Proc. Natl. Acad. Sci. USA
Vol. 77, No. 4, pp. 1832–1836, April 1980
Biochemistry

Sizes, locations, and directions of transcription of two genes on a cloned maize chloroplast DNA sequence

(ribulosebisphosphate carboxylase/2.2-kilobase gene/plasmid)

GERHARD LINK* AND LAWRENCE BOGORAD†

The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

Contributed by Lawrence Bogorad, December 26, 1979

ABSTRACT **mRNA for the large subunit (LS) of ribulose-1,5-bisphosphate carboxylase [3-phospho-D-glycerate carboxylase (dimerizing), EC 4.1.1.39] of *Zea mays* is complementary to an uninterrupted 1600-base-pair-long chloroplast DNA sequence that has been mapped precisely within the 4350-base-pair-long chloroplast DNA fragment *Bam* 9 to which it had been traced earlier [Bedbrook, J. R., Coen, D. M., Beaton, A. R., Bogorad, L. & Rich, A. (1979) *J. Biol. Chem.* 254, 905–910]. An additional 1400-base-pair-long uninterrupted region that is colinear with a chloroplast RNA has been detected on *Bam* 9. The transcript from this region is part of a 2200-nucleotide-long RNA. The remainder of the DNA sequence for the 2200-base-pair RNA maps outside *Bam* 9. The 1600-base-pair *LS* gene and the gene for the 2200-nucleotide transcript are close to one another. They are separated by an untranscribed intercistronic "gap" about 330 base pairs long. These two closely packed genes are inverted on the chromosome—i.e., their 3′ termini are at opposite ends of the untranscribed gap and they map on opposite strands.**

The organization of genes for rRNAs in the chloroplast chromosome of maize and a few other plants is known in detail (1–4) but genes for only two chloroplast proteins have been mapped physically to relatively small regions of chloroplast chromosomes. The gene for the 52,000-dalton large subunit (LS) of ribulosebisphosphate carboxylase [3-phospho-D-glycerate carboxylase (dimerizing), EC 4.1.1.39] in maize has been mapped to within a 2.5-kilobase-pair (kbp) portion of the 4.35-kbp chloroplast DNA sequence *Bam* 9 that has been cloned (5–7). Photogene 32 which codes for a 34,500-dalton precursor of a chloroplast thylakoid protein has been located on a 2.1-kbp maize chloroplast DNA fragment which has also been cloned (8, 9). The present research was undertaken to obtain detailed information about the spacing and arrangement of chloroplast genes for proteins.

In the present work we have determined that mRNA for maize LS is 1.6 kilobases (kb) in length and is colinear with a chloroplast DNA sequence. A DNA sequence coding for a part of a 2.2-kb transcript has been located 0.33 kbp from the 3′ end of the gene for LS. The two genes are transcribed in opposite directions from one another.

EXPERIMENTAL PROCEDURES

Preparation of RNA. Maize chloroplast RNA capable of directing the synthesis of LS in a rabbit reticulocyte cell-free translation system was prepared as described (10).

Recombinant DNA; Preparation of DNA Fragments. DNA of recombinant plasmids pZmc 37 (6, 7) and pZmc 3711, both of which contain the structural sequence of LS, was prepared by the method of Clewell and Helinski (11). pZmc 3711 was constructed by Linda Scrafford-Wolff by inserting DNA fragment B 2.7 (Fig. 1) isolated from pZmc 37 into one of the two recognition sites for *Bgl* II within RSF 1030 (12). Work with recombinant plasmids was carried out at the P2 level of physical containment as specified in National Institutes of Health Guidelines on Recombinant DNA Research, 1976.

Digestion with restriction endonucleases was carried out as described (5, 10). DNA fragments were isolated either after electrophoresis in agarose slab gels (13) or by two cycles of sucrose density gradient centrifugation (6, 7). DNA fragments were extracted with phenol/chloroform and concentrated by precipitation with ethanol.

Radioactive Labeling of DNA and RNA. DNA fragments were labeled with ^{32}P at their 3′ termini by using terminal deoxynucleotidyltransferase (14) and [α-^{32}P]UTP (520 Ci/mmol; Ci = 3.7×10^{10} becquerels; New England Nuclear) according to Roychoudhury *et al.* (15). Labeling of dephosphorylated 5′ ends with ^{32}P from [γ-^{32}P]ATP (1000 Ci/mmol) by using polynucleotide kinase was as described by Maxam and Gilbert (16). Nick translation of DNA fragments (17) was performed in 0.1-ml volumes. Each of the four dXTPs (350 Ci/mmol; New England Nuclear) was present at a concentration of 1.8 μM. Labeled DNA fragments were separated from unincorporated nucleotides by filtration on Sephadex G-50.

^{32}P-Labeled copy RNAs (cRNAs) from DNA fragments were synthesized by using *Escherichia coli* DNA-dependent RNA polymerase (18). The cRNA synthesis procedure (5) was modified by increasing the DNA concentration 5- to 10-fold (1–2 μg) and by using 150 μCi of [α-^{32}P]UTP (150 Ci/mmol; New England Nuclear). The specific activity of the cRNA was $1–5 \times 10^7$ cpm/μg.

