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Nucleotide sequence of the gene for the M_r 32,000 thylakoid membrane protein from *Spinacia oleracea* and *Nicotiana debneyi* predicts a totally conserved primary translation product of M_r 38,950

(chloroplast DNA/*psbA* mRNA/amino acid sequence)

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ABSTRACT **The gene for the so-called M_r 32,000 rapidly labeled photosystem II thylakoid membrane protein (here designated *psbA*) of spinach (*Spinacia oleracea*) chloroplasts is located on the chloroplast DNA in the large single-copy region immediately adjacent to one of the inverted repeat sequences. In this paper we show that the size of the mRNA for this protein is ≈1.25 kilobases and that the direction of transcription is towards the inverted repeat unit. The nucleotide sequence of the gene and its flanking regions is presented. The only large open reading frame in the sequence codes for a protein of M_r 38,950. The nucleotide sequence of *psbA* from *Nicotiana debneyi* also has been determined, and comparison of the sequences from the two species shows them to be highly conserved (>95% homology) throughout the entire reading frame. Conservation of the amino acid sequence is absolute, there being no changes in a total of 353 residues. This leads us to conclude that the primary translation product of *psbA* must be a protein of M_r 38,950. The protein is characterized by the complete absence of lysine residues and is relatively rich in hydrophobic amino acids, which tend to be clustered. Transcription of spinach *psbA* starts about 86 base pairs before the first ATG codon. Immediately upstream from this point there is a sequence typical of that found in *E. coli* promoters. An almost identical sequence occurs in the equivalent region of *N. debneyi* DNA.**

One of the most rapidly labeled products of protein synthesis in chloroplasts is a thylakoid membrane protein of photosystem II whose M_r has variously been estimated as being 32,000–36,000 (1–7). This protein, originally referred to as peak D (1) but now usually referred to as the M_r 32,000 protein, is characterized by its abundant synthesis in mature light-grown tissue (6) and by its rapid turnover rate (5). Precursors of M_r 34,500 and 33,500 for the M_r 32,000 membrane protein have been identified in maize (4) and *Spirodela* (5), respectively. Interest in this protein has recently increased because of the accumulating evidence that it is the protein that binds the herbicides 3-(3,4,-dichlorophenyl)-1,1-dimethylurea (DCMU) and atrazine (8) and is involved in electron flow from photosystem II (9).

The mRNA for the M_r 32,000 membrane protein is relatively abundant in chloroplasts (10), and its synthesis is light dependent (7). The gene for the M_r 32,000 membrane protein (here designated *psbA*) of a number of plant species (7, 10–13) has been mapped to the chloroplast DNA in the large single-copy region, immediately adjacent to one of the inverted repeat regions. In this paper we define the spinach (*Spinacia oleracea*) gene in terms of its transcript, nucleotide sequence, and predicted translation product and compare it with the corresponding gene from *Nicotiana debneyi*.

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MATERIALS AND METHODS

Procedures used for the isolation of spinach chloroplast DNA and RNA, for restriction enzyme digestions and agarose gel electrophoresis have been described (14). Details of the electrophoresis of RNA under denaturing conditions, transfer of the RNA to diazotized aminothiophenol paper (reverse Southern blots), and hybridization with radioactive probes also have been published (15). 3′-End-labeling (16), 5′-end-labeling (17), and strand-separation of DNA fragments (17), DNA sequence determination (17, 18), and S1 nuclease mapping (19) were carried out as specified.

