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Cotranscription of genes encoding the small and large subunits of ribulose-1,5-bisphosphate carboxylase in the cyanobacterium *Anabaena* 7120

(*rbcS* gene sequence/promoter mapping/transcription regulation)

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ABSTRACT **In the filamentous cyanobacterium *Anabaena*, the gene for the small subunit (*rbcS*) of ribulose-1,5-bisphosphate carboxylase is linked to and transcribed together with the gene encoding the large subunit (*rbcL*) of the same enzyme. The two genes are separated by a spacer of 545 base pairs (bp) that does not contain an open reading frame. Both genes hybridize with a predominant 3.1-kilobase transcript that initiates 414 bp upstream from the *rbcL* coding region. The nucleotide sequence 14–8 bp preceding the transcription start site resembles a good *Escherichia coli* promoter, but the sequence in the −35 region does not. There is no obvious relationship between the sequence flanking the amino terminus of the cyanobacterial small subunit gene and the transit peptide of eukaryotic small subunit genes. The *Anabaena rbcS* gene is 61% homologous at the amino acid level with the gene from the unicellular cyanobacterium *Anacystis* and 37–38% homologous with the corresponding nuclear genes from eukaryotes. In contrast, the *Anabaena rbcL* gene is ≈86% homologous at the amino acid level with the *rbcL* genes from plant chloroplasts. Cotranscription satisfies one of the requirements for coordinate expression of the two genes whose products are needed in equimolar amounts in the mature enzyme. The *rbcL–rbcS* transcript is equally abundant in *Anabaena azollae* grown in the light or on fructose in the dark.**

Ribulose-1,5-bisphosphate (RuP_2) carboxylase catalyzes the incorporation of CO_2 into carbohydrate in plant and algal chloroplasts as well as in photosynthetic prokaryotes. The enzyme contains eight identical large subunits of M_r = 56,000 and eight (nearly) identical small subunits of M_r ≈14,000 (1). In plants, a unique gene in chloroplast DNA encodes the large subunit, while a family of eight or more nuclear genes encodes the small subunit (2, 3). The nuclear genes are not identical and many or all are expressed, leading to some microheterogeneity in the small-subunit amino acid sequence (4). Moreover, the nuclear genes contain intervening sequences, some of which separate domains of the mature small-subunit protein (3). All of the eukaryotic small-subunit genes studied thus far contain an intervening sequence located between amino acid residues 2 and 3 of the mature protein and a leader region that codes for a transit peptide (5, 6). A precursor of the small subunit is synthesized on cytoplasmic ribosomes and then transported into the chloroplast accompanied by cleavage of the transit peptide at a Cys-Met bond to yield the mature protein (3).

In this report, we show that the cyanobacterium *Anabaena* contains a unique small-subunit gene located downstream from the large-subunit gene. There is no transit peptide in the small-subunit protein sequence nor are intervening sequences present in the prokaryotic gene. The genes coding for the two subunits of RuP_2 carboxylase appear to be transcribed in a single mRNA in *Anabaena*.

MATERIALS AND METHODS

Isolation of DNA and Preparation of Southern Blots. DNA from *Anabaena* 7120 was prepared as described (7). *Escherichia coli* plasmid DNA was isolated as described by Davis *et al.* (8). Chromosomal and plasmid DNA were digested with restriction endonucleases, subjected to agarose gel electrophoresis, and transferred to nitrocellulose filters as described (9–11).

Isolation of RNA and Preparation of RNA Transfer Blots. Total RNA was isolated from *Anabaena* cells grown on complete medium as described (12). Aliquots containing 20 μg of the RNA were denatured with dimethyl sulfoxide and glyoxal, fractionated on 1% agarose gels, and transferred to nitrocellulose paper as described (12). Size markers, consisting of DNA fragments of known size, were similarly denatured and fractionated.

