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Functional Architecture of the Light-Responsive Chalcone Synthase Promoter from Parsley

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We have combined in vivo genomic footprinting and light-induced transient expression of chalcone synthase promoter derivatives in parsley protoplasts to identify *cis* sequences regulating light activation. The parsley chalcone synthase promoter contains two *cis* "units" that are light-responsive. Each unit is composed of short DNA stretches of approximately 50 base pairs, and each contains two in vivo footprints. One of the footprints in each unit covers a sequence that is highly conserved among other light- and stress-regulated plant genes. The other footprinted sequences in each unit are not related to each other. The TATA distal light-responsive unit is inherently weak but can compensate partially for the loss of the stronger TATA proximal unit. Levels of light-induced expression from either can be influenced by the presence of a region of approximately 100 base pairs located upstream of the TATA distal light-responsive unit. Combination of the light-responsive units and upstream region generates a synergistic response to light. We speculate that functional compensation generated by nonidentical, but sequence-related, *cis* units foreshadows combinatorial diversity of cognate *trans* factors.

INTRODUCTION

Rapid protective response to the damaging effects of UV irradiation is paradigmatic of active defense mechanisms in plants. In many cases, protection is thought to derive from the induced accumulation of strongly UV-absorbing flavonoids. Biosynthesis of various flavonoids from phenylalanine is brought about via transcriptional activation of genes of phenylpropanoid metabolism (Hahlbrock, 1981; Scheel and Hahlbrock, 1989). In addition to their probable role as UV protectants, diverse structural classes of flavonoids serve ubiquitous roles as fruit or flower pigments and potential insect repellants or attractants. As well, flavones have been shown to function as bacterial chemoattractants, and isoflavonoids are antimicrobial phytoalexins in legume species (Dixon, 1986; Ebel, 1986; Firmin et al., 1986; Peters, Frost, and Long, 1986; Redmond et al., 1986).

Chalcone synthase (CHS) is the first committed enzyme in the flavonoid-specific branch from the general phenylpropanoid pathway (Hahlbrock and Grisebach, 1979). As its activity is regulated by a constellation of environmental and developmental stimuli, CHS is an appealing candidate for the identification of *cis*-linked DNA sequences involved in plant gene regulation (see Dangl, Hahlbrock, and Schell, 1989, for review).

Treatment of dark-grown suspension cultured parsley cells (*Petroselinum crispum*) with UV-containing white light (referred to throughout as light) triggers the series of transcriptional and biosynthetic steps culminating in vacuolar deposition of flavonoids (Kreuzaler and Hahlbrock, 1973; Heller and Hahlbrock, 1980; Kreuzaler et al., 1983; Chappell and Hahlbrock, 1984; Schmelzer, Jahnen, and Hahlbrock, 1988). Wavelengths of light other than UV are thought to modulate CHS expression (Duell-Pfaff and Wellman, 1982; Bruns, Hahlbrock, and Schäfer, 1986; Ohl, Hahlbrock and Schäfer, 1988). These events are cell-type-specific in parsley plants since vacuolar deposition of flavonoids and accumulation of CHS mRNA and protein are restricted to leaf epidermal cells (Schmelzer, Jahnen, and Hahlbrock, 1988). As well, developmental cues influence CHS expression since CHS protein is no longer present in mature light-grown parsley cotyledons (although flavonoids are), but is found in unfolding primary leaves on the same plantlet (Jahnen and Hahlbrock, 1988). This complex pattern of regulation is controlled by one promoter and its associated *cis*-acting DNA sequences since parsley contains only one CHS gene per haploid genome (Herrman, Schulz, and Hahlbrock, 1988).

