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A DNA Binding Activity for One of Two Closely Defined Phytochrome Regulatory Elements in an *Lhcb* Promoter Is More Abundant in Etiolated Than in Green Plants

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The *Lhcb2*1* gene of *Lemna gibba* is regulated positively by phytochrome, and two separate, 10-bp regions of this promoter have been shown to be necessary for phytochrome regulation. We have now analyzed the effects of one and two base pair mutations to define exactly two *cis* elements within these regions that are necessary for phytochrome regulation. These elements, designated REα and REβ, consist in part of sequences highly conserved among promoters of genes encoding light-harvesting chlorophyll *a/b* proteins of photosystem II (*Lhcb* genes). They are located −134 to −129 bp and −114 to −109 bp from the transcription start site, respectively. REα has the sequence AACCAA and was found to interact specifically in vitro with a DNA binding activity in whole-cell extracts of plants. This activity was high in etiolated plants but much lower in green plants. REβ has the sequence CGGATA. A GATA sequence created at a position six nucleotides upstream could replace the function of REβ. We conclude that the phytochrome regulation of *Lhcb2*1* is mediated by at least two *cis* elements. These elements are likely to function by repression of the promoter activity in darkness, although the REβ region also may be able to play a role in the activation of transcription.

INTRODUCTION

The regulation of gene expression by the photoreceptor phytochrome has been a subject of extensive studies. A number of nuclear genes have been studied with regard to this photoreceptor; among them are members of the gene families for the light-harvesting chlorophyll *a/b* proteins of photosystem II (*Lhcb* genes, formerly known as *cab* genes [Jansson et al., 1992]), ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit (*rbcS* genes), and phytochrome itself (*PHY* genes). These genes have been shown to be regulated by phytochrome at the level of transcription (reviewed in Tobin and Silverthorne, 1985). Phytochrome is converted photochemically by red light (R) from its inactive form (Pr) into its active state (Pfr). This process can be reversed substantially by illumination with far-red light (FR). The signal transduction pathway from the activated phytochrome to the regulation of these genes remains mostly unknown, although many components of other signal transduction pathways have been implicated (reviewed in Bowler and Chua, 1994; Deng, 1994; Millar et al., 1994; Tobin and Kehoe, 1994; Quail et al., 1995).

In an attempt to understand the final steps of the phytochrome signal transduction pathway, several promoters have been examined to characterize the *cis* elements specifically necessary for mediation of phytochrome regulation (reviewed in Tobin and Kehoe, 1994; Terzaghi and Cashmore, 1995). For the *PHYA* gene, which is regulated negatively by phytochrome, a *cis* element involved in repression of activity was found in a 10-bp fragment of its promoter (Bruce et al., 1991). Several studies have been able to delineate larger promoter fragments involved in positive regulation by phytochrome in members of the *rbcS* and *Lhcb* gene families.

A 166-bp fragment of the pea *rbcS-3A* promoter was found to be involved in phytochrome regulation (Gilmartin and Chua, 1990a). This region includes the elements box II and box III, which are conserved among pea *rbcS* genes. Box II was shown to act as a positive element for expression in white light (Lam and Chua, 1990), and a factor (GT-1) binding in vitro to this box has been studied extensively (Green et al., 1987, 1988). Clones for a factor binding to box II have been isolated (Gilmartin et al., 1992; Perisic and Lam, 1992; Hiratsuka et al., 1994). However, neither the GT-1 binding activity nor the level of RNA encoded by these clones has been found to change in response to dark or light treatments, and the exact role of the box II sequence in phytochrome regulation has not been established. The *rbcS SSU5B* promoter of *Lemna gibba* lacks elements similar to boxes II and III but contains a −165 to −134 region that is necessary for phytochrome regulation (J. Degenhardt and E.M. Tobin, unpublished data) and is bound in vitro by a factor, light-regulated factor-1 (LRF-1), that binds in a light-dependent manner (Buzby et al., 1990).

Extensive studies have also been conducted on members of the *Lhcb* gene family. We have found (Kehoe et al., 1994)

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that a 1.6-kb fragment of the *Lhcb2*1* (formerly called *cabAB19*) promoter of *L. gibba* conferred phytochrome regulation on a reporter gene in a transient transformation assay. A 5′ deletion analysis located sequence elements critical for phytochrome regulation between −174 and −105 from transcription start. Internal deletion constructs did not reveal additional regulatory elements upstream of −184. Linker substitution analysis of the region from −174 to −105 demonstrated that mutations in two separate 10-bp sequences, from −134 to −125 and from −114 to −105, could abolish phytochrome responsiveness. These two regions contain conserved CCAAT and GATA motifs found in *Lhcb* promoters from many species (Castresana et al., 1987; Gidoni et al., 1989; Piechulla et al., 1991; Kehoe et al., 1994). The Arabidopsis *Lhcb1*1* (formerly called *cab165* and *CAB2*) gene was found to retain phytochrome regulation and circadian responsiveness in a promoter fragment from −111 to −33 (Anderson et al., 1994), and mutations in the region from −74 to −58, which contains multiple GATA elements, abolished phytochrome responsiveness (Anderson and Kay, 1995). For the Arabidopsis *Lhcb1*3* (formerly called *cab140*) gene, sequences downstream of −183 were able to confer phytochrome responsiveness (Sun et al., 1993). A promoter region from −138 to −99 is bound in vitro by a protein factor, CA-1 (Sun et al., 1993), and this region is necessary for phytochrome regulation and contains an element involved in maintaining a high level of transcription (Kenigsbuch and Tobin, 1995).

The goal of this study was to define exactly the *cis* elements necessary for phytochrome regulation in the region from −134 to −105 of the promoter of the *Lhcb2*1* gene. We have now utilized two base pair mutations in the context of a 592-bp promoter fragment to define closely two such elements necessary to mediate phytochrome regulation of this promoter. We also found a DNA binding activity specific for one of these elements, and we have shown that this activity is more abundant in etiolated seedlings than in light-grown plants.

