The Plant Cell, Vol. 1, 201–208, February 1989, © 1989 American Society of Plant Physiologists

Sequences Downstream of Translation Start Regulate Quantitative Expression of Two Petunia *rbcS* Genes

Caroline Dean,[1] Mitchell Favreau, Diane Bond-Nutter, John Bedbrook, and Pamela Dunsmuir [2]

Advanced Genetic Sciences, 6701 San Pablo Avenue, Oakland, California 94608

We investigated the basis for quantitative differences in leaf expression of the petunia genes (*rbcS*) encoding the small subunit of ribulose bisphosphate carboxylase. The most abundantly, *SSU301*, and the most weakly, *SSU911*, expressed petunia *rbcS* genes maintained their differential expression when transferred to tobacco, indicating that the determinants of quantitative expression are intrinsic to these *rbcS* genes. Analysis of chimeric genes in which the sequences of *SSU301* and *SSU911* had been exchanged at the translation start showed that sequences both 5′ and 3′ to the start codon contribute to differences in steady-state mRNA levels. The sequences 3′ to the translation initiation codon were investigated by preparing chimeric genes in which sequences of the *SSU301* and *SSU911* were exchanged between each intron and at the translation termination codon. The results showed that sequences downstream of the coding region contribute to quantitative differences in expression of *SSU301* and *SSU911*, and nuclear run-on transcription experiments indicated that the 3′ sequences affect transcription rates of the *rbcS* genes.

INTRODUCTION

Ribulose bisphosphate carboxylase (RuBPCase) is the primary enzyme of carbon fixation in the chloroplast. The holoenzyme is composed of eight large and eight small subunits. The large subunit is encoded by chloroplast DNA and is synthesized in the chloroplast (Coen et al., 1977). The small subunit is encoded by a small gene family in nuclear DNA (Kawashima and Wildman, 1972; Dunsmuir et al., 1983), synthesized as a higher molecular weight precursor in the cytoplasm, and then imported into chloroplasts (Chua and Schmidt, 1978; Highfield and Ellis, 1978).

We are studying *Petunia rbcS* genes. The eight petunia *rbcS* genes are divided into three subfamilies that encode slightly different versions of the *rbcS* polypeptide (Dean et al., 1987a). One of the subfamilies contains six genes, five of which are closely linked; the other two subfamilies contain single genes (Dean et al., 1985a). Although all the *rbcS* genes show a high degree of nucleotide sequence similarity both within the coding and the 5′- and 3′-flanking regions (Dean et al., 1985a; Dean et al., 1987a), there is a 100-fold range in expression levels between the genes in leaf tissue (Dean et al., 1985b; Dean et al., 1987b). The most abundantly expressed gene, *SSU301*, contributes about 50% of the *rbcS* mRNA in leaf tissue. The other *rbcS* genes are expressed at levels that are 2- to 100-fold lower than *SSU301*.

Many studies have characterized sequences regulating temporal and spatial expression of multi-gene families (Firtel, 1981; Fyrberg et al., 1983; Hee Kim et al., 1983; Hightower and Meagher, 1985; Sugita and Gruissem, 1987). By contrast, we have characterized sequences responsible for quantitative differences in expression of members of the petunia *rbcS* multi-gene family in leaf tissue.

The basis of the differential expression between the most abundantly expressed petunia *rbcS* gene, *SSU301*, and the least abundantly expressed, *SSU911*, was investigated by analysis of transgenic tobacco plants containing intact or chimeric genes constructed from the two petunia *rbcS* genes. Sequences both 5′ and 3′ to the translation start codon were found differentially to affect transcription of these genes. Here we have dissected the regions 3′ to the start codon, and in the following paper (Dean et al., 1989) we have characterized the regions 5′ to the start codon.

RESULTS

Differential Expression of *SSU301* and *SSU911* Is Intrinsic to the Genes

The most abundantly expressed petunia *rbcS* gene, *SSU301*, is not closely linked (≥10 kb) to other *rbcS* genes (Dean et al., 1985a) or any gene that is moderately expressed in leaf tissue (C. Dean, unpublished results). By contrast, the weakly expressed *rbcS* gene, *SSU911*, lies downstream in a tandem array of five *rbcS* genes (Dean et al., 1985a). To test whether position in the petunia

[1] Current address: John Innes Institute, Colney Lane, Norwich NR4 7UH, United Kingdom.

[2] To whom correspondence should be addressed.

genome affected the expression of *SSU301* and *SSU911*, we transferred genomic regions carrying the genes *SSU301* and *SSU911* with 5′- and 3′-flanking DNA (1.2 kb and 2.5 kb 5′, respectively, and 1.5 kb and 2.4 kb 3′, respectively) into tobacco using *Agrobacterium*-mediated transformation. Multiple independently transformed tobacco plants were regenerated and the expression levels of the *SSU301* and *SSU911* genes were assayed at the stage of leaf development with maximal *rbcS* mRNA levels (Dean et al., 1988a). Although there was considerable variability between transformants in the expression of the introduced gene, several of the transformed tobacco plants carrying the *SSU301* gene had levels of *SSU301* mRNA equivalent to those in petunia leaf tissue, as seen in Figure 1. Similarly, the tobacco plants carrying the *SSU911* gene had levels of *SSU911* mRNA that are comparable with the levels measured in petunia (75- to 100-fold lower than *SSU301* mRNA: Dean et al., 1985b). These data indicate that the differential expression of the petunia *rbcS* genes is an intrinsic property of the transferred *SSU301* or *SSU911* genomic fragments and not a function of their position in the petunia genome.

