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Biochemistry

Cloning, mapping, and *in vitro* transcription–translation of the gene for the large subunit of ribulose-1,5-bisphosphate carboxylase from spinach chloroplasts

(molecular cloning/chloroplast DNA)

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ABSTRACT **An 11.2-kilobase pair (kbp) *Bam*HI restriction nuclease fragment from spinach chloroplast DNA has been found to contain the gene for the large subunit (LS) of ribulose-1,5-bisphosphate carboxylase [RuP_2 carboxylase; 3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39]. The gene was located by hybridization of cloned chloroplast DNA fragments containing the maize LS gene (Bedbrook, J. R., Coen, D. M., Beaton, A. R., Bogorad, L. & Rich, A. (1979) *J. Biol. Chem.* 254, 905–910) to spinach chloroplast DNA cleaved with restriction nucleases. The 11.2-kbp *Bam*HI fragment has been inserted into the *Bam*HI site of the plasmid pBR322. The resulting recombinant plasmid, pSoe3101, was used to direct the synthesis of a protein, which was immunoprecipitable with antibody to RuP_2 carboxylase, in a partially defined *in vitro* transcription–translation system derived from *Escherichia coli*. The product synthesized *in vitro* has a molecular weight identical to that of authentic spinach LS. By using pSoe3101 DNA cleaved at various positions with restriction nucleases, and the *in vitro* transcription–translation system, the LS gene has been mapped to a 1.5-kbp region located at one end of the 11.2-kbp *Bam*HI fragment. The direction of transcription of the LS gene on the plasmid as well as on the chloroplast chromosome has also been determined. The position of the LS gene on circular spinach chloroplast DNA is approximately 27 kbp from the start of one of the inverted repeat regions and 180° from one of the rRNA-coding regions.**

In most plants the chloroplast enzyme ribulose-1,5-bisphosphate carboxylase [RuP_2 carboxylase; 3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39] is composed of eight identical large subunits (LS) and eight identical small subunits (1). Various methods have shown that the small subunit is coded by nuclear DNA and LS is coded by chloroplast DNA (cpDNA) (2). Although the LS of RuP_2 carboxylase appears to be a highly conserved protein (2–5), its synthesis can vary considerably. For example, the chloroplasts of leaf mesophyll cells in C4 plants, such as maize, contain little or no RuP_2 carboxylase (6, 7), whereas in C3 plants, such as spinach, mesophyll cell chloroplasts contain abundant quantities of the enzyme (5). Link *et al.* (8) were also able to demonstrate that chloroplasts of bundle sheath cells in maize contain high levels of mRNA coding for the LS and that mesophyll cell chloroplasts lack this mRNA species. In addition, other studies have shown that chloroplasts in different tissues or cell types contain identical genomes (8, 9). Together these studies clearly indicate the existence of regulatory processes that control the expression of the LS gene. Because the LS genes from spinach and maize produce nearly identical proteins, but have much different modes of regulation, a direct comparison of the coding and noncoding sequences of these two genes might provide important clues as to what types of regulation are involved in their expression. We have used the LS gene from maize, which has previously been inserted into a plasmid and cloned (10–13), to determine the location of the LS gene on spinach cpDNA. In this report we describe the initial characterization of the LS gene in spinach.

MATERIALS AND METHODS

Plants and Bacterial Strains. Spinach leaves were obtained from a local wholesaler. *Zea mays* var. 125 Jubilee (Harris Seed, Rochester, NY) was grown under continuous illumination for 9–14 days before harvesting.

Escherichia coli K-12 strain RR1 (14) was used as the host in transformation experiments. The plasmid pBR322, constructed by Bolivar *et al.* (15), was used as the cloning vector.

Isolation of Chloroplast and Plasmid DNA. Spinach and maize cpDNA were isolated according to the method of Kolodner and Tewari (16) except that proteinase K (EM Biochemicals, Darmstadt, Federal Republic of Germany) at 200 μg/ml was substituted for Pronase.

For preparation of plasmid DNA, transformed RR1 cells were grown in M9 medium as described (14). Plasmid was isolated by the cleared lysate method and centrifuged in CsCl/ethidium bromide equilibrium gradients (17). Plasmids and cpDNA were further analyzed on agarose gels as described (18).

Ligation and Transformation. DNA fragments were ligated into the *Bam*HI site of pBR322 by using previously described conditions (14, 19). The total ligation reaction mixture (10 μl) was added to 1 ml of competent RR1 cells, following the procedure of Kushner for transformation of SK1592 cells (14, 20). Transformed cells were screened for ampicillin resistance and tetracycline sensitivity. Clones containing potential inserts were further examined by using the mini-lysate technique for plasmid isolation (21).

