Sequences in the pea *rbcS-3A* gene have homology to constitutive mammalian enhancers but function as negative regulatory elements

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The pea *rbcS-3A* gene, which codes for a key component of the photosynthetic machinery, requires light for its expression. Analysis of chimeric constructs in transgenic tobacco plants has shown that a 280-bp fragment from the 5' noncoding region can act as a light-inducible transcriptional enhancer. Further characterization of this enhancer identifies a 58-bp sequence containing two regulatory elements that can decrease transcription in the dark. One has a high degree of homology to the SV40 core enhancer, the other to the adenovirus 5 E1A enhancer and the constitutive part of the human interferon- β gene enhancer. Deletion of the 58-bp sequence uncovers additional light-responsive elements (LREs) located further upstream and downstream.

[Key Words: *rbcS-3A* gene; enhancers; light-responsive elements; transgenic tobacco plants]

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The pea *rbcS-3A* gene encodes the small subunit of ribulose-1,5-bisphosphate carboxylase, the key enzyme in the photosynthetic fixation of carbon dioxide. The expression of this gene is highly regulated, transcripts being most abundant in leaves and lower or undetectable in other organs (Coruzzi et al. 1984; Fluhr et al. 1986b). Moreover, the expression is at least 20-fold induced by light. The effective wavelength depends on the developmental stage of the plant. In immature pea plants, the photoreceptor phytochrome mediates the induction of the *rbcS-3A* gene, since transcription is turned on by a pulse of red light, whereas the effect can be reversed by far-red light. In mature plants, however, there is evidence for an additional blue photoreceptor acting in concert with phytochrome (Fluhr and Chua 1986a).

Using transgenic tobacco plants as an assay system, we recently showed that a 280-bp upstream sequence in the pea *rbcS-3A* gene from -330 to -50 bp relative to the transcription start site was capable of increasing the level of transcription in the light, but not in the dark when fused to a constitutively expressed reporter gene (Fluhr et al. 1986a). Moreover, the enhancement was organ-specific, i.e., most pronounced in leaves, much less so in stems and absent in roots.

In many genes upstream sequences are important for regulated expression (for reviews, see Voss et al. 1985; Gluzman 1985; Sassone-Corsi and Borelli 1986). Most

often, such enhancers act by increasing the rate of transcription, but in yeast (Brand et al. 1985), and recently also in mammals, so-called silencer elements have been identified that decrease transcription. The human interferon- β enhancer consists of a constitutive enhancer and a negative regulatory element. Removal of the negative element leads to increased transcription under noninducing conditions, without affecting the induced level (Goodbourn et al. 1985, 1986). Very closely linked to this negative element is the constitutive enhancer that has sequence homology to constitutive enhancer motifs from SV40 and adenovirus. Similarly, in the rat insulin gene silencer elements are thought to act in concert with positive elements, leading to regulated enhancement of transcription (Laimins et al. 1986; Nir et al. 1986).

In a number of animal genes evidence has been obtained for the presence of multiple regulatory elements. In the case of the *Drosophila* heat shock genes, several copies of a 14-bp consensus heat shock element (HSE) were identified in the upstream region. Depending on the particular gene and the assay system, it was shown that one or two HSEs were sufficient to confer heat inducibility upon a constitutive promoter (Pelham and Bienz 1982; Dudler and Travers 1984; Pauli et al. 1986). The SV40 enhancer contains two copies of a 72-bp element; deletion of one of these two elements does not interfere with viability of the virus (Herr and Gluzman 1985). Furthermore, the 72-bp element and surrounding sequences comprise at least three dissimilar domains (Zenke et al. 1986; Herr and Clarke 1986). Structural and sometimes functional redundancy of transcriptional ele-