RESULTS

To identify the exact sequence elements important for phytochrome regulation, we introduced one and two base pair mutations into a 592-bp promoter fragment of the *Lhcb2*1* promoter fused to a luciferase (*luc*) reporter gene. The sequence of the region in which mutations were made is shown in Figure 1. The 10-bp regions LS5 and LS7, identified in previous work as necessary for phytochrome regulation, and LS6, which affects expression levels (Kehoe et al., 1994), are indicated. The sequence alterations made in the mutants are shown in Figures 2 to 5. Both wild-type and mutant promoter constructs were assayed in the homologous transient assay system for their expression and phytochrome responsiveness.

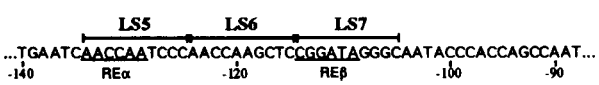

Figure 1. Partial Sequence of the *Lhcb2*1* Promoter in the Region in Which Mutations Were Made.

Numbers indicate nucleotide positions relative to transcription start. LS5, LS6, and LS7 refer to linker scan mutations examined by Kehoe et al. (1994). Motifs REα and REβ are underlined.

An AACCAA Sequence in the −134 to −125 (LS5) Region Is Required for Phytochrome Regulation

Figure 2 shows results with eight different mutations within the 592-bp promoter fragment from −136 to −126 relative to transcription start. The activity and phytochrome responsiveness of each construct are shown. The wild-type promoter was fully responsive to R, and this effect could be partially reversed by FR, demonstrating that the induction was mediated by phytochrome. Mutant 1 had reduced activity compared with the wild type, but the ratio of expression of the reporter gene under R and dark conditions was not altered substantially. However, mutations in the nucleotides AACCAA, from −134 to −129 (Figure 2, mutants 2 to 5), resulted in a decreased degree of phytochrome regulation due to increased expression in dark-treated plants. Among these, mutant 4 exhibited completely abolished phytochrome regulation of the promoter. Although mutant 7, with altered nucleotides T and C at positions −128 and −127, showed a slightly elevated dark level, the change of a T residue alone (mutant 6) did not have any effect. The T was also altered to a G in another case (Figure 4, mutant B), with no effect on phytochrome regulation.

We conclude that although the T residue is a part of the conserved CCAAT sequence, it is not critical for phytochrome responsiveness. The mutation of the residue at −127 and the following nucleotide (mutant 8) had no significant effect on phytochrome regulation. We have therefore designated the six nucleotides AACCAA as a *cis* element, designated REα, that is essential for phytochrome regulation of the *Lhcb2*1* gene. The higher dark activity in the mutant constructs suggests that REα is involved in repression of transcription in darkness and that repression is relieved in the presence of Pfr.

A CGGATA Sequence in the Region from −114 to −105 (LS7) Is Required for Phytochrome Regulation and Affects Promoter Activity

Figure 3 shows the results of mutations within the region −116 to −90 from transcription start. Mutant 9 showed increased expression of the reporter gene under both R and dark conditions. Mutations 10 to 13, each changing two nucleotides between −114 and −109, affected the extent of phytochrome regulation of the promoter. Mutations 11 and 12 also resulted in an increase of promoter activity in the dark-treated plants,

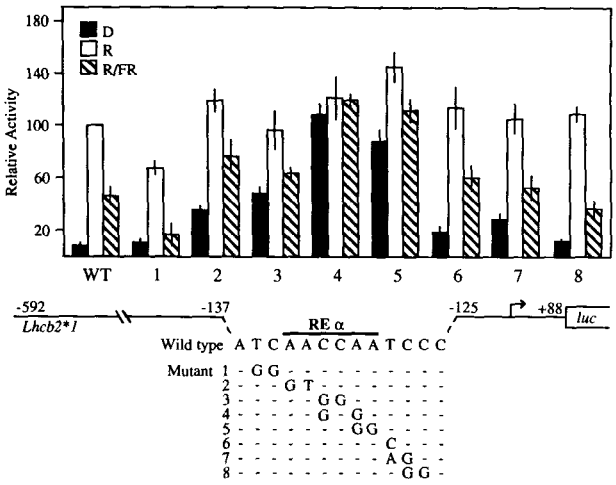

Figure 2. Mutations Define an Element (REα) Necessary for Phytochrome Regulation within the −136 to −126 Region of the *Lhcb2*1* Promoter.

The relative activities of wild-type (WT) and mutant (1 to 8) constructs after different light treatments are shown along with the standard error. All values were normalized to the wild-type R value. The one and two base pair changes introduced into the mutant constructs and a partial sequence of the wild-type promoter are also shown, along with a diagram of the fusion constructs used. Numbers above the diagram indicate nucleotide positions in the *Lhcb2*1* gene relative to transcription start; *luc*, luciferase coding region. The REα element is overlined. Dashes indicate identity with the wild-type sequence. D, no light treatment; R, 2 min of R; R/FR, 2 min of R plus 2 min of FR (see Methods).

suggesting that this region is involved in repressing transcription in darkness. The levels of expression in mutant 10, which has nucleotides −114 and −113 changed from CG to GC, were reduced substantially, but the extent of phytochrome regulation was more affected when these nucleotides were changed to AA (mutant 11). The element defined by these mutations, CGGATA, was designated REβ. Because mutant constructs 10 and 13 had a somewhat lower activity after R than did the wild-type promoter, it is possible that the REβ region is also important for activation of the promoter, and we conclude that the function of this region is complex.

Function of the REα Element Is Position Dependent

Figure 3 also shows that the *Lhcb2*1* promoter contains a second sequence similar to the REα region downstream of the REβ motif (six of seven identical residues). This sequence, AGCCAAT, occurs from positions −94 to −88. We tested whether this sequence might have a function similar to that of REα. Mutation 16 introduced nucleotide changes similar to mutation 4 (Figure 2), which had abolished phytochrome regulation. However, the phytochrome responsiveness was not affected by this mutation, suggesting that the REα element is not redundant and raising the possibility that its function is dependent on its position in the promoter.