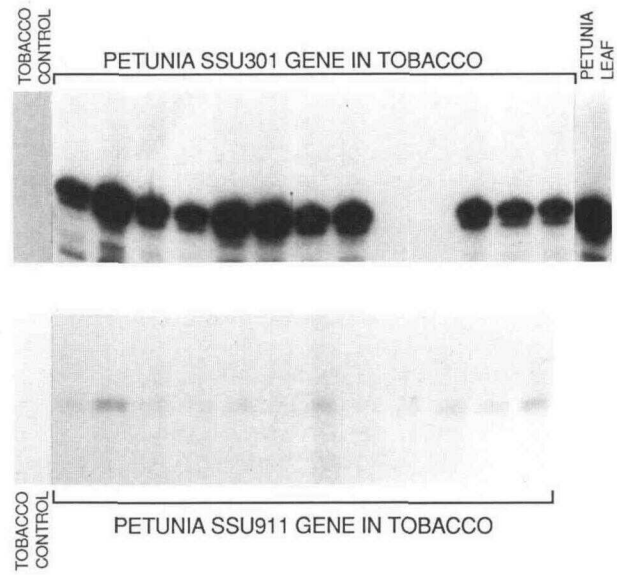

Figure 1. Petunia *SSU301* and *SSU911* Gene Expression in Tobacco RNA.

Primer extension analysis of total RNA isolated from transgenic tobacco plants containing the petunia *rbcS* genes *SSU301* and *SSU911*. Total RNA from untransformed tobacco and petunia were included as controls.

Sequences 5′ and 3′ to the Translation Start Contribute to the Quantitative Differences in Expression of *SSU301* and *SSU911*

We introduced NcoI restriction sites at the translation start codons of *SSU301* and *SSU911*; the mutations were shown to have no effect on the steady-state mRNA levels specified by either gene in gene transfer experiments (data not shown). Sequences of *SSU301* and *SSU911* were then exchanged at these restriction sites, producing chimeric genes with the *SSU301* and *SSU911* sequences combined at the start of the coding region. These genes were introduced into tobacco and their expression was assayed in multiple independently transformed plants.

Figure 2 shows that, although there was considerable variability between transformants in the expression levels of the fusions, the average level of expression of the fusion carrying the 5′ region of *SSU911* and the 3′ region of *SSU301* (the 911/301 fusion) was approximately 15-fold higher than that for the intact *SSU911* gene, Thus, sequences downstream of the translation initiation codon of *SSU301* have a major effect on the steady state mRNA level. The average level of expression of the reciprocal 301/911 fusion was approximately 8-fold lower than that for the intact SSU301 gene, but was still higher than that for the intact *SSU911* gene, showing that sequences 5′ to the translation initiation codon of *SSU301* also contribute to the high mRNA levels of *SSU301.*

To ensure that this result was not an artifact of variable expression between transformed plants, we repeated the experiment and assayed an additional 15 independently transformed plants for each introduced gene or gene fusion. The second analysis confirmed our original findings; the 301/911 fusion gave 5-fold lower levels of mRNA than the intact *SSU301* gene, and the 911/301 fusion gave 30-fold higher levels of mRNA than the intact *SSU911* gene. Our analysis of sequences 5′ to the translation start codon, which influence the quantitative levels of expression of *SSU301* and *SSU911*, is described in the accompanying paper (Dean et al.,1989).

Removal of Intron Sequences from *SSU301* Reduces the Steady-State mRNA Levels

The *SSU301* gene is unique among the eight petunia *rbcS* genes in that it contains a third intron within the coding region 51 bp downstream of the second intron (Dean et al., 1987a). To test whether *SSU301* intron sequences contributed to the expression of this gene, we substituted the coding region and intron sequences from the genomic clone with the coding region from the cDNA clone. The construct was introduced into tobacco and the steady-state mRNA levels were assayed in independently transformed plants. Results are shown in Figure 3. Removal of the intron sequences from the *SSU301* gene reduced the steady-state mRNA level by approximately 5-fold, and this result was confirmed in three independent transformation experiments.

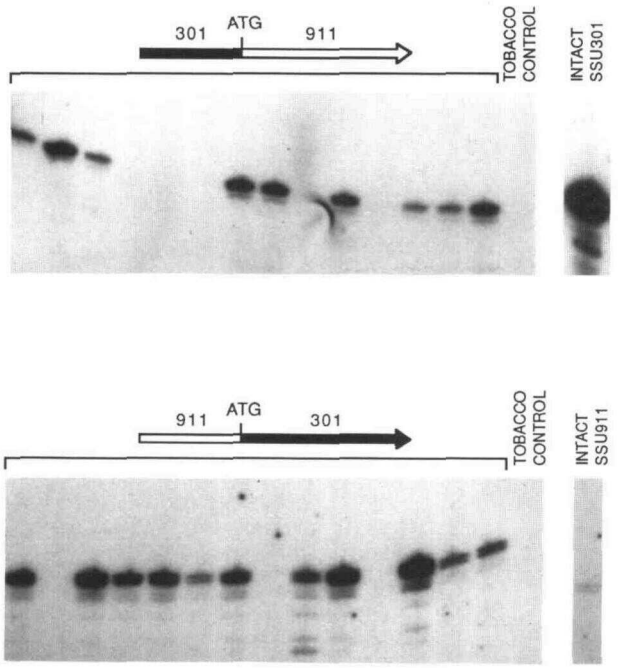

Figure 2. Chimeric *SSU301/SSU911* Gene Expression in Tobacco RNA.