Hybridization in Solution; Digestion with Nuclease S1. DNA·RNA hybridizations were carried out in 80% (vol/vol) formamide/0.4 M NaCl/0.04 M 1,4-piperazinediethanesulfonic acid, pH 6.4/1 mM EDTA (19, 20); 25–50 ng of DNA and 10 μg of RNA were present in a total volume of 10 μl. The mixture was heated at 65°C for 10 min, incubated at 52°C for 12 hr, and then rapidly cooled.

Digestions of DNA·RNA hybrids with nuclease S1 were as described by Berk and Sharp (19) but at 37°C for 30 min with 0.1–20 units of nuclease S1 (as indicated in the figure legends). After precipitation with ethanol and centrifugation, samples were taken up in 25 μl of 0.03 M NaOH/2 mM EDTA/10% (vol/vol) glycerol/0.01% bromophenol blue.

Alkaline Gel Electrophoresis; DNA Transfer; Hybridization. Electrophoresis was in $14 \times 12 \times 0.4$ cm alkaline agarose slab gels (21) run at 50 mA for 7 hr and briefly stained with eithidium bromide (4 μg/ml in 0.3 M sodium acetate, pH 6). DNA fragments were then transferred (22) from gels to nitro-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: LS, large subunit of ribulose-1,5-bisphosphate carboxylase; cRNA, copy RNA; kb, kilobase(s); kbp, kilobase pair(s).

* Present address: Biologisches Institut II der Universitaet Freiburg, Schaenzlestrasse 1, D-7800 Freiburg i. Br.m FRG.
† To whom correspondence should be addressed.

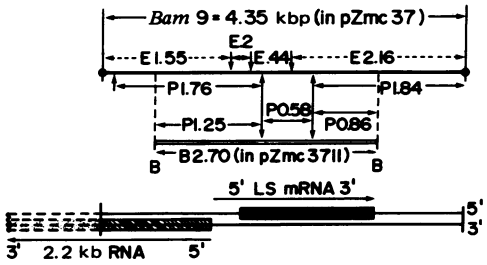

FIG. 1. (*Upper*) The orientation of fragments generated from chloroplast DNA sequence *Bam* 9 by restriction endonucleases *Eco*RI, *Bgl* II, and *Pst* I (7). Fragments of *Bam* 9 are designated by the first letter of the restriction enzyme used for the cleavage—B for *Bgl* II, E for *Eco*RI, P for *Pst* I—followed by the size of the fragment in kilobase pairs. DNA fragment B 2.7 has been cloned separately as part of recombinant plasmid pZmc 3711 (see *Experimental Procedures*); accordingly, DNA fragments P 1.25 and P 0.86 have a recognition sequence for *Bgl* II at one end. (*Lower*) The arrangement of genes for LS and a 2.2-kb RNA on chloroplast DNA *Bam* 9. The 2.2-kb gene extends 0.8 kb beyond the left-hand end of *Bam* 9.

cellulose sheets (Schleicher & Schull BA 85, 0.45 μm; or BA 83, 0.20 μm).

Hybridization with [^{32}P]cRNA in sealed plastic bags was as described (5, 10).

Gel Electrophoresis of RNA and Transfer to Diazobenzyloxymethyl-Paper; Hybridization with Labeled DNA. Denatured (23) chloroplast RNAs were separated electrophoretically (100 V, 2.5 hr) in 14 × 12 × 0.4 cm slabs of 1.5% (wt/vol) agarose gel in 10 mM sodium phosphate buffer at pH 7.0. Adjacent tracks containing glyoxylated DNA size marker fragments were cut off and stained with acridine orange (30 μg/ml).

Gel portions containing chloroplast RNA were soaked in 50 mM KOH and neutralized in pH 6.5 potassium phosphate buffer. RNA was then transferred to diazobenzyloxymethyl-paper and hybridized with nick translated DNA fragments essentially as described by Alwine *et al.* (24).

RESULTS

Two Stable Chloroplast RNAs Are Colinear with DNA Sequences on *Bam* 9. The sizes of stable transcripts complementary to the chloroplast DNA sequence *Bam* 9 (5–7) were determined by hybridizing chloroplast RNA with pZmc 37 DNA denatured after digestion with *Bam*HI (this digestion yields *Bam* 9 plus the vehicle RSF 1030), digesting away unhybridized DNA and RNA with nuclease S1 (19), electrophoretically separating nuclease S1-resistant DNA sequences on alkaline agarose gels (21) together with size markers, and transferring the DNA to nitrocellulose filter sheets. The DNA was located by hybridization with [^{32}P]cRNA transcribed from B 2.7 obtained from pZmc 3711 (Fig. 1).

The chloroplast RNA preparation contained transcripts that hybridized to and protected 1.6- and 1.4-kb chloroplast DNA sequences in *Bam* 9 from digestion with nuclease S1 over a 200-fold range of nuclease concentrations (Fig. 2, lanes 2–5). A band was visible at the 4.35-kb position at the lowest nuclease concentration used (lane 2); this is the size of undigested *Bam* 9. The 3.5- and 2.6-kb DNAs are probably partial nuclease S1 digestion products. At the highest nuclease S1 concentrations (lanes 4 and 5), the 1.6- and 1.4-kb bands were less intense and a band of 1.1-kb DNA was present. The latter could result from scission of the 1.4-kb DNA—e.g., at a short A + T-rich region (27).

