form complex D. This protected region included the two AAG sequence repeats that are boxed in Figure 8. In addition, the sequence GACC was also protected. Small differences in the degree and sites of protection were observed between the two strands. For example, the A residue marked by an asterisk between the protected reiterative AAG sequences was not protected from DNase I digestion in the case of the upper strand. This observation suggests that this A nucleotide is not critical for protein binding. Interestingly, the sequence downstream of the protected region in the lower strand and a few nucleotides in the corresponding region in the upper strand showed hypersensitivity to DNase I cleavage. This result suggests that protein binding to the AAG box region induced the downstream DNA to alter its structure.

AGF Binding to the AAG Box Is Required for Transcription from the *psbD* LRP

The relationship between AGF (for AAG binding factor) binding to the AAG box and transcription from the *psbD* LRP was

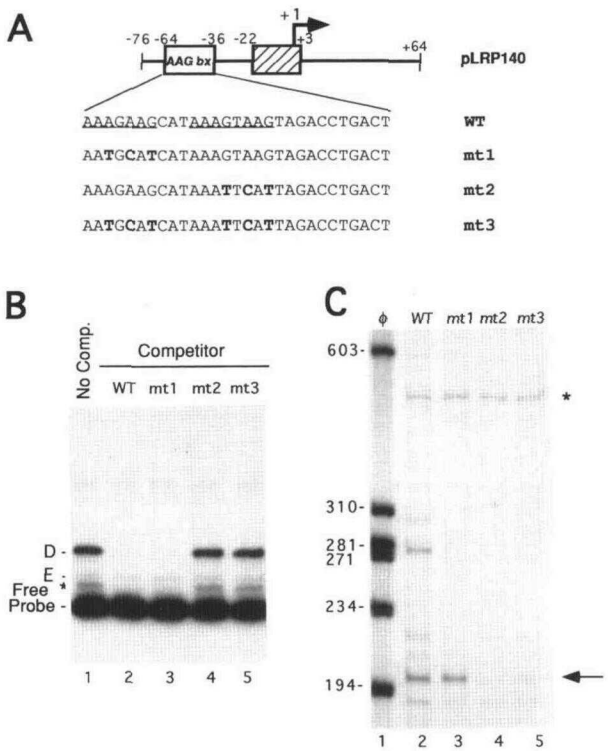

Figure 9. Gel Retardation Competition and In Vitro Transcription Experiments Using AAG Box Mutants.

(A) Schematic representation of the recombinant plasmids used for the preparation of competitor DNA fragments for competition binding experiments shown in **(B)** and in vitro transcription experiments shown in **(C).** The wild-type construct (WT) is the original pLRP140 described in Figure 5A whose AAG box sequences are not changed. The AAG repeats are underlined. Constructs mt1, mt2, and mt3 are modified pLRP140 plasmids containing AAG box mutations that are shown in boldface letters.

(B) Competition binding experiments with the radiolabeled LRP136 DNA fragment and proteins enriched in AGF by heparin-agarose chromatography. Binding reactions were performed in the presence of 100 ng of nonradiolabeled competitor DNA fragment containing either the native AAG box sequences or mutated AAG sequences indicated above the lanes (see **[A]** for sequences). Each competitor DNA fragment was prepared by restriction digestion of corresponding recombinant plasmid. A control reaction (lane 1) was performed in the absence of any competitor DNA. Complexes identified as D and E are designated based on direct comparison of migrations with those formed by unfractionated plastid extracts on the same gel (data not shown). A previously undetected complex is marked with an asterisk. The migration of the free probe is indicated.

(C) In vitro transcription from modified *psbD* LRP constructs containing point mutations in the AAG box. The constructs described in **(A)** were transcribed in vitro in barley plastid extracts. Transcripts were analyzed by primer extension analyses (lanes 2 to 5). The plastid high-salt extract (5.2 × 10^8 plastids per reaction) used for the experiments was obtained from 7.5-day-old dark-grown barley plants. Similar results were obtained using extracts of 4.5-day-old plants. The arrow indicates the primary transcript synthesized from the *psbD* LRP in vitro. An asterisk marks the position of a signal produced from extracts in the absence of template. DNA length markers are indicated at left in base pairs.