Homologous and Heterologous Hybridizations. The cDNA clone kindly provided by J. Bedbrook, pSSU160, contains 70% of the pea small subunit coding sequence (13) and was used as a heterologous hybridization probe to locate the *Anabaena* small-subunit gene. pSSU160 DNA was digested with *Hin*dIII and electrophoresed on a 5% acrylamide gel. A 230-base-pair (bp) fragment, containing the last 7 amino acids of the small-subunit transit peptide and the first 70 amino acids of the mature small-subunit coding sequence (13), was purified and nick-translated as described (9). This probe was hybridized with Southern blots containing *Hin*dIII-cut pAn600 and pAn606 (see Fig. 1). Filters were prehybridized at 40°C for 2 hr in 100 ml of 6× concentrated SSPE buffer (SSPE buffer = 0.15 M NaCl/0.01 M NaH_2PO_4/0.001 M Na_2EDTA, pH 7.0) containing 0.5% $NaDodSO_4$, Denhardt's solution (14), and 0.03 μg of pBR322 DNA per ml and then hybridized for 12 hr at 40°C in 10 ml of 6× concentrated SSPE buffer containing 0.5% $NaDodSO_4$, Denhardt's solution, and ≈1 × 10^6 cpm of ^{32}P-labeled probe DNA. Filters were rinsed at 40°C three times for 1 hr each in 6× concentrated SSPE buffer/0.5% $NaDodSO_4$, air dried, and autoradiographed. Subsequently, filters were rewashed two times for 2 hr each in 6× concentrated SSPE buffer at 42°, 44°, 46°, and 50°C. Following each wash at the higher temperature, filters were air dried and autoradiographed.

In the homologous hybridizations, DNA fragments specific for the *Anabaena* large subunit and small subunit genes were purified and hybridized with RNA transfer blots or Southern blots. For the large-subunit probe, the plasmid pAn602 was digested with *Hin*dIII and *Hpa* I and the 1.0-kbp

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Abbreviations: RuP_2, ribulose-1,5-bisphosphate; bp, base pair(s); kb, kilobase(s).

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*Hin*dIII/*Hpa* I fragment internal to the large-subunit coding region (Fig. 1) was purified after electrophoresis on preparative agarose gels according to Maniatis *et al.* (15). A 0.95-kbp *Hin*dIII/*Xba* I fragment containing the entire small-subunit gene was isolated in an analogous manner from a *Hin*dIII and *Xba* I double digest of the plasmid pAn606 (Fig. 1).

DNA Sequence Analysis. *Anabaena* 7120 DNA fragments were prepared from plasmid pAn606, which contains a 1.5-kbp *Hin*dIII fragment (Fig. 1). DNA sequence analysis was carried out according to Maxam and Gilbert (16), with the exception of the piperidine cleavage step, which was modified as described by Smith and Calvo (17). The modified DNAs were analyzed on 5%, 8%, and 20% polyacrylamide gels (16) and autoradiographed with Kodak XAR-5 film using intensifier screens. All restriction sites except one were overlapped. The sequence at this site was determined by reading up to the labeled end on adjacent fragments by using 20% acrylamide gels (16). Sequencing ladders were transcribed manually, entered into an IBM computer, and translated by using the program of Queen and Korn (18).

S1 Nuclease Mapping and Primer Extension Assays. The start site of *rbcL* transcription was roughly determined by isolating *Hpa* I fragments containing sequences upstream from the *rbcL* coding region and subjecting these to S1 nuclease protection assays (see Fig. 1). The 450-bp *Hpa* I fragment that includes the 5′ end of the coding region was fully protected, whereas the next *Hpa* I fragment upstream (400 bp) was only partially protected. To define transcription initiation more precisely, the 310-bp *Hin*fI fragment that overlaps the partially protected *Hpa* I fragment was labeled at its 5′ ends and used for primer extension and S1 nuclease assays. For S1 nuclease protection, the *Hin*fI fragment was cut with *Hpa* I to yield a 280-bp fragment with the desired labeled end. The 280-bp fragment was hybridized with 120 μg of RNA and treated with S1 nuclease as described (12). For the primer extension assay, the end-labeled *Hin*fI fragment was cut with *Dde* I to yield a 90-bp primer. The primer was hybridized with 120 μg of RNA and extended with reverse transcriptase as described (12). The primer extension and S1 nuclease protection products were run on denaturing acrylamide gels with sequence ladders of the *Hin*fI fragment. See Fig. 6 for location of the sites used in these assays.

RESULTS AND DISCUSSION

Previous work by Heinhorst and Shively indicated that genes related to the large and small subunits of RuP_2 carboxylase of *Cyanophora* were located on the same restriction fragment of cyanelle (chloroplast) DNA of that organism (19). We also knew from preliminary experiments that the *Anabaena* large-subunit gene was transcribed to give a message large enough to include the small subunit and that the small-subunit gene sequence was not included in the nucleotides on the 5′ side of the large-subunit coding region. We therefore directed our attention to the DNA on the 3′ side of the large-subunit coding region—in particular, the 1.5-kbp *Hin*dIII fragment cloned in pAn606 (Fig. 1).