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Figure 1. Salient features of the intermediate vectors and of the *rbcS*-3A upstream sequence. (A) The SEV transfer system and intermediate vector pMON200 (Fraley et al. 1985) were used for transferring constructs into tobacco plants. The structure of the transferred DNA after integration into the plant genome is shown. The *rbcS*-3A reference gene was inserted into site 1 and the test gene into site 2, which was created by replacing an *Sst*III site with a *Bgl*III-*Cla*I linker. All test genes contained a 3' tail derived from the *rbcS*-E9 gene, whereas the reference gene had its normal 3' tail. This allows discrimination between transcripts from test and reference genes by using a probe derived from the *rbcS*-E9 gene. For a detailed description see Fluhr et al. (1986b). When no reference gene was present, the test genes were inserted into site 1. The direction of transcription was always as indicated by the arrows. (LB) Left border; (nos-neo) nopaline synthase-neomycin phosphotransferase gene; (Spc/Str) bacterial selectable marker; (nos) nopaline synthase gene; (RB) right border. (B) Sequence of the *rbcS*-3A upstream region. Boxes I–V are conserved between the four sequenced pea *rbcS* genes (Fluhr et al. 1986a). Box IV is the TATA box. Boxes II* and III* have a high degree of homology with boxes II and III, respectively. To facilitate cloning, the sequence ATAGA (–50 through –46) was replaced by TTCGTCGAC creating *Eco*RI and *Sal*I sites. The trinucleotide TCT was inserted between nucleotides +15 and +16 creating an *Xba*I site. The initiation codon ATG is underlined. The major transcription start site is at +1. The sequence of the complete *rbcS*-3A gene has been published by Fluhr et al. (1986b).

ments has also been found in the metallothionein gene (Carter et al. 1984; Stuart et al. 1984), in the rat insulin gene (Edlund et al. 1985), and in the human β -interferon gene (Zinn et al. 1983).

Here we show that the light-regulated transcription of the *rbcS*-3A gene is mediated by a complex array of regulatory elements. Two of these light regulatory elements (LREs) act by decreasing transcript levels in the dark. Deletion analysis indicates the presence of additional LREs.

Results

A 58-bp sequence with homology to mammalian constitutive enhancing elements has a silencer activity

As pointed out previously (Fluhr et al. 1986a; see also Fig. 1), the DNA sequence of the *rbcS*-3A gene between –169 and –112 contains three elements (boxes I, II, III) that are completely conserved between the four sequenced pea *rbcS* genes. Therefore, we chemically synthesized a 58-bp DNA fragment containing these conserved boxes and placed it in front of a constitutive reporter gene. The reporter gene consisted of the bacterial chloramphenicol acetyl transferase (CAT) gene preceded by a fragment from the cauliflower mosaic virus (CaMV) 35S gene (from –46 to +6) containing the TATA box and the transcriptional start site (Odell et al. 1985). The proper spacing between this 35S promoter and the *rbcS*-3A fragment was preserved by the insertion of a 60-bp synthetic “stuffer” of unrelated sequence (for exact sequence, see legend to Fig. 2). The resulting chimeric gene was inserted into plasmid pMON200 and by

Agrobacterium-mediated gene transfer introduced into tobacco plants (Fig. 1). Figure 2 (lane 1) shows that this 58-bp sequence fails to direct any expression of the CAT gene in the transgenic plants; neither does a 16-bp DNA fragment containing the *rbcS*-3A sequence from –152 to –137 (box II), neither in one nor in three copies (Fig. 2, lanes 2 and 3). Thus, the 58-bp sequence fails to act as a positive regulatory element. Only when further upstream segments are fused back to the 58-bp sequence, together reconstituting the region from –330 to –112, could a positive effect on transcription be observed (Fig. 2, lanes 4 and 5).

Since this 58-bp sequence was unable to enhance transcription, we decided to combine it with a constitutive enhancer to determine whether it might modulate transcription in a negative way. To this end we made use of a chimeric gene containing the complete transcription unit of the CaMV 35S gene from –943 to +6 fused to the CAT gene. Sequences of the 35S gene between –943 and –31 have all the characteristics of a constitutive enhancer, that is, they function in either orientation, at the 3' end of a gene, and with a heterologous promoter (F. Nagy et al., unpubl.). We have previously shown that this 35S–CAT gene is not regulated by light (Fluhr and Chua 1986). The 58-bp sequence from the *rbcS*-3A gene was inserted into this 35S–CAT construct, between the promoter and the enhancer. As before, the synthetic DNA fragment of unrelated sequence was inserted to maintain the proper spacing between the 58-bp element and the 35S promoter. This spacer segment alone does not result in a significant light regulation of transcription but the additional presence of the 58-bp sequence