Primer extension analysis of total RNA isolated from transgenic tobacco plants containing fusions of *SSU301* and *SSU911* where their sequences have been exchanged at the translation initiation codon. *SSU301* sequences are represented in black and *SSU911* sequences are represented in white. Total RNA from representative tobacco plants containing the intact *SSU301* and *SSU911* genes and untransformed tobacco were included as controls.

Sequences Downstream of the Stop Codon Impact Steady-State mRNA Levels

To define which sequences 3′ to the translation start are important for *SSU301* expression, we constructed a series of fusions that exchanged the sequences of *SSU301* and *SSU911* between each intron and also at their translation termination codons, as shown in Figure 4. One exchange point was located 4 bp from the 5′ end of the second intron. A second exchange point was located 9 bp into the third exon, and the third exchange point was at an introduced site immediately downstream of the translation termination codon in both genes. (Subsequently, we showed that these introduced mutations do not affect the steady-state mRNA levels of either gene.) The different fusions were introduced into tobacco plants in two independent transformation experiments, and the expression of each fusion was assayed in 15 plants in each experiment. The primer extension analysis from the first transformation experiment is shown in Figure 5. The expression of the introduced fusion varied between plants, which makes accurate quantitation of the relative expression level of these gene fusions difficult. To obtain an approximate value for the relative expression of the different fusions, we pooled equal amounts of RNA from the 30 tobacco plants containing the same fusion (15 plants from each transformation experiment) and repeated the primer extension analysis. In this way, the 30 tobacco plants containing the same fusion were considered to be one population.

The primer extension analysis on pooled RNA samples from the 11 different fusions is shown in Figure 6, together with a 2-fold dilution series that aided in the estimation of relative expression values given as a percentage of the expression level of the *SSU301* gene in tobacco. Relative expression values, estimated from the individual plants showing expression of the introduced fusion, were used to calculate standard error values.

Expression of the intact *SSU911* gene in tobacco was approximately 75-fold lower than expression of *SSU301*. When the sequences of *SSU911* 3′ to the translation start were replaced with those from *SSU301* (fusion 8), the resulting steady-state mRNA levels increased approximately 15-fold (as discussed earlier). A similar increase was observed when smaller fragments of *SSU301* were used to replace corresponding regions of *SSU911* (fusions 9, 10, and 11). It appears that *SSU301* sequences down-

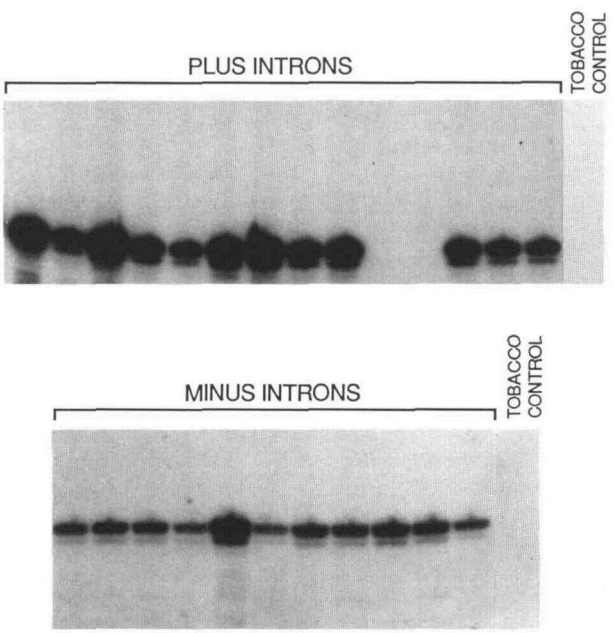

Figure 3. Effect of Introns on *SSU301* Expression.

Primer extension analysis of total RNA isolated from transgenic tobacco plants containing the petunia *SSU301* gene with and without introns. Total RNA from untransformed tobacco was included as a control.

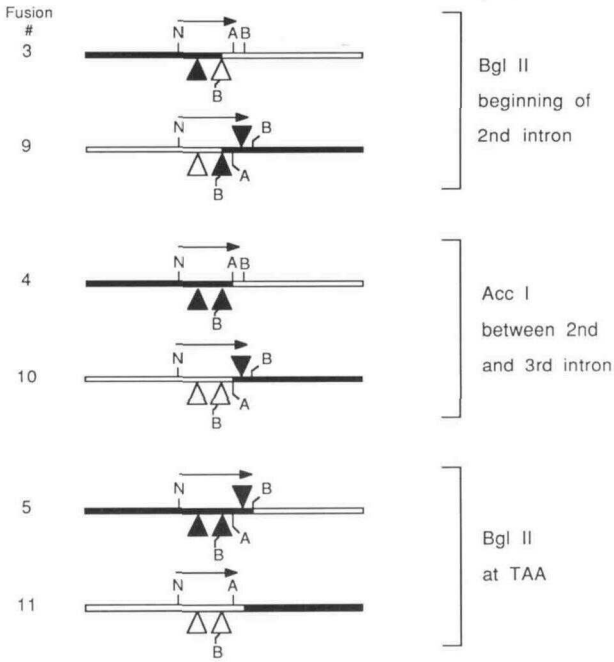

Figure 4. Summary of *SSU301/SSU911* Fusion Genes Transferred to Tobacco.