Radioactive Labeling and Hybridization Conditions. Plasmid DNAs and fragments were radioactively labeled by nick-translation (22) and hybridization followed the protocol of Southern (23).

***In Vitro* Synthesis and Assay for the LS.** The components of the *in vitro* system have been described (24–26) and the conditions used were similar to those of Zarucki-Schulz *et al.* (24). The amount of plasmid DNA used for the *in vitro* studies is described in the appropriate figure legends. In experiments using plasmid DNA digested with restriction nucleases, the DNA was digested with a 4-fold excess of the restriction enzyme for 4 hr, then precipitated with ethanol in the presence of 0.1

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Abbreviations: cpDNA, chloroplast DNA; RuP_2 carboxylase, ribulose-1,5-bisphosphate carboxylase; LS, large subunit of RuP_2 carboxylase; kbp, kilobase pair(s); kDal, kilodalton(s).

M NaOAc, pH 5.0. The pellet was dried and the *in vitro* components were added directly. L-[^{35}S]Methionine was used as the radioactive label (25,000 cpm/pmol). At the end of the incubation (60 min at 37°C), samples were electrophoresed on $NaDodSO_4$ slab gels (27) in a 7.5–15% acrylamide gradient. The radioactive products on the slab were detected by fluorography (28). The amount of [^{35}S]LS formed was quantitated as described previously to measure the formation of several gene products coded by λ*rif*[d]18 and λ*fus*3 phage DNAs (24). Immunoprecipitation was done in the presence of [^{3}H]LS, which was prepared by reductive methylation (29). Samples were solubilized and then electrophoresed in $NaDodSO_4$/10% polyacrylamide disc-gel tubes (29) to separate [^{35}S]LS from low molecular weight contaminants. The inclusion of [^{3}H]LS allowed us to accurately compare the LS synthesized *in vitro* with authentic LS.

RESULTS

Preparation of Maize and Spinach cpDNA Clones. Previous workers (11–13) have determined that the LS gene of RuP_2 carboxylase on the maize chloroplast genome is contained on a 2.7-kilobase pair (kbp) *Bgl* II restriction endonuclease fragment that is within a 4.3-kbp *Bam*HI fragment. We have prepared recombinants of the plasmid pBR322 that contain these maize cpDNA inserts. The plasmids pZme9019 and pZme1502 contain the 4.3-kbp *Bam*HI fragment and the 2.7-kbp *Bgl* II fragment, respectively. Both inserts were ligated into the *Bam*HI site of pBR322. Fig. 1 shows the orientation of the 4.3-kbp *Bam*HI insert with respect to restriction sites within pBR322. The 2.7-kbp *Bgl* II insert in pZme1502 was found to have the opposite orientation. Because of the close similarity between the LS produced by spinach and maize, it seemed likely that sufficient homology would exist between the two genes so that it would be possible to detect the LS genes on the spinach chloroplast chromosome by hybridization of the pZme9019 clone to total spinach cpDNA. Fig. 2 shows the hybridization of ^{32}P-labeled pZme9019 DNA to *Bam*HI digested cpDNA isolated from maize and spinach. As expected, one band (lane A) of *Bam*HI-digested maize cpDNA hybridized with pZme9019. In addition,

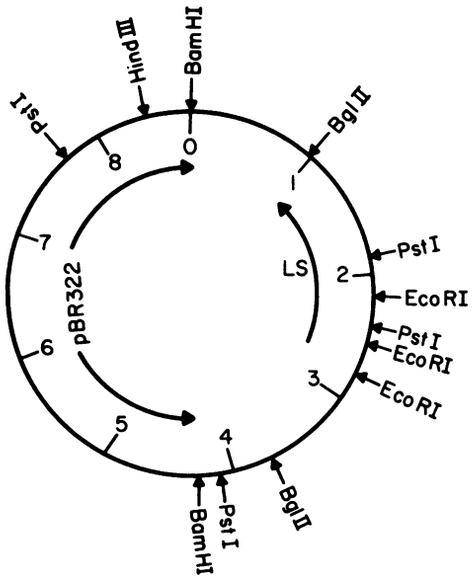

FIG. 1. Restriction map of plasmid pZme9019. The plasmid pZme9019 contains the 4.35-kbp *Bam*HI fragment from maize cpDNA ligated into the *Bam*HI site of pBR322. Total length is 8.7 kbp. Restriction sites and the coding region of maize LS are from the data of Bedbrook *et al.* (11).