Figure 2. A conserved 58-bp sequence has no positive effect on transcription. (Lane 1) The *rbcS-3A* sequence from -169 to -112 was chemically synthesized with appropriate restriction sites and inserted upstream of a 35S–promoter CAT fusion gene, which contained the 35S region from -46 to $+6$ (Odell et al. 1985). A 60-bp random sequence (66 after cloning) was cloned between the 58-bp element and the 35S promoter region to preserve the proper spacing. This synthetic sequence resulted in replacement of nucleotides -111 through -46 of *rbcS-3A* by 5′-GAATTCGTCGACTACAAATTCAGAGTTAT-TACGACTGTAGAATACAGAAATGATCAGTACTATCGAC-3′, exactly conserving the distance between upstream elements and TATA box. (Lane 2) A 21-mer oligonucleotide containing 16 bp of the *rbcS-3A* sequence from -152 to -137 was inserted in front of the 35S promoter region. No spacer DNA was present. (Lane 3) Three copies of the 21-mer elements were inserted, the middle one in the opposite direction. (Lane 4) The *rbcS-3A* DNA sequence from -330 to -169 was linked 5′ of the construct described in lane 1. A single base pair change was introduced at base 225 to create a *Bgl*III site. (Lane 5) The *rbcS-3A* sequence from -410 to -98 was fused to the 35S–promoter CAT gene. All chimeric genes were cloned into site 1 (Fig. 1A). No reference gene was present. Fifty micrograms of total RNA was analyzed by 5′ S_1 protection using a uniformly labeled probe. (D) Dark; (L) light; 35S sequences are hatched; the 60-bp stuffer is dotted.

has a dramatic negative effect on the mRNA level in the dark (Fig. 3A, lanes 1 and 2).

A high-resolution 5′ S_1 mapping (Fig. 3B) reveals a set of bands that is identical regardless of which upstream sequences are present. The main band represents an mRNA with a 5′ end identical to the one determined by Odell et al. (1985).

When the 16-bp sequence (box II) is inserted between the 35S enhancer and promoter, at most a modest light-

regulation is observed. However, three copies of this element lead to a distinct down-regulation of the dark mRNA level (Fig. 3A, lanes 3 and 4).

We have not tested box I or box III individually for light-regulatory activity, but there is strong evidence that box III constitutes an additional negative regulatory element. When the *rbcS-3A* sequence between -100 and -50 is inserted into the 35S–CAT construct between the enhancer and the promoter, the transcript levels are the same in the light and in the dark. When a slightly larger fragment, from -136 to -50 and thus including box III, is tested in the same 35S–CAT background, a negative influence on the transcript levels in the dark is observed (Fig. 4).

Location of positive elements

Since the 58-bp sequence between -169 and -112 has only silencing activity and fails to turn on transcription in a positive way, the question remains where the positive elements are located.

A series of 5′ deletions of the *rbcS-3A* gene was constructed and inserted into the intermediate vector pMON200. To assess the effect of these 5′ deletions in a quantitative manner, we inserted an additional intact *rbcS-3A* gene into the same vector, separated from the deleted gene by approximately 5 kb of intervening DNA. The RNAs from these two genes can be distinguished by 3′ S_1 analysis due to the fact that the reference gene has its cognate 3′ end while the 5′ deleted genes (the “test” genes) have a 3′ end derived from the pea *rbcS-E9* gene. Figure 5 shows that 5′ deletions down to -166 bp relative to the transcription start site have no effect on the level of expression (compare signals from reference and test genes) nor on the light-regulatory properties of the gene. Further deletion to -149 , severely impedes expression. From this we conclude that sequences downstream of -166 are sufficient for light-regulated expression at wild-type levels in transgenic tobacco, and that the 5′ end of a positive element is located between -166 and -149 .

Although 5′ deletions beyond -166 lead to a dramatic decrease of the level of expression, even the -100 deletion does not abolish expression completely, and the remaining low level of transcription is light-dependent (Fig. 5). This suggests that additional positive elements are located downstream of -100 . It is theoretically possible that this residual activity is caused by readthrough from the *rbcS-3A* reference gene, which is present in the same orientation at approximately 5 kb from the test gene (see Fig. 1A).