Plasmids. pSocS15 was constructed as detailed (20) by inserting *Sal* I fragment F [8.7 kilobases (kb)] of spinach (*Spinacia oleracea*) chloroplast DNA into the *Sal* I site of pBR322. Single-stranded forms of *Sal* I fragment F were prepared by ligating the fragment into the single-stranded fd phage as follows. *Sal* I-digested pSocS15 (9 μg) and *Xho* I-digested fd 106 RF (replicative form) DNA (21) (0.5 μg) were incubated for 16 hr with T4 DNA ligase in 50 μl of 50 mM Tris·HCl, pH 7.8/10 mM $MgCl_2$/20 mM dithiothreitol/10 mM ATP/50 μg of bovine serum albumin per ml at 16°C. The ligated DNA was added to $CaCl_2$-treated (100 mM; 1–24 hr at 0°C) *Escherichia coli* RR1 (*pro, leu, lac Y, thi, strA,* $r_k m_k$, end A, F^-) cells; the mixture was left for 1 hr at 0°C, heated at 42°C for 90 sec, and diluted 1:10 with Luria broth, after which the cells were grown for 1 hr at 37°C. Two chloramphenicol-resistant (at 25 μg/ml) and kanamycin-sensitive (at 30 μg/ml) transformants were recovered (fd 106.154 and fd 106.1514) with *Sal* I fragment F inserted in opposite orientations into the *Xho* I cloning site of fd 106. *Sma* I digestion of fd 106.154 RF DNA generated two fragments of about 16 kb and 2 kb and of fd 106.1514 RF DNA generated two fragments of about 10 kb and 8 kb. This defined the orientation of *Sal* I fragment F (see refs. 10 and 21) and indicated that fd 106.154 packages the 3′-to-5′ strand left-to-right as drawn in Fig. 1A and fd 106.1514 packages the 5′-to-3′ strand.

fd 106.1514Δ*Sma* I-4 was constructed by digesting fd 106.1514 RF DNA with *Sma* I and religating. One chloramphenicol-resistant transformant from this ligation lacked the smaller *Sma* I fragment. Therefore, the only chloroplast DNA contained in this plasmid was the fragment lying between the

Abbreviations: kb, kilobase(s); bp, base pair(s); RF, replicative form.

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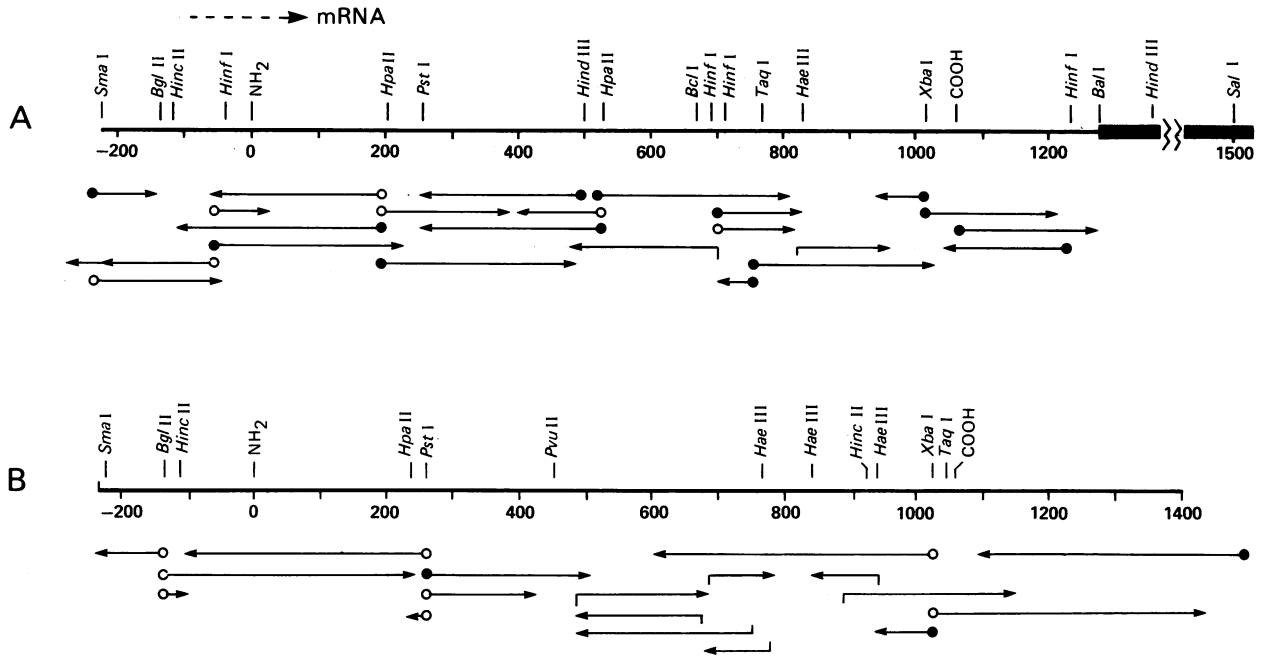

