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Identification and cloning of the chloroplast gene coding for the large subunit of ribulose-1,5-bisphosphate carboxylase from *Chlamydomonas reinhardi*

(CO_2-fixation enzyme/gene localization/chloroplast DNA/DNA cloning/algae)

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ABSTRACT **mRNA coding for the large subunit (LS) of ribulose-1,5-bisphosphate carboxylase [3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39] from *Chlamydomonas reinhardi* has been isolated from small polyribosomes immunoadsorbed to column-bound anti-LS antibody. ^{32}P-Labeled LS mRNA was used as a hybridization probe to detect LS genes. The probe hybridized to *C. reinhardi* chloroplast DNA and at hybridization saturation revealed that there are approximately 75 LS genes per chloroplast. When chloroplast DNA was digested with the restriction endonuclease *Eco*RI and the fragments were transferred to a nitrocellulose filter, the LS mRNA probe hybridized to a DNA fragment of molecular weight 3.2 $\times 10^6$. This same fragment codes (in part) for 16S and 23S chloroplast rRNAs, which are also coded (in part) by fragments of molecular weights 9.0, 2.3, and 0.4 $\times 10^6$. The restriction fragment containing the LS gene has been cloned in the *Escherichia coli* plasmid pMB9.**

Ribulose-1,5-biphosphate carboxylase [3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39], the enzyme responsible for CO_2 fixation in a wide variety of photosynthetic organisms, is the major protein found in the chloroplast. In higher and lower plants, including the green algae, the holoenzyme [*ca* 560,000 molecular weight (M_r)] is composed of eight large subunits (M_r *ca* 55,000) and eight small subunits (M_r *ca* 15,000). The large subunit (LS), which apparently possesses the catalytic site (1), is translated on chloroplastic polyribosomes (2, 3), whereas the small subunit (SS) is translated on cytoplasmic polyribosomes (4). It is thought that SS is coded for by the nuclear genome in plants and LS, by the chloroplast genome. This arrangement of coding sites has been put forward by Chan and Wildman (5) from studies of the mode of inheritance of large and small subunit polymorphisms in tobacco hybrids and by Kung *et al.* (6) from studies of the transmission of polymorphisms in parasexual hybrids. In this study, we have used LS mRNA as a hybridization probe to detect LS genes. We have previously shown (7, 8) that LS mRNA: (*i*) sediments at 14 S in nondenaturing sucrose gradients, (*ii*) does not bind to oligo(dT)-cellulose, (*iii*) is translated *in vivo* on small (n = 2–5 ribosomes) chloroplast polyribosomes, and (*iv*) can be translated into peptides, precipitable by antibodies to LS, in an *in vitro* protein synthesizing system derived from *Escherichia coli*. In this paper, we describe the use of purified LS mRNA as a hybridization probe to identify which fragment, from the digestion of *Chlamydomonas reinhardi* chloroplast DNA by the restriction endonuclease *Eco*RI, codes for LS. This DNA fragment also codes (in part) for chloroplast 16S and 23S rRNA. These results are physical proof that the chloroplast genome is the coding site for LS.

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MATERIALS AND METHODS

Strains and Growth Conditions. *C. reinhardi* wild-type strain *137C* mt$^+$ (from the collection of W. Ebersold) was used for enzyme isolation. For RNA and polyribosome isolation, a detergent-sensitive cell wall mutant *CW2* mt$^-$ (a gift of D. R. Davies) was used. Cell cultures were grown autotrophically at 25° in a high-salt medium, 3/10 HSM (9), bubbled with 3% CO_2/97% air and illuminated by fluorescent light at 4000 lux (measured at the culture surface). For labeling of RNA, cells were sedimented and resuspended in a phosphate-lacking medium [3/10 HSM lacking phosphate and including 20 mM Tris·HCl (pH 7.5)]. Cells were grown for a short period of time as above but with $H_3{}^{32}PO_4$, 5–50 μCi/ml.

Polyribosome Isolation. Polyribosomes were prepared as described (8). The clarified lysate in buffer A (25 mM Tris·HCl, pH 7.5/50 mM $Mg(OAc)_2$/25 mM Na_2EDTA/200 mM KCl/1 mM 2-mercaptoethanol/polyvinyl sulfate, 10 μg/ml/0.25 M sucrose) was loaded onto 11.6-ml 15–30% (wt/vol) sucrose gradients in buffer B (25 mM Tris·HCl, pH 7.5/10 mM $Mg(OAc)_2$/200 mM KCl/1 mM 2-mercaptoethanol/polyvinyl sulfate, 10 μg/ml) and sedimented at 36,000 rpm for 90 min in a Beckman SW-41 rotor at 4°. The tubes were pierced and fractions were collected. Polyribosomes not fractionated on the anti-LS–Sepharose affinity column were precipitated by the addition of two volumes of 95% ethanol and stored at −20°.