We conclude that the mRNA for LS is colinear with an uninterrupted 1.6-kb DNA sequence completely within B 2.7.

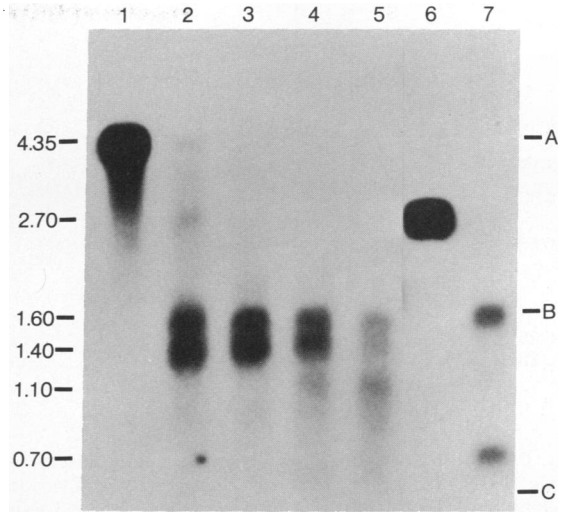

FIG. 2. Autoradiograph of a nitrocellulose filter sheet showing hybridization of B 2.7 [^{32}P]cRNA (5×10^6 cpm in 5 ml) with fragments of DNA separated electrophoretically in a 1% agarose gel. Lanes: 1, 25 ng of pZmc 37 DNA digested with *Bam*HI (no nuclease S1); 2–5, *Bam*HI digest of 50 ng of pZmc 37 DNA hybridized with 8 μg of chloroplast RNA and treated with 0.1 (lane 2), 1 (lane 3), 5 (lane 4), or 20 (lane 5) units of nuclease S1 before electrophoresis; 6, B 2.7 DNA (50 ng of pZmc 3711 digested with *Bgl* II) (no nuclease S1); 7, fragments of B 2.7 DNA protected from digestion with 5 units of nuclease S1 by hybridization with chloroplast RNAs (with nuclease S1). DNA fragment sizes in kilobases are based on pBR322 (25) digested with *Hin*fI or *Pst* I as markers. Marker fragments were detected by hybridization with pBR322 [^{32}P]cRNA: A, 4.362 kb [(26) linearized pBR322]; B, 1.731 kb; C, 0.517 and 0.506 kb (unresolved).

Hybridization of B 2.7 DNA (produced from pZmc 3711 digested with *Bgl* II) with chloroplast RNA followed by nuclease S1 digestion of unhybridized nucleic acids also yielded 1.6-kb protected DNA sequences (with the same B 2.7 cRNA probe as in the previous experiment). However, the 1.4-kb DNA sequence was replaced by one of 0.7 kb (Fig. 2, lanes 6 and 7). The 0.7-kb DNA is judged to be that part of the 1.4-kb sequence at the left-hand edge of *Bam* 9 (Fig. 1) retained in B 2.7; these two DNA sequences were protected by parts of a 2.2-kb transcript (see below).

Mapping DNA Sequences on *Bam* 9 Colinear with Stable Chloroplast RNAs. Coding sequences have been mapped closely on *Bam* 9 (and B 2.7) by two modifications of the nuclease S1 mapping technique. First, instead of hybridizing unfractionated chloroplast RNA with intact *Bam* 9 or B 2.7 DNA for nuclease S1 digestion, subfragments of *Bam* 9 or B 2.7 generated by digestion with *Pst* I were used; in this way, *Pst*-generated fragments fully protected from nuclease S1 digestion by hybridization with chloroplast RNA could be easily identified. Second, after electrophoresis, the location of nuclease S1-resistant chloroplast DNA sequences was established by hybridization with [^{32}P]cRNA transcribed from individual *Pst* I- or *Eco*RI-generated fragments of *Bam* 9 or B 2.7 rather than with *in vitro* transcripts of the entire B 2.7 DNA; in this way, those *Pst*-generated fragments decreased in length by nuclease S1 digestion because of only partial hybridization to chloroplast RNA could be assigned. The results of this detailed mapping analysis are summarized in Fig. 1 *Lower*. The data are shown in Fig. 3 and discussed below. The *LS* gene includes 0.22 kb of P 1.76 [and also of P 1.25 (Fig. 1)] and continues without interruption through all of P 0.58 plus 0.80 kb of P 0.86 (and also P 1.84).