FIG. 1. Restriction maps and sequence assay strategy diagrams of the *psbA* region of chloroplast DNA. (*A*) The 1.4-kb *Sma* I–*Bal* I interval of *Sal* I fragment F adjacent to the end of the inverted repeat region of *S. oleracea* chloroplast DNA. The direction of transcription, starting from the proposed point of transcription initiation, is indicated by a dashed arrow above the map. (*B*) The 1.4-kb segment starting at the *Sma* I site in the 5-kb *Bam*HI fragment of *N. debneyi* chloroplast DNA. The locations of the amino-terminal and carboxyl-terminal coding regions are marked above the map (NH_2 and COOH, respectively). Numbering is in base pairs relative to the first ATG codon in the open reading frame. The direction and extent of the individual DNA sequence assays used to determine the sequence in Fig. 3 are indicated by arrows under the restriction maps. Arrows commencing from solid dots indicate that the DNA was labeled at the 3′ end by using [α-^{32}P]dNTP and the Klenow fragment of *E. coli* DNA polymerase (16); the sequence was determined by the method of Maxam and Gilbert (17). Arrows commencing from open dots indicate that the DNA was labeled at the 5′ end by using [γ-^{32}P]ATP and T4 polynucleotide kinase after dephosphorylation with calf alkaline phosphatase (17); the sequence was determined by the method of Maxam and Gilbert (17). Arrows commencing from bars specify that the sequence was determined by the dideoxy method (18) with single-stranded DNA as template and restriction fragments as primer DNA.

Sma I site in *Sal* I fragment F and the *Sal* I site just within the inverted repeat region (Fig. 1A). fd 106.1514Δ*Sma* I-4 and pSocS15 were used to generate DNA fragments for sequence analysis.

Plasmid pNdcB76, a 5-kb *Bam*HI fragment of *N. debneyi* chloroplast DNA cloned into the *Bam*HI site of a modified ColE1 vector, was selected from a library of *N. debneyi* chloroplast DNA fragments (provided by J. B. Langridge) by screening with labeled *Sal* I fragment F of spinach chloroplast DNA. The 2.4-kb *Pst* I fragment and the two *Bam*HI–*Pst* I fragments derived from the 5-kb *Bam*HI fragment by digestion with *Pst* I were subcloned into pBR322 and pUC8 (Bethesda Research Laboratories, Gaithersburg, MD) for nucleotide sequence analysis.

Labeling of Single-Stranded fd DNA. Single-stranded fd 106.154 and fd 106.1514 were prepared (22), and 5 μg of each DNA was digested with 10 units of *Hae* III and *Hha* I in 6 mM Tris·HCl, pH 7.4/6 mM NaCl/6 mM 2-mercaptoethanol/100 μg of bovine serum albumin per ml for 2 hr at 37°C. The fragments were then dephosphorylated and 5′ end-labeled by using [γ-^{32}P]ATP and polynucleotide kinase (17). The specific activity of the probes was $\approx 10^7$ cpm/μg of DNA.

RESULTS

Size and Direction of Transcription of the M_r 32,000 Thylakoid Membrane Protein mRNA from Spinach. Previous studies (10) have shown that *in vitro* synthesis of the M_r 32,000 spinach chloroplast membrane protein is programmed by a 14S mRNA species and that this RNA hybridizes to the *Sal* I fragment F of spinach chloroplast DNA. In order to define the size and direction of transcription of the mRNA, recombinant phage fd 106.154 and fd 106.1514 containing single strands of *Sal* I fragment F were hybridized to reverse Southern blots of spinach chloroplast RNA. The M_r 32,000 membrane protein mRNA, detected as the most abundant of the hybridizing RNA species, was estimated to be $\approx$1.25 kb long and it hybridized only to the fd 106.154 probe (Fig. 2). Restriction analysis of the replicative form of this phage showed that the single-stranded phage DNA

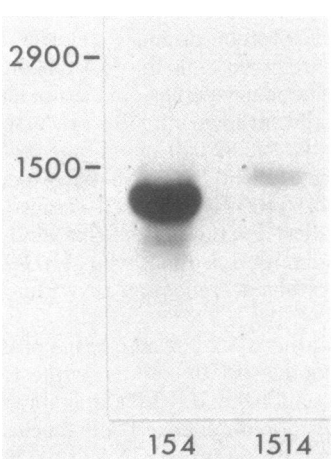

FIG. 2. Determination of the size and direction of transcription of the mRNA for the spinach M_r 32,000 membrane protein. Spinach chloroplast RNA (30 μg per slot) was electrophoresed, transferred to diazotized aminothiophenol paper, hybridized with fd 106.154 or fd 106.1514 single-stranded [^{32}P]DNA and autoradiographed. The size of the RNA (in nucleotides) was calculated by reference to the mobility of *E. coli* ribosomal RNAs (2,900 and 1,500 nucleotides).