Fragments of pAn606, prepared by cutting with *Hin*dIII alone or with *Hin*dIII and *Xba* I, were fractionated by electrophoresis, blotted, and probed with the 230-bp fragment from the cloned pea small subunit gene (ref. 13; see *Materials and Methods*). Under low stringency conditions, the probe hybridized with the 1.5-kbp *Hin*dIII band and both *Hin*dIII–*Xba* I bands. When the blots were washed at increasing temperatures, the labeled band corresponding to the smaller *Hin*dIII–*Xba* I fragment (520 bp) disappeared, whereas the band corresponding to the larger *Hin*dIII–*Xba* I fragment (980 bp) remained (data not shown; see Fig. 1 for the map). Though washing blots at increasing temperatures allowed us to distinguish specific from nonspecific hybridization with cloned DNA, this procedure was not effective with total *Anabaena* DNA, at the DNA concentration used (4 μg of DNA per lane). After determining the complete nucleotide sequence of the corresponding *Anabaena* DNA fragments (see below) it became apparent that, although the pea DNA probe and the *Anabaena* gene have average nucleotide homology of 55%, all of the hybridization observed is due to a single stretch in which 17 of 18 bp are identical.

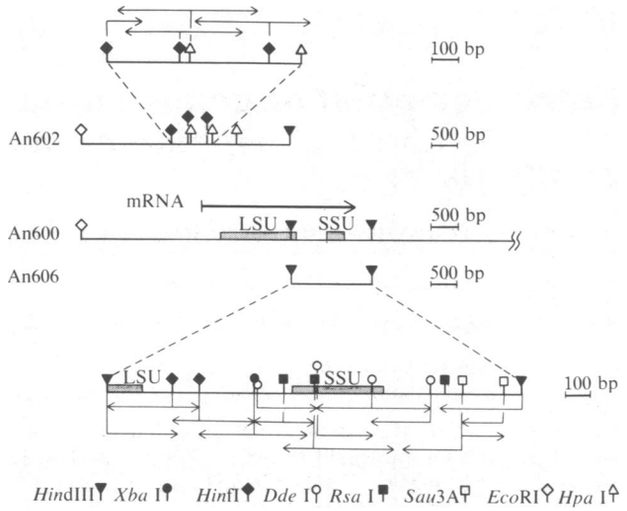

FIG. 1. Physical map of the region containing the *Anabaena* large-subunit (LSU) and small-subunit (SSU) genes. The 17.0-kbp *Eco*RI fragment of An600 had previously been shown to contain the large-subunit gene (7). The subclones An602 and An606 were used to determine the nucleotide sequences of the large-subunit 5′ flanking region and the small-subunit gene, respectively, by using the strategies indicated. The thick bars represent the coding regions of the small-subunit and large-subunit genes. The two genes are transcribed into a message that initiates 414 bp upstream from the large-subunit coding region. The large-subunit and small-subunit genes are separated by a 545-bp spacer.

We next determined the nucleotide sequence of the small-subunit gene following the strategy outlined in the lower portion of Fig. 1. Fig. 2 displays the nucleotide sequence of the noncoding strand of the small-subunit gene and its flanking regions. The *Anabaena* small-subunit coding sequence is 330 nucleotides long and terminates with a single ochre codon. The sequence flanking the open reading frame on the 5′ side does not contain a typical prokaryotic ribosome-binding sequence (20). A possible prokaryotic transcription terminator, an inverted repeat followed by a stretch of T residues (21), was observed 32–63 bp beyond the ochre codon (underlined in Fig. 2).