Fig. 1 *Lower* shows the position of DNA complementary to the 5′ end of a 2.2-kb transcribed sequence that starts 0.33 kb

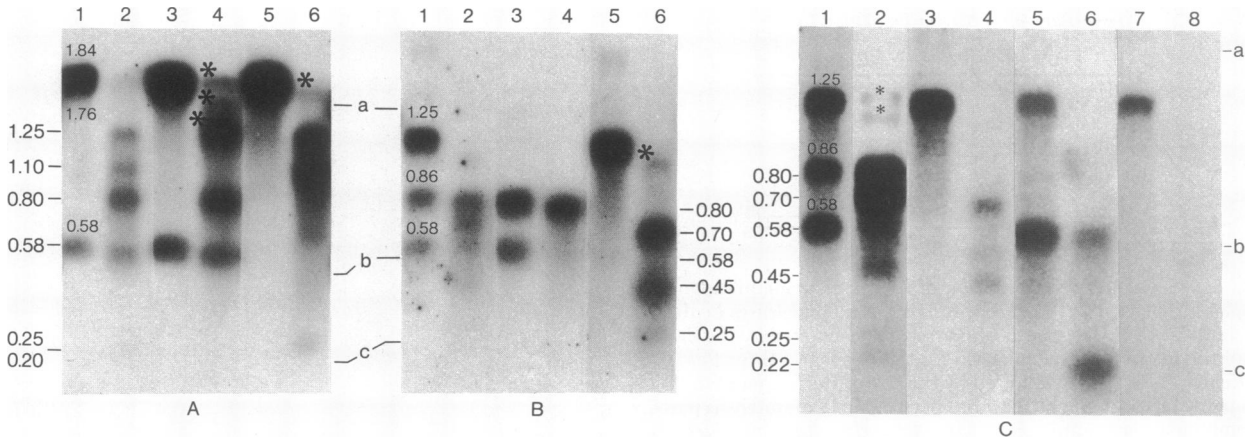

FIG. 3. Autoradiographs showing sizes of chloroplast DNA sequences in *Bam* 9 or B 2.7 complementary to chloroplast RNAs. (*A*) Lanes: 1, pZmc 37 DNA (25 ng) digested with *Bam*BI and *Pst* I, no nulcease S1 treatment, B 2.7 [^{32}P]cRNA probe; 2, as in lane 1 but DNA hybridized with chloroplast RNA (9 μg) and treated with nuclease S1; 3, as in lane 1 but with E 2.16 [^{32}P]cRNA probe; 4, as in lane 2 but with E 2.16 ^{32}P-cRNA probe; 5, as in lane 1 but with E 1.55 [^{32}P]cRNA probe; 6, as in lane 2 but with E 1.55 [^{32}P]cRNA probe. Electrophoresis was in 1% agarose gels. Where used, nuclease S1 concentration was 20 units per reaction. (*B*) Lanes 1–6 as in *A* except pZmc 3711 DNA was digested with *Bgl* II and *Pst* I. (*C*) pZmc 3711 DNA digested as in *B*. Lanes: 1, same as lane 1, in *B*; 2, same as lane 2 in *B* but 5 units of nuclease S1 used; 3, as in lane 1 but with P 1.25 [^{32}P]cRNA probe; 4, same as in lane 2 but 20 units of nuclease S1 used and P 1.25 [^{32}P]cRNA probe; 5, same as in lane 1 but with E 0.44 [^{32}P]cRNA probe; lane 6, as in lane 4 but with E 0.44 [^{32}P]cRNA probe; 7, as in lane 1 but with E 0.20 [^{32}P]cRNA probe; 8, as in lane 4 but with E 0.20 [^{32}P]cRNA probe. For lanes 5–8, 0.20-μm nitrocellulose was used. Bands interpreted as partial digestion products of nuclease S1 are marked by asterisks. Letters refer to the positions of pBR322 marker fragments generated by *Hin*fI (26): a, 1.631 kb; b, 0.517/0.506 kb; c, 0.221/0.220 kb.

from the *LS* gene, spans the left-hand side of *Bam* 9, and continues on an adjacent segment of the chloroplast chromosome. Data are presented below.

Digestion of pZmc DNA with *Bam*HI and *Pst* I yielded three fragments: P 1.84, P 1.76, and P. 0.58 (Fig. 1). P 1.84 and P 1.76 were not resolved in Fig. 3*A*, lane 1. Hybridization with chloroplast RNA protected all of P 0.58 from digestion with nuclease S1 (Fig. 3*A*, lane 2) but P 1.84 and P 1.76 were lost—instead, new 1.25-, 1.0-, 0.8-, and (faintly) 0.22- to 0.25-kb DNAs were seen. The new 0.8-kb DNA apparently was derived from P 1.84 because it hybridized to [^{32}P]cRNA transcribed from E 2.16, a fragment from the right-hand end of *Bam* 9 (Fig. 1; Fig. 3*A*, lanes 3 and 4). DNAs of 1.5 and 1.2 kb also hybridized E 2.16 cRNA and are believed to be partial nuclease S1 digests of P 1.84. Hybridization with E 2.16 cRNA again showed that P 0.58 was fully protected.

The origin of the 0.8-kb fragment from P 1.84 was confirmed by its failure to hybridize E 1.55 [^{32}P]cRNA (Fig. 3*A*, lanes 5 and 6). However, this cRNA was complementary to 1.25-, 1.0-, and 0.25-kb DNAs, showing that these three species are derived from P 1.76—i.e., arise from the left-hand part of *Bam* 9 (Fig. 1*A*). The 1.0-kb DNA in Fig. 3*A* (lane 6) is judged to be a scission product of the 1.25-kb species.