We developed a parsley protoplast system that responds accurately to light in terms of both transcriptional activation of the endogenous CHS gene and flavonoid biosynthesis (Dangl et al., 1987). Light-induced transient expression of CHS promoter-reporter gene fusions al-

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lowed identification of light-responsive *cis* elements from both a heterologous *Antirrhinum majus* CHS promoter and the homologous parsley CHS promoter (Lipphardt et al., 1988; Schulze-Lefert et al., 1989). For the parsley CHS promoter, in vivo footprinting defined three short nucleotide sequences exhibiting light-dependent changes indicating putative protein-DNA interactions. Two footprints appear on a 126-bp fragment shown in the protoplast transient assay system to be part of the smallest tested promoter capable of light activation. Clustered point mutation of 10 bp within either of these two footprints abolished light responsiveness. Therefore, we concluded that sequences defined by these two light-dependent footprints function in concert as a light-responsive *cis*-acting unit.

Here, we extend these findings, by in vivo footprinting and functional analysis, to include 615 bp of the parsley CHS promoter. Our data show that a second, TATA-distal, light-responsive unit exists on the CHS promoter, and that it is separable from the previously analyzed TATA-proximal unit. Each light-responsive unit contains a set of two light-induced footprints. We also show that an upstream region of about 100 bp strongly increases regulated expression mediated through the TATA-distal, light-responsive *cis* unit, and can weakly affect the TATA-proximal unit. We discuss the possible roles for two functionally compensatory, separable *cis* element systems in terms of both the light activation and tissue-specific regulation of CHS and the more general need for regulatory diversity in plant stress gene systems.

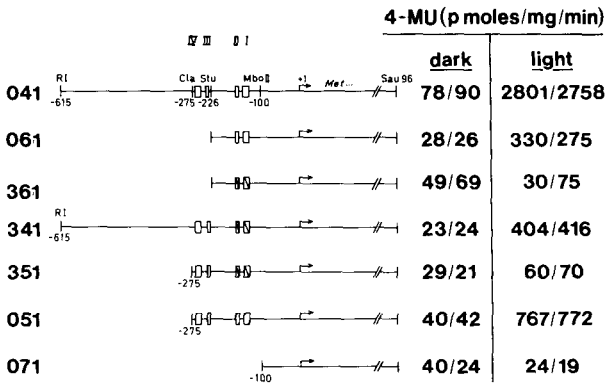

Figure 1. The Parsley CHS[a] Promoter Contains Two Separable Light-Responsive *cis* Element Regions.

Names of GUS fusion constructs are listed on the left and GUS assay data on the right. Each parsley protoplast transformation was done in duplicate, and the results from these two were averaged. Data from two independent experiments (four data points) are given as specific β-glucuronidase activity measured as the conversion rate of 4-methylumbelliferyl glucuronide to 4-methylumbelliferone (4-MU). Landmark restriction sites used in cloning steps and their positions relative to the transcription start point are noted. Boxed regions and Roman numerals refer to the position of in vivo footprints; boxes containing a diagonal line through them indicate 10-bp clustered point mutations in footprints I and II (Schulze-Lefert et al., 1989). Each construct shares an identical translation fusion consisting of 20 amino acids from the parsley CHS protein and four from polylinker sequences. See Schulze-Lefert et al. (1989) for footprint data not included in Figure 2, and for the details of the original construction from which all new promoter mutants are derived. Note that the CHS[a] promoter was previously referred to as the CHS-1 promoter (Herrmann, 1987; Herrmann, Schulz, and Hahlbrock, 1988; Schulze-Lefert et al., 1989).

RESULTS

Experiments described here were motivated by the finding that an operationally defined minimal parsley CHS promoter did not recapitulate the entire light response of a larger promoter fragment (Schulze-Lefert et al., 1989). Repeated expression data from the key constructs are shown as part of Figure 1. A 615-bp promoter directs high levels of light-induced β-glucuronidase (GUS) expression (construct 041), whereas truncation to a 226-bp promoter, containing sequences covered by in vivo footprints I and II, results in the smallest tested promoter that remains light-regulated (construct 061). Further deletion to a 100-bp promoter abrogates light-induced GUS expression (construct 071). Clustered point mutation of 10 bp within either or both footprinted sequences in this minimal promoter abolishes light-regulated GUS expression (for example, construct 361). We concluded that sequences covered by light-inducible footprints I and II define a *cis* unit necessary for light-induced GUS expression in the context of a truncated CHS promoter (Schulze-Lefert et al., 1989). Note, however, that this light-responsive *cis* unit generates only 15% to 20% of the maximal GUS activity (construct 041).