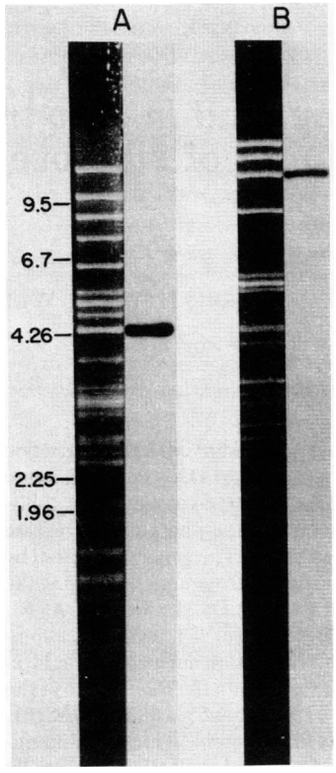

FIG. 2. Hybridization of ^{32}P-labeled pZme9019 to *Bam*HI-digested total maize and spinach cpDNA. One microgram of cpDNA was digested with *Bam*HI restriction nuclease and electrophoresed on 0.75% agarose gels. Bands were transferred to nitrocellulose paper and hybridized with pZme9019, ^{32}P-labeled by nick-translation (22). (*A*) Maize cpDNA; (*B*) spinach cpDNA. The left lane in each pair is a photograph of the gel stained with ethidium bromide and the right lane is an autoradiograph of ^{32}P-labeled hybrids. *Hin*dIII fragments of λ phage DNA were used as size markers (expressed in kbp).

only one band (Fig. 2, lane b) of *Bam*HI-digested spinach cpDNA hybridized with pZme9019 DNA. This band was estimated to be 11.2 kbp in length. The 11.2-kbp *Bam*HI fragment (referred to here as *Bam* 3) was isolated from total spinach cpDNA digested with *Bam*HI, ligated into the *Bam*HI site of pBR322, and used to transform *E. coli* K-12 strain RR1 cells. The plasmid, pSoe3101, was isolated, and restriction analysis showed that it contained an insert of the correct size. To demonstrate that this plasmid bears the gene for the LS, an *in vitro* transcription–translation system derived from *E. coli* was utilized to direct the synthesis of plasmid-coded gene products. Fig. 3 is a fluorograph of the [^{35}S]methionine-labeled protein products synthesized in the coupled system, programmed with plasmid DNA containing either maize or spinach cpDNA inserts. The parent plasmid, pBR322, directs the synthesis of one predominant protein of approximately 30 kilodaltons (kDal), which is most probably β-lactamase (lane A) (30, 31). Products coded for by the tetracycline gene on pBR322 are apparently not synthesized in this system (see ref. 30). The plasmids pZme9019, pZme1502, and pSoe3101 all direct the synthesis of additional polypeptides, with one especially prominent at 52–53 kDal (lanes, B, C, and D, respectively). Further evidence that this product is the LS is shown in Fig. 4. Immunoprecipitation of the ^{35}S-labeled products synthesized *in vitro* by using anti-RuP_2 carboxylase yielded one major protein at about 53 kDal on polyacrylamide gels (Fig. 4) that comigrates with the [^{3}H]LS standard. No ^{35}S-labeled proteins were immunoprecipitated by anti-RuP_2 carboxylase when the *in vitro* incubations were programmed with pBR322 alone (data not shown).

Estimates of the size of the LS synthesized *in vitro*, using either the maize or spinach DNA clones, indicate that the LS produced with pSoe3101 is slightly larger than the LS produced with pZme9019 or pZme1502 plasmids (Fig. 3). The LS in maize is reported to be a 52-kDal polypeptide (8). A molecular mass for spinach LS of 55 kDal has been reported (32), although in the gel electrophoresis system used here we estimate it to be approximately 53 kDal. The ability of the *in vitro* system to synthesize a complete LS is also evidence that the LS gene lacks

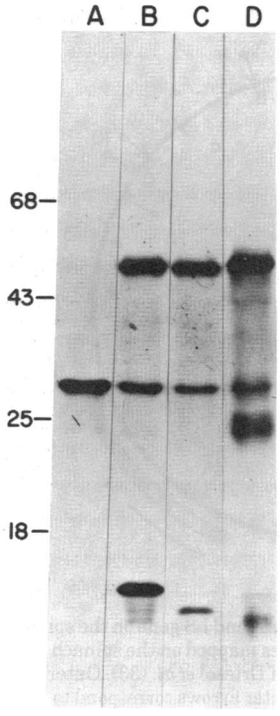

FIG. 3. Fluorograph of $NaDodSO_4$ slab gel analysis of the ^{32}S-labeled products synthesized *in vitro* by using recombinant plasmids. Plasmids (1.5 μg of DNA) were added to the *E. coli* partially defined *in vitro* transcription–translation system and after incubation a 2.5-μl aliquot was removed and mixed with 2 vol of sample buffer [0.25 M Tris·HCl, pH 6.8/2.5% (vol/vol) 2-mercaptoethanol/5% (vol/vol) glycerol/1.5% $NaDodSO_4$]. After heating, the samples were electrophoresed on $NaDodSO_4$/7.5–15% acrylamide gradient gels. Lanes correspond to *in vitro* products synthesized from the following templates: A, pBR322; B, pZme1502; C, pZme9019; and D, pSoe3101. Molecular mass markers were bovine serum albumin (68 kDal), ovalbumin (43 kDal), α-chymotrypsinogen (25 kDal), and β-lactoglobulin (18.4 kDal).