Redundancy of regulatory elements

Redundancy of *cis*-acting elements has been demonstrated in a number of animal genes (Pelham and Bienz 1982; Pelham 1986; Herr and Gluzman 1985), thus we investigated whether additional LREs could be present in the *rbcS-3A* gene. The 5′ deletion experiments (Fig. 5) suggest that there are one or more elements downstream of -100 , but these are only capable of low levels of expression. To test for additional major LREs, each of the

Figure 3. The 58-bp sequence decreases the transcription in the dark of a constitutive gene. (A) (Lane 1) The CaMV 35S upstream sequence from -943 to -31 was placed upstream of the 35S promoter–CAT gene, and the 60-bp spacer (Fig. 2, lane 1) was positioned between the enhancer and the promoter. (Lanes 2–4) As in Fig. 2, lanes 1–3, but with the CaMV 35S enhancer placed upstream. Chimeric genes were in site 1 (Fig. 1A) and no reference gene was present. Fifty micrograms of total RNA was analyzed by 5' S_1 nuclease digestion, as in Fig. 2. The 35S sequences are hatched and the 60-bp stuffer is dotted. (B) The 58-bp sequence does not affect the transcription start site. (Lanes 1 and 2) RNAs from light-grown plants containing the constructs described in Fig. 3A (lanes 1 and 2) were subjected to 5' S_1 nuclease analysis. The same primer used for preparing the probe was used for dideoxy sequencing (lanes T, C, G, A). The transcription start site of the 35S DNA as determined by Odell et al. (1985) is indicated by an arrow.

three conserved boxes within the 58-bp sequence was replaced with an unrelated linker sequence. Figure 6 shows that such substitutions have no discernable effect, neither on the dark : light ratio nor on the absolute transcript levels in the light. Even a complete deletion of the 58-bp element is without effect. To confirm that the additional LREs are located in the upstream sequence, the region from -410 to -50 with the $-162/-116$ internal deletion was fused to the 35S promoter–CAT reporter gene. Analysis of the transgenic plants shows that this construct is still fully light-regulated (Fig. 7). Thus, there is a redundancy of LREs in the *rbcS-3A* upstream region.

Discussion

Position effects

The experiments described here involved the analysis of several hundreds of transgenic plants. Transcript levels in the dark and in the light were individually assayed by S_1 analysis. From this large sample of data it became clear that the DNA sequences flanking the integration site exert a relatively minor influence on expression of the transgenes. For constructs containing both the test gene and the reference gene, both genes were affected similarly by chromosomal position. We observed a variation in transcript levels of 5- to 10-fold maximally. This

Figure 4. A sequence containing box III is a negative LRE. (Lane 1) The *rbcS-3A* sequence from -136 to -50 was inserted between the 35S enhancer and promoter. Insertion was into site 2 (see Fig. 2A) with the *rbcS-3A* reference gene in site 1. (Lane 2) As lane 1 but with *rbcS-3A* sequence from -100 to -50 . Fifty micrograms of total RNA was analyzed by S_1 nuclease digestion using the 5' probe.

is in contrast with other reports (e.g., Jones et al. 1985) indicating far greater variation between individual plants. In general, we see far less variation with the pMON200 and pMON505 vectors than with previously used vectors, such as pMON120 or pMON145. This decreased variability might be correlated with the superior selectable marker present in pMON200 and pMON505, which allows for selection on higher concentrations of kanamycin. Only in rare cases were plants with an aberrant pattern of regulation encountered. These were not further analyzed.

Negative regulatory elements

We previously showed that a 280-bp sequence between -330 and -50 bp of the pea *rbcS-3A* gene can direct the fully regulated expression of a 35S promoter–CAT reporter gene. Thus, the expression was induced by light, and this induction was conveyed by the photoreceptor phytochrome (Fluhr et al. 1986a). Inspection of this 280-bp sequence led us to focus on a 58-bp sequence in the distal part of this element, between -169 and -112 bp. Comparison of the sequenced *rbcS* genes identified three short conserved sequences (labeled box I, II, III; Fluhr et al. 1986a; Fig. 1). Moreover, box II was shown to have a strong resemblance to the SV40 core enhancer sequence, further encouraging us to test its biological significance. The results shown in Figure 2 make clear that this 58-bp fragment has no detectable enhancing activity. However, when this sequence was placed between the enhancer and the TATA box of a strong constitutive gene, a dramatic decrease of the steady-state mRNA levels in dark-adapted plants was observed (Fig. 3A). From the large numbers of individual plants analyzed, we have

gained confidence that this element indeed does not significantly influence mRNA levels in the light but acts to suppress transcription in the dark. Therefore, we conclude that this 58-bp element has a negative influence on the steady-state mRNA level in dark-adapted plants.