Chloroplast RNA hybridization and nuclease S1 digestion experiments comparable to those with *Bam* 9 DNA but with B 2.7 confirmed the assignments made above. Digestion of B 2.7 DNA with *Pst* I yielded P 1.25, P 0.58, and P 0.86, as revealed by hybridization with B 2.7 [^{32}P]cRNA (Fig. 3*B*, lane 1). P 0.58 was fully protected against nuclease S1 by hybridization with chloroplast RNA but P 1.25 and P 0.86 were lost; new DNAs of 0.8, 0.7, 0.45, and (faintly) 0.22–0.25 kb were produced. Hybridization with E 2.16 cRNA revealed which *Pst*-generated fragments arose from the right-hand portion of B 2.7 (Fig. 1). P 0.86 and P 0.58 were visualized from a preparation not treated with nuclease S1 (Fig. 3*B*, lane 3); the nuclease S1-generated 0.80-kb DNA was shown to be derived from P 0.86 (Fig. 3*B*, lane 4) and the 0.58-kb DNA also hybridized, although poorly. Probing with E. 1.55 [^{32}P]cRNA (Figs. 1 and 3*B*, lanes 5 and 6) revealed that the 0.7-, 0.45-, and (faintly) 0.25-kb DNAs are derived from P 1.25. The 0.45- and 0.25-kb DNAs seen in Fig. 3*B* (lane 6), most likely were produced by scission of 0.7-kb DNA.

The adjacent nuclease S1-resistant 0.8- and 0.58-kb DNA fragments of B 2.7 accounted for most of the chloroplast DNA colinear with the 1.6-kb LS mRNA. The remaining 0.22 kb of this mRNA should map within fragment P 1.25 adjacent to the *Pst* I site. A weak band at 0.22–0.25 kb was found (Fig. 3 *A* and *B*, lane 2) with B 2.7 cRNA as a probe. However, DNA in this size range was also detected (Fig. 3 *A* and *B*, lane 6) by hybridization with E 1.55 [^{32}P]cRNA although E 1.55 does not overlap the 0.38-kb portion of P 1.25 near the *Pst* I site.

To resolve ambiguities regarding the location of DNA complementary to the left-hand limit of the 1.6-kb *LS* transcript, we used [^{32}P]cRNAs transcribed from DNA fragments overlapping this region as hybridization probes (Fig. 3*C*). pZmc 3711 DNA digested with *Bgl* II plus *Pst* I was either applied directly to the gel or first hybridized with chloroplast RNA and treated with nuclease S1 as described. The positions of control DNAs, P 1.25, P 0.86, and P 0.58 after gel electrophoresis were revealed by hybridization with B 2.7 [^{32}P]cRNA (Fig. 3*C*, lane 1). In Fig. 3*C* (lane 2) bands at 0.8, 0.7, 0.58, 0.45, and (faintly) 0.22–0.25 kb were revealed with the same cRNA. In addition, there were two bands (at about the position where P 1.25 normally occurs) that were not detected in the corresponding experiment (Fig. 3*B*, lane 2) (these latter bands are probably due to incomplete digestion at the low nuclease S1 concentration used here). In lanes 3 and 4 of Fig. 3*C*, the probe was P 1.25 cRNA. Without hybridization to chloroplast RNA and nuclease S1 treatment (lane 3) only P 1.25 was hybridized. Hybridization to chloroplast RNA and nuclease S1 treatment (lane 4) resulted in the appearance of 0.7-, (faintly) 0.5-, 0.45-, and (faintly) 0.22–0.25-kb DNAs. When nuclease S1 treatment was omitted, fragments P 1.25 and P 0.58 were hybridized by E 0.44 [^{32}P]cRNA (Fig. 3*C*, lane 5). After hybridization to chloroplast RNA and nuclease S1 digestion (lane 6), P. 0.58 was unaltered and a 0.22-kb fragment defined the left-hand limit of the 1.6-kb transcript. Finally, when E 0.20 cRNA was used as a probe, hybridization to P 1.25 was detected in the control (Fig. 3*C*, lane 7) but there was none to any DNA after chloroplast RNA hybridization and nuclease S1 digestion (Fig. 3*C*, lane 8).

These results place the left-hand limit of the DNA sequence colinear with the 1.6-kb *LS* transcript on fragment P 1.25 about 0.22 kb from the junction with fragment P 0.58. The transcribed region extends perhaps 40 bases into fragment E 0.20 but such a short sequence is unlikely to be detected by hybridization with E 0.20 cRNA (Fig. 3C, lane 8).

There was a 1.4-kb DNA sequence complementary to chloroplast RNA at the left-hand end of *Bam* 9 (Fig. 1*B*). Thus, a 0.33-kb intercistronic "gap" exists between the 1.6- and 1.4-kb regions colinear with stable chloroplast RNAs.