We also noted that the region containing the REα element (−135 to −125; CAACCAATCCC) is almost identically (9 of 11 residues) repeated just downstream (−125 to −115; CAAC CAAGCTC). We had shown previously (Kehoe et al., 1994) that mutations affecting the AACCAA sequence in this downstream region (see Figure 1, LS6) did not alter phytochrome responsiveness, although they increased expression levels. The same effect on expression levels was also seen with mutant 9 (Figure 3). To test whether the two nucleotides that differ between these regions are responsible for the observed difference in the function of these two regions, we tested the effects of making mutations such that either the upstream (Figure 4, mutant A) or downstream (Figure 4, mutant B) sequence was repeated exactly. Mutant A did not have an altered phytochrome response, but it did show increased expression, similar to the other mutations in this LS6 region. In contrast, mutant B had virtually no effect. Because nucleotides outside of the REα element were altered, it was not unexpected that the phytochrome response was unchanged, but an altered level of expression might have been predicted by a duplication of the LS6 region. These results confirm the different functions

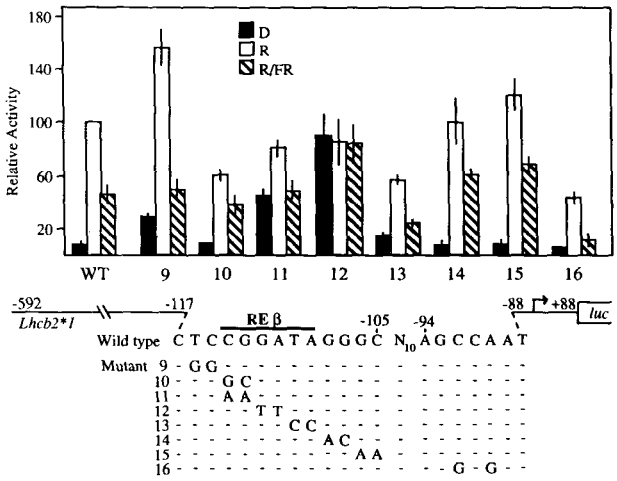

Figure 3. Mutations Define an Element (REβ) Necessary for Phytochrome Regulation within the −116 to −90 Region of the *Lhcb2*1* Promoter.

The relative activities of wild-type (WT) and mutant constructs (9 to 16) after different light treatments are shown along with the standard error. All values were normalized to the wild-type R value. The two base pair changes introduced into the mutant constructs and a partial sequence of the wild-type promoter are also shown, along with a diagram of the fusion constructs used. Numbers above the diagram indicate nucleotide positions in the *Lhcb2*1* gene relative to transcription start; N_{10}, 10 nucleotides not shown; *luc*, luciferase coding region. The REβ element is overlined. Dashes indicate identity with the wild-type sequence. D, no light treatment; R, 2 min of R; R/FR, 2 min of R plus 2 min of FR (see Methods).

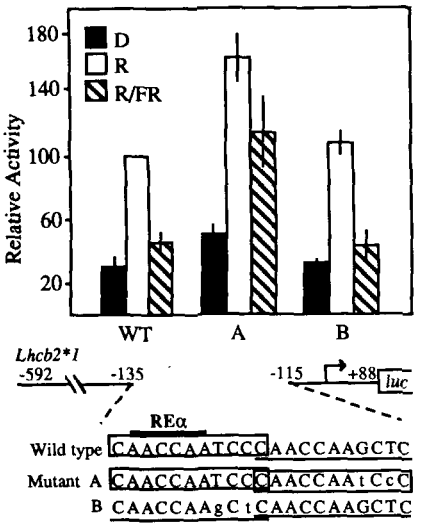

Figure 4. The Functions of the LS6 and LS7 Regions Differ and Are Position Dependent.

The relative activities of wild-type (WT) and mutant (A and B) constructs after different light treatments are shown along with the standard error. All values were normalized to the wild-type R value. The two base pair changes introduced into the mutant constructs (A and B) are shown in lowercase letters, and the sequence of the wild-type promoter in this region is also shown, along with a diagram of the fusion constructs used. Numbers on the diagram indicate nucleotide positions in the *Lhcb2*1* gene relative to transcription start; *luc*, luciferase coding region. The REα element is overlined, the sequence that occurs in the wild-type promoter from −133 to −125 is boxed, and the sequence that occurs in the wild-type promoter from −125 to −115 is underlined. D, no light treatment; R, 2 min of R; R/FR, 2 min of R plus 2 min of FR (see Methods).

of the two regions and support the idea that the proper functioning of the REα element and of the similar sequence immediately downstream is position dependent.

The GATA Sequence of the REβ Element Can Function as a Phytochrome Regulatory Element in an Altered Position

Because a number of *Lhcb* genes have a second GATA sequence just upstream of the CGGATA sequence found in the REβ element, we tested whether the position or number of GATA sequences affected phytochrome responsiveness. The results of these experiments are shown in Figure 5. The change of the REβ GATA motif to TTTA caused loss of phytochrome regulation (mutant C). Mutant D contained not only the mutation of construct C but also an additional mutation of nucleotides −117 and −115, which created a new GATA motif upstream of the original one. This construct showed phytochrome responsiveness, and it had a higher activity compared with the wild type. Thus, the newly created GATA sequence could substitute for the regulatory function of the REβ element. A third mutation, which resulted in the promoter containing two GATA sequences (mutant E), showed normal phytochrome regulation (in terms of the ratio of reporter gene expression under R and dark conditions), but it had reduced overall activity, supporting the notion that this region of the promoter may also be involved in activation of transcription (Figure 3, cf. mutant constructs 10 and 13).

The REα and REβ Regions Are Necessary for Normal Expression Levels in Green Plants

The *Lhcb2*1* gene is transcribed at high levels in green plants grown in continuous light. If the function of the REα and REβ elements were only to repress transcription in etiolated or dark-treated plants, then alterations in these regions might have no effect on transcription in light-grown plants. We investigated this possibility by testing the effects of two mutations, one in REα (Figure 2, mutant 4) and the other in REβ (Figure 3, mu-

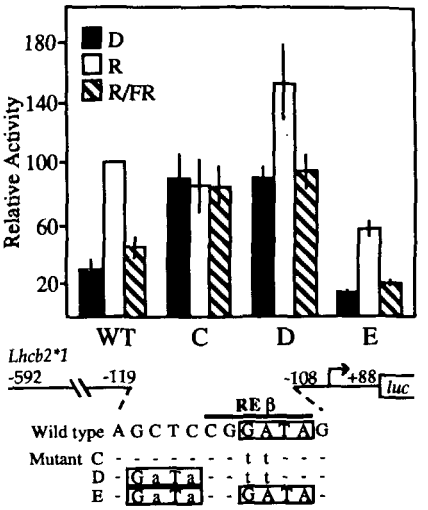

Figure 5. A GATA Sequence Immediately Upstream of REβ Can Restore Phytochrome Regulation in a Construct with an REβ Mutation That Is Not Phytochrome Responsive.