will hybridize with RNA molecules transcribed towards the inverted repeat unit. fd 106.154 also hybridized to a minor RNA species of size 0.95 kb and to a tRNA (not shown). A low abundance RNA of about 1.4 kb hybridized to the fd 106.1514 probe (Fig. 2), indicating that transcription of some sequences in the opposite strand also must occur.

Sequence Analysis of the M_r 32,000 Membrane Protein Gene (*psbA*). The sequence of the 1.4-kb interval of DNA adjacent to the inverted repeat region in *Sal* I fragment F of spinach chloroplast DNA was determined as indicated in Fig. 1*A*. The nucleotide sequence commencing at the *Sma* I site is shown in Fig. 3. Translation of the sequence towards the inverted repeat unit revealed the presence of only one open reading frame of a length sufficient to encode a protein of M_r 32,000. The deduced amino acid sequence, commencing at the first available ATG (methionine) translation start codon in the open reading frame and ending at a TAA translation stop codon, is shown below the nucleotide sequence (Fig. 3). There are no lysines present, and the predicted M_r of the 353-residue protein is 38,950.

The size of the gene product is significantly larger than that anticipated (M_r 35,000) on the basis of the measured electrophoretic mobility of the *in vitro* synthesized protein (2, 24). However, the predicted size of the protein would be approximately that expected if translation were to commence at the second ATG in the open reading frame (amino acid residue 37; Fig. 3). In order to establish whether translation starts at the first or second methionine codon, the sequence of the *psbA* gene from another plant species, *Nicotiana debneyi*, was also determined (Figs. 1*B* and 3). The size of the open reading frame