A Second Light-Responsive *cis* Unit

CHS promoter sequences from −226 to −615, in the context of an inactivated TATA-proximal, light-responsive *cis* unit, partially restore light-activated GUS expression (construct 341, Figure 1). This level of induced GUS expression is approximately the same as that observed with the TATA-proximal, light-regulated *cis* unit defined by construct 061. Therefore, one or more *cis* elements between −226 and −615 partly compensate for the loss of light responsiveness acting through footprints I and II. It is also readily apparent that the additive response to light of the TATA-distal unit (construct 061) and the sequences from −226 to −615 (construct 341) still does not regenerate the complete activity of the wild-type promoter (construct 041). This suggests that synergistic sets of *cis* elements operate to control CHS gene expression.

The entire region from −226 to −615 was analyzed for the light-dependent appearance of additional in vivo footprints. Only two, labeled III and IV in all figures, were found. Figure 2 shows an autoradiogram used to define footprint IV (see Schulze-Lefert et al., 1989, for the definition of footprint III). Although the intensities of light-induced changes in dimethyl sulfate reactivity are subtle, they have been observed in three independent experiments. The definition of footprint IV is also strengthened by its absence 1 hr after onset of light treatment and subsequent appearance and maintenance during an 8 hr time course. These kinetics parallel the previously established kinetics of transcriptional activation of the CHS gene (Chappell and Hahlbrock, 1984; Ohl, Hahlbrock, and Schäfer, 1988; Schulze-Lefert et al., 1989).

Figure 3 shows the sequences of footprints III and IV and their position relative to footprints I and II. Footprint III is a degenerate form of the previously defined "box II" sequence found to be highly conserved among a plethora of light- and stress-regulated promoters [Giuliano et al., 1988; Schulze-Lefert et al., 1989 (see discussion)]. Footprint IV covers a sequence of 16 bp that, although nearly identical in size, shares no homology to footprint I. As with footprint I, footprint IV is characterized by both hyper- and hyporeactive guanine residues on both DNA strands. The spacing between the four light-inducible footprints on the CHS promoter is striking in that all are separated by approximately even turns of the DNA helix. As well, the total distance covered by the four footprints (approximately 137 bp) is the length required to wrap around one nucleosome.

Since footprints I and II function together to generate high-level, light-activated GUS expression from construct 061, we reasoned that footprint regions III and IV may also form a light-responsive *cis* unit. A 49-bp fragment containing sequences covered by footprints III and IV compensates partially for the loss, through mutation, of the TATA-proximal, light-responsive unit (construct 351, Figure 1). The twofold to threefold activation of construct 351 is significant, as it has been observed in 12 independent transformations. The conclusion that these 49 bp are weakly light-responsive is strengthened by the observation that they can influence light-induced GUS expression mediated through an unmutated TATA-proximal, light-responsive unit (compare constructs 051 and 061, Figure 1).

Are sequences containing footprints III and IV, in fact, necessary components of the strongly light-responsive *cis* element set defined by construct 341? We first generated constructs in which the TATA-proximal *cis* unit between −100 and −226 was either deleted or replaced. These changes have no effect on the magnitude of light induction mediated by the sequences between −226 and −615 (constructs 0101 and 0131, Figure 4). In addition, constructs 0101 and 0131 show that the TATA-distal, light-responsive region can be slid a short distance up- or downstream with no apparent effect. The necessity of the