The relative activities of wild-type (WT) and mutant (C to E) constructs after different light treatments are shown along with the standard error. All values were normalized to the wild-type R value. A partial sequence of the wild-type promoter and the two base pair changes introduced into the mutant constructs (lowercase letters) are also shown, along with a diagram of the fusion constructs used. Numbers on this diagram indicate nucleotide positions in the *Lhcb2*1* gene relative to transcription start; *luc*, luciferase coding region. GATA motifs in the promoter and created by mutations are boxed, and the REβ element is overlined. Dashes indicate identity with the wild-type sequence. D, no light treatment; R, 2 min of R; R/FR, 2 min of R plus 2 min of FR (see Methods).

tant 12), using green rather than etiolated plants for the transient expression assay. Figure 6 shows that the mutation of the REα element decreased the transcriptional activity in continuous white light. This result contrasts with the finding in etiolated plants, in which this mutation led to a slight increase in promoter activity. If the REα element is indeed bound by a repressing activity in etiolated plants, this activity does not bind or must have an altered function in green plants. The mutation of the REβ element also reduced the *Lhcb2*1* expression in the green plants, supporting the finding in etiolated plants that this *cis* element also might have a role in activating transcription.

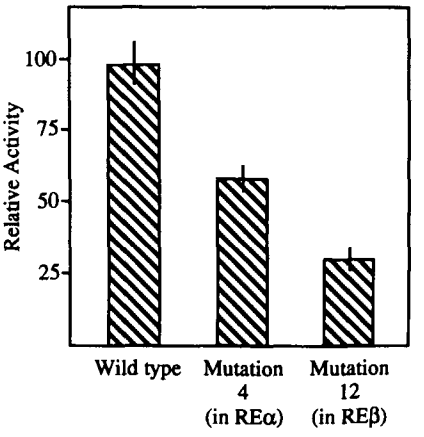

Figure 6. Functions of the REα and REβ Elements in Light-Grown Plants Differ from Those Observed in Etiolated Plants.

The relative activities of wild-type and mutant constructs in green plants are shown along with the standard error. All values were normalized to the wild-type value. Mutations 4 and 12 and their effects on phytochrome regulation in etiolated plants are as described in Figures 2 and 3, respectively.

Etiolated Plants Contain a Binding Activity Specific for REα

We attempted to detect specific binding of proteins to the promoter regions REα and REβ by means of in vitro gel electrophoresis mobility shift assays. We used an *Lhcb* promotor fragment from −170 to −106 to test whole-cell extracts for such binding activities. This fragment and two similar fragments containing mutations in the REα and REβ regions are diagrammed in Figure 7A. Figure 7B shows DNA–protein complexes, using a whole-cell extract of etiolated plants fractionated with a KCl step gradient on a heparin–agarose column. Three complexes (a to c) of lower mobility than the free probe were detected. Under different buffer conditions, an additional complex was detected in the 500 mM fraction (data not shown). We then conducted competition experiments with the wild-type and mutated fragments (Figure 7A) to test whether any of these binding activities were specific for either the REα or REβ regions. We found that the activities in complexes a and c and the activity in the 500 mM fraction did not show any such specificity (data not shown). However, Figure 7C demonstrates that the complex observed with protein from the 200 mM KCl fraction (complex b) bound specifically to the REα element. A fragment that was mutated in the REβ element as well as the wild-type fragment competed effectively for binding at low molar ratios, but a fragment altered in the REα element was not an effective competitor. Thus, this activity shows specificity for the REα region.

The Specific Activity Binding to the REα Region Is More Abundant in Etiolated Than in Green Plants

Figure 8 shows that the REα binding activity found in etiolated plants (lane 2) was at a much lower level in a similarly prepared extract of plants grown under continuous white light (lane 6). To study a possible relationship between light treatment and the decrease of the REα binding activity, we examined fractionated whole-cell extracts from etiolated plants that were treated with 1, 4, and 12 hr of white light before harvesting (lanes 3 to 5). The amount of the bound complex in the 200 mM KCl fraction did not change during the 12 hr of light treatment. Another binding activity in these same fractions, which interacts with the box II element of the *rbcS-3A* promoter of pea (Buzby et al., 1990) and does not change in response to light and dark treatments (Green et al., 1988), was also assayed as an internal control (data not shown). These results show that no change in the amount of binding activity in response to a light treatment could be detected within 12 hr by this in vitro assay.

DISCUSSION

We have defined two specific DNA sequence elements, REα and REβ, that are necessary to mediate the phytochrome response of the *L. gibba Lhcb2*1* promoter. Mutations in either of these 6-bp elements resulted in an increased promoter activity in dark-treated plants and reduced or absent phytochrome regulation. These results suggest that the *cis* elements are involved in repression of gene activity in darkness and that phytochrome action relieves this repression. The results suggest that the REβ region is also important in activating gene expression and are consistent with the findings of Gidoni et al. (1989) and Anderson and Kay (1995) that GATA elements in *Lhcb* promoters are involved in activating transcription in green tissue.

The possibility that the LS7 region can be involved both in repression in the dark and activation after a light treatment or in green plants may help to explain the apparent discrepancy in some of the findings presented here and for the LS7 mutation