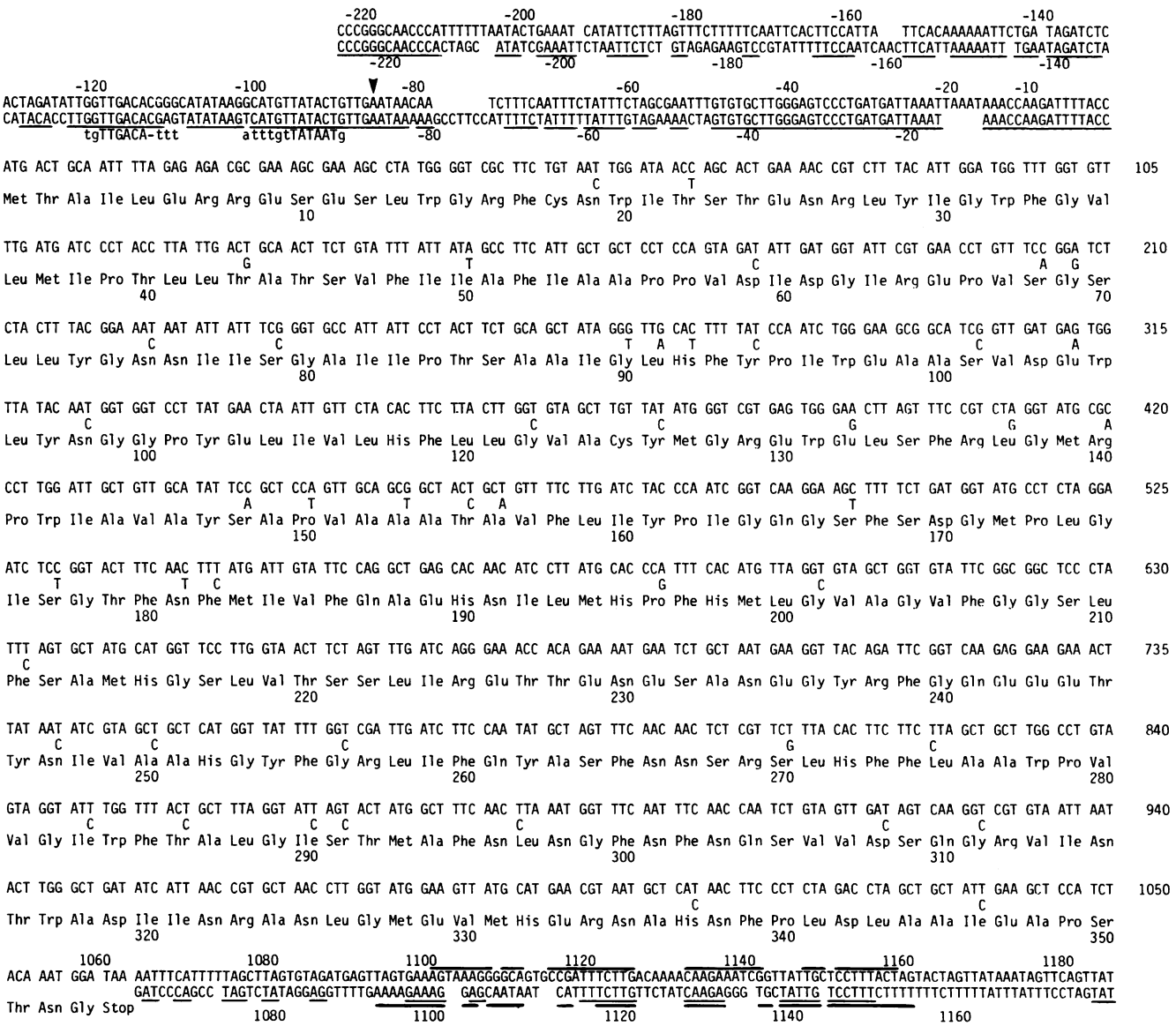

FIG. 3. Nucleotide sequence and deduced amino acid sequence of *psbA* from *S. oleracea* and *N. debneyi*. The upper sequence is for spinach and the lower is for *N. debneyi*. The deduced amino acid sequence is identical for both species. Numbering starts at the first ATG in the open reading frame. Within the protein-coding region, only nucleotide differences are indicated for the *N. debneyi* sequence; in the flanking regions, the sequences from both species are given in full. The extensive regions of homology in the flanking sequences have been aligned by introducing gaps at appropriate points; where the sequences are identical, they are lightly underlined. The vertical arrow marks the approximate start site for transcription of the spinach mRNA. The *E. coli* consensus promoter sequence (23) is printed below the *psbA* sequence between nucleotides −100 and −125 approximately; upper case letters indicate that a base appears more frequently in that position in promoters than do bases indicated by lower case letters. The thick lines above and below parts of the 3′-flanking sequences mark those nucleotides which base-pair to form the stem-and-loop structures shown in Fig. 4.

in the *N. debneyi* nucleotide sequence was found to be identical to that in spinach, and the homology of the two sequences in the protein coding region, including the 108 base pair(s) (bp) between the first and second ATG codons, was greater than 95% (Fig. 3). In view of this high sequence conservation, we concluded that the full reading frame must be functional and that the primary translation product of *psbA* must be a protein of M_r 38,950.

This conclusion is reinforced by the observation that there are no differences in the deduced amino acid sequences of the spinach and *N. debneyi* proteins (Fig. 3). All 47 nucleotide differences in the protein coding region of *psbA* are silent. The flanking regions are far less conserved than the open reading frame, although the 50 bp immediately preceding the first ATG codon are identical in the two species except for a 5-bp deletion in *N. debneyi* (Fig. 3). When the sequences were aligned to maximize the homology by including small deletions where appropriate (Fig. 3), the overall similarity was 75% for the 224 bp between the *Sma* I site and the translation initiation codon and 38% for the 120 bp downstream from the TAA stop codon.

Mapping of the 5′ End of the M_r 32,000 Membrane Protein mRNA from Spinach. The region coding for the 5′ end of spinach *psbA* mRNA was defined by the S1 nuclease mapping procedure of Berk and Sharp (19). A 188-bp *Hin*fI fragment spanning the *Sma* I site (Fig. 1) was 5′ end-labeled and strand-separated (17). Only one of the strands was protected (for 46–49 residues) from S1 nuclease digestion by prior hybridization to chloroplast RNA. DNA sequence analysis of this strand confirmed that it corresponded to the transcribed strand of *psbA* and positioned the region coding for the 5′ end of the mRNA about 86 bp prior to the first methionine codon (Fig. 3).