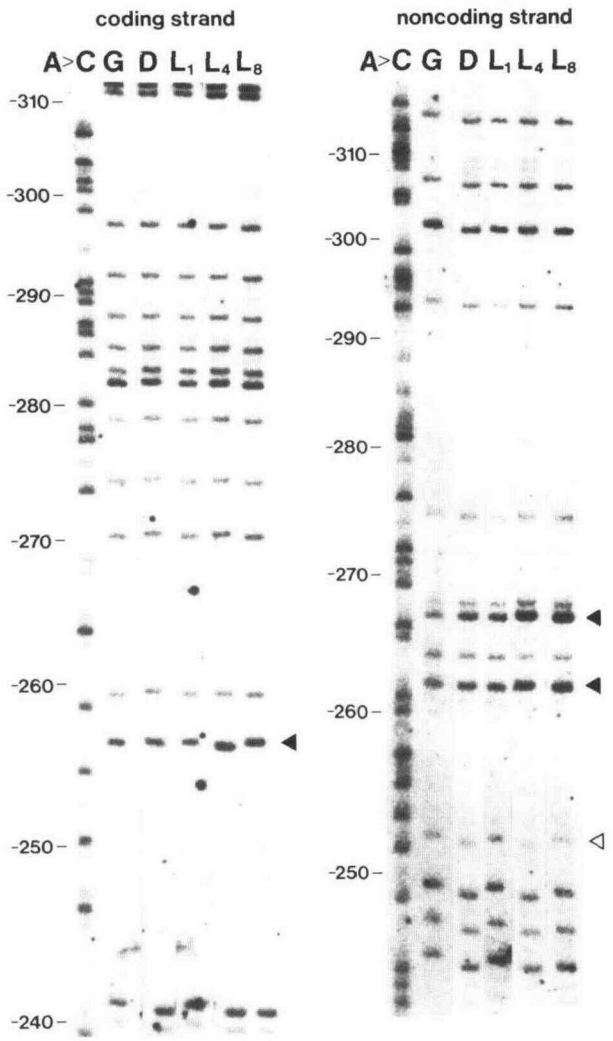

Figure 2. In Vivo Footprints on a TATA-Distal Region of the CHS Promoter.

Genomic DNA sequence G ladders of the parsley CHS[a] allele from −240 to −310 are presented. Light-induced changes in the reactivity of DNA to dimethyl sulfate in vivo as analyzed in suspension cultured parsley cells kept in the dark (lanes D), or continuously treated with light for 1 hr, 4 hr, or 8 hr (lanes L_1, L_4, L_8). Protections from or enhancements of methylation are shown as open and closed triangles, respectively. Numbers refer to the start of transcription. Reference sequence ladders for G and A > C reactions with in vitro treated cloned DNA are also shown. A total of eight single-strand probes were used to generate overlapping in vivo genomic sequencing data on both strands between −226 and −615.

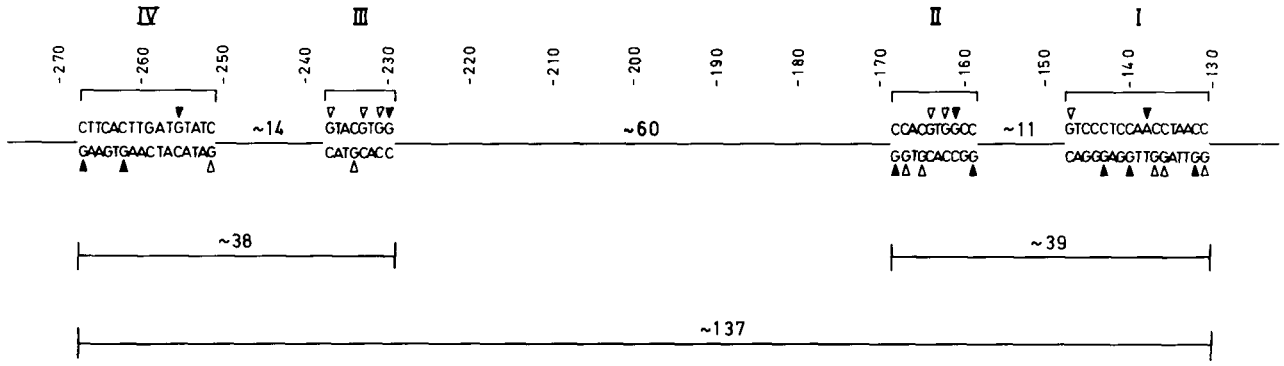

Figure 3. *Schematic Summary of the Position and Spacing of Light-Induced in Vivo Footprints on the CHS[a] Promoter.*