any intervening sequences, because it is unlikely that a transcript containing introns would yield an immunoprecipitable protein that is also the same size as authentic LS.

Map Position of the LS Gene on the 11.2-kbp *Bam* 3 Insert in pSoe3101. In order to clearly define the position of the LS gene on pSoe3101, the cleavage sites for a number of restriction nucleases were determined. The restriction sites for *Sma* I, *Sal* I, *Kpn* I, *Bgl* II, *Bam*HI, *Hin*cII, *Pst* I, and *Hin*dIII are presented in Fig. 5. As shown, *Pst* I, *Sma* I, and *Sal* I all yielded single cleavage sites within the 11.2-kbp insert. Only the sites at the ends of the insert were determined for *Hin*cII, and one *Bgl* II site remains partially ambiguous. To determine the boundaries of the LS gene, we again utilized the cloned maize DNA. A 0.2-kbp *Eco*RI fragment was isolated from the plasmid pZme1502 containing the 2.7-kbp *Bgl* II insert (see Fig. 1 and above). The 0.2-kbp *Eco*RI fragment was ^{32}P-labeled by nick-translation and hybridized to the 11.2-kbp *Bam* 3 insert from pSoe3101 and to various restriction fragments derived from this insert. The 0.2-kbp *Eco*RI maize DNA segment hybridized to a 2.3-kbp *Bgl* II and to a 1.5-kbp *Kpn* I fragment (data not shown). Both of the fragments generated by *Pst* I hybridized to the 0.2-kbp *Eco*RI DNA (data not shown). The *Pst* I site as shown on the restriction map in Fig. 5 is within both the 2.3-kbp *Bgl* II and 1.5-kbp *Kpn* I fragments. Link and Bogorad (12) have presented evidence indicating that the 0.2-kbp *Eco*RI fragment contains a short stretch of the amino-terminal coding sequence of LS (about 40 base pairs). It is likely that an additional 50–100 base pairs, which could include any RNA polymerase binding site and possible control sequences, are part of the LS gene. Hybridization of the 0.2-kbp *Eco*RI fragment to both *Pst* I fragments indicates that the *Pst* I site is at or very near the amino terminus of the spinach LS gene. If the start of the LS gene is at the *Pst* I site, then there is a sufficient amount of DNA in either direction to code for the LS protein, although the entire length of the smaller *Pst* I fragment (≈1.45 kbp) would be needed to code for a 53-kDal protein.

Direction of Transcription of the Spinach LS Gene. Because the LS is efficiently synthesized by the *in vitro* transcription–translation system programmed with pSoe3101, we have