Affinity Chromatography of Small Polyribosomes Bearing LS Nascent Chains. Approximately 10 mg of highly purified rabbit anti-LS IgG (8) was linked to Sepharose 4B by the method of Porath *et al.* (10). The beads were washed with 50 ml each of: (*i*) 0.1 M NaOAc, pH 5.4/1.0 M NaCl; (*ii*) 0.1 M $NaHCO_3$, pH 8.5/1.0 M NaCl; (*iii*) 1.0 M NaOAc, pH 4.0/1.0 M NaCl; (*iv*) 20 mM $NaPO_4$, pH 7.0/0.15 M NaCl; and (*v*) buffer A containing 2.0% Nonidet P-40. All further operations were performed at 0–4°. Small (n = 2–5 ribosomes) polyribosomes taken directly from sucrose gradients were passed over the column. The column was washed further with buffer A containing 2.0% Nonidet P-40 and the bound polyribosomes

Abbreviations: M_r, molecular weight; *Eco*RI, *E. coli* restriction enzyme RI; LS, large subunit of D-ribulose-1,5-bisphosphate carboxylase; SS, small subunit of D-ribulose-1,5-bisphosphate carboxylase; anti-LS, rabbit antiserum against LS; $NaDodSO_4$, sodium dodecyl sulfate; SSC, 0.15 M NaCl/0.015 M sodium citrate; 2× and 4× SSC, concentration of the solution used is 2 and 4 times that of the standard saline–citrate solution, respectively.

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were eluted with 1.0 M NaOAc (pH 4.0). Polyribosomes from the original flow-through fractions and those that had been immunoadsorbed were precipitated by the addition of two volumes of 95% ethanol and stored at −20°.

Extraction and Fractionation of RNA. All operations were performed at 0–4°. Polyribosomes precipitated by ethanol were collected by centrifugation, dried in an air stream, resuspended in 2 ml of buffer C [50 mM NaOAc, pH 5.2/50 mM NaCl/10 mM $Mg(OAc)_2$] containing 1% sodium dodecyl sulfate ($NaDodSO_4$) and extracted by the addition of 2 ml of phenol/chloroform/isoamyl alcohol (2:1:0.13, vol/vol). The organic phase was extracted once again by the addition of 1 ml of buffer C. The aqueous phases were combined and the RNA was precipitated by the addition of three volumes of 95% ethanol. When RNA was extracted directly from cells, the cells were first washed in 25 mM Na_2EDTA (pH 7.2), pelleted, and extracted as above except that 1.0% diethyl pyrocarbonate was added to the first extraction solution.

RNA was fractionated by sucrose density-gradient centrifugation by loading 1 ml of sample (up to 4 mg of RNA) in buffer D (10 mM NaOAc, pH 5.2/0.1 M NaCl) containing 0.1% $NaDodSO_4$ onto 11.6-ml 15–30% (wt/vol) sucrose gradients in buffer D containing 0.01% $NaDodSO_4$ and sedimenting for 20 hr at 27,000 rpm in a Beckman SW-41 rotor at 4°. The tubes were pierced and fractions were collected. RNA was monitored either by A_{260} or by ^{32}P detection in a Beckman LS-230 scintillation counter. Under these sedimentation conditions, 25S rRNA was pelleted, allowing better resolution of the 4–18S region of the gradient.

RNA was fractionated by electrophoresis on 2-mm 2.5% acrylamide–0.5% agarose slab gels containing 40 mM Tris·acetate, pH 7.5/20 mM NaOAc/2 mM Na_2EDTA/0.2% ($NaDodSO_4$) [a modification of Summers' method (11)]. Electrophoresis was carried out at 4° at a constant voltage of 6 V/cm. The bromphenol blue tracking dye migrated 14 cm. The gels were dried and subjected to autoradiography with Kodak RP X-Omat x-ray film. Appropriate bands of RNA in gel slices were eluted in one of the following ways: (*i*) For filter hybridization to *Eco*RI digestion fragments, elution was carried out with 4 × SSC during the hybridization to the filter. (*ii*) For the other filter hybridizations, elution was performed using 0.6 M LiCl/0.2% $NaDodSO_4$ at 37° for 24 h. The RNA was pelleted by centrifugation for 24 hr at 40,000 rpm in a type 50 Ti rotor at 20°. (*iii*) For translation, RNA bands were cut from an undried gel and electroeluted at 5 mA per slice for 1 hr at 4°.