analyzed by introducing point mutations in the AAG box. As shown in Figure 9A, one or both of the repeated AAG sequences were selectively changed. Figure 9B shows that nonradiolabeled DNA fragments containing mutations in the AAG sequence regions (mt2 and mt3) blocked their ability to compete for binding complex D. Complex D is the major complex formed when radiolabeled LRP136 is added to proteins in 0.3 to 0.4 M KCl fraction of heparin-agarose chromatography (mentioned previously; also see Figure 9B, lane 1). However, several minor complexes, including complex E, were observed. The abundance of complex D, along with the fastest migrating complex below complex E (marked with an asterisk), which was enriched by heparin chromatography, was reduced by the addition of DNA fragments containing either native chloroplast AAG sequences or point mutations in the upstream AAG box (Figure 9B, lanes 2 and 3). In contrast, the addition of DNA fragments containing point mutations either in the downstream AAG (mt2) or in both AAG repeats (mt3) were unable to compete for binding with any of the DNA–protein complexes (Figure 9B, lanes 4 and 5). These results show that the downstream AAG sequences are important for AGF binding activity.

Figure 9C shows that mutations of specific downstream AAG sequences in plasmid pLRP140 inhibited transcription from the *psbD* LRP in barley extracts (lanes 4 and 5). Point mutations in the upstream AAG box (pLRP140-mt1) that did not affect AGF binding (Figure 9B, lane 3) did not alter transcription from the *psbD* LRP (Figure 9C, lane 3). Together, these results indicate that AGF binding to the AAG box is required for transcription from the *psbD* LRP.

DISCUSSION

Earlier studies have shown that pea and barley chloroplast membrane high-salt extracts accurately transcribe *rbcL* and *psbA* promoters (Orozco et al., 1985). In this study, similar extracts were found to transcribe accurately the *psbD* LRP. Transcription in the plastid extracts was blocked by tagetitoxin, an inhibitor of plastid transcription (Mathews and Durbin, 1990). This result is consistent with tagetitoxin's ability to inhibit light-induced accumulation of *psbD* transcripts in barley seedlings (Sexton et al., 1990b). Transcription of the *psbD* LRP required supercoiled templates similar to transcription of other plastid genes (Jolly and Bogorad, 1980; Orozco et al., 1985; Chen and Orozco, 1988).

Transcription of the *psbD* LRP occurred when templates containing 76 or 121 bp of DNA upstream of the transcription initiation site were added to in vitro transcription extracts. However, removal of sequences 39 to 68 bp (AAG box) upstream of the initiation site completely inhibited transcription. DNA from this same region interacted with proteins to form the sequence-specific binding complex D (Figure 7). Complex D formed when LRP136 was added to extracts, and this complex could be competed specifically by AAG39 but not by other DNA fragments from the *psbD* LRP (Figures 6 and 7). Moreover,

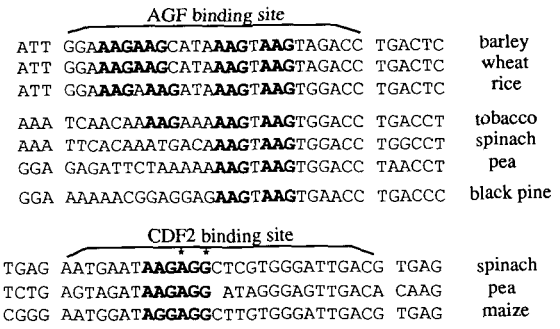

Figure 10. Sequence Comparison of the AGF and CDF2 Binding Sites.

The AGF binding site in the barley psbD LRP is aligned with similar sequences located upstream of *psbD* in other plants. AAG repeat sequences in the AGF binding site are shown in boldface letters. The CDF2 binding site from the spinach 16S rDNA promoter, shown below, is compared with similar sequences in pea and maize. The location of mutations that block binding of CDF2 to the spinach 16S rDNA promoter is indicated by asterisks (Iratni et al., 1994).

DNase I protection assays showed that proteins in barley plastid transcription extracts bind to and protect the region from −40 to −66 in the *psbD* LRP. Together, these results indicate that a transcription factor, AGF, binds in a sequence-specific manner to the −40 to −66 region upstream of the *psbD* site of transcription initiation. Furthermore, point mutations in the AAG boxes revealed that the downstream AAG sequences are required for AGF binding and transcription from the *psbD* LRP. Based on this information, we speculate that AGF is a specificity factor required for transcription of the *psbD* LRP. To our knowledge, AGF is a newly identified sequence-specific DNA binding protein that stimulates transcription in plastids. This factor is distinct from plastid sigma-like factors (Tiller and Link, 1993b) that bind to DNA only in the presence of RNA polymerase to modulate promoter recognition.