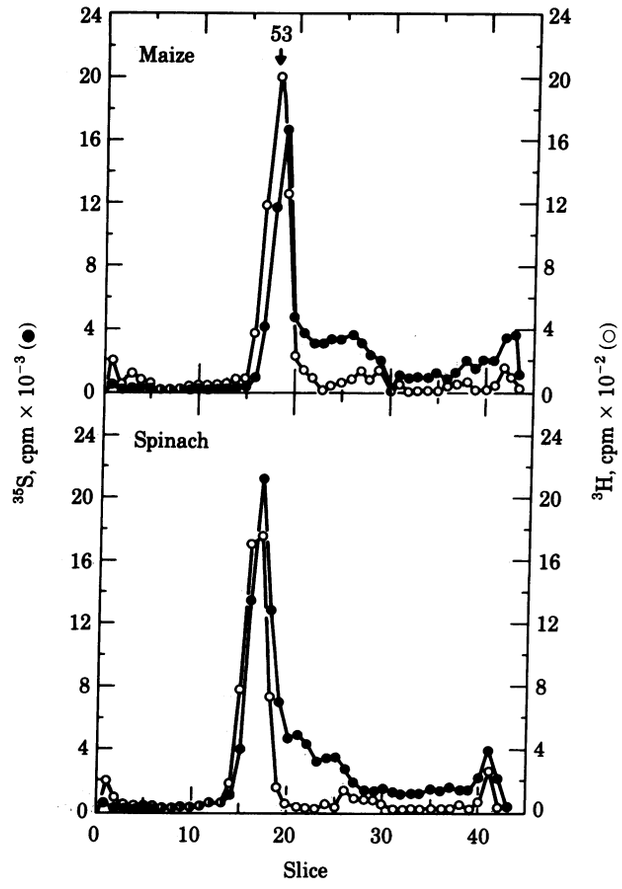

FIG. 4. $NaDodSO_4$ disc gel analysis of anti-RuP_2 carboxylase immunoprecipitated product synthesized *in vitro* by using recombinant plasmids. The *in vitro* system was programmed with plasmid DNA as indicated in the legend to Fig. 2. Aliquots (20 μl) were removed and immunoprecipitated with anti-RuP_2 carboxylase in the presence of ^{3}H-labeled RuP_2 carboxylase. Samples were electrophoresed on 10% acrylamide/$NaDodSO_4$ disc gels. The gels were sliced and ^{35}S and ^{3}H radioactivity were determined. Products were synthesized with pZme9019 (*Upper*) or pSoe3101 (*Lower*) as template.

utilized this assay to further define the region of DNA that contains the LS gene. The design of this experiment was to digest pSoe3101 with restriction enzymes that have defined cleavage sites on pSoe3101. Then, after cleavage of the plasmid with specific restriction nucleases, the formation of the LS protein was analyzed by immunoprecipitation of the ^{35}S-labeled product synthesized *in vitro*. Fig. 6 shows the results of such an experiment. *Bam*HI or *Hin*cII digestion (Fig. 6, traces b and c) does not affect the size of LS produced as compared to uncut plasmid (Fig. 6, trace a). The digestion of pSoe3101 with *Bgl* II, *Hin*dIII,

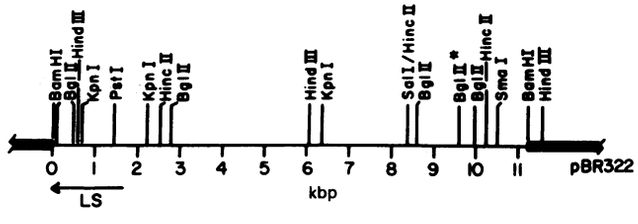

FIG. 5. Restriction map of pSoe3101. The *Bgl* II site at the position indicated by the asterisk has not been unambiguously determined. The site could alternatively map 8.9 kbp from the left *Bam*HI site. Only the *Hin*cII cleavage sites at the ends of the *Bam* 3 insert have been determined, with the exception of the *Sal* I site, which is also cleaved by *Hin*cII.

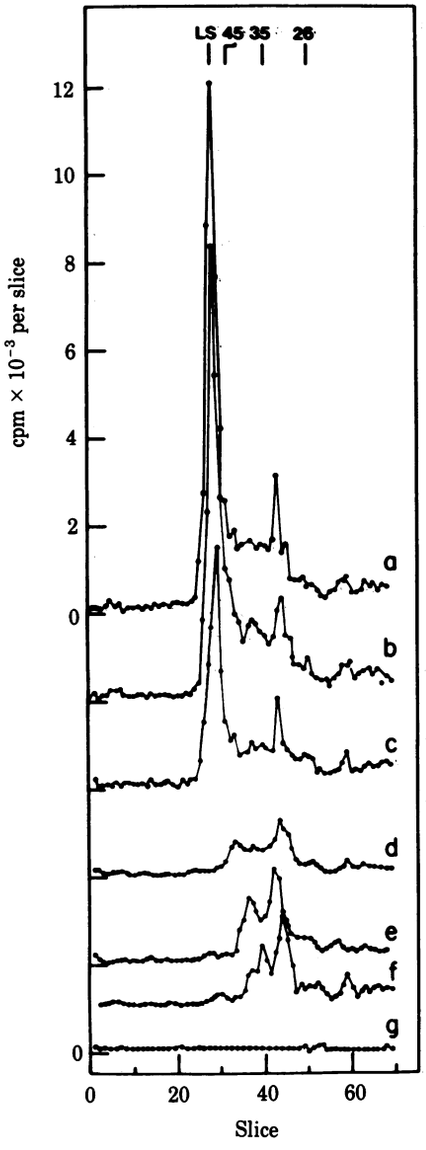

FIG. 6. $NaDodSO_4$ disc gel analysis of the ^{35}S-labeled product immunoprecipitated with anti-RuP_2 carboxylase from *in vitro* incubations programmed with pSoe3101 after digestion with restriction nucleases. The plasmid pSoe3101 was digested with restriction nucleases and the resulting fragments were used to direct the *in vitro* synthesis of ^{35}S-labeled proteins. Aliquots (20 μl) from incubations were immunoprecipitated with anti-RuP_2 carboxylase in the presence of [^{3}H]LS. The precipitates were electrophoresed on $NaDodSO_4$/10% polyacrylamide disc gels. Gels were sliced and radioactivity was determined. Undigested pSoe3101 is shown in trace a; pSoe3101 was digested with: b, *Bam*HI; c, *Hin*cII; d, *Bgl* II; e, *Hin*dIII; f, *Kpn* I; g, *Pst* I. Gels were aligned according to the position of the internal standard, [^{3}H]LS. Molecular mass markers are in kDal.