***In Vitro* Translation of LS mRNA.** Translation of LS mRNA *in vitro* was performed in a system derived from *E. coli* as described (7). LS synthesis was detected by immunoprecipitation of the [^3H]arginine-labeled peptides by a double immune system using first rabbit anti-LS and then goat anti-rabbit-IgG (7).

Isolation of Chloroplast DNA. *C. reinhardi* CW 15^+ cells were harvested from 5 liters of culture by centrifugation, washed once with 250 ml of DNA extraction buffer (50 mM Na_2EDTA/1 mM Tris·HCl, pH 8.0) and resuspended in 25 ml of DNA extraction buffer. $NaDodSO_4$ was added to a final concentration of 1.0% and the cells were incubated for 5 min at 37°. An equal volume of DNA extraction buffer was added and the cells were incubated at 37° for 30 min with RNase A at 10 μg/ml. $NaDodSO_4$ and NaCl were added to final concentrations of 2.0% and 0.5 M, respectively. Protein was removed first by incubation with Pronase, 500 μg/ml, and then by extraction with an equal volume of phenol/chloroform, 1:1, (vol/vol). DNA was precipitated by the addition of two volumes of 95% ethanol and resuspended in 100–200 ml of DNA extraction buffer. The solution was brought to a density of 1.7 g/cm^3 by the addition of CsCl and the ethidium bromide concentration was adjusted to 2 μg/ml. Gradients were centrifuged to equilibrium in a type 50 Ti rotor at 42,000 rpm for 40–48 hr at 20°. The upper band of chloroplast DNA was collected and the ethidium bromide was removed by extraction with isoamyl alcohol and dialysis against 10 mM Tris·HCl, pH 8.0/1 mM Na_2EDTA.

Filter Hybridization. [^3H]Adenine-labeled chloroplast DNA (11,800 cpm/μg) was separated from nuclear main band and ribosomal satellite DNA by equilibrium centrifugation in CsCl. About 50 μg of denatured DNA was loaded onto 25-mm nitrocellulose filters (Schleicher and Schuell B-6) and the filters were dried under reduced pressure. From each filter, seven ¼-inch (0.63 cm) "minifilters" were punched with a paper punch. Hybridization of ^{32}P-labeled RNA with unlabeled competitor RNA was carried out as indicated at 65° in 4 × SSC. The filters were incubated with RNase A (25 μg/ml, 30 min, 37°), washed extensively with 2 × SSC, dried, and assayed for radioactivity.

^{32}P-Labeled RNA was hybridized to filter-bound *Eco*RI fragments. Chloroplast DNA was digested fully by the restriction endonuclease *Eco*RI (100 mM NaCl/7 mM $Mg(OAc)_2$/10 mM Tris·HCl, pH 7.5; 37°, 30 min) and the fragments were separated by electrophoresis on 0.7% agarose gels. The DNA was denatured and transferred to nitrocellulose filters according to Southern (12). ^{32}P-Labeled RNA species, together with unlabeled competitor species, were hybridized to strips cut from these filters in 4 × SSC at 65° for 24 hr. The strips were digested with RNase A (25 μg/ml, 37°, 30 min) and washed extensively with 2 × SSC. The filters were dried and subjected to autoradiography with Kodak RP/R2 Royal X-Omat x-ray film.