Figure 10 shows that the sequence of the *psbD* AGF binding site is highly conserved among higher plant plastid genomes. In the cereals, this region contains two AAG repeats, whereas only one pair of AAG sequences is present in most dicots except for tobacco. A recently described sequence-specific DNA binding factor, termed CDF2 (for chloroplast DNA binding factor 2) interacts with the 16S rDNA promoter (Iratni et al., 1994). The binding site for CDF2 is shown in Figure 10 to facilitate comparison with the AGF binding site. Both sites are ~25 bp, are AG rich, and contain AAG sequences. Mutations in the sequence AAGAGG result in a loss of CDF2 binding (Figure 10; sequence changes are marked by asterisks). CDF2 is capable of binding to DNA in the absence of RNA polymerase, and binding can occur at NaCl concentrations as high as 0.6 M (Iratni et al., 1994). Similarly, AGF is able to bind to DNA in the absence of RNA polymerase, and binding can occur in high KCl (0.75 M; data not shown). The AGF binding site is located ~40 to 60 bp upstream from the *psbD* transcription initiation site. Similarly, the CDF2 binding site is located in approximately this position relative to one 16S rDNA promoter in pea (Sun et al., 1989) and in maize (Strittmatter et al., 1985), although in spinach it is within 20 nucleotides of the transcription initiation site (Baeza et al., 1991). Both factors elute from heparin columns in a similar salt range, and both form relatively small gel shift complexes.

These factors have many similar properties and may be members of a larger family of DNA binding proteins; however, it is likely that CDF2 and AGF have different functions for several reasons. First, transcription of 16S rDNA is highest in young barley plastids and decreases dramatically as chloroplasts mature (Baumgartner et al., 1993). In contrast, transcription of the *psbD* LRP is light inducible, and high levels of the *psbD* LRP transcripts are maintained in mature chloroplasts (Christopher and Mullet, 1994). Second, CDF2 has been shown to inhibit transcription of the 16S rDNA by the *E. coli*–like plastid RNA polymerase and proposed to stimulate transcription of the 16S rDNA by another type of plastid RNA polymerase (Iratni et al., 1994). In contrast, AGF was required for transcription from the *psbD* LRP. Clearly, additional binding site, competition, and cloning studies are needed to elucidate fully the relationship between AGF and CDF2.

Illumination of 4.5-day-old dark-grown barley plants for 4 hr activates transcription from the *psbD* LRP (Sexton et al., 1990b). However, extracts from these two plastid populations transcribed the *psbD* LRP equally well in vitro. Transcription from the *psbD* LRP was dependent on AGF and an RNA polymerase, suggesting that these factors may not be the target of blue light regulation. Alternatively, the influence of light may not be observed in vitro due to the stoichiometry of template and DNA binding factors or changes in protein modification during isolation of the extract. Other protein factors, such as those forming the PGT complex, may modulate light regulation. The PGT binding factor interacts specifically with sequences upstream of the AGF binding site. In a previous study by Wada et al. (1994), it was reported that transcription from the wheat *psbD* LRP could be stimulated by addition of flow-through fractions from a heparin-Sepharose column to RNA polymerase fractions. It is possible that positive transcription factors such as AGF are present in the flow-through fractions and can activate transcription from the *psbD* LRP when mixed with fractions containing plastid RNA polymerase.

Plastids contain at least two different RNA polymerases (for a review, see Igloi and Kössel, 1992). The first is *E. coli*–like, and some of its subunits are encoded by plastid genes (*rpoA*, *rpoB*, *rpoRC1*, and *rpoC2*). This polymerase recognizes −10/−35 promoter elements and transcribes genes such as *rbcL* and *psbA*. A second RNA polymerase has been observed under conditions where expression of the plastid-encoded RNA polymerase is low (Morden et al., 1991; Hess et al., 1993) or after RNA polymerase purification (Narita et al., 1985; Lerbs-Mache, 1993). The promoter specificity of this second RNA polymerase is not known, but −35 promoter sequences are not thought to be required for transcription (Lerbs-Mache, 1993). The *psbD* LRP contains potential −10 (TATTCT) and −35 (TTGAAT) promoter sequences upstream of the site of transcription initiation (located at position −7 to −12 and −28 to

−33, respectively). However, the *psbD* LRP was not recognized by *E. coli* RNA polymerase with the same specificity as plastid RNA polymerase, even though this enzyme utilized the *psbA* promoter with high fidelity. Furthermore, promoters that lack or have point mutations in AGF binding site but retain the −35/−10 sequences were not transcribed in plastid extracts. Further information is required to determine which plastid RNA polymerase is mediating transcription from the *psbD* LRP.