DISCUSSION

Features of *psbA* mRNA. The mRNA for the M_r 32,000 thylakoid membrane protein is one of the most abundant messenger species in chloroplasts (5, 7, 10). Of the RNAs that hybridized to the 8.7-kb *Sal* I fragment F of spinach chloroplast DNA (Fig. 2), the 1.25-kb RNA gave by far the strongest signal, and we concluded that it was the mRNA for the M_r 32,000 protein. Subsequent experiments (not shown) with restriction fragments derived from *Sal* I fragment F as hybridization probes confirmed earlier data (10) that the RNA was encoded in the 1.4-kb segment between the *Sma* I and *Bal* I sites (Fig. 1).

We have noted (15) that the proposed promoter region for spinach *rbcL* (the gene for the large subunit of ribulose bisphosphate carboxylase) shares sequence similarities with *E. coli* promoters. As can be seen in Fig. 3, regions of the spinach *psbA* sequence prior to the mRNA transcription initiation point also have a striking similarity to the consensus sequence for *E. coli* promoters (23). Sequences identical to the spinach "Pribnow box" and "recognition site" sequences occur in the same region in *N. debneyi* chloroplast DNA also, reinforcing the view that they are functionally important. The close similarity of the *E. coli* and chloroplast DNA sequences upstream from the transcription initiation site suggests that the recognition signal for the chloroplast RNA polymerase will prove to be very similar to that for the bacterial enzyme. It also explains why prebound *E. coli* RNA polymerase protects the *Hin*cII site at −118 in the spinach sequence (Fig. 3) from cleavage (25).

Knowing the length of the spinach mRNA (1.25 kb) and the transcription initiation point, one could calculate that transcription must terminate 80–90 bp distal to the translation stop codon. Examination of the sequence immediately upstream from this transcription termination point revealed that it coded for an RNA which would be capable of forming a stable stem-and-loop structure (Figs. 3 and 4). The analogous region of *N. debneyi psbA* also is able to form a stable stem-and-loop structure (Fig. 4). Similar stem-and-loop structures occur immediately prior to *E. coli* transcription termination sites (23), and one occurs at the 3′ end of spinach chloroplast *rbcL* mRNA (15).

Size of the Primary Translation Product of *psbA*. Despite the high rate of synthesis of the M_r 32,000 membrane protein by chloroplasts, a physical characterization of the protein has been hindered by its high rate of turnover and resulting lack of accumulation. Correlation of the labeled protein with a stained protein band on a polyacrylamide gel has not been observed for spinach. The estimated size of the protein, whether synthesized in isolated spinach chloroplasts or by *in vitro* translation of chloroplast RNA, ranges from M_r 32,000 to 36,000 (2, 3, 10, 24). There is no published evidence showing the conversion of a precursor to a mature form of the spinach protein, but processing is likely to be comparable to that of the maize or *Spirodela* proteins (5, 7). The discrepancy between the predicted M_r of the spinach protein specified by the open reading frame shown in Fig. 3 (38,950) and the maximum observed size (36,000) may be due simply to an anomaly associated with M_r determinations based on electrophoretic mobility in sodium dodecyl sulfate/polyacrylamide gels. Inconsistencies between observed M_r values calculated ones are common, an extreme example being that of beef heart mitochondrial cytochrome *c* subunits I and III, where the observed values are 35,700 and 21,000, but the values predicted from the nucleotide sequences are 57,000 and 29,900 (28).

Features of the M_r 38,950 Primary Translation Product. The two most outstanding characteristics of the M_r 38,950 protein revealed by our studies are the total conservation of the amino acid sequence between spinach and *N. debneyi* and the complete absence of lysine residues. The fact that there are no amino acid differences between the protein from a member of the Chenopodiaceae family and that from a member of the Solanaceae family suggests that the constraints on the primary structure are very rigid. The phylogenetic similarity of the mature M_r 32,000 chloroplast protein has recently been noted