The amino acid sequence of the *Anabaena* small subunit, determined by translating the gene sequence, is compared with the sequences of small subunits from other species in Fig. 3. The *Anabaena* small-subunit protein is 2 amino acid residues shorter than the *Anacystis* small subunit protein and 11–19 amino acid residues shorter than eukaryotic small-subunit proteins. As shown in Fig. 3, the amino acid residues at positions three and four of the eukaryotic mature small-subunit proteins are deleted in the *Anabaena* small subunit. Also, 12 amino acid residues have been deleted somewhere between positions 45–65. This deletion has been arbitrarily positioned at residues 54–65, since the exact location cannot be determined from the available data. Highly conserved regions in small-subunit proteins of *Anabaena* and other species, allowing conservative amino acid replacements, occur at positions 10–22, 66–76, and 98–116. Also, many individual or small groups of homologous amino acid residues are present (boxed, in Fig. 3). Small-subunit proteins of prokaryotes differ considerably from those of eukaryotes at the NH_2 ter-

FIG. 2. Nucleotide sequence of the noncoding strand of the small-subunit gene from *Anabaena* 7120 and its flanking regions. The deduced amino acid sequence of the small subunit is printed below the nucleotide sequence. Numbering of residues begins with the first A of the methionine codon. The sequence shown begins with the first nucleotide following the ochre codon of the large subunit gene. Underlined residues indicate an inverted repeat that may function as a termination signal.

minus (residues 3–9) and the area adjacent to the deletion (residues 45–65).

At the protein level, the *Anabaena* small subunit is 61% homologous with that of *Anacystis* and 37–38% homologous with eukaryotic small-subunit proteins (excluding deletions). In contrast, the *Anabaena* large-subunit protein is ≈84% homologous to both *Anacystis* and eukaryotic large-subunit proteins (7). It is interesting to note, however, that if the NH_2-terminal region and the region adjacent to the deletion are excluded and conservative amino acid replacements are allowed, the *Anabaena* small-subunit and the eukaryotic small-subunit proteins are ≈86% homologous. At the nucleotide level, the *Anabaena* small-subunit coding sequence is 60% homologous with that of *Anacystis* (22) and 53–56% homologous with those of eukaryotic small-subunit genes (excluding deletions) (5, 6, 13). Hence, small-subunit nucleotide homologies of eukaryotes and *Anabaena* are ≈17% greater than protein homologies.

To determine how many copies of sequences related to the small-subunit gene are present in *Anabaena*, the 980-bp *Hin*dIII–*Xba* I fragment of pAn606 containing the small-subunit gene was nick-translated and hybridized to *Eco*RI or *Hin*dIII digests of total *Anabaena* DNA. In the latter digest, only the 1.5-kbp fragment corresponding to pAn606 was identified. In the *Eco*RI digest, only the 17-kbp fragment corresponding to pAn600 (7) was identified (data not shown). Therefore, *Anabaena* contains only one copy each of the large- and small-subunit genes.

To determine whether a single transcript contains both genes in *Anabaena*, RNA was prepared from cells grown on medium containing ammonia, denatured, fractionated by electrophoresis, blotted onto nitrocellulose paper, and hybridized with either an *rbcL*- or *rbcS*-specific probe from *Anabaena*. Since RNA transfer blot hybridizations with each of these probes identified a preponderant message species of ≈3.1 kilobases (kb) (Fig. 4), this message appears to contain both gene transcripts. The message length estimate varied from 2.8 to 3.1 kb on different gels, but the intensity of the hybridization bands was similar in all cases. RNA species of ≈2.7, ≈2.25, and ≈1.7 kb were also identified by using the large-subunit probe. The small-subunit probe hybridized with some of these species (Fig. 4). Since the large-subunit probe hybridized with some species smaller than 1 kb (see the map of Fig. 1), the RNA preparation must contain some small fragments from the 5′ end of the mRNA; presumably, these result from nonspecific degradation, since specific processing events are not expected to occur within the coding region of the *rbcL* gene.

In some plant species, transcription of the small-subunit genes is much more abundant in the light than in the dark. To determine whether transcription of the *rbcL–rbcS* genes is light regulated in *Anabaena*, RNA was prepared from *Anabaena azollae* grown either phototrophically or in the dark, using fructose as a carbon source. The two RNA prepara-