or *Kpn* I (Fig. 6, traces d, e, and f, respectively) results in the *in vitro* synthesis of truncated products that are immunoprecipitable by anti-RuP_2 carboxylase, and cleavage with *Pst* I (Fig. 6, trace g) abolishes the synthesis of any immunoprecipitable product. The lack of synthesis of LS or truncated LS after digestion of pSoe3101 with *Pst* I again indicates that the *Pst* I site is at or near the start site of the LS gene. Also because *Hin*cII does not affect the synthesis of LS, transcription could not proceed in this direction. A *Hin*cII site maps 1.1 kbp to the right of the *Pst* I site (Fig. 5), which is an insufficient amount of DNA to code for LS. Consistent with this interpretation is the size of the immunoprecipitated products produced by DNA cleaved with *Bgl* II, *Hin*dIII, or *Kpn* I. As seen in Fig. 6, trace f, *Kpn* I-digested

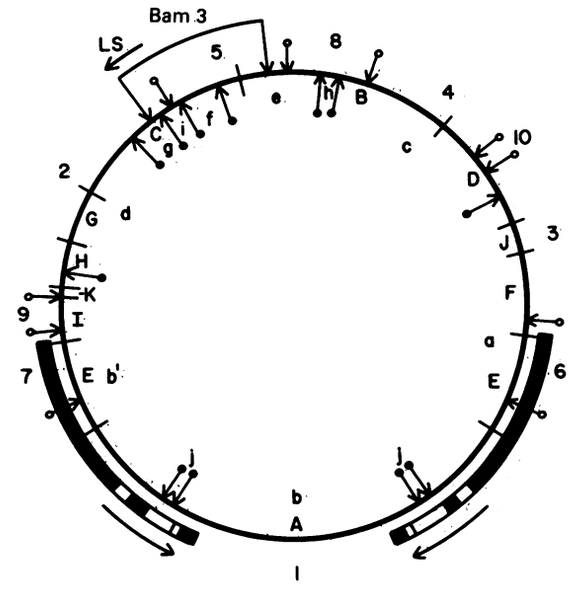

FIG. 7. Position of the *Bam* 3 fragment and LS gene on the spinach chloroplast chromosome. Restriction sites mapped on the spinach chloroplast chromosome are from the data of Driesel *et al.* (33). Outer bars represent inverted repeat regions and outer arrows correspond to transcription units of rRNA genes and the LS gene on *Bam* 3. Arrows perpendicular to the circle are *Kpn* I sites (●) with fragments designated alphabetically (lower case), or *Pst* I sites (○) with fragments designated numerically. Bars intersecting the circle are *Sal* I sites with fragments designated alphabetically (upper case).

pSoe3101 directs the synthesis of truncated products smaller than those produced after either *Bgl* II or *Hin*dIII digestion (Fig. 6, traces d and e). The restriction map (Fig. 5) shows that a *Kpn* I site maps about 0.7 kbp to the left of the *Pst* I site, whereas *Hin*dIII and *Bgl* II sites are 0.85 and 0.9 kbp to the left, respectively. Taken together, these data indicate that the LS gene starts at or slightly to the right of the *Pst* I site and proceeds leftward to probably within less than 100 base pairs of the *Bam*HI site (Fig. 5).

Position of LS Gene on the Chloroplast Genome. Driesel *et al.* (33) have previously mapped a number of restriction sites on the total spinach chloroplast chromosome. Because the *Bam* 3 fragment overlaps some of these sites, we are now able to precisely determine the position and orientation of the 11.2-kbp *Bam* 3 fragment on the 145-kbp chloroplast chromosome (Fig. 7). The LS gene is approximately 27 kbp from the start of one of the inverted repeat regions and almost exactly 180° from one of the rRNA-coding regions. The positioning of the LS gene on the spinach chloroplast chromosome is therefore very similar to that found in maize chloroplasts (11–13).