Insertion of the 58-bp element into the constitutive 35S–CAT gene between the promoter and the enhancer causes this gene to become light-regulated. Figure 3B shows that the insertion has no effect on the transcriptional start site. Similarly, the 3' end of the message is not affected (data not shown). Thus, the constitutive and the light-regulated constructs direct the production of identical mRNAs. This rules out that the 58-bp element decreases the steady-state mRNA levels in the dark by a post-transcriptional mechanism, but rather indicates that the effect is at the level of transcription initiation.

An 87-bp sequence from -136 to -50 containing box III is capable of down-regulating transcript levels in the dark (Fig. 4). The sequence between -100 and -50 , which does not contain box III, is inert with respect to light regulation. This strongly suggests that box III is, or is part of, a negative LRE, which can function in a single copy and independently of box II.

Box II is inactive as a positive element, whether present in one or three copies (Fig. 2). One copy has at most a weak negative activity, but three copies have the effect of strongly down-regulating expression in the dark (Fig. 3). There are several ways to explain this remarkable effect. First, when box II is present in three copies, the middle copy is positioned in reverse orientation. It

Figure 5. Effect of 5' deletions on the transcript levels of the *rbcS-3A* gene. The *rbcS-3A* gene and flanking sequences (Fluhr et al. 1986b) were inserted into site 1 of the intermediate vector (see Fig. 1A). The 5' deletions were created by digestion with *Bal31* exonuclease and the end points were verified by sequence analysis. S_1 analysis was with the 3' probe, which protects 160 nucleotides of the reference gene transcripts and 231 nucleotides of the test gene transcripts. Ten micrograms of total RNA were used for each lane, except for the last two lanes, where 50 μ g were used. (D) Dark; (L) light.

Figure 6. Substitution/deletion mutations in the 58-bp sequence reveal redundancy of regulatory elements. Substitution mutations were created via oligonucleotide-mediated site-directed mutagenesis using the plasmid template method of Inouye and Inouye (1986). The background is the pseudo wild-type (5' –410) described in Fig. 5 with the wild-type *rbcS-3A* gene as a reference. Twenty micrograms of total RNA was analyzed by 3' S₁ as in Fig. 5. (Lane 1) No substitution/deletions; (lane 2) Box I (TTTCAAAT) was replaced by AGAATTCA; (lane 3) Box II (TGTGGTTAATAT) was replaced by ATCTAAGAGCTC; (lane 4) Box III (TCATTTTC) was replaced by CTCGAGAA; (lane 5) Box I and Box III were replaced by AGAATTCA and CTCGAGAA, respectively; (lane 6) the sequence between the *EcoRI* and *XhoI* sites in the construct of lane 5 was deleted by double digestion and filling in with Klenow polymerase. Substitutions are indicated by the solid black boxes.

should be noted that there is some sequence homology between box III and the reverse box II (Table 1), and therefore it cannot be ruled out that box II only functions as a negative LRE in the reverse orientation. Second, the 35S enhancer is a relatively strong constitutive element, and one copy of box II may be insufficient to turn off transcription in the dark, whereas multiple copies may override the constitutive elements in the 35S enhancer. We are presently testing this hypothesis by using a weak constitutive enhancer. Third, the well-studied SV40 core enhancer consists of separate, dissimilar domains (Zenke et al. 1986). Inactivation of one domain abolishes enhancer function, but the mutation can

Figure 7. Redundancy in the upstream region of the *rbcS-3A* gene. A fragment from –410 to –50 containing the deletion of Fig. 6, lane 6, was fused upstream of the 35S–promoter CAT gene and the chimera was inserted into site 1. No reference gene was present. Fifty micrograms of total RNA were subjected to 5' S₁ analysis. The hatched area represents the 35S promoter.

be suppressed by duplication of other domains (Herr and Clarke 1986). Thus, a sequence motif is inactive by itself but gains a function when present in multiple copies. A similar situation could exist in the *rbcS-3A* gene: Box II is inactive by itself and needs either box III or additional copies of box II to become functional.