Sizes of Chloroplast RNAs Complementary to Maize Chloroplast *Bam* 9 DNA. In Fig. 2 (lane 2) 3.5- and 2.6-kb DNAs were detected in small amounts. These could have been protected from nuclease S1 digestion by precursors of the 1.6- or 1.4-kb transcripts or could be intermediates in nuclease S1 digestion. The sizes of all transcripts complementary to either *LS* DNA or total B 2.7 DNA were determined (24).

Electrophoretically separated chloroplast RNAs were transferred to diazobenzyloxymethyl-paper (24) and hybridized with [^{32}P]DNA nick-translated (17) from P 0.58. RNA (1.6 kb) was hybridized (Fig. 4*A*, lanes 1, 2, and 3; Fig. 4*C*, lane 1). The smaller RNAs hybridized by this probe are likely to be breakdown products. No hybridizable larger chloroplast RNA that might be a stable precursor of *LS* mRNA was detected over a 100-fold RNA concentration range.

With [^{32}P]DNA nick-translated (12) from B 2.7 DNA, a 2.2-kb chloroplast RNA was hybridized in addition to the 1.6-kb species (Fig. 4 *B* and *C*). The 2.2-kb RNA apparently was complementary to the 0.7-kb DNA within B 2.7 of the "1.4-kb" transcribed region. Part of the coding sequence for the 2.2-kb chloroplast RNA mapped outside of fragment *Bam* 9.

Directions of Transcription from The *LS* and 2.2-kb Genes. The following approach was used to determine the directions of transcription from the *LS* and 2.2-kb genes.

B 2.7 DNA was isolated electrophoretically from a *Bgl* II digest of pZmc 3711 and digested with *Pst* I. The resulting fragments, P 1.25, P 0.86, and P 0.58, were labeled with ^{32}P at their 5′ (16) or 3′ ends (15). Terminally labeled DNA fragments were then either applied directly to an alkaline agarose gel for electrophoresis or were first hybridized with chloroplast RNA and treated with nuclease S1. Fragments with radioactive termini protected from nuclease S1 by hybridization with chloroplast RNA were located by autoradiography of gels.

Lane 1 of Fig. 5*A* shows the positions of all three 5′-labeled fragments. In lane 2, 5′-labeled fragments of 0.70, 0.58 (faint), and 0.22 kb were detected. In lanes 1 and 3 of Fig. 5*B*, the positions of the 3′-labeled fragments P 1.25, P 0.86, and P 0.58 are shown. Lane 2 reveals that hybridization with chloroplast RNA protected the label at the 3′ ends of the 0.80- and 0.58-kb DNA fragments during nuclease S1 digestion.

Hybridization of *Pst*-generated fragments of B 2.7 DNA with LS mRNA in chloroplast RNA prevented removal of ^{32}P-labeled nucleotides at the 3′ ends of P 0.58 and P 0.86 (0.80-kb [3′-^{32}P]DNA is derived from [3′-^{32}P]P 0.86) but not P 1.25 (Fig. 5*B*, lane 2) by nuclease S1 action. The conclusion that the *LS* gene is transcribed from left to right, with the 3′ terminus of the gene in P 1.25 as illustrated in Fig. 1 *Lower*, is supported by the observation (Fig. 5*A*, lane 2) that hybridization with LS mRNA prevented removal, by nuclease S1, of ^{32}P-labeled nu-

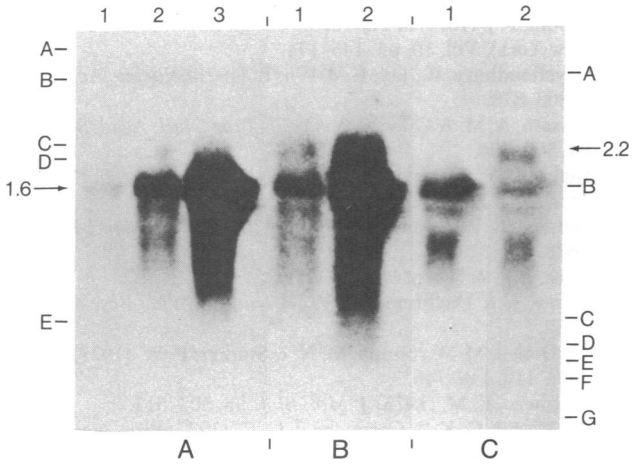

FIG. 4. Sizes of chloroplast RNA transcripts complementary to chloroplast DNA sequence *Bam* 9. Glyoxylated chloroplast RNA was separated electrophoretically in a 1.5% agarose gel (23) and transferred to diazobenzyloxymethyl-paper (24) for hybridization by [^{32}P]DNAs. (*A*) Lanes: 1, 0.1 μg of chloroplast RNA; 2, 1 μg; 3, 10 μg. DNA nick translated from P 0.58 was used as a hybridization probe (24). Exposure time during autoradiography was 16 hr. (*B*) Diazobenzyloxymethyl-paper used in *A* was washed with 99% (vol/vol) formamide to remove hybridized DNA. No signal was detected by autoradiography after 2 days of exposure. DNA nick-translated from B 2.7 was used as a hybridization probe. Lanes: 1, portion of paper corresponding to lane 2 in *A*; portion of lane 3 in *A*. Exposure was for 16 hr. (*C*) Lanes: 1, same as lane 3 in *A* but 4-hr exposure; 2, same as lane 2 in *B* but 4-hr exposure. Size markers were glyoxylated DNA fragments generated from pBR322 with *Pst* I or *Hin*fI and from phage λ DNA by using *Hin*dIII. Sizes of the pBR322 fragments (right-hand margin) in kbp are: A, 4.362; B, 1.731; C, 0.517/0.506; D, 0.396; E, 0.344; F, 0.298; G, 0.221/0.220 (26). Sizes of the λ DNA fragments (left-hand margin) in kbp are: A, 6.67; B, 4.26; C, 2.25; D, 1.96; E, 0.59 (28).