METHODS

Plant Growth

Barley (*Hordeum vulgare* var Morex) seedlings were grown in controlled environmental chambers at 23°C as described by Kim et al. (1993). Seedlings were germinated and grown in complete darkness. After 4.5 days, the dark-grown seedlings were either harvested or transferred to a continuously illuminated chamber (fluorescent plus incandescent light, light intensity of 250 $\mu E\ m^{-2}\ sec^{-1}$) for an additional 4 or 48 hr before harvesting. Plastids were isolated from the top 4 cm of primary leaves of barley seedlings by Percoll gradient (35 to 75%) centrifugation (Klein and Mullet, 1986). The concentration of plastids was quantitated (plastids per microliter) by phase contrast microscopy, using a hemacytometer.

Preparation of Transcriptionally Active Extracts from Plastids

The transcriptionally active high-salt extracts from plastids were prepared essentially according to Orozco et al. (1985). However, 45% ammonium sulfate was used for the precipitation of the proteins after high-salt extraction. After the final dialysis step against 1 × DEAE buffer (Orozco et al., 1985), protein concentrations were determined by the method of Bradford (1976) using the Bio-Rad protein assay kit. The extract was divided into aliquots, frozen in liquid nitrogen, and stored at −80°C.

Transcription of Exogenous DNA Templates

RNA polymerase activity in high-salt extracts of 1.2×10^8 plastids (if not specified otherwise) was assayed on 200 ng of exogenous superhelical DNA templates in 40 μL of reaction mixture as follows: 12 mM Hepes-KOH, pH 8.0, 60 mM KCl, 10 mM $MgCl_2$, 10.25 mM DTT, 25 mM Tricine-KOH, pH 8.0, 0.25 mM EDTA, 2.5% glycerol, 0.25 mM ε-amino-η-caproic acid, 0.5 mM GTP, 0.5 mM CTP, 0.05 mM ATP, 0.05 mM UTP, and 12.5 units of RNAsin (Promega). After a 60-min incubation at 30°C, the reaction mixture was extracted with phenol-chloroform and precipitated with ethanol. As a control reaction, several in vitro transcription reactions were further treated with 10 μg/mL of RNase A for another 10 min at 30°C before phenol-chloroform extraction. The resulting nucleic acids were treated with RQ1-DNase (Promega), followed by phenol extraction and ethanol precipitation, and then subjected to primer extension analyses.

Primer Extension Analyses

Primer extension analysis of the in vitro–transcribed RNA was performed as described by Christopher et al. (1992). However, a total of 5×10^5 cpm of ^{32}P-labeled primers and 50 units of Superscript II RNase H–reverse transcriptase (GIBCO BRL) was used. The T3 primer was used to analyze the transcripts originated from the plasmid p*psb*A138; the (−) 40 primer was used for other recombinant plasmids, including pLRP185 and pLRP140. Results were analyzed on 6% (5% for Figure 4) polyacrylamide 8.3 M urea gels.

Recombinant Plasmid Construction

Oligonucleotides used for cloning were purchased from the Texas A & M University Oligonucleotide Synthesis Service. pLRP185 and p*psb*A138, the recombinant plasmids used for in vitro transcription experiments, were constructed by inserting the polymerase chain reaction (PCR)-amplified barley chloroplast DNA fragments, LRP185 and psbA138, into SmaI and EcoRV of pBluescript SK+ (Stratagene), respectively. LRP185 and *psb*A138 fragments extend from −121 to +64, flanking the transcription initiation site of the *psbD* light-responsive promoter (LRP) in vitro in this study, and −80 to +58, flanking the *psbA* transcription initiation site (Boyer and Mullet, 1988), respectively.