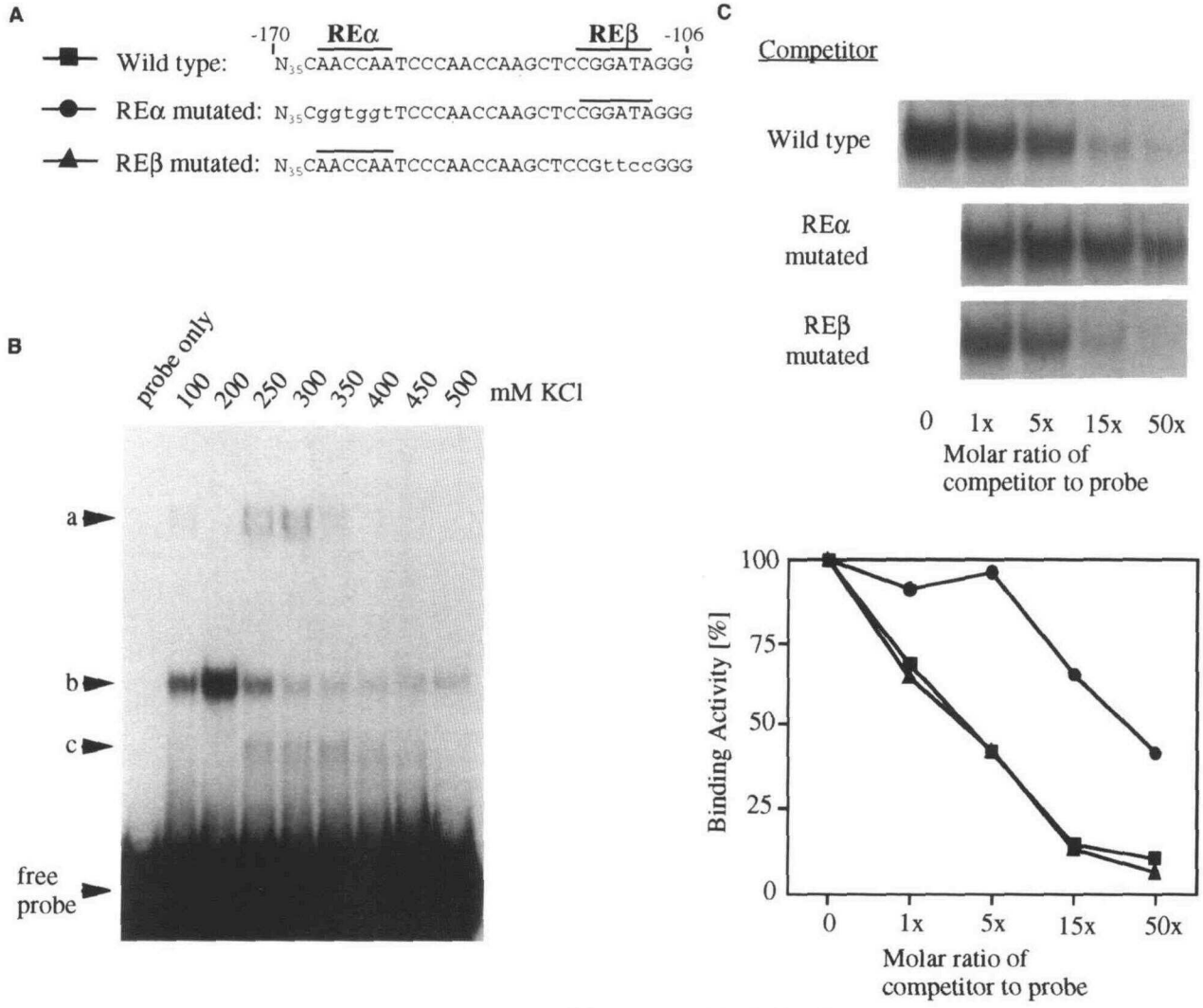

Figure 7. Identification of a DNA Binding Activity Specific for the REα Element of the *Lhcb2*1* Promoter.

(A) The partial sequences of the wild-type fragment from −170 to −106 used as the probe and the wild-type competitor and the mutated fragments used as competitors. Each fragment also contained eight additional nucleotides with a BamHI site at the 5′ end (see Methods). The sequences comprising REα and REβ are overlined; mutated nucleotides are shown in lowercase letters. N, nucleotides.

(B) Electrophoretic mobility shift assays with whole-cell extracts of etiolated plants fractionated on a heparin–agarose column and eluted with a KCl step gradient. The labeled arrowheads (a to c) mark the positions of complexes of lower mobility than the free probe (probe only).

(C) Competition of wild-type and mutant fragments for binding to the activity in the 200 mM KCl fraction from the heparin–agarose column. The autoradiograph of the probe–protein complexes with the indicated concentrations of unlabeled competitor DNAs is shown at the top, and the quantitation of the relative amounts of radioactivity in these complexes is shown below.

examined by Kehoe et al. (1994). In the LS7 mutant, 8 of 10 bp were altered, and the construct consisted of a 171-bp fragment of the *Lhcb2*1* promoter (−239 to −69) fused to a minimal ubiquitin promoter from maize and a *luc* reporter gene. In that case, the expression level in dark-treated plants was not increased compared with the equivalent wild-type construct, and R did not induce any increase, suggesting that this region is involved in induction of expression in response to phytochrome action. In this work (see Figure 3), two base pair mutations in this region, in the context of the wild-type promoter, resulted in some cases in an increase in expression in the dark, with little or no further increase after R (mutants 11 and 12) or in slightly reduced induction by R (mutants 10 and 13). The results with mutants 11 and 12 suggest that this region is involved in repression of expression that is relieved by phytochrome action, whereas the results with mutants 10 and 13 suggest

that it is also involved in activation in response to phytochrome action. Although it is possible that the difference in the constructs and the differing extents of the mutations are responsible for the differing results, we favor the idea that this region is, in fact, not a simple repressing or activating region but can serve either function, depending on the exact circumstances. For example, it might be able to bind two different activities—one of which might be involved in inhibiting transcription and the other in activating transcription.

It has been reported previously that most known *Lhcb* genes have highly conserved sequences ~100 bp from the transcription start. The consensus sequence is $CCAATN_{15}GATA$ (Castresana et al., 1987; Gidoni et al., 1989; Piechulla et al., 1991; Kehoe et al., 1994). Previous studies on the *Lhcb2*1* promoter (Kehoe et al., 1994) revealed that two 10-bp regions containing these elements were necessary for phytochrome regulation of the promoter. The importance for phytochrome responsiveness of a region that contains a repeated GATA sequence in an Arabidopsis *Lhcb* promoter also has been demonstrated (Anderson and Kay, 1995). In this study, we showed that both the CCAAT and GATA sequences are part of phytochrome regulatory elements. The two additional nucleotides that are upstream of the conserved motifs in both REα and REβ and that we have shown are important for regulation in the *Lhcb2*1* promoter are not conserved among different *Lhcb* genes. The differences may account, at least in part, for differences in phytochrome responsiveness (and the overall activity) among members of *Lhcb* gene families (e.g., see White et al., 1995). Interestingly, the highly conserved nucleotide T within the CCAAT sequence from −132 to −128 could be altered to either a G or C without affecting phytochrome responsiveness and so does not seem to be an essential part of the element.