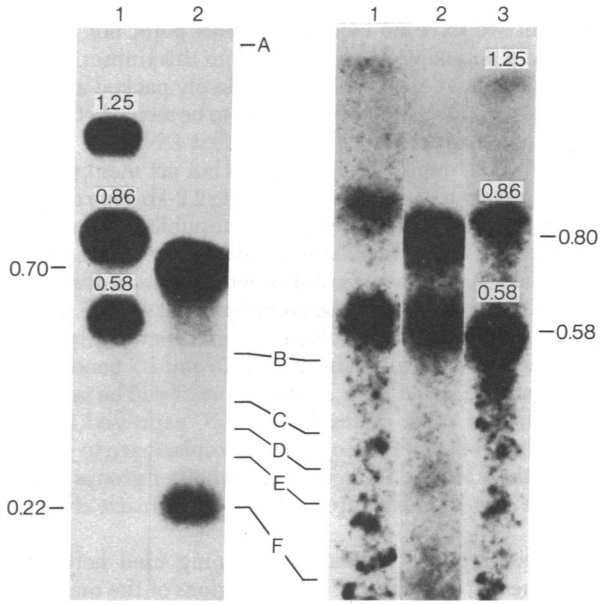

FIG. 5. Determining the orientation of protected regions (from nuclease S1) on *Bam* 9. For DNA·RNA hybridization, 0.25 μg of terminally labeled DNA fragments (see text) was incubated with 30 μg of chloroplast RNA in 50 μl. After digestion with 25 units of nuclease S1 in 0.55 ml and precipitation with ethanol, the sample was applied to an alkaline 1.5% (wt/vol) agarose gel for electrophoresis. The positions of labeled nuclease S1-resistant DNA fragments were determined by autoradiography of dried gels. (*Left*) Lanes: 1, chloroplast RNA fragments P 1.25, P 0.86, and P 0.58 (25 ng) labeled with ^{32}P at their 5′ termini (no nuclease S1); 2, 5′-labeled nuclease S1-resistant DNA fragments. (*Right*) Lanes: 1 and 3, 3′-labeled P 1.25, P 0.86, and P 0.58 DNA (25 ng); 2, 3′-labeled nuclease S1-resistant DNA fragments. Positions of pBR322 fragments resulting from *Hin*fI digestion are indicated by letters: A, 1.631 kb; B, 0.517/0.506 kb; C, 0.396 kb; D, 0.344 kb; E, 0.298 kb; F, 0.221/0.220 kb (26).

cleotides at 5′ ends of P 1.25 (0.22-kb DNA was produced by nuclease S1 digestion of LS mRNA·P 1.25 DNA hybrids) and P 0.58. The low level of radioactivity in 5′-terminally labeled P 0.58 (Fig. 5*A*, lane 2) compared to the high retention of 3′-terminal ^{32}P (Fig. 5*B*, lane 2) during nuclease S1 treatment of RNA·DNA hybrids may result from sequence differences near the two ends that could influence local stability of the hybridization and consequently the degree of protection against nuclease digestion.

The 2.2-kb RNA complementary to 1.4 kb of DNA at the left end of *Bam* 9 (Fig. 1 *Lower*) is apparently transcribed from the opposite strand and thus in the opposite direction from the *LS* gene. The 0.70-kb DNA in Fig. 5*A* (lane 2) was derived by nuclease S1 digestion from one 5′-^{32}P-labeled strand of P 1.25 hybridized to 2.2-kb RNA; the 0.22-kb 5′-^{32}P-labeled DNA in this experiment was produced by nuclease S1 digestion of the hybrid of LS mRNA and the opposite strand of P 1.25. On the other hand, the 3′ ends of P 1.25 were not protected against nuclease S1 digestion by hybridization with chloroplast RNA (Fig. 5*B*, lane 2).

DISCUSSION

The deduced map positions and orientation of the two stable chloroplast transcripts complementary to maize chloroplast DNA sequences in *Bam* 9 are presented in Fig. 1 *Lower*. The 1.6-kb transcript for *LS* is colinear with an equally long segment of chloroplast DNA. The 1.6-kb mRNA for the 52,000-dalton LS has little space for noncoding sequences.

The 2.2-kb transcript in maize chloroplast RNA is colinear with 1.4 kb of the chloroplast DNA sequence in *Bam* 9 and may be colinear with a chloroplast DNA sequence for its entire length but we have no evidence on this point nor have we identified a polypeptide translated from this transcript.