Cloning of the LS Gene. The chloroplast DNA restriction fragment containing the LS gene was cloned in the nonconjugative tetracycline-resistant *E. coli* plasmid pMB9 (13). The cloning experiments were carried out under P2–EK1 containment conditions (14). Ten micrograms of *C. reinhardi* chloroplast DNA and 3 μg of pMB9 DNA were digested with 10 units of *Eco*RI endonuclease for 30 min at 37° and the molecules were ligated with T4 DNA ligase by a modification of the procedure of Dugaiczyk *et al.* (15). Increasing the time of incubation at 0–4° after ligation to 5–10 days improved the results. *E. coli* strain C600 Δ trpE was transformed by the method of Hershfield *et al.* (16) and plated on M9 medium (plus 0.1% casamino acids and 0.001% tryptophan) containing tetracycline, 25 μg/ml. Clones were picked and grown in the above medium, and 25 μl of the crude cell lysates was analyzed directly on 0.5% agarose slab gels containing ethidium bromide, 0.5 μg/ml (17). Clones with plasmid bands migrating slower than the parental pMB9 plasmid were presumed to be clones containing chloroplast DNA inserted into the pMB9 plasmid DNA. Cells from a 250-ml culture grown to stationary phase were harvested, resuspended in 3 ml of 0.25 M sucrose/0.05 M Tris·HCl, pH 7.5, and spheroplasts were made by incubating the cells on ice for 20 min with 0.2 volumes of freshly prepared lysozyme (10 mg/ml) and 0.4 volumes of 0.4 M Na_2EDTA (pH 8.0). The spheroplasts were lysed by the addition of an equal volume of lysis solution (0.1% Triton X-100/0.083 M Na_2EDTA, pH 8.0/0.05 M Tris·HCl, pH 8.0) and the solution was incubated at room temperature for 15 min. The cells were spun at 10,000 rpm (SS-34 rotor) for 20 min. The cleared lysates were adjusted to an ethidium bromide concentration of 500 μg/ml and brought to a density of 1.57 g/cm^3 by the addition of solid CsCl. The gradients were centrifuged to equilibrium at 35,000 rpm

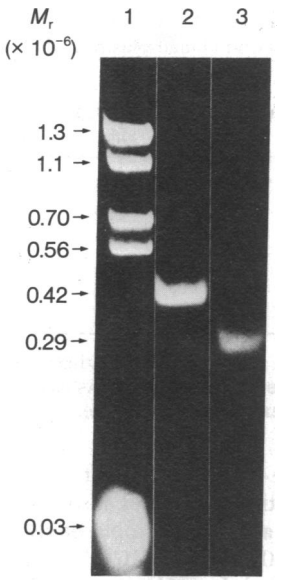

FIG. 1. Polyacrylamide gel electrophoresis of ^{32}P-labeled RNA species. *C. reinhardi* cells were labeled *in vivo* with $H_3{}^{32}PO_4$, 20 μCi/ml for 12 hr. Lanes: (*1*) total cell RNA; (*2*) 0.42 × 10^6-M_r species eluted from gel and resubjected to electrophoresis; (*3*) 0.29 × 10^6-M_r species eluted from gel and resubjected to electrophoresis.

at 20° for 40–48 hr in a 40 Ti rotor. The DNA bands were visualized under ultraviolet illumination and the lower supercoiled plasmid band was gently removed with a wide-bore pipet. The ethidium bromide was removed and the DNA was digested with *Eco*RI endonuclease (to relax the supercoiled molecules), denatured, and loaded onto nitrocellulose filters for hybridization with LS mRNA or 16S or 23S rRNA as described above.

RESULTS

Isolation of the ^{32}P-labeled LS mRNA hybridization probe

We have shown previously that LS mRNA from *C. reinhardi* sediments at about 14 S and is translated on small polyribosomes ($n < 5$) which can be immunoadsorbed by anti-LS IgG (7, 8). We have taken advantage of these properties to isolate ^{32}P-labeled LS mRNA as a hybridization probe from cells labeled *in vivo*. When total RNA extracted from cells labeled for 12 hr with $^{32}PO_4{}^{3-}$ was subjected to electrophoresis on polyacrylamide gels, the most prominent bands seen in autoradiographs (Fig. 1, lane 1) were 18S and 25S cytoplasmic rRNA (M_r 0.70 and 1.3 × 10^6, respectively), 16S and 23S chloroplastic rRNA (M_r 0.56 and 1.1 × 10^6, respectively), and low-molecular-weight rRNA and tRNAs. Between these high- and low-molecular-weight forms were other less prominent bands (seen in other overexposed autoradiographs) including a band migrating at a position expected for LS mRNA (14 S, equivalent to M_r 0.42 × 10^6). When small polyribosomes obtained from ^{32}P-labeled cells were bound to and eluted from an anti-LS IgG affinity column (as described in *Materials and Methods*) and the RNA extracted from eluted polyribosomes was subjected to gel electrophoresis, a labeled band corresponding to that expected for LS mRNA was observed. By electroeluting from the gel, this band could be obtained in pure form as shown when subjected again to electrophoresis (Fig. 1, lane 2). Another labeled RNA band migrating with an equivalent M_r of 0.29 × 10^6 was also found in RNA extracted from affinity column-bound polyribosomes. The RNA in this band similarly was purified by electroelution from the first polyacrylamide gel (Fig. 1, lane 3).