Schematic diagram illustrating the fusions of *SSU301* and *SSU911*, where the sequences of the two genes were exchanged at the beginning of the second intron (fusions 3 and 9), between the second and third introns (fusions 4 and 10), and at the translation termination codon (fusions 5 and 11). Intron sequences are shown as triangles and the arrow indicates the direction of transcription. N = NcoI, A = AccI, B = BglII.

stream of the stop codon have an effect on expression parallel to that observed from sequences 3′ to the start codon (cf. fusion 8 and 11). The results from the reciprocal fusions 2, 3, 4, and 5 support this conclusion. When the sequences of *SSU301* 3′ to the start codon were replaced with those from *SSU911* (fusion 2), the resulting steady-state mRNA levels decreased 7-fold. Addition of progressively more *SSU301* sequences (until only the 3′-flanking sequences were missing) did not result in restoration of expression levels equivalent to the intact *SSU301* gene.

The reduction in mRNA levels after removal of intron sequences (fusion 6) appears to be due to the absence of introns rather than to loss of specific *SSU301* intron sequences, as the expression of fusion 8 (containing the three *SSU301* introns) is approximately the same as fusion 11, which contains the two *SSU911* introns. Alternately, sequences within the *SSU301* introns may interact with those in the *SSU301* 5′-untranslated region and influence transcript stability in the nucleus.

The simplest interpretation of the results from the exchange fusions is that the sequences that have a major impact on steady-state mRNA levels and that lie 3′ to the start of the *rbcS* coding region are actually located 3′ to the coding region. We cannot rule out that sequence interactions between different regions of the *rbcS* genes affect transcript stability.

Sequences 3′ to the Translation Start Affect Transcription

We used nuclear run-on transcription experiments to test whether sequences 3′ to the translation start influence transcription rates of the different genes. Populations of transgenic tobacco plants containing the different petunia *rbcS* genes and gene fusions were used for nuclear run-on transcription experiments to overcome the problem of variability between transformants. Equal amounts of leaf tissue were harvested from 15 tobacco plants containing the same construct, and nuclei were isolated. The nuclei were allowed to extend previously initiated transcripts for 10 min in the presence of ^{32}P-UTP; total RNA was then isolated and used as a probe for slot blots containing single-stranded DNA. The hybridization and washing conditions used in the experiment allowed hybridization of transcripts from both the endogenous tobacco *rbcS* genes and the introduced petunia *rbcS* transcripts. Hence, the cDNA clone of *SSU301* (slot 2, antisense strand and slot 3, sense strand) was included as an internal control for tobacco *rbcS* gene expression in the different populations. Clones containing the 3′ regions of *SSU301* and *SSU911*

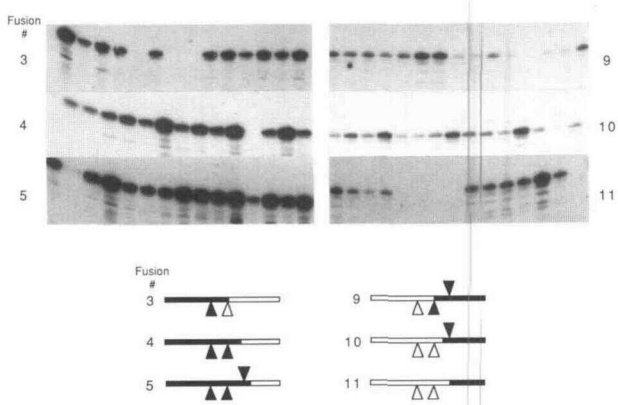

Figure 5. Expression of Chimeric Genes in Populations of Transformed Tobacco Plants.

Primer extension analysis of total RNA isolated from 15 transgenic tobacco plants containing fusions of *SSU301* and *SSU911*. Sequences have been exchanged at the beginning of the second intron (fusions 3 and 9), between the second and third introns (fusions 4 and 10), and at their translation termination codons (fusions 5 and 11). Schematic illustrations of the fusions are included at the base of the figure. *SSU301* sequences are shown in black, *SSU911* sequences are shown in white, and intron sequences are shown as triangles.

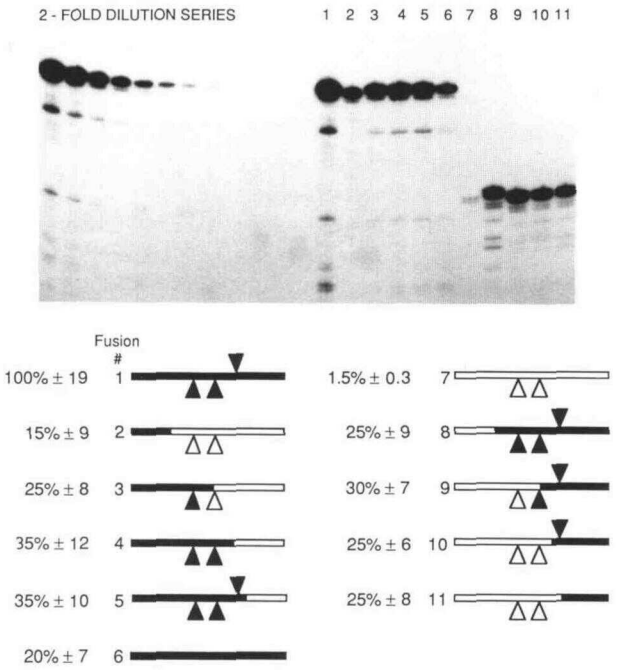

Figure 6. Expression of Chimeric Genes in Tobacco RNA.

Each numbered lane represents the primer extension analysis of RNA samples pooled from 30 transgenic tobacco plants carrying different petunia *rbcS* genes or gene fusions. The lanes labeled 1 to 11 show the primer extension analyses of the pooled RNA samples from plants containing fusions 1 to 11. These fusions are illustrated schematically at the base of the figure. *SSU301* sequences are shown in black, *SSU911* sequences are shown in white, and intron sequences are shown as triangles. A twofold dilution series was included to aid quantification of the relative expression levels. The expression levels of fusions 2 to 11 were estimated relative to the expression of *SSU301* and are shown as percentages, alongside the schematic illustrations. Standard error values are also shown on the figure. These were calculated from expression levels of individual plants that showed expression of the introduced fusion (>20).

were used as specific probes for the introduced petunia *rbcS* fusions.