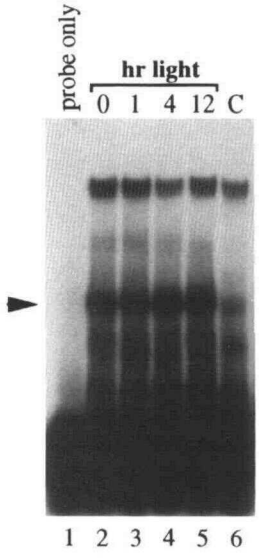

Figure 8. The REα-Specific Binding Activity Is Present at Lower Levels in Plants Grown in White Light Than in Etiolated Plants.

Electrophoretic mobility shift assays were conducted with extracts from etiolated and light-grown plants. Etiolated plants were treated with white light for 0, 1, 4, and 12 hr (lanes 2 to 5) before extraction; green plants (C) were grown in continuous white light. Lane 1 contains the probe only. The labeled DNA probe consisted of a fragment of the wild-type *Lhcb2*1* promoter extending from −170 to −106 relative to the transcription start site. Aliquots from the 200 mM KCl heparin–agarose column fraction containing 2 μg of protein were used for each sample. The complex specific for the REα element is marked with an arrowhead.

An earlier study that deleted a region including the conserved CCAAT and GATA motifs or mutated these elements in the petunia *cab-22R* promoter demonstrated a loss of promoter activity in transformed, light-grown tobacco (Gidoni et al., 1989). This finding is consistent with our observations on expressing mutant REα and REβ constructs in green plants. However, the effect of this deletion on phytochrome regulation of the petunia gene was not tested. A 5-day dark treatment of the plants led to additional reduction in expression. However, dark treatment of green plants can have physiological effects that can suppress *Lhcb* gene expression (Williams et al., 1994; S.C. Weatherwax, J. Degenhardt, and E.M. Tobin, unpublished data), so the possible relationship of the observations on the petunia gene to phytochrome regulation is not known.

We found that the location of the REα element within the promoter is important for its function. However, the GATA sequence that is part of the REβ element could function in an altered position. An upstream GATA sequence restored the phytochrome responsiveness of a construct with a nonfunctional REβ element (Figure 5, mutants C and D). This result also suggests that the spacing between the REα and REβ elements can be varied to some extent. A similar finding was reported for the pea *rbcS-3A* promoter, in which a 5- or 7-bp deletion between box II and box III did not affect the function of the promoter (Gilmartin and Chua, 1990b). We also observed that an additional GATA sequence did not alter phytochrome regulation but decreased the expression level of the promoter. This is rather surprising because many other *Lhcb* genes have an additional GATA sequence immediately upstream of the conserved GATA sequence found in the REβ element. Anderson and Kay (1995) have suggested that the repeated GATA elements in an Arabidopsis *Lhcb* gene are needed for phytochrome responsiveness; however, the roles of the individual elements (and the role of a nearby GATT element that was also mutated) were not tested. Earlier work (Gidoni et al., 1989) suggested that of three GATA repeats in a petunia *Lhcb* promoter, only the one most proximate to transcription start played a role in enhancing expression.

Because mutants that disrupted the REα element did not show repression of the gene in darkness, we propose that a factor that normally binds to this site in etiolated seedlings functions as a repressor of transcription. This hypothesis is consistent with the transformation experiments using light-grown plants and showing that the REα element is no longer involved in repression of gene activity. Because REα was

necessary for the full expression of the gene in light-grown plants, the function of the REα element seems to differ at different times during development. This finding suggests the possibilities that a repressing activity binding to REα in etiolated tissue is absent or altered in green plants or that another binding activity that binds to this element and that enhances transcription is present in the green plants.

We identified a DNA binding activity that can bind in vitro specifically to the REα element. This factor was detected in extracts of etiolated plants but was found at much lower levels in green plants grown in continuous light, consistent with its postulated function as a repressor of transcription. However, the REα binding activity detected in the in vitro assay did not decrease substantially during a 12-hr white light treatment of dark-grown plants, although this treatment is sufficient to increase transcription of the *Lhcb* gene (Tobin, 1981a). It is possible that phytochrome might affect the localization or activity of this factor in vivo in a way that might not be reflected in the in vitro assay. Alternatively, the observed difference in binding activities might be related to the presence or absence of fully developed chloroplasts.

The REα binding protein might be expected to have similarity with CCAAT binding proteins from other species. Despite reports of CCAAT binding proteins in maize (Grasser et al., 1990; Li et al., 1992; Brignon and Chaubet, 1993), specific CCAAT box binding transcription factors have been well characterized only in yeast (HAP2/HAP3) and animals (e.g., NF-Y, C/EBP, CTF/NF-1, and mYB-1). NF-Y has been implicated in having a role in basal transcription (Mantovani et al., 1992; Milos and Zaret, 1992) and in facilitating the binding of upstream transcription factors (Wright et al., 1994). In in vitro competition experiments, NF-Y and mYB-1 were shown to bind most strongly to a promoter that contains an inverted AACCAAT motif (Dorn et al., 1987; Gai et al., 1992). It might be expected that the REα binding protein will prove to be similar to these factors in its sequence requirements.

We were unable to identify a specific REβ binding activity, although the analysis of the promoter suggested that one should be present in both etiolated and green plants. This might be due to the particular binding conditions tested, the methods of cell extract preparation, or components in the extracts (e.g., proteases and phosphatases) that might render such a factor unable to bind. Another positively phytochrome-regulated promoter in *L. gibba*, *rbcS SSU5B*, contains a 26-bp region with multiple GATA sequences and was shown to bind in vitro to an *L. gibba* nuclear protein, LRF-1 (Buzby et al., 1990). Deletion of the LRF-1 binding region abolished phytochrome regulation in transient transformation experiments (J. Degenhardt and E.M. Tobin, unpublished data). However, no binding to the REβ probe was detected in *L. gibba* extracts that were shown to contain LRF-1 (S.C. Weatherwax and E.M. Tobin, unpublished data), indicating that the GATA-containing elements of the *rbcS SSU5B* and the *Lhcb2*1* genes are recognized by different binding factors.