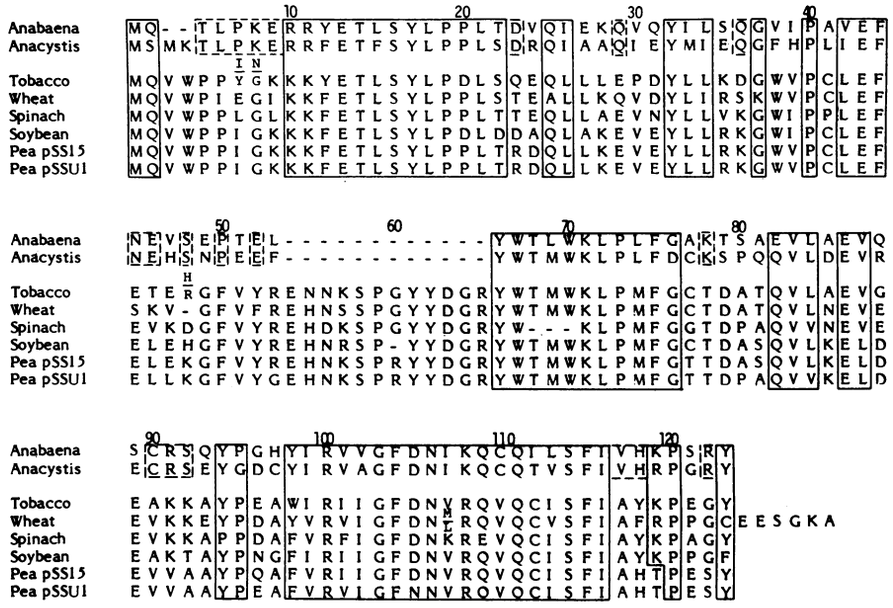

FIG. 3. Comparison of amino acid sequences of the small subunit of RuP_2 carboxylase from *Anabaena*, *Anacystis* (22), tobacco (23), wheat (6), spinach (24), soybean (5), pea pSS15 (25), and pea pSSU1 (13). Residue numbering refers to the pea sequence. Solid boxes surround positions that are conserved in all of the proteins, allowing for conservative amino acid replacements. Dashed boxes surround residues that are identical in the two prokaryotic sequences but differ in the eukaryotic sequences.

tions were fractionated as in Fig. 4, blotted, and probed with *rbcS* DNA. The concentrations of *rbcS*-specific RNA in the two preparations were indistinguishable (data not shown). We conclude that transcription of the *rbcL–rbcS* genes in *Anabaena* is not regulated by light.

The *Anabaena* 7120 RNA preparation was also used for S1 nuclease protection and primer extention experiments to determine the start site for transcription. Transcription initiation was first roughly localized to a 310-bp *Hin*fI fragment upstream from the *rbcL* coding region. This *Hin*fI fragment was labeled at its 5′ ends and the coding strand was hybridized to the RNA. The RNA·DNA hybrids were treated with S1 nuclease and electrophoresed, along with sequencing cleavage products of the fragment. The results of Fig. 5 indicate that the start site of transcription begins with the A residue at position −414 in Fig. 6. This result was confirmed by extension of a primer derived from the same *Hin*fI fragment (Fig. 5). The −10 and −35 sequences upstream from the transcription start are T-A-T-A-T-T and G-A-A-T-A-A, respectively. The −10 sequence is an almost perfect *E. coli* promoter (21), but the −35 sequence is not. The first two positions must be T·T for the −35 sequence to function without specific activation in *E. coli* (21). We do not know enough yet about *Anabaena* promoters to say whether this one needs activation in *Anabaena*.

The complete nucleotide sequence of the DNA corresponding to the *Anabaena* RuP_2 carboxylase mRNA has now been determined. For the 5′ flanking sequence, the strategy outlined in the upper portion of Fig. 1 was followed, and Fig. 6 displays the nucleotide sequence up to the ATG start codon for the *Anabaena* large subunit. A sequence capable of forming a stem-and-loop structure is located 32–63 bp downstream from the TAA stop codon of the small-subunit gene (Fig. 2). If this actually represents the termination site of the message, then the message contains 2816 bases, of which there is an untranslatable leader of 414 bases followed

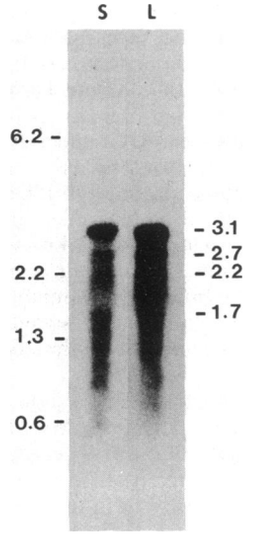

FIG. 4. Identification of *rbcL* and *rbcS* RNA species. Total *Anabaena* RNA (20 μg) was denatured, electrophoresed, blotted, and hybridized with *rbcL*- or *rbcS*-specific probes: a 1.0-kbp *Hpa* I–*Hin*dIII fragment for *rbcL* and a 0.95-kbp *Xba* I–*Hin*dIII fragment for *rbcS* (see Fig. 1). Sizes (kb) of the DNA fragments run as standards are indicated. The two larger markers were pBR322 and an *Eco*RI fragment of *Anabaena* DNA; the two smaller markers were from an *Hae* III digest of φX174 replicative form DNA.