Three modified pLRP185 plasmids were constructed as follows: pLRP140 was constructed by inserting a PCR-amplified DNA fragment, extending from −76 to +64, flanking the transcription initiation site of the *psbD* LRP, into a SmaI site of pBluescript SK+. For the construction of pLRP185/MCS (multicloning site), the previously cloned pBE3-5, which contains 837-bp EcoRI-EcoRI DNA fragment spanning the LRP185 region (5372 to 6244; Sexton et al., 1990a) in pTZ18 vector (Pharmacia), was first linearized by cutting a BstXI site, which is located 65 to 76 bp upstream of the transcription initiation site of the *psbD* LRP in vitro. Then, the BstXI 3′ overhangs were removed by S1 nuclease digestion and ligated with 175-bp BssHII-BssHII MCS DNA fragment, which was released from pBluescript KS+, gel purified, and 3′ filled in by the Klenow DNA polymerase I. The resulting recombinant plasmid, pBE3-5/MCS, turned out to contain the MCS fragment in an orientation opposite the one in pBluescript KS+. The pLRP185/MCS was then constructed by inserting PCR-amplified DNA fragment LRP185/MCS, which originated from the DNA template pBE3-5/MCS, into a SmaI site of pBluescript SK+. The LRP185/MCS fragment, which contains the 175-bp BssHII-BssHII MCS, extends from −121 to +64, flanking the transcription initiation site of the *psbD* LRP in vitro. For preparation of pLRP(185-AAG bx), the pBE3-5/MCS was digested at both BstXI and XhoI sites present in the MCS fragment, then unidirectional digestion was done from the XhoI site toward the transcription initiation site of the *psbD* LRP by using exonuclease III (Erase-A-Base kit; Promega). The resulting deletion product, which contains a deletion spanning from −70 to −39, was used as a DNA template to generate PCR-amplified DNA fragment LRP(185-AAG bx). This DNA fragment, extending from −121 to +64 region with the aforementioned deletion, was then inserted into a SmaI site of pBluescript SK+ to give rise to pLRP(185-AAG bx).

Recombinant plasmids pLRP140-mt1, pLRP140-mt2, and pLRP140-mt3, which contain point mutations in the AAG box of pLRP140, were constructed based on PCR cloning. First, DNA fragments that contain point mutations in the AAG box and span the −87 to +64 region of the *psbD* LRP were obtained by PCR amplification using pLRP185 (described previously) as a DNA template. The 5′ end primers used for PCR amplification were designed based on mRNA-like sequences

as follows: AAG-mt1, 5′-TATCATCTAGACCATAAAATTGGAAtGcAtCAT (−87 to −45 region of the *psbD* LRP); AAG-mt2, 5′-TATCATCTAGA CCATAAAATTGGAAAGAAGCATAAAtTcAtTA (−87 to −45 region of the *psbD* LRP); AAG-mt3, 5′-TATCATCTAGACCATAAAATTGGAAtGcAtCATAAAtTcAtTA (−87 to −45 region of the *psbD* LRP). Engineered XbaI sites are underscored and nucleotide changes from the native chloroplast sequences are designated by lowercase letters. The PCR products were digested by XbaI, gel purified, and ligated into XbaI and SmaI sites of pBluescript SK+. The resulting plasmids were named pLRP140-mt1, pLRP140-mt2, and pLRP140-mt3, respectively.

Recombiant plasmid pLRP136 was constructed from the previously cloned recombinant plasmid pLRPI3, which contains the PCR DNA product extending from −391 to −30 upstream of the *psbD* LRP transcription initiation site in vitro to the SmaI site of pBluescript KS+ vector. After restriction digestion of the multiple cloning site in pLRPI3 with ClaI and EcoRI, a unidirectional DNA deletion from the EcoRI site was done using exonuclease III to generate the upstream deletion clone pLRP136, containing 136 bp (−165 to −30) of the *psbD* LRP upstream region. Recombinant plasmid pAAG39, representing 39 bp (−68 to −30) of the *psbD* LRP upstream region, was obtained by inserting a 67-bp BstXI-BstXI restriction fragment of pLRP136 (Figure 1) into the SmaI site of pBluescript KS+ after removing 3′ overhangs by S1 nuclease digestion. The orientation of all the inserted DNA fragments described previously, except *psb*A138, was the same as that of the *lac*Z gene transcription. Superhelical recombinant plasmids were obtained according to Sambrook et al. (1989).

pGT30 and pLRP20, representing four tandem copies of the −100 to −71 region (GT30) and three tandem copies of the −16 to +4 region (LRP20) of the *psbD* LRP, respectively (Figure 1), were obtained by inserting either the GT30 or LRP20 DNA fragment into either the SmaI or BamHI site of pBluescript KS+, respectively. For the preparation of multimerized DNA fragments GT30 and LRP20, each monomeric unit of annealed synthetic oligonucleotides was self-ligated, and ligation products were separated on a 10% polyacrylamide gel. Subsequently, DNA bands corresponding to a tetramer or trimer of the oligonucleotide were excised and eluted from the gel. DNA sequences of the mRNA-like strand of monomeric oligonucleotides for GT30 and LRP20 are as follows: GT30, 5′-CAAAAAGGGTTCTTATCAAATCCACCATAA; LRP20, 5′-GATCCCCGCTATTCTGATATATAAAG (native chloroplast sequence underscored with the additional sequence at the 5′ and 3′ ends to produce a BamHI overhang site after annealing). The nucleotide sequences of all the cloned DNA fragments described previously were confirmed by dideoxy sequencing reactions, using the Sequenase 2.0 kit from the U.S. Biochemical Corporation.