An autoradiograph of a slot blot probed with run-on transcripts from different tobacco populations is shown in Figure 7A. Hybridization to DNA in slot 2 (the internal control slot) was similar for each sample. Nuclear transcripts from the nontransformed W38 tobacco plants did not hybridize to slots containing DNA from the 3′ regions of *SSU301* or *SSU911*, demonstrating that the petunia gene-specific probes (in slots 4 to 7) did not cross-hybridize to the endogenous tobacco *rbcS* transcripts. Nuclear transcripts from the tobacco plants containing fusion 1 (the intact *SSU301* gene) hybridized strongly to DNA in slot 4 (the slot containing the antisense DNA of the 3′ region of *SSU301*); however, transcripts from the tobacco plants containing fusion 7 (the intact *SSU911* gene) showed no detectable specific hybridization to DNA in slot 6 (the slot containing the antisense strand of the *SSU911* probe). Transcripts from the 911/301 fusion 9, containing 3′ sequences from *SSU301*, hybridized strongly to DNA in slot 4, indicating that the relative transcription rate of fusion 9 was considerably higher than that of fusion 7, and, thus, that the *SSU301* sequences contained in fusion 9 influence the transcription rate of the fusion.

We repeated the run-on transcription experiments using populations of tobacco plants carrying fusions 1, 2, 6, 7, 8, and 11. Hybridization to DNA in slot 2 (the internal control slot) was similar for each of the RNA samples. The hybridization signal with slots containing the gene-specific probes was quantitated by densitometric scanning, and the data are shown in Figure 7B. The relative transcription rates of fusions 1, 2, and 6, which all contain the *SSU301* promoter region, are similar. The relative transcription rate of fusion 7 (the intact *SSU911* gene) is considerably lower. When sequences of *SSU301* either 3′ to the start codon (fusion 8) or 3′ to the stop codon (fusion 11) were contained in the chimeric gene, the relative transcription rate increased. These data suggest that sequences of *SSU301* lying downstream of the coding region and affecting mRNA levels exert their effect at the transcriptional level.

It is not clear from these results whether the relative transcription rate of fusion 6, the *SSU301* gene lacking introns, is reduced significantly compared with that of fusion 1, the intact *SSU301* gene. As the observed differences in steady-state mRNA levels are relatively small (5-fold), more run-on experiments are necessary to determine whether the reduction in steady-state mRNA levels is due to transcriptional or posttranscriptional effects.

DISCUSSION

To understand the basis of the quantitative differences in expression of petunia *rbcS* genes in leaf tissue, we conducted experiments with the most abundantly expressed (*SSU301*) and the least abundantly expressed (*SSU911*) *rbcS* genes. Both genes maintained their relative expression levels after transfer to tobacco, showing that the expression of *SSU911* in petunia is not affected by its location at the end of the tandem array of four other expressed *rbcS* genes.

Experiments in which different regions of *SSU301* and *SSU911* were exchanged and the expression of the fusions assayed in transgenic tobacco plants showed that sequences 3′ to the translation initiation codon contribute more to the quantitative differences in expression of *SSU301* and *SSU911* than sequences 5′ to the coding region.

Reciprocal fusions between *SSU301* and *SSU911*, where the exchange points were located between intron sequences and at the end of the coding region, showed that sequences 3′ to the coding region significantly influenced steady-state mRNA levels. These sequences appear to increase the transcription rate of the fusion.

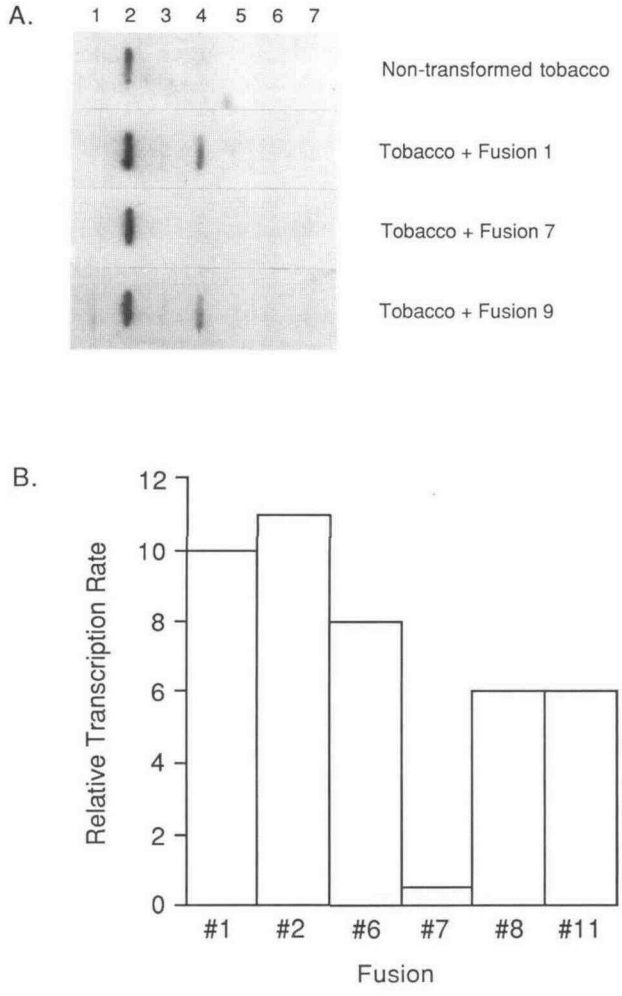

Figure 7. Nuclear Run-On Transcription Experiments.