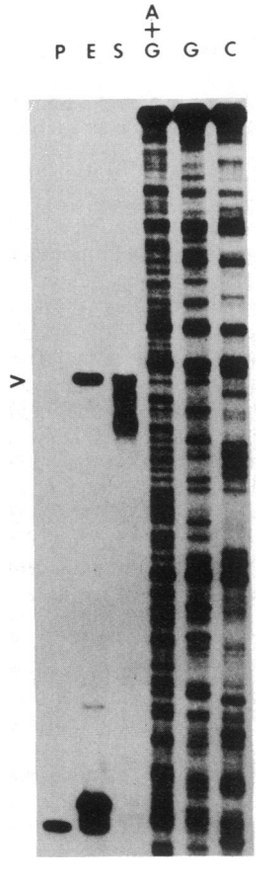

FIG. 5. Identification of the start site for *rbcL* transcription. The coding strand of a *Hin*fI fragment upstream from the *rbcL* coding region was labeled, hybridized with RNA, and digested with S1 nuclease. The S1 nuclease protected fragment(s) was electrophoresed on a denaturing acrylamide gel alongside sequencing reaction products A+G, G, and C of the same *Hin*fI fragment. The S1 protection result was confirmed (arrowhead) by primer extension (E) of a primer (P) derived from the same *Hin*fI fragment. The lower molecular weight bands in the S lane are probably artifacts of the S1 nuclease digestion resulting from an A+T-rich region of the coding strand.

FIG. 6. Transcription initiation site of the *Anabaena rbcL* gene. The nucleotide sequence of the 630 bp preceding the *rbcL* coding region is shown. Numbering indicates the distance upstream from the *rbcL* coding region. Transcription initiation was mapped to nucleotide −414, indicated by the arrowhead (Fig. 5). Sequences −10 and −35 nucleotides from transcription initiation are underlined. The restriction sites used for the experiments of Fig. 5 are also indicated.

by the large-subunit coding region of 1428 bases (7), a spacer of 545 bases, the small-subunit coding region of 330 bases, and a 3′ extension of 68 bases.

In *Cyanophora paradoxa* and both cyanobacteria studied to date, the *rbcS* and *rbcL* genes are linked and are probably cotranscribed. In contrast, these genes in plants are physically separated in the nuclear and chloroplast genomes, and *rbcS* is multicopy. The *rbcS* genes of plants contain a transit peptide required for transport of the small subunit into the chloroplast. The transit peptide is not encoded in the prokaryotic genes, as expected. In addition, there is no obvious relationship between the sequence flanking the terminus of the cyanobacterial *rbcS* genes and the transit peptide encoding regions of the plant genes. All of the plant *rbcS* genes sequenced to date also contain an insertion of 12 amino acid residues preceding the highly conserved region at residues 66–76, as well as an intervening sequence between residues 2 and 3 of the mature protein. It is possible that the 12 amino acid insert in the eukaryotic genes is necessary for chloroplast membrane crossing. This possibility can be tested by fusing the eukaryotic transit peptide coding sequence to the *Anabaena* small-subunit gene and examining the processing and transport behavior of the resulting polypeptide *in vitro*.

Mature RuP_2 carboxylase contains equimolar amounts of the large- and small-subunit protein. How this stoichiometry is obtained in plants is a mystery, since the total number of chloroplast *rbcL* genes vastly exceeds the number of nuclear *rbcS* genes. Cotranscription of the two genes in *Anabaena* makes it possible that equimolar amounts of the two proteins are produced by equal translation of the two messages. However, the *rbcL* gene has what appears to be a good ribosome-binding site just before its open reading frame (Fig. 6, A-G-G-A at residues −6 to −9), whereas the *rbcS* gene has none (Fig. 2). The distance between the genes, 545 bases, seems too great to expect ribosomes to read through from the *rbcL* message so the yield of small subunit obtained by translation of the 3-kb message could be low. It is also possible that processing and differential stability modify the relative levels of the two gene messages *in vivo*, but the problem of preventing nonspecific degradation of the RNA during isolation (Fig. 4) makes tests of those possibilities difficult.

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