The sequence of the newly defined footprint IV is shown relative to previously defined footprints (Schulze-Lefert et al., 1989) to demonstrate the organization of all four light-induced footprints. Footprinting data are interpreted as follows: open triangles refer to hypo- and closed triangles to hypermethylated G residues. Numbers above the sequences are relative to the start of transcription. Sequences are shown that include all observed putative protein-DNA contacts at G residues, and should not be construed as representing the functional limits of putative protein-DNA contacts. Lines with numbers above them refer to the distances between various sequences in the light-responsive *cis* units described in the text.

49 bp containing footprints III and IV for light activation driven by the region between −226 and −615 is demonstrated in Figure 4 by construct 091, where their removal from construct 0101 is sufficient to destroy light responsiveness. Therefore, 49 bp containing footprints III and IV alone form only a weakly light-responsive *cis*-acting unit, and these 49 bp are required for the relatively strong light activation directed by the entire TATA-distal promoter region from −226 to −615. It is also apparent from Figure 4 that sequences upstream of footprint IV (−275) are not independently light-responsive, although they do play a regulatory role (compare also constructs 351 and 341, Figure 1).

Localization of an Element That Enhances Light Responsiveness

We used 5′ endpoint deletion analysis to identify the promoter region upstream of −275 that boosts the light response mediated through footprints III and IV. Figure 5 shows that deletion from −615 up to −510 does not influence light-induced GUS activity compared with construct 341. Further truncation to −381, however, clearly diminishes light-activated GUS expression. This enhancing effect is only apparent after light treatment, as dark control values are not influenced by these sequences. Thus, sequences between −381 and −510 serve a light-dependent quantitative role in CHS regulation. The 3′ border of this region is illustrated by the observation that all other 5′ endpoint mutants between −381 and −275 are similar to construct 351, directing twofold to threefold light-induced GUS expression. We observed no in vivo footprints in this region, although there are many guanine residues on both DNA strands. We stress that this result illustrates the present limits of the in vivo genomic sequencing technique, which detects potential protein-DNA contact at only guanine, and rarely adenine, residues. Probable protein-DNA interactions from −381 to −510 may be mediated by contacts at residues other than guanine, or, alternatively, these sequences may play a structural role.

The light-dependent quantitative effect of sequences between −427 and −510 was specifically uncovered by its ability to influence the level of light-activated GUS expression mediated through footprints III and IV. Can these sequences also enhance light-inducible expression from the relatively strong TATA-proximal *cis* unit? To answer this question, four of the most critical 5′ endpoint deletions were transferred onto construct 061. Figure 6 shows that the −510 mutant, the largest 5′ deletion retaining a full influence on the TATA-distal *cis* unit, also increases the induced response from the TATA-proximal *cis* unit (compare 0141-Δ4 to 061). Truncation to the 3′ border of this region abolishes the effect. The influence of this enhancing region on the TATA-proximal *cis* unit is low (twofold) compared with its effect on the TATA-distal *cis* unit (fivefold).

Silent, Far Upstream Light-Responsive System(s)

To what extent are compensatory *cis* element sets reiterated on the CHS promoter? We began to address this question by analyzing whether or not sequences upstream of −615 influenced light-induced CHS expression. Figure 7 compares the expression of GUS fusions made with

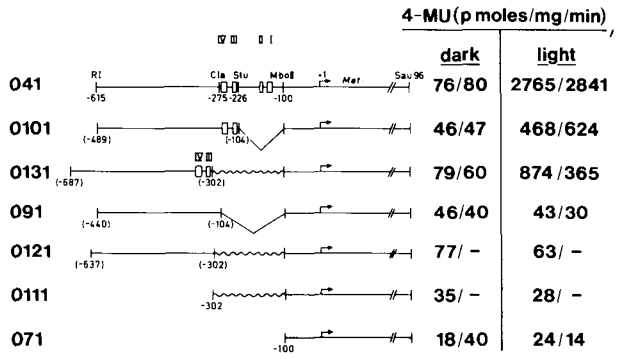

Figure 4. A 49-bp Fragment Containing Footprints III and IV Is Required for the Light Response Mediated Through Sequences from −226 to −615.