(A) Slot blot analysis of nuclear run-on products from nontransformed tobacco plants, tobacco plants containing fusion 1 (intact *SSU301* gene), fusion 7 (intact *SSU911* gene), or fusion 9 (the *SSU911/SSU301* exchange fusion, where the sequences were exchanged at the beginning of the second intron). Ten micrograms of single-stranded DNA was applied to each slot: 1, pUC118; 2, cDNA clone of *SSU301*, antisense orientation; 3, cDNA clone of *SSU301*, sense orientation; 4, *SSU301* gene-specific probe, antisense orientation; 5, *SSU301* gene-specific probe, sense orientation; 6, *SSU911* gene-specific probe, antisense orientation; 7, *SSU911* gene-specific probe, sense orientation.

(B) Histogram showing results from a nuclear run-on transcription experiment. The run-on products were analyzed using a slot blot analysis and quantitated by scanning the autoradiograph of the slot blot. The fusions contained in the different tobacco populations are indicated underneath the histogram.

Removal of intron sequences from *SSU301* also decreased the steady-state mRNA levels 5-fold. Callis et al. (1987) have recently shown that the addition of heterologous intron sequences to gene fusions increased their expression after electroporation in cultured maize protoplasts. They suggested that the presence of introns affect posttranscriptional processes in the maize cells. In a similar way, the intron sequences may be increasing the level of expression of *SSU301*. As the cytoplasmic mRNA structure from the *SSU301* gene with and without introns is identical, stability of the cytoplasmic mRNA cannot be a cause of the lower mRNA levels. Differential stability of the nuclear transcripts with and without introns may cause the differences in mRNA levels (Leys et al., 1984). Introns have been shown to increase the transcriptional efficiency of four sets of gene fusions in transgenic mice (Brinster et al., 1988); however, removal of intron sequences from a pea *rbcS* gene did not appear to reduce the expression level of the fusion (Kuhlemeier et al., 1988).

The most novel result emerging from this study is that sequences 3′ to translation start, which may all be located 3′ to the *rbcS* coding region, have a significant impact on the relative transcription rate of the *rbcS* genes. This is the first example of plant sequences that are located 3′ to the coding region having an effect on transcription rate. Sequences 3′ to the coding region have been shown to affect the qualitative expression of a proteinase inhibitor II gene in potato (Sanchez-Serrano et al., 1987; Thornberg et al., 1987), but whether they exert their effect at the transcriptional level is not yet known.

We are investigating whether the observed effects are due to sequences 3′ to the *SSU301* coding region having a positive effect on transcription rate or the sequences 3′ to the *SSU911* coding region having a negative effect. There are now many examples of sequences downstream of animal genes that act as "enhancer" elements (Choi and Engel, 1986; Fischer and Maniatis, 1986; Bodine and Ley, 1987; Owen and Kuhn, 1987; Trainor et al., 1987). These can be quite short regions of DNA and have been shown to exert their positive effect on transcription irrespective of their orientation or position in the gene. They often exert their effects in a tissue-specific or developmentally regulated fashion. Currently, we are defining in more detail which *rbcS* sequences are important for the effects on transcription and whether their effects are light-dependent or tissue-specific.

METHODS

Details of Cloning Procedures

Cloning the SSU301 and SSU911 Genes into an Agrobacterium Vector

Genomic clones carrying the genes *SSU301* and *SSU911* with 5′- and 3′-flanking DNA (1.2 kb and 2.5 kb 5′, respectively, and 1.5

kb and 2.4 kb 3′, respectively) in pUC plasmids (Vieira and Messing, 1982) were linearized at a unique BamHI site located 1.5 kb 3′ to the *SSU301* gene and 1.8 kb 3′ to the *SSU911* gene. These were then cloned into the BamHI site of the *Agrobacterium* binary vector pAGS135 (Dean et al., 1988a).

Construction of Fusions Where the SSU301 and SSU911 Sequences Were Exchanged at the Translation Initiation Codon

NcoI sites were introduced into both genes at the translation initiation codon by site-directed mutagenesis (Kunkel, 1985). This required a 1-bp change in both genes ACATGG to CCATGG. The 301/911 and 911/301 ATG exchange fusions were cloned into the *Agrobacterium* binary vector pAGS140 (Dean et al., 1988b).

Construction of an SSU301 Gene Lacking Introns

We substituted the coding region and intron sequences from the genomic clone with the coding region from the cDNA clone, utilizing restriction sites found in both a cDNA clone and the genomic clone *pSSU301* (a PstI site 20 bp 3′ to the ATG codon and a SacI site located 108 bp 5′ to the TAA codon). The *SSU301* gene without introns was linearized with BamHI and cloned into the BamHI site of pAGS135 (Dean et al., 1988a). The same orientation of the genes within the binary vectors was chosen for all the constructions.