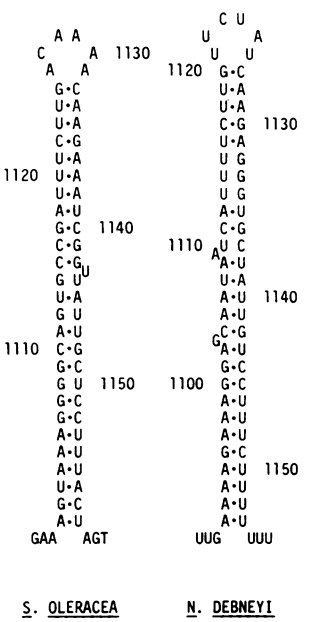

FIG. 4. Proposed stem-and-loop secondary structure in the vicinity of the 3′ end of the mRNA coding for the spinach and *N. debneyi* M_r 32,000 thylakoid membrane protein. Numbering of the nucleotides is as in Fig. 3. The calculated free energy of formation, ΔG (26, 27), of the spinach structure is −34.9 kcal and that of the *N. debneyi* structure is −27.4 kcal.

by Hoffman-Falk *et al.* (29), who found that the proteins from several diverse angiosperms and from *Chlamydomonas* have a similar size, yield similar partial peptide cleavage patterns, and have a similar orientation in the thylakoid membranes. It will be interesting to see whether this extensive similarity holds true at the level of amino acid sequence.

On the basis of radioactive labeling studies, it has been suggested that the M_r 32,000 protein from *Spirodela* chloroplasts lacks lysine residues (5). In view of our demonstration that the protein from both spinach and *N. debneyi* also lacks lysine residues, it seems probable that this will prove to be a general property of the protein regardless of its source. Such a feature should be a useful criterion for monitoring the purification of the protein from thylakoid membranes. For example, it clearly serves to distinguish the M_r 32,000 protein from the M_r 33,000 membrane protein isolated from spinach chloroplasts by Kuwabara and Murata (30), which is characterized by a relatively high lysine content.

The mature M_r 32,000 protein is located in the chloroplast thylakoid membrane. Calculation of the hydrophobicity (31) of the M_r 38,950 primary translation product shows it to be far more hydrophobic than, for example, the large subunit of ribulose bisphosphate carboxylase which is a stroma-located protein. The hydrophobic amino acids tend to be clustered, yielding a number of hydrophobic regions interspersed with hydrophilic ones (Fig. 5). It is known that limited digestion of thylakoid membranes with trypsin results in the partial cleavage of the M_r 32,000 protein to give initially a M_r 19,500 polypeptide, which is slowly converted to a M_r 17,000 polypeptide (4, 9, 29, 32). Examination of the distribution of the arginine residues in the deduced protein sequence (see Fig. 5) shows that there is only one possible cleavage pattern that could give such a result. The peptide flanked by Arg-64 and Arg-238 would have a M_r of 18,909. Subsequent cleavage of this peptide at Arg-225 would yield a M_r 17,410 peptide, with Arg-129, Arg-136, and Arg-140, presumably being protected from digestion.

Our elucidation of the structure of *psbA* should facilitate the *in vitro* construction of *E. coli* strains that might express the M_r 38,950 protein at a relatively high level. This should then enable a detailed study of the interaction of DCMU and atrazine with the protein to be made.

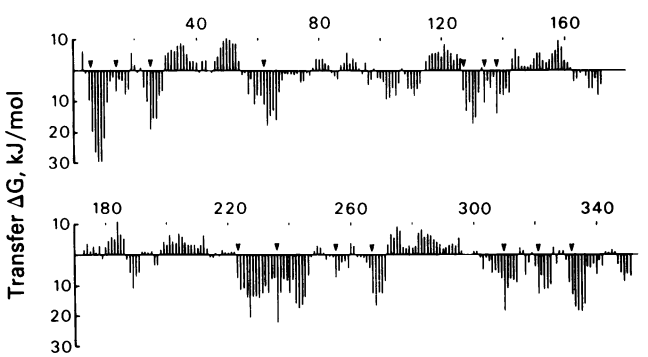

FIG. 5. Estimated free energies of transfer of the amino acid residues presented in Fig. 3 from a helix in water to a helix in a nonpolar phase lacking hydrogen-bonding capacity. The values represent the sum of the hydrophobic contribution, the H-bond contribution, and the charge contribution of each residue (31). Each bar represents the free energy of transfer of each residue averaged with the two preceding and the two succeeding residues, starting from the first methionine shown in Fig. 3. Arrows indicate the location of arginine residues.

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