Several GATA binding factors that can interact with light-regulated genes have been described in other plant species. The factors IBF-2a in tomato (Borello et al., 1993) and CGF-1 in tobacco (Anderson et al., 1994) seem to require at least a GATAAPu consensus sequence for binding. This sequence was named the I box (Giuliano et al., 1988) and has been implicated in positive regulation by white light (Borello et al., 1993). Because the REβ element does not contain an I box–like sequence, *L. gibba* homologs of CGF-1 and IBF-2a are not likely to interact with REβ. Protein factors that could be homologous to the putative factor binding to the REβ element are GA-1 and ASF-2. A factor designated GA-1 was shown to bind to triple or less strongly to single GATA elements in the tobacco *cabE* promoter (Schindler and Cashmore, 1990). The factor ASF-2 from tobacco, which binds to the as-2 site of the cauliflower mosaic virus 35S promoter, was also shown to bind in vitro to a GATA-containing site of the petunia *cab-22L* promoter (Lam and Chua, 1989).

Additional studies defining the nucleotide requirements of GA-1 and ASF-2 upstream of the GATA sequence would allow a comparison with the specific characteristics of the putative REβ binding site. An activity (CGF-1) that could bind to the repeated GATA region in an Arabidopsis *Lhcb* gene could also bind to sequences similar to box II of a pea *rbcS* promoter (Anderson et al., 1994), and conversely, an activity (GT-1) that could bind to box II could also bind to the repeated GATA region of the Arabidopsis *Lhcb1*1* promoter (Hiratsuka et al., 1994). However, the interaction of *cis* elements and transcription factors between different species has to be interpreted carefully because possible evolutionary changes altering both site and factor have to be taken into account. This consideration is especially important when comparing distantly related plants such as *L. gibba* (a monocotyledon) with dicotyledons such as tobacco, Arabidopsis, or tomato.

Our work has shown that the phytochrome response system leading to increased transcription of the *Lhcb2*1* gene includes at least two *cis* elements and, most likely, at least two *trans*-acting factors. A model for the mechanism of gene regulation that is compatible with the data presented is shown in Figure 9. In dark-grown plants, a complex of REα and REβ binding activities would act to repress the initiation of transcription. A brief R treatment would activate phytochrome, and the activated signal transduction cascade would result in modification of the complex of the REα and REβ binding factors. This modification would somehow relieve the repression of transcription. In light-grown plants, the REα binding activity is postulated to be reduced or modified in a way that would prevent it from binding to the promoter. It is also possible that it could be replaced by a different binding protein involved in activating the promoter. In the REβ region, a postulated activity would bind and be involved in repression in the dark but would be modified or displaced by another activity in green plants, leading to activation of transcription. Further experiments to characterize and clone the REα and REβ binding activities will lead to an understanding of the last steps of the phytochrome signal transduction pathway and to insights into the mechanism of the complex regulation of transcription of *Lhcb* genes by many developmental and environmental stimuli.

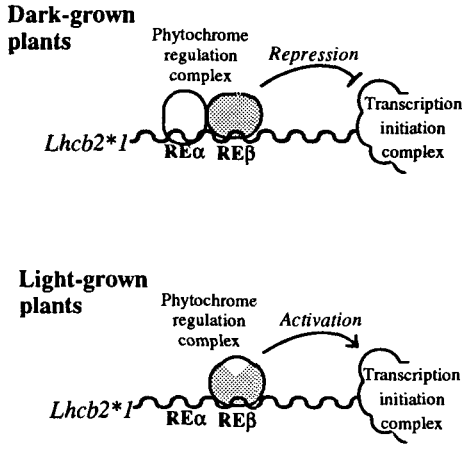

Figure 9. A Model for a Possible Mechanism of Phytochrome Regulation of Transcription of the *L. gibba Lhcb2*1* Promoter.

In dark-grown (etiolated) plants, a phytochrome regulation complex with proteins binding to the REα and REβ elements represses transcriptional activity of the promoter. The activation of the phytochrome system acts to relieve this repression. In light-grown plants, the REα binding activity is reduced or less able to bind; this renders the phytochrome regulation complex unable to repress gene activity. Instead, the remaining complex, which may involve an altered or different REβ binding activity, activates transcription.

METHODS

Plant Material

Green plants (*Lemna gibba*; L.G-3) were grown according to Tobin (1981b) in E medium in continuous white light (77 μE m^{-2} sec^{-1}) and harvested 4 weeks after transfer of the culture. Etiolated plants were cultivated for 6 to 8 weeks in darkness with intermittent red light (R) illumination (2 min of R [17 μE m^{-2} sec^{-1}/8 hr]) in E medium supplemented with 30 μM kinetin (Tobin, 1981b).

Transient Transformation Assays

Approximately 2 g of etiolated *L. gibba* fronds (grown with intermittent R) was plated on agar plates on E medium with 30 μM kinetin as described by Rolfe and Tobin (1991). Plants were treated with 10 min of far-red light (FR; 12 μE m^{-2} sec^{-1}) to convert phytochrome to its Pr form and then kept in total darkness for 2 days before transformation. Alternatively, 2 g of green plants was plated 48 hr before transformation. The transient transformation with a helium-driven Biolistics particle delivery system (Bio-Rad), using 3 μg of the plasmid DNA and 50 ng of an actin::β-glucuronidase control construct (McElroy et al., 1990), as well as the assays for luciferase (LUC) and β-glucuronidase activity, was described previously (Kehoe et al., 1994). Directly after transformation, the plants were maintained in darkness or treated with 2 min of R (17 μE m^{-2} sec^{-1}) or 2 min of R followed by 2 min of FR (12 μE m^{-2} sec^{-1}). Light-grown plants were transformed using the same protocol, but transformation and incubation were conducted in white light.

The assay reaction for β-glucuronidase activity was extended to a 1-hr incubation at 37°C. The activity and phytochrome responsiveness of every construct were determined by at least six separate transformations per light treatment in two experiments with DNA from two independent plasmid preparations. Analysis of covariance was used to calculate the LUC activity levels of each construct, using the β-glucuronidase activity levels as the dependent variable. Each experiment contained the wild-type construct, with dark and R treatments as control groups. Student's *t* test was used to assess the significance of differences between groups.