Construction of Fusions Where the SSU301 and SSU911 Sequences Were Exchanged Within and Downstream of the Coding Region

The sequences of *SSU301* and *SSU911* were exchanged at three positions downstream of the translation start. The first was a BglII site located in both genes at the beginning of the second intron (4 bp from the exon/intron junction). As there were two other BglII restriction sites in both genes, the clonings required the use of partial restriction enzyme digestions. The second exchange point was an AccI site in exon sequences (9 bp from the intron/exon junction in the third exon of both genes). The genomic clone carrying *SSU301* contained one other AccI site and the genomic clone carrying *SSU911* contained six other AccI sites, so partial restriction enzyme digests were required in the cloning steps. The third exchange point was a BglII site introduced by site-directed mutagenesis adjacent to the TAA translation termination codon. *SSU301* sequence was mutated from TAAGTTAT to TAAGATCT. A BglII site was introduced into the *SSU911* gene by insertion of 3 bp to TAAATT to TAAAGATCT. Partial BglII restriction digests were used in the cloning reactions. The plasmids were then linearized with BamHI and cloned into pAGS140.

Generation and Analysis of Transgenic Plants

All binary vector plasmids were mobilized into *Agrobacterium tumefaciens* LBA4404/pAL4404 (Hoekema et al., 1983) by triparental mating (Figurski and Helinski, 1979). The *Agrobacterium* strains were co-cultivated with tobacco protoplasts (van den Elzen et al., 1985) or tobacco leaf discs (Horsch et al., 1985). Kanamycin-resistant shoots were rooted and the plants were transferred to soil and into the greenhouse. Total RNA was isolated (Dean et al., 1985b) from 10-cm leaves of plants 25 days after transfer to the greenhouse and analyzed by primer extension analysis with oligonucleotides 301T and 911T (Dean et al., 1987b). These oligonucleotides anneal to a region in the *rbcS* transit sequence that is divergent between *SSU301* and *SSU911*. The major fragments resulting from extension of 301T and 911T are 161 bp and 126 bp, respectively.

The relative expression values for the different fusions were estimated by comparing the signal on the autoradiograph to the signals from the twofold dilution series made from RNA isolated from plants containing fusion 1.

Nuclear Run-On Transcription Experiments

Nuclei were isolated from tobacco leaves by a combination of the protocols described by Luthe and Quatrano (1980), Walling et al. (1986), and D. Burgess (personal communication). Details of the procedure are available upon request.

Slot blots carrying 10 μg of single-stranded DNA were hybridized to equal amounts of the labeled RNA in 50% formamide, 10% dextran sulfate, Denhardt's solution, 0.1% SDS, 50 mM phosphate buffer, pH 6.8, and 0.5 M NaCl. Prehybridizations were carried out for 12 hr and hybridizations for 48 hr at 42°C. The filters were washed in 2 × SSC, 0.1% SDS at 42°C and then in 0.1 × SSC, 0.1% SDS at 50°C. The last wash contained 1 μg/ml RNase A. Only the hybridization signal present in the slot containing the antisense DNA, not present in the slot containing the sense DNA, was measured to estimate relative transcription rates.

A 3′ 1.5-kb BglII (at the translation stop codon)-BamHI fragment from *SSU301* was cloned and used as the *SSU301* gene-specific probe. A similarly positioned 1.8-kb BglII-BamHI fragment was cloned and used as the gene-specific probe for *SSU911*. Four-hundred-base pair probes extending 3′ from the translation stop codon were also cloned from each gene. These showed the same relative hybridization signals as the longer probes.

ACKNOWLEDGMENTS

We thank Diane Burgess for unpublished protocols and advice on the nuclear run-on transcription experiments. We also thank Tom Lemieux and Cara Robinson for care of the tobacco plants, Connie Stephens and Rob Narberes for preparing the figures, and Gary Warren and Diane Burgess for critical reading of the manuscript.

Received December 2, 1988.

REFERENCES

Bodine, D.M., and Ley, T.J. (1987). An enhancer element lies 3′ to the human gamma globin gene. EMBO J. **6**, 2997–3004.

Brinster, R.L., Allen, J.M., Behringer, R.R., Gelinas, R.E., and Palmiter, R.D. (1988). Introns increase transcriptional efficiency in transgenic mice. Proc. Natl. Acad. Sci. USA **85**, 836–840.

Callis, J., Fromm, M., and Walbot, V. (1987). Introns increase gene expression in cultured maize cells. Genes Dev. **1,** 1183–1200.

Choi, O.R., and Engel, J.D. (1986). A 3′ enhancer is required for temporal and tissue-specific transcriptional activation of the chicken adult beta-globin gene. Nature **323,** 731–734.

Chua, N.-H., and Schmidt, G.W. (1978). Post-translational transport into intact chloroplasts of a precursor to the small subunit of ribulose 1, 5-bisphosphate carboxylase. Proc. Natl. Acad. Sci. USA **75,** 6110–6114.

Coen, D.M., Bedbrook, J.R., Bogorad, L., and Rich A. (1977). Maize chloroplast DNA fragment encoding the large subunit of ribulose bisphosphate carboxylase. Proc. Natl. Acad. Sci. USA **74,** 5487–5491.

Dean, C., van den Elzen, P., Tamaki, S., Dunsmuir, P., and Bedbrook, J. (1985a). Linkage and homology analysis divides the eight genes for the small subunit of petunia ribulose-1,5-bisphosphate carboxylase into three gene families. Proc. Natl. Acad. Sci. USA **82,** 4964–4968.

Dean, C., van den Elzen, P., Tamaki, S., Dunsmuir, P., and Bedbrook, J. (1985b). Differential expression of the eight genes of the petunia ribulose bisphosphate carboxylase small subunit multi-gene family. EMBO J. **4,** 3055–3061.

Dean, C., van den Elzen, P., Tamaki, S., Black, M., Dunsmuir, P., and Bedbrook, J. (1987a). Molecular characterization of the *rbcS* multi-gene family of *Petunia* (Mitchell). Mol. Gen. Genet. **206,** 465–474.