Additional Genes Coded on the *Bam* 3 Fragment. The studies presented here are primarily concerned with the initial characterization of the spinach LS gene. It is evident, however, that the 11.2-kbp *Bam* 3 fragment contains sufficient information to code for as many as seven polypeptides the size of the LS. Examination of products synthesized *in vitro*, using the *E. coli* coupled transcription–translation system, programmed with pSoe3101, indicates that at least two additional proteins may be coded by the *Bam* 3 segment. These are in the range of 20 to 25 Kd (see Fig. 3, lane 3). These proteins are not immunoprecipitated by anti-RuP_2 carboxylase, and they are not present when pBR322 is used in the *in vitro* assay (Fig. 3, lane A).

DISCUSSION

Bottomley and Whitfield (32) used an unfractionated *E. coli* extract to demonstrate that the LS could be synthesized *in vitro*

by using as templates total cpDNA isolated from different plant species. In addition, they showed that predigestion of spinach cpDNA with restriction nucleases in some cases abolished the synthesis of the complete LS. The present results have verified and extended their findings, using the cloned 11.2-kbp *Bam* 3 fragment, which we have shown here to contain the gene for the LS. The use of the partially defined *in vitro* transcription–translation system (24) in this study, coupled with the use of an immunoprecipitation assay, has allowed us not only to detect and map the position of the LS gene on pSoe3101 but also to determine the direction of its transcription. Similar to the finding with maize LS, the spinach LS transcript appears to be colinear with its coding sequence of approximately 1.5 kilobases and oriented such that it is approximately 27 kbp from the start of one of the inverted repeat regions. Our hybridization data indicate that there is only one copy of the LS gene on the spinach chloroplast chromosome, as has been also found in maize (11) and *Chlamydomonas reinhardi* (34).

Link and Bogorad (12) have presented evidence, based on S1 nuclease mapping, for part of an additional gene on the 4.35-kbp *Bam*HI fragment from maize cpDNA that is approximately 300 base pairs from the start of the LS gene and reading in the opposite direction. The entire gene is not on the 4.35-kbp *Bam*HI fragment, however, and no protein synthesized *in vitro* has been observed that would correspond to it. The 11.2-kbp *Bam* 3 fragment from spinach also appears to contain additional coding sequences, on the basis of the appearance of several small polypeptides produced *in vitro* (Fig. 3), although these have not been mapped to a precise region. In addition, Driesel *et al.* (33) have mapped the positions of 21 to 25 tRNA genes on the spinach chloroplast chromosomes. Coincidentally, at least 8 and possibly as many as 12 of these tRNA genes are found on the *Bam* 3 fragment cloned in pSoe3101. In the restriction map defined by Driesel *et al.* (33), the *Bam* 3 fragment would overlap a region that would place the majority of these tRNA genes from 5 to 9 kbp to the right of the LS gene (see Fig. 5).

The ability to transcribe and translate chloroplast genes *in vitro* by using a system derived from *E. coli* illustrates their prokaryotic nature. Even more surprising is the efficiency with which the chloroplast gene products are synthesized compared to the synthesis of β-lactamase, a product coded by the parent plasmid pBR322 (30). This is seen qualitatively by the intensity of the radioactive bands in Fig. 3. Because we used [^{35}S]methionine as the radioactive label, this difference is even more pronounced when it is considered that β-lactamase contains 10 methionine residues per polypeptide chain (0.37 methionine residue per kDal of protein) (35), whereas the LS contains only 7 to 8 methionine residues per chain (0.15 methionine residue per kDal of protein) (3–5).

The demonstration here that the LS gene from maize will hybridize specifically with the LS gene of spinach allows for a facile method of detecting the LS gene in other plants, providing they are not too distantly related. We have failed to detect the LS gene on *Euglena* cpDNA by using the maize LS gene as a probe. Halleck *et al.* (36), however, have reported that the LS gene from *Chlamydomonas* hybridizes to a specific *Euglena* cpDNA restriction fragment that appears to code for the LS protein.

While this manuscript was in preparation, Whitfeld and Bottomley (37) reported results similar to those reported here. The data presented here, however, are more conclusive with respect to the direction and boundaries of transcription of the LS gene due to the use of a specific immunoprecipitation assay.

We thank Mrs. Betty Redfield for performing *in vitro* transcription–translation assays and Dr. Tanya Zarucki-Schulz for providing protocols for molecular cloning.