See Figure 1 for description of all symbols except the following: Numbers in parentheses indicate new positions of promoter fragments relative to transcription start created by cloning. Wavy line indicates a stuffer sequence derived from a similar position in a promoter known not to be light-regulated (I. Sommssich and K. Hahlbrock, unpublished data).

either of the two CHS promoter alleles that occur in parsley (constructs 041 and 011). They are sequence identical to position −584 (Herrmann, 1987; Herrmann, Schulz, and Hahlbrock, 1988). Comparison of light-activated GUS expression revealed that the 615-bp promoter is, if anything, slightly more light-responsive than a promoter of 2.0 kb. Differences between the two are uncovered, however, when the strongly light-responsive, TATA-proximal *cis* unit is destroyed by mutation. The subsequent diminution of light-induced GUS expression is much less (40%) in the context of the longer promoter than in the context of the shorter promoter (80%) (compare constructs 311 and 341, Figure 7). This result suggests the existence of still additional compensatory elements whose influence on light activation is only revealed when the strong, TATA-proximal, light-responsive *cis* unit is nonfunctional.

DISCUSSION

Our analysis of functional *cis*-acting sequences contained on the parsley CHS promoter reveals two separable light-responsive *cis* units. Each is characterized by the light-induced appearance of two in vivo DNA footprints, and each can be quantitatively influenced by a region of approximately 100 bp located further upstream. The TATA-proximal and TATA-distal *cis* units operate with inherently different strengths. As well, one footprint in each unit delineates a sequence highly conserved in genes involved in photosynthesis [the small subunit of ribulose biphosphate carboxylase (SSU) and the chlorophyll *a*/*b* binding protein (CAB)] as well as various stress responses (Castresana, et al., 1988; Giuliano et al., 1988; Schulze-Lefert et al., 1989). The two light-responsive units are, therefore, related through a shared footprinted sequence, but are functionally separable in our assay system. We note that this promoter architecture was unravelled in a simplified system, namely in protoplasts. We suggest that the apparent redundancy of light-responsive units belies a more subtle differentiation of functional roles for these sequences and their cognate *trans*-acting factors in the plant.

Precedent for functional reiteration exists in the SSU system (see Kuhlemeier, Green, and Chua, 1987, for review; Aoyagi, Kuhlemeier, and Chua, 1988; Kuhlemeier et al., 1988). These authors showed that sequences downstream of −170 in a pea SSU promoter directed tissue-specific and light-regulated expression of a reporter gene. Distal elements could compensate for the loss, through deletion or mutation, of this TATA-proximal, light-responsive element. Importantly, they showed that such operational redundancy was not strictly maintained through development. We postulate that similar separation of regulatory functions, mediated by the two light-responsive *cis* units, may be used to establish the exquisite tissue-specific regulation of CHS expression triggered in early development (Jahnen and Hahlbrock, 1988; Schmelzer, Jahnen, and Hahlbrock, 1988).