The two genes described here are closely packed and alternate on the two DNA stands. It remains to be seen whether these are common characteristics of chloroplast DNA.

LS mRNA is found in bundle sheath but not mesophyll cells of maize leaves (10) but transcripts of the 2.2-kb gene described here are present in both cell types (unpublished data). The present work will permit detailed analysis of the region between the *LS* and 2.2-kb genes (as well as determination of sequence of the DNA now known to be complementary to LS mRNA). If DNA sequences are involved in developmentally related regulation of transcription of the 2.2-kb and *LS* genes, differences should be found here. Furthermore, it will be interesting to see whether other genes, besides *LS*, expressed in maize bundle sheath but not mesophyll chloroplasts are to the right (Fig. 1 *Lower*) of the *LS* gene—i.e., whether groups of genes in a single expression class are clustered on the maize chloroplast genome.

The techniques of transcript mapping used here should permit the analysis of other cloned regions of the maize chloroplast genome without purification of specific mRNAs. RNA mapping permits the genome to be screened for transcribed regions. Structural genes can then be identified by linked transcription translation analysis followed by immunoprecipitation as in our previous studies (6).

We thank Mrs. L. Scrafford-Wolff and Mrs. B. Link for expert technical assistance and Dr. A. J. Berk for helpful discussions. G.L. was initially supported by a grant from the Deutsche Forschungsgemeinschaft and later held a Long-Term Fellowship from the European Molecular Biology Organization. This research was supported in part by grants from the National Science Foundation, the National Institute of General Medical Sciences, and the Competitive Research Grants Office of the U.S. Department of Agriculture. It was also supported in part by the Maria Moors Cabot Foundation of Harvard University.

1. Bedbrook, J. R., Kolodner, R. & Bogorad, L. (1977) *Cell* **11**, 739–749.
2. Gray, P. W. & Hallick, R. B. (1978) *Biochemistry* **17**, 284–289.
3. Rochaix, J. D. & Malnoe, P. (1978) *Cell* **15**, 661–670.
4. Whitfeld, P. R., Hermann, R. G. & Bottomley, W. (1978) *Nucleic Acids Res.* **5**, 1741–1751.
5. Bedbrook, J. R. & Bogorad, L. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 4309–4313.
6. Coen, D. M., Bedbrook, J. R., Bogorad, L. & Rich, A. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5487–5491.
7. Bedbrook, J. R., Coen, D. M., Beaton, A. R., Bogorad, L. & Rich, A. (1979) *J. Biol. Chem.* **254**, 905–910.
8. Bedbrook, J. R., Link, G, Coen, D. M., Bogorad, L. & Rich. A. (1978) *Proc. Natl. Acad. Sci. USA.* **75**, 3060–3064.
9. McIntosh, L., Jolly, S. O. & Bogorad, L. (1980) in *Emergent Techniques for the Genetic Improvement of Crops*, ed. Rubenstein, I. (Plenum, New York), in press.
10. Link, G., Coen, D. M. & Bogorad, L. (1978) *Cell* **15**, 725–731.
11. Clewell, D. D. & Helinski, D. R. (1969) *Proc. Natl. Acad. Sci. USA* **62**, 1159–1166.
12. Heffron, R., Sublett, R., Hedges, R. W., Jacob, A. E. & Falkow, S. (1975) *J. Bacteriol.* **122**, 250–256.
13. Thuring, R. W. J., Sanders, J. P. M. & Borst, P. (1975) *Anal. Biochem.* **66**, 213–220.
14. Bollum, F. J. (1974) in *The Enzymes*, ed. Boyer, P. D. (Academic, New York), Vol. 10, pp. 145–171.
15. Roychoudhury, R., Jay, E. & Wu, R. (1976) *Nucleic Acids Res.* **3**, 863–878.
16. Maxam, A. M. & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 560–564.
17. Rigby, P. W., Dieckmann, M., Rhodes, C. & Berg, P. (1977) *J. Mol. Biol.* **113**, 237–251.
18. Burgess, R. R. & Jendrisak, J. J. (1975) *Biochemistry* **14**, 4634–4638.
19. Berk, A. J. & Sharp, P. A. (1977) *Cell* **12**, 721–732.
20. Casey, J. & Davidson, N. (1977) *Nucleic Acids Res.* **4**, 1539–1552.
21. McDonell, M. W., Simon, M. N. & Studier, F. W. (1977) *J. Mol. Biol.* **110**, 119–146.
22. Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503–517.
23. McMaster, G. K. & Carmichael, G. G. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5350–5354.
24. Alwine, J. C., Kemp, D. J. & Stark, G. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5350–5354.
25. Bolivar, F., Rodriquez, R. L., Green, P. J., Betlach, M. C., Heyneker, H. L., Boyer, H. W., Crosa, J. H. & Falkow, S. (1977) *Gene* **2**, 95–113.
26. Sutcliffe, J. G. (1978) *Nucleic Acids Res.* **5**, 2721–2728.
27. Berk. A. J. & Sharp, P. A. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 1274–1278.
28. Murray, K. & Murray, N. E. (1975) *J. Mol. Biol.* **98**, 551–564.