We tested whether these RNA species, obtained from bulk preparations and purified by gel electrophoresis, could code for LS in an *in vitro* synthesizing system derived from *E. coli*. Unlabeled RNA was extracted from either whole cells or small polyribosomes and fractionated on 15–30% (wt/vol) sucrose gradients. The 7–15S region was pooled, and the RNA was concentrated and subjected to polyacrylamide gel electrophoresis along with marker ^{32}P-labeled RNA from small polyribosomes. The gel was autoradiographed and the bands corresponding to the 0.42 and 0.29 × 10^6-M_r species were eluted.

As can be seen from Table 1, neither RNA species appreciably stimulated total incorporation of [^{3}H]arginine into acid-precipitable material. Both species, however, stimulated the synthesis of immunoprecipitable material, although the 0.42 × 10^6-M_r species was more efficient on a per-RNA basis. In the presence of the 0.42 × 10^6-M_r species obtained from whole cell RNA, 15% of the total [^{3}H]arginine incorporation was specifically immunoprecipitated by anti-LS IgG, whereas only 3% was immunoprecipitable in the presence of the 0.29 × 10^6-M_r species. Thus, the 0.42 × 10^6-M_r species efficiently codes for LS whereas the 0.29 × 10^6-M_r species appears to be either a breakdown product of LS that is less efficiently translated or a different RNA species that is somewhat contaminated with LS mRNA.

Identification of LS and chloroplast rRNA cistrons

Digestion of chloroplast DNA with the restriction enzyme *Eco*RI yielded approximately 30 separable DNA bands by

Table 1. *In vitro* synthesis of LS (anti-LS-precipitable material)

M_r of RNA species ×10^{-6}	Source of RNA	Amount of RNA, μg	Total acid-precipitable, cpm	Anti-LS-precipitable cpm	Anti-LS-precipitable cpm/μg RNA	%*
0.42	Whole cell	5.0	19,800	816	163	15.5
	Small polyribosomes	5.0	17,600	793	158	26.1
0.29	Whole cell	5.0	27,600	411	82	3.1
—	—	0	14,500	0	—	—

Translation of RNA obtained from polyacrylamide gel electrophoresis fractions of whole cell RNA or RNA obtained from small polyribosomes as described in *Materials and Methods*. Anti-LS precipitable cpm were corrected for cpm precipitable by rabbit preimmune IgG in the same reaction.

* Calculated as: (cpm anti-LS preciptable/cpm total acid precipitable) × 100. cpm for reactions without RNA were subtracted from the total acid-precipitable cpm.

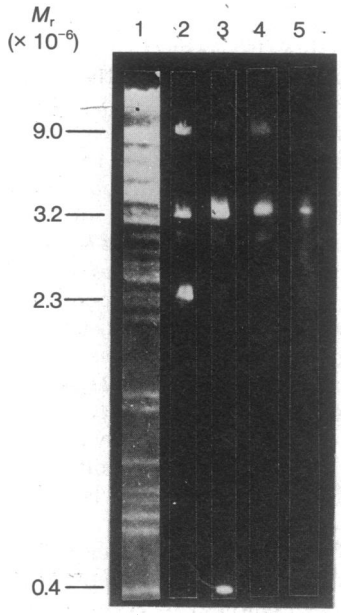

FIG. 2. Filter hybridization of ^{32}P-labeled RNA species to *Eco*RI restriction fragments of *C. reinhardi* chloroplast DNA according to Southern (12). Lanes: (*1*) reference gel of *Eco*RI digestion fragments; (*2*) ^{32}P-labeled 23S rRNA plus 100 μg of unlabeled 25S, 18S, and 16S rRNA; (*3*) ^{32}P-labeled 16S rRNA plus 100 μg of unlabeled 25S, 23S, and 18S rRNA; (*4*) ^{32}P-labeled 14S (LS) mRNA (0.42 × 10^6-M_r species) derived from affinity column-purified small polyribosomes plus 100 μg of unlabeled 25S, 23S, 18S, and 16S rRNA; (*5*) ^{32}P-labeled 0.29 × 10^6-M_r species derived from affinity column-purified small polyribosomes plus 100 μg of unlabeled 25S, 23S, 18S, and 16S rRNA.