Dean, C., Favreau, M., Dunsmuir, P., and Bedbrook, J. (1987b). Confirmation of the relative expression levels of the *Petunia* (Mitchell) rbcS genes. Nucleic Acids Res. **15,** 4655–4668.

Dean, C., Favreau, M., Tamaki, S., Bond-Nutter, D., Dunsmuir, P., and Bedbrook, J. (1988a). Expression of tandem gene fusions in transgenic tobacco plants. Nucleic Acids Res. **16,** 7601–7617.

Dean, C., Jones, J., Favreau, M., Dunsmuir, P., and Bedbrook, J. (1988b). Influence of flanking sequences on variability in expression levels of an introduced gene in transgenic tobacco plants. Nucleic Acids Res. **16,** 9267–9283.

Dean, C., Favreau, M., Bedbrook, J., and Dunsmuir, P. (1989). Sequences 5′ to translation start regulate expression of petunia *rbcS* genes. Plant Cell **1,** 209–215.

Dunsmuir, P., Smith, S.M., and Bedbrook, J.R. (1983). A number of different nuclear genes for the small subunit of RuBPCase are transcribed in petunia. Nucleic Acids Res. **11,** 4177–4183.

Figurski, D., and Helinski, D.R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. USA **76,** 1648–1652.

Firtel, R.A. (1981). Multigene families encode actin and tubulin. Cell **24,** 6–7.

Fischer, J.A., and Maniatis, T. (1986). Regulatory elements involved in *Drosophila Adh* gene expression are conserved in divergent species and separate elements mediate expression in different tissues. EMBO J. **5,** 1275–1289.

Fyrberg, E.A., Mahaffey, J.W., Bond, B.J., and Davidson, N. (1983). Transcripts of the six *Drosophila* actin genes accumulate in a stage and tissue specific manner. Cell **33,** 115–123.

Hee Kim, K., Rheinwald, J.G., and Fuchs, E.V. (1983). Tissue specificity of epithelial keratins: Differential expression of mRNAs from two multi-gene families. Mol. Cell. Biol. **3,** 495–502.

Highfield, P.E., and Ellis, R.J. (1978). Synthesis and transport of the small subunit of chloroplast ribulose bisphosphate carboxylase. Nature **271,** 420–424.

Hightower, R.C., and Meagher, R.B. (1985). Divergence and differential expression of soybean actin genes. EMBO J. **4,** 1–8.

Hoekema, A., Hirsch, P.R., Hooykaas, P.J.J., and Schilperoot, R.A. (1983). A binary plant vector strategy based on separation of *vir*- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature **303,** 179–180.

Horsch, R.B., Fry, J.E., Hoffman, N.L., Eichholtz, D., Rogers, S.G., and Fraley, R.T. (1985). A simple and general method for transferring genes into plants. Science **227,** 1229–1231.

Kawashima, N., and Wildman, S.G. (1972). Studies on fraction 1 protein IV. Mode of inheritance of primary structure in relation to whether chloroplast or nuclear DNA contains the code for a chloroplast protein. Biochim. Biophys. Acta **262,** 42–49.

Kuhlemeier, C., Fluhr, R., and Chua, N.-H. (1988). Upstream sequences determine the differences in transcript abundance of pea *rbcS* genes. Mol. Gen. Genet. **212,** 405–411.

Kunkel, T.A. (1985). Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82,** 488–492.

Leys, E.J., Crouse, G.F., and Kellems, R.E. (1984). Dihydrofolate reductase gene expression in cultured mouse cells is regulated by transcript stabilization in the nucleus. J. Cell. Biol. **99,** 180–187.

Luthe, D.S., and Quatrano, R.S. (1980). Transcription in isolated wheat nuclei. 1. Isolation of nuclei and elimination of endogenous ribonuclease activity. Plant Physiol. **65,** 305–308.

Owen, D., and Kuhn, L.C. (1987). Non-coding 3′ sequences of the transferring receptor gene are required for mRNA regulation by iron. EMBO J. **6,** 1287–1293.

Sanchez-Serrano, J., Keil, M., Pera-Cortes, M., Rocha-Sosa, M., and Willmitzer, L. (1987). Wound-induced expression of proteinase inhibitor II in potato and transgenic tobacco. In Plant Gene Systems and Their Biology, J. Key and L. McIntosh, eds (New York: Alan R. Liss), pp. 331–338.

Sugita, M., and Gruissem, W. (1987). Developmental, organ-specific, and light-dependent expression of the tomato ribulose-1, 5-bisphosphate carboxylase small subunit gene family. Proc. Natl. Acad. Sci. USA **84,** 7104–7108.

Thornberg, R.W., An, G., Cleveland, T.E., Johnson, R., and Ryan, C. (1987). Wound-inducible expression of a potato inhibitor II-chloramphenicol acetyl transferase gene fusion in transgenic tobacco plants. Proc. Natl. Acad. Sci. USA **84,** 744–748.

Trainor, C.D., Stamler, S.J., and Engel, J.D. (1987). Erythroid-specific transcription of the chicken histone H5 gene is directed by a 3′ enhancer. Nature (Lond.) **328,** 827–830.

van den Elzen, P., Lee, K.Y., Townsend, J., and Bedbrook, J. (1985). Simple binary vectors for DNA transfer to plant cells. Plant Mol. Biol. **5,** 149–154.

Vieira, J., and Messing, J. (1982). The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene (Amst.) **19,** 259–268.

Walling, L., Drews, G.N., and Goldberg, R.B. (1986). Transcriptional and post-transcriptional regulation of soybean seed protein mRNA levels. Proc. Natl. Acad. Sci USA **83,** 2123–2127.