Preparation of Radiolabeled DNA Probes and Competitor Fragments

Probes for gel retardation assays and DNase I footprinting were 3′-end labeled with α-^{32}P-deoxynucleotide triphosphates using the Klenow DNA polymerase I at the first restriction digestion site (XhoI) of recombinant plasmids, followed by a second restriction digestion at the SacI site. However, for labeling the pLRP136 upper strand for DNase I footprinting, NotI and KpnI sites were used as the first and second restriction sites. Labeled probes and competitor DNA fragments were isolated on a 6% polyacrylamide gel and eluted by the "crush-and-soak" method as described by Sambrook et al. (1989). Total incorporation of radioactivity was measured on DE 81 paper (Whatman) and used to quantitate the purified probes. The concentration of nonradiolabeled DNA fragments was determined spectrophotometrically and confirmed further by comparison with standard DNA fragments on ethidium bromide–stained agarose gels.

Gel Retardation Assay

A typical binding reaction in 20 μL of total reaction volume contained 5 × 10^4 cpm (~1 ng) radiolabeled DNA probe, 1 μg poly(dI-dC)·(dI-dC) (Pharmacia), 1 × DEAE buffer (50 mM Tricine-KOH, pH 8.0, 50 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 5% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 mM ε-amino-η-caproic acid, 10 mM $MgCl_2$), and 5.0 μg plastid high-salt extract (1.0 μg of protein in the case of 0.3 to 0.4 M fractions from heparin-agarose chromatography). For competition binding experiments, 100 or 200 ng of specific competitor DNA was included in the reaction. The mixture was incubated for 10 min at room temperature and loaded onto a 5% polyacrylamide gel (29:1 acrylamide/bisacrylamide, 0.5 × TBE [1 × TBE is 90 mM Tris-borate, 2 mM EDTA, pH 8.0], 5% glycerol). The gel was pre-run for at least 3 to 4 hr, until the current was stabilized, before loading the mixture. Electrophoresis was performed at 4°C at 220 V in 0.5 × TBE running buffer (Sambrook et al., 1989) using a Protean II xi apparatus (Bio-Rad). Gels were dried and exposed to x-ray film.

Fractionation of Plastid High-Salt Extracts by Heparin-Agarose Chromatography

Plastid high-salt extracts, obtained from barley seedlings illuminated for 48 hr after 4.5 days growth in darkness, were fractionated through a heparin-agarose column (type I; Sigma). After the binding step in the presence of 1 × DEAE buffer including 5 mM $MgCl_2$, protein elution was performed stepwise with 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1.0, and 1.5 M KCl in DEAE buffer. Peak fractions were pooled, dialyzed against 1 × DEAE buffer, then concentrated using a Centricon-10 microconcentrator (Amicon, Beverly, MA).

DNase I Footprinting Experiments

DNase I footprinting was performed as described by Ausubel et al. (1987). The 0.3 to 0.4 M KCl fraction of heparin-agarose chromatography was used for DNA binding reactions. Incubation of the proteins (5 μg) with DNA probe was performed as described for the gel retardation assays using 8000 cpm of radiolabeled probe. However, no poly(dI-dC)·(dI-dC) was added in the reaction, because most of the non-specific proteins that bind to the probe were eliminated due to chromatography. One millimol $CaCl_2$ was included in the reaction for optimal DNase I digestion. Binding saturation and migration of the DNA/protein complexes were checked by gel retardation. DNase I digestion was started by adding 0.4 units per 2 μL of the enzyme (Boehringer Mannheim) and stopped by adding 220 μL of stop buffer (50 mM Tris-HCl, pH 8.0, 1% SDS, 5 mM EDTA, 25 μg/mL tRNA) after 1 or 2 min. The mixture was immediately extracted by phenol-chloroform and precipitated by ethanol. DNA samples were resuspended in formamide loading buffer and separated on 8% denaturing polyacrylamide gels.

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