1. Baker, T. S., Eisenberg, D., Eiserling, F. A. & Weissman, L. (1975) *J. Mol. Biol.* **91**, 391–399.
2. Kirk, J. T. O. (1978) in *The Plastids*, eds. Kirk, J. T. O. & Tilney-Bassett, R. A. E. (Elsevier, Amsterdam), pp. 560–607.
3. Kawashima, N., Kwok, S.-Y. & Wildman, S. G. (1971) *Biochim. Biophys. Acta* **236**, 578–586.
4. Kawashima, N. & Wildman, S. G. (1971) *Biochim. Biophys. Acta* **229**, 749–760.
5. Wildman, S. G. (1979) *Arch. Biochem. Biophys.* **196**, 598–610.
6. Kirchanski, S. J. & Park, R. B. (1976) *Plant Physiol.* **58**, 345–349.
7. Huber, S. C., Hall, T. C. & Edwards, G. E. (1976) *Plant Physiol.* **57**, 730–733.
8. Link, G., Coen, D. M. & Bogorad, L. (1978) *Cell* **15**, 725–731.
9. Walbot, V. (1977) *Cell* **11**, 729–737.
10. Coen, D. M., Bedbrook, J. R., Bogorad, L. & Rich, A. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5487–5491.
11. Bedbrook, J. R., Coen, D. M., Beaton, A. R., Bogorad, L. & Rich, A. (1979) *J. Biol. Chem.* **254**, 905–910.
12. Link, G. & Bogorad, L. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 1832–1836.
13. Bogorad, L., Link, G., McIntosh, L. & Jolly, S. O. (1979) in *Extrachromosomal DNA*, ICN–UCLA Symposia on Molecular and Cellular Biology, eds. Cummings, D. J., Borst, P., Dawid, I. G., Weissmann, S. M. & Fox, C. F. (Academic, New York), Vol. 15, pp. 113–136.
14. Bolivar, F. & Backman, K. (1979) *Methods Enzymol.* **68**, 245–267.
15. Bolivar, F., Rodriguez, R., Greene, P. J., Betlack, M., Heyneker, H. L., Boyer, H. W., Crosa, J. & Falkow, S. (1977) *Gene* **2**, 95–113.
16. Kolodner, R. & Tewari, K. K. (1975) *Biochim. Biophys. Acta* **402**, 372–390.
17. Kahn, M., Kolter, R., Thomas, C., Figurski, D., Meyer, R., Remaut, E. & Helinski, D. R. (1979) *Methods Enzymol.* **68**, 268–280.
18. Southern, E. M. (1979) *Methods Enzymol.* **68**, 152–176.
19. Dugaiczyk, A., Boyer, H. W. & Goodman, H. M. (1975) *J. Mol. Biol.* **96**, 171–184.
20. Kushner, S. R. (1978) in *Genetic Engineering*, eds. Boyer, H. W. & Nicosida, S. (Elsevier, Amsterdam), p. 17.
21. Birnboim, H. C. & Doly, J. (1979) *Nucleic Acids Res.* **7**, 1513–1523.
22. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. (1977) *J. Mol. Biol.* **113**, 237–251.
23. Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503–517.
24. Zarucki-Schulz, T., Jerez, C., Goldberg, G., Kung, H.-F., Huang, K. H., Brot, N. & Weissbach, H. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 6115–6119.
25. Kung, H.-F., Redfield, B. & Weissbach, H. (1979) *J. Biol. Chem.* **254**, 8404–8408.
26. Kung, H.-F., Redfield, B., Treadwell, B. V., Eskin, B., Spears, C. & Weissbach, H. (1977) *J. Biol. Chem.* **252**, 6889–6894.
27. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
28. Bonner, W. M. & Laskey, R. G. (1974) *Eur. J. Biochem.* **46**, 83–88.
29. Jentoft, N. & Dearborn, D. G. (1979) *J. Biol. Chem.* **254**, 4359–4365.
30. Sutcliffe, J. G. (1979) *Cold Spring Harbor Symp. Quant. Biol.* **43**, 77–90.
31. Yang, H.-L., Zubay, G. & Levy, S. B. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 1509–1512.
32. Bottomley, W. & Whitfeld, P. R. (1979) *Eur. J. Biochem.* **93**, 31–39.
33. Driesel, A. J., Crouse, E. J., Gordon, K., Bohnert, H. J., Herrmann, R. G., Steinmetz, A., Mubumbila, M., Keller, M., Burkard, G. & Weil, J. H. (1979) *Gene* **6**, 285–306.
34. Malnoe, P., Rochaix, J.-D., Chua, N. H. & Spahr, P.-F. (1979) *J. Mol. Biol.* **133**, 417–434.
35. Sutcliffe, J. G. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3737–3741.
36. Hallick, R. B., Rushlow, K. E., Orozco, E. M., Jr., Stiegler, G. L. & Gray, P. W. (1979) in *Extrachromosomal DNA*, ICN–UCLA Symposia on Molecular and Cell Biology, eds. Cummings, D. J., Borst, P., Dawid, I. B., Weissman, S. M. & Fox, C. F. (Academic, New York), Vol. 15, pp. 127–141.
37. Whitfeld, P. R. & Bottomley, W. (1980) *Biochem. Intl.* **1**, 172–178.