Homology between the LREs and mammalian constitutive enhancer elements

Sequence comparison between light-responsive elements from the *rbcS-3A* gene and transcriptional elements from animal genes reveals striking homologies with constitutive enhancers from mammalian viral and cellular genes (Table 1). As pointed out previously (Coruzzi et al. 1984), box II resembles the SV40 core enhancer GT motif. Box III, in the reverse orientation, has strong homology with both the adenovirus 5 E1A –200 enhancer sequence (Hearing and Shenk 1985) and with the constitutive enhancer in the human interferon- β upstream region (Goodbourn et al. 1986). Thus, both box II and box III resemble constitutive mammalian enhancers rather than regulatory elements. It is possible that the switch that turns the *rbcS-3A* gene off in the dark does not operate at the level of differential protein binding to the LREs, but rather by a light-dependent interaction of another agent with constitutively bound DNA-binding proteins. Alternatively, despite their resemblance to constitutive elements from animal genes, in plants they may be true regulatory *cis*-elements to which repressors bind only in the dark.

Multiple regulatory elements

Substitutions of parts of the 58-bp element or even a complete deletion do not affect expression of the *rbcS-3A* gene. Similarly, when the 58-bp element is deleted from the –410 to –50 upstream region fused to the 35S promoter–CAT gene, no effect can be measured (Figs. 6 and 7). These results indicate that other up-

Table 1. Homology among box II, box III, and other regulatory elements

Sequence	Name	Homology	Reference
GTGTGGTTAATATG	<i>rbcS</i> -3A box II		Fluhr et al. (1986a)
GTG a GG- TAATAT c	<i>rbcS</i> -3A box II*	11/13	Fig. 1B
TGTGG ^{AA} _{TT} AG	SV40 core enhancer consensus		Sassone-Corsi and Borrelli (1986)
AGATAGTGAAAATGATAAAGTT	<i>rbcS</i> -3A box III reverse		Fluhr et al. (1986a)
AGTG t AAATG	<i>rbcS</i> -3A box III* reverse	9/10	Fig. 1B
AGA-AGTGAAA g TG g g AAA-TT	β -interferon constitutive element	17/22	Goodbourn et al. (1986)
g GA-AGTGAAAAT	Ad5 E1A-200	11/12	Hearing and Shenk (1985)
g t g t g GT t AA t ATG	<i>rbcS</i> -3A box II	7/9	Fluhr et al. (1986a)
g t g a g G- t AA t AT c	<i>rbcS</i> -3A box II*	5/8	Fig. 1B
AG-AGTGAAA-TGTGTG	<i>rbcS</i> -3A box III* reverse		Fig. 1B
AG a AGTGAAA g TG	β -interferon constitutive element	11/13	Goodbourn et al. (1986)
GTG g AA-TGTGTG	SV40 enhancer core type II element	11/12	Zenke et al. (1986)

stream sequences must have both enhancing and regulatory capacity. The far upstream region contains a number of SV40 core enhancer-like sequences as pointed out before (Fluhr et al. 1986a). When a direct sequence comparison is made between the 58-bp element and the rest of the upstream sequence, two sequences (labeled box II* and box III*) with striking homology to box II and box III stand out. Box II* and box III* also have strong sequence homology to the SV40 core enhancer and adenovirus 5 E1A enhancer (Table 1). DNase I footprinting experiments with pea nuclear extracts indicate the significance of these homologies (P. Green et al., unpubl.).

A deletion of the 5' end of the *rbcS*-3A gene to -100 bp retains light-regulated expression, albeit at a low level (Fig. 5). This is in agreement with previous results obtained for two other pea *rbcS* genes which locate a weak LRE in the vicinity of the TATA box (Morelli et al. 1985; Timko et al. 1985). In addition, we are currently testing the significance of an "Sph" motif (Zenke et al. 1986) in the transit sequence close to the first intron.