Each of the CHS promoter's light-responsive regions contains two short nucleotide sequences defined by light-

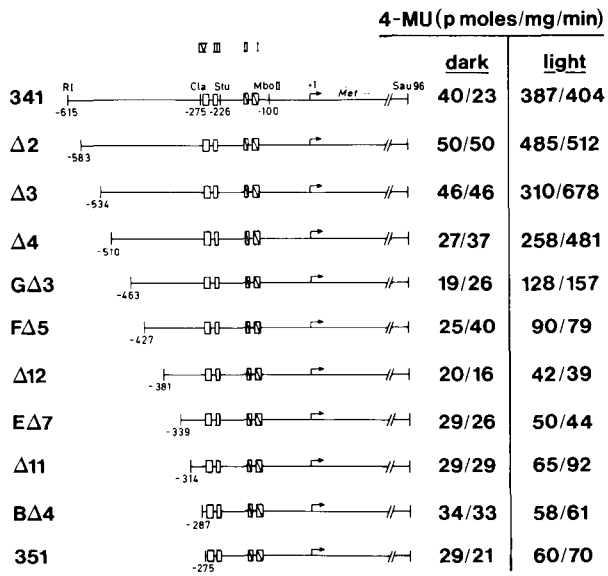

Figure 5. Deletion Analysis Identifies Regulatory Sequences That Influence Light Activation from the TATA-Distal *cis* Unit.

Construct 341 was used as a starting point for Bal31 deletions (see Methods). All symbols are as in legend to Figure 1.

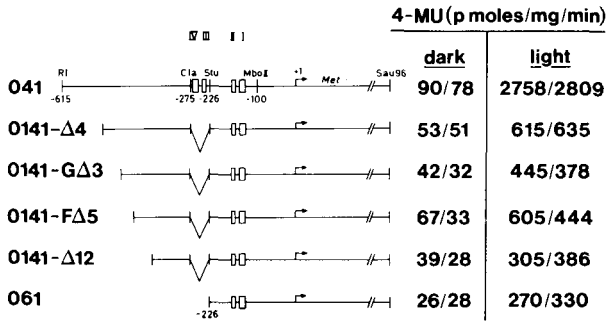

Figure 6. Upstream Regulatory Sequences Can Influence Light Activation from the TATA-Proximal *cis* Unit.

Relevant 5′ endpoint deletion mutants were transferred onto construct 061. Names of 5′ endpoint deletion clones are derived from Figure 5. Symbols are as in the legend to Figure 1.

inducible footprints. The sequences of one of the two footprints in each unit are highly related to each other (footprints II and III), but the corresponding "partner" sequences share no homology (footprints I and IV) (Figure 3). We do not know whether the factors binding the conserved, but nonidentical, sequence defined by footprints II and III are the same. Subtle differences in binding sites could reflect that related but distinct factors recognize them. In the SSU promoter system, however, this is not the case. There, one factor, designated GT-1, binds to several variations of a functionally necessary sequence element reiterated on the SSU promoter (Kuhlemeier, Green, and Chua, 1987; Kuhlemeier et al., 1987, 1988; Green, Kay, and Chua, 1987; Green et al., 1988). On the other hand, there are also several examples of cell-type specificity generated by different factors, encoded by related genes, binding to identical or overlapping *cis* elements (see McKnight and Tjian, 1986; Ptashne, 1986, 1988, for reviews; Zinn and Maniatis, 1986; Poellinger, Yoza, and Roeder, 1989, for examples).

What is perhaps more intriguing is the seeming requirement for two distinct *cis* elements in each light-responsive unit. We assume that more than one factor is bound to each unit since Giuliano et al. (1988) have shown that a 12-bp oligonucleotide nearly identical to the footprint II/III sequence is sufficient to form a stable protein-DNA complex. Each light-responsive unit, then, would be bound by the combination of a common, perhaps related, factor and a second protein idiosyncratic for sequences defined by footprint I or IV. Implicit in this organization is the potential for distinctive protein-protein interactions within each unit, generating a more rapid and/or flexible response to different environmental or developmental stimuli (see Fischer and Maniatis, 1988, for an example of the latter). A particularly illustrative example of regulatory diversity mediated through combinations of *cis* elements was provided by Miller, MacKay, and Nasmyth (1985). These authors showed that two related but distinct sequences bound different repressors, and that a naturally occurring combination of the two *cis* elements dictated a different set of regulatory outcomes than a dimer of one. Further refinement of the in vivo requirements for the CHS light-responsive *cis* units, combined with isolation and characterization of their cognate *trans* factors, will clarify how combinations of *cis* elements regulate CHS transcription.