Protein Extract Preparation and Chromatography

Whole-cell extracts were prepared according to the method of Green et al. (1989). Thirty grams of green or etiolated plants was homogenized in 3.5 mL/g cold extraction buffer (40 mM Tris, pH 7.5, 5 mM $MgCl_2$, 0.5 M sucrose, 25 mM β-mercaptoethanol, 0.8 mM phenylmethylsulfonyl fluoride [PMSF], 10 μM benzamidine, 50 μM ε-aminocapronic acid, 5 μg/mL leupeptin, 5 μg/mL antipain) with a Polytron (Kinematica, Lucerne, Switzerland). After 60% ammonium sulfate precipitation, the precipitate was resuspended in NEB buffer (25 mM Hepes–KOH, pH 7.2, 40 mM KCl, 0.1 mM EDTA, 10% glycerol, 5 mM β-mercaptoethanol, 0.2 mM PMSF). The extract was purified with four consecutive spin columns containing 4 mL of Sephadex G25 in NEB buffer. Extracts were fractionated through heparin–agarose chromatography (Bio-Rad) and eluted stepwise with 100, 200, 300, 400, and 500 mM KCl in NEB buffer. The fractions were concentrated and washed with NEB buffer in a microconcentrator (Centricon 30; Amicon, Beverly, MA). Protein concentrations were determined using Bradford protein assay reagent (Bio-Rad), with BSA as a standard.

Probes and DNA Fragments

For the preparation of probe, 0.14 μg of an oligonucleotide containing the sense strand of the promoter fragment from −170 to −106 and a 3′ BamHI site (5′-GACAGCATACAGAAAATCTGCCATCGAAATTGAATCAACCAATCCCAACCAAGCTCCGGATAGGGATCCTGCC-3′) was annealed to an oligonucleotide containing the antisense strand from the BamHI site to position −127. The Klenow fragment of DNA polymerase I was used to fill in the overhanging ends in the presence of 10 μCi each of α-^{32}P–labeled dATP, dCTP, dGTP, and TTP. Unlabeled nucleotides were added after 30 min to ensure complete synthesis. The fragment of the correct length was electroeluted after PAGE of the reaction. Unlabeled competitor fragments were synthesized similarly, using 50 times more oligonucleotide. The oligonucleotides used for the mutated competitor fragments are as follows: REα mutated, GACAGCATACAGAAAATCTGCCATCGAAATTGAATCGGTGGTTCCCAACCAAGCTCCGGATAGGGATCCTGCC; and REβ mutated, GACAGCATACAGAAAATCTGCCATCGAAATTGAATCAACCAATCCCAACCAAGCTCCGTTCCGGGATCCTGCC.

Electrophoretic Mobility Shift Assays

If not indicated otherwise, the protein–DNA binding reaction was performed in 20 μL of buffer (20 mM Hepes, pH 7.5, 50 mM KCl, 1 mM $MgCl_2$, 0.2 mM DTT, 10% glycerol), using 5 × 10^4 cpm of labeled probe, 0.5 μg of poly(dI-dC), and 2 μg of whole-cell extract. After a 10-min incubation at room temperature, the samples were electrophoresed

for 3 hr at 30 mA in Tris-acetate buffer on a 4.2% polyacrylamide gel at 4°C. Gels were dried and autoradiographed using Kodak X-Omat AR film.

Construction of Plasmids and Site-Directed Mutagenesis

The plasmid pDR19PS, which is described in detail by Kehoe et al. (1994), contains a PstI-SalI fragment of *Lhcb2*1* from −592 to +85 fused to a *luc* reporter gene. This fragment was cloned into M13mp19 and subjected to site-directed mutagenesis according to Kunkel (1985) with some modifications (Kunkel et al., 1987). The sequences of the oligonucleotides used to create the constructs in Figure 2 are 1, 5′-TCTGCCATCGAAATTGAAGGAACCAATCCCAACCAAGC-3′; 2, 5′-GCCATCGAAATTGAATCGTCCAATCCCAACCAAGC-3′; 3, 5′-CCATCGAAATTGAATCAAGGAATCCCAACCAAGCTCCGG-3′; 4, 5′-GCCATCGAAATTGAATCAAGCGATCCCAACCAAGCTCCGG-3′; 5, 5′-CCATCGAAATTGAATCAACCGGTCCCAACCAAGCTCCGG-3′; 6, 5′-GAAATTGAATCAACCAACCCCAACCAAGCTC-3′; 7, 5′-GCCATCGAAATTGAATCAACCAAAGCCAACCAAGCTC-3′; and 8, 5′-CGAAATTGAATCAACCAATGGCAACCAAGCTCCGG-3′.

Oligonucleotides used for the constructs in Figure 3 are 9, 5′-CCAATCCCCAACCAAGCGGCGGATAGGGCAATACCC-3′; 10, 5′-CCCAACCAAGCTCGCGATAGGGCAATACCCACC-3′; 11, 5′-CCCAACCAAGCTCCGTTTAGGGCAATACCC-3′; 12, 5′-CCCAACCAAGCTCCGGACCGGGCAATACCCACCAG-3′; 13, 5′-CCAAGCTCCGGATAACGCAATACCCACC-3′; 14, 5′-CAAGCTCCGGATAGGAAAATACCCACCAGCCAA-3′; and 15, 5′-GCAATACCCACCAGGCGATAGGGTGGCTCCCCCG-3′.

Oligonucleotides used for the constructs in Figure 4 are A, 5′-GAATCAACCAATCCCAACCAATCCCCGGATAGGGCAATACC-3′; and B, 5′-CGAAATTGAATCAACCAAGCTCAACCAAGCTCCGG-3′. Oligonucleotides used for the constructs in Figure 5 are C, see oligonucleotide 4; D, 5′-CCAATCCCAACCAAGATACGGATAGGGCAATACCC-3′; and E, 5′-CCAATCCCCAACCAAGATACGTTTAGGGCAATACCC-3′. The second primer was 5′-CCACTAAAACGACCGACCGAGATTAAGGG-3′. The mutated PstI-SalI fragments were cloned back into pDR19PS and sequenced completely.

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