Positive elements

The experiments described here identify at least two short elements (box II and box III) that can function as negative LREs, but do not precisely locate the positive element(s). The following observations are relevant for determining their position. First, 5' deletions of the intact *rbcS*-3A gene define a positive element with a 5' boundary between -166 and -149. Second, a 5' deletion to -100 retains a low level of transcriptional activity, suggesting that a positive element may be located downstream of -100. Third, the 58-bp sequence between -169 and -112 has no enhancing activity (Fig. 2), but also deletion of this sequence has no effect on the absolute transcript levels (Figs. 6 and 7). We suggest that a positive element is located between -166 and extends beyond -100. A 5' deletion to -149 severely, but not completely, inactivates this element. A 3' deletion

beyond -112 totally abolishes its activity. A second positive element most likely is present in the far upstream region between -410 and -170.

The complexity of light regulation

These experiments make it clear that the light-regulated expression of the pea *rbcS*-3A gene is mediated by an extremely complex array of *cis*-acting DNA elements. There is, in fact, an excess of these LREs, because deletion of some of them is without phenotypic effect. Such functional redundancy may be a means to protect the cell against mutational loss of these important elements. However, it should be kept in mind that the assay conditions employed in these studies involve transfer of the plants from total darkness to bright white light, which is a gross simplification of the variety of changes in environmental and endogenous parameters that the intact plant is exposed to under natural conditions. It is conceivable that, for instance, under very dim light or under light of specific wavelengths, a deletion of one set of LREs does have a measurable impact on *rbcS*-3A expression. In addition, expression of *rbcS* genes is known to be sensitive to the concentrations of the plant hormones auxin and cytokinin (Shinkle and Briggs 1984, 1985; Lerbs et al. 1985). It will be interesting to test the effect of hormones on the gene constructs that are assayed here in simple dark-to-light transitions.

Clearly, the only way to determine the physiological importance of the various *cis*-acting elements is to study the mutant gene constructs in the background of the intact organism. The relative ease with which transgenic plants can be generated in large numbers is a great asset for further unraveling the complex regulation of the pea *rbcS*-3A gene.

Experimental procedures

DNA cloning

All cloning procedures were done using established methods. Oligonucleotides were chemically synthesized with an Applied

Biosystems Model 380A DNA synthesizer. Full-length products were purified by electrophoresis in denaturing acrylamide gels. Strands were hybridized and cloned into a derivative of pEMBL8(+) (Dente et al. 1983), and their sequence was verified by dideoxy sequence analysis. Details of the constructions are available upon request.

DNA transfer

Plasmids were transferred by triparental mating into *Agrobacterium tumefaciens* GV3111 SE (Fraley et al. 1985). Exconjugants were purified and used for leaf disc transformation. A slight modification of the published procedure (Horsch et al. 1985) was employed. Leaves from axenically grown *Nicotiana tabacum* plants were placed on solid Murashige and Skoog (MS) medium containing 0.2 mg/liter indole-3-acetic acid (IAA) and 1 mg/liter benzyl adenine purine (BAP). The leaves were wounded by scratching them with a syringe needle dipped into the *Agrobacterium*. After 4 days at 22°C in the light, the leaves were transferred to the same medium containing carbenicillin (0.5 mg/ml) and kanamycin (0.2 mg/ml). Shoots appearing after 2–6 weeks were transferred to MS medium without hormones and with carbenicillin (0.5 mg/ml) and kanamycin (0.1 mg/ml). Plantlets that formed roots (up to 90% of the total) were transferred to soil.

Light induction

Young transgenic plants were transferred into complete darkness for 70–74 hr. A sample of 2–10 grams of leaves was taken in the dark and the plants were transferred to continuous bright white light and, after 23–25 hr, another sample of the leaves was taken. Total RNA was prepared as described (Fluhr et al. 1986b). An average of eight plants per construct were analyzed individually and representative examples are shown.

S₁ analyses

Analysis of 3' termini was performed as described (Fluhr et al. 1986b). The single-stranded probe, prepared by labeling the 3' end of the *rbcs-E9* gene, will protect 230 nucleotides of the *rbcs-E9* transcript and 160 nucleotides of the *rbcs-3A* transcript. For 5' analysis of the 35S–CAT constructs a uniformly labeled probe was prepared by primer extension. A 208-nucleotide fragment was synthesized with its 5' end in the CAT gene and its 3' end at an artificially introduced *SalI* site upstream of the 35S TATA box. Hybridization and S₁ digestion conditions were the same as for the 3' analysis, except that hybridization was in 80% formamide.

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Sequences in the pea *rbcS-3A* gene have homology to constitutive mammalian enhancers but function as negative regulatory elements.

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