METHODS

Protoplast Transformation and GUS Enzymatic Assay

Protoplasts were prepared from 5-day-old suspension cultured parsley cells as described (Dangl et al., 1987). Supercoiled plasmid DNA was used for all transformations at 1 mg/mL; 20 μg/10^6 protoplasts. DNA transfer was done by a modification (Lipphardt et al., 1988; Schulze-Lefert et al., 1989) of original procedures (Krens et al., 1982; Hain et al., 1985). Each construct was transferred in duplicate, and each transfer was split into two samples. One sample was irradiated continuously with UV-containing white light for 9 hr (Phillips TL20W18 lamps) and the other kept in the dark. Preparation of extracts and GUS enzyme assay was performed as described (Jefferson, 1987; Schulze-Lefert et al., 1989).

Genomic Sequencing

In vivo footprinting was done according to modifications (Schulze-Lefert et al., 1989) of the original procedure (Church and Gilbert, 1984). To analyze the region including footprint IV, DNA enriched for the 1.05-kb EcoRI fragment containing the CHS^a promoter was cut with HaeIII (position −149) to create the reference cut. Two synthetic 17-mer oligonucleotides homologous to either the coding strand (5′ end at −273) or the noncoding strand (5′ end at −149) were used to prime probe synthesis over an M13 template containing the 1.05-kb EcoRI fragment. Synthesis products were cut with either HaeIII (coding strand probe) or ClaI (noncoding strand probe) and separated on a 6% polyacrylamide

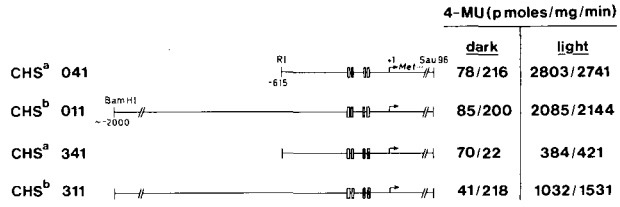

Figure 7. Sequences Upstream from −615 Influence Light Activation Only in the Absence of the TATA-Proximal *cis* Unit.

Symbols are as in legend to Figure 1. CHS^b is a second CHS allele, previously called CHS-2 (Herrmann, 1987; Herrmann, Schulz, and Hahlbrock, 1988).

gel containing 8 M urea, and probes were recovered by isotachophoresis (Öfverstedt et al., 1984).

Plasmid Constructions

5′ endpoint deletions in the base construct 341 were made as follows: 8 μg of plasmid was HindIII-digested (40 units, 20 min) and purified by phenol-chloroform extraction and isopropanol precipitation. The linearized plasmid was resuspended in Bal31 nuclease buffer (containing 600 mM NaCl end concentration) and digested with 30 units of Bal31 (Bethesda Research Laboratories) at 30°C. Aliquots were removed at 30-sec intervals and extracted with phenol-choloroform before isopropanol precipitation. Ends were repaired with the Klenow fragment of *Escherichia coli* DNA polymerase I, and 10-mer HindIII polylinkers were ligated onto the repaired ends. After extensive HindIII digestion, deleted plasmids were recircularized and transformed into *E. coli* MC1061recA⁻. Candidate endpoint mutants were identified by restriction analysis and then transferred back into the pRT99 expression plasmid (Töpfer, Schell, and Steinbiss, 1988) as HindIII-EcoRI fragments containing the entire promoter-GUS gene fusion. In this way, the point of translational fusion was never altered. Endpoints were sequenced from plasmid DNA using the 15-mer polylinker primer to extend dideoxy reactions across the HindIII site. Details of other constructions are in Schulze-Lefert et al. (1989). All plasmids for protoplast transformation were prepared by the cleared lysate procedure, and were twice banded through CsCl gradients. Standard enzymatic and purification procedures were as described by Maniatis, Fritsch, and Sambrook (1982).

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