agarose gel electrophoresis. The same basic pattern has been obtained by Rochaix (18). The DNA fragments in this gel pattern were transferred to a nitrocellulose filter according to Southern (12). ^{32}P-Labeled LS mRNA (M_r 0.42 × 10^6), obtained from polyacrylamide gel slices of RNA extracted from small polyribosomes immunoadsorbed to column-bound anti-LS antibody, was hybridized to filter-bound chloroplast DNA fragments in the preserce of 100 μg of all four species of unlabeled competitor rRNA. An autoradiograph of the hybridization pattern and a reference photograph of the *Eco*RI digestion fragments of chloroplast DNA in the original agarose gel are shown in Fig. 2. ^{32}P-Labeled LS mRNA (M_r 0.42 × 10^6) hybridized almost exclusively to a chloroplast DNA fragment of

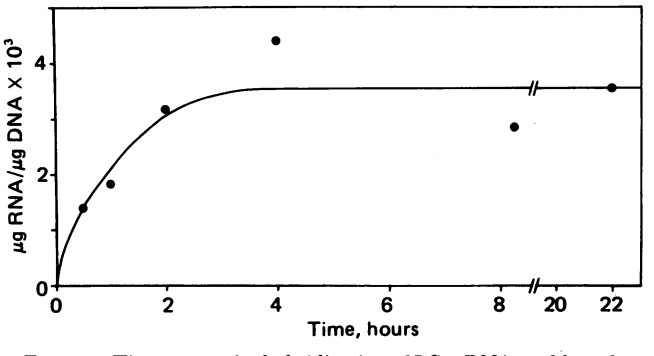

FIG. 3. Time course for hybridization of LS mRNA to chloroplast DNA. ^{32}P-Labeled LS mRNA derived from affinity column-purified small polyribosomes was hybridized to [^{3}H]adenine-labeled chloroplast DNA on nitrocellulose "minifilters" in the presence of 100 μg of unlabeled 25S, 23S, 18S, and 16S rRNA. The filters were treated as described in *Materials and Methods*. Zero time background has been subtracted.

Table 2. Hybridization of ^{32}P-labeled RNA species to filter-bound cloned plasmid DNA

DNA source	DNA/filter, μg	LS mRNA, cpm	Chloroplast ribosomal species 16S, cpm	Chloroplast ribosomal species 23S, cpm
pMB9	1	56	74	184
	20	460	ND	ND
Clone 12	1	ND	1601	610
	20	3634	ND	ND
Clone 19	1	82	93	61
Clone 34	1	77	61	54

^{32}P-Labeled RNA species were hybridized in the presence of the appropriate unlabeled competitor RNAs as described in *Materials and Methods*. ND, experiment not done.

M_r 3.2 × 10^6 (lane 4). This fragment, or one with similar electrophoretic migration properties, also hybridized (though not exclusively) to 23S and 16S chloroplastic rRNA. DNA fragments with M_r of 9.0 × 10^6 and 2.3 × 10^6 also hybridized with labeled 23S rRNA (lane 2) and a DNA fragment of M_r 0.4 × 10^6 also hybridized with labeled 16S rRNA (lane 3). Hybridizations with ^{32}P-labeled 16S or 23S rRNAs were carried out in the presence of 100 μg each of the other three unlabeled rRNA species obtained from acrylamide gels. For example, ^{32}P-labeled 23S rRNA was hybridized in the presence of competitor 16S chloroplast rRNA and 18S and 25S cytoplasmic rRNA. The presence of competitor RNA was necessary to ensure specificity of hybridization which can be lessened by the presence of contaminating fragments of the other rRNA species. Cross contamination is a problem in plant rRNA preparations because gradient and gel separations of RNA must be carried out under nondenaturing conditions to preserve the integrity of the rRNA species, especially 23S rRNA (19).

Quantitation of the number of LS genes

^{32}P-Labeled LS mRNA (M_r 0.42 × 10^6) isolated from immunoadsorbed small polyribosomes was hybridized to filter-bound chloroplast DNA. Hybridizations were again carried out in the presence of a large excess (100 μg) of the four unlabeled rRNA species. As can be seen from Fig. 3, at saturation, LS mRNA hybridized to chloroplast DNA to a level of 3.3 ng of RNA per μg of DNA. Chloroplast DNA homologous to LS mRNA therefore comprises 0.66% of the total chloroplast DNA. Assuming an analytical complexity of 1 × 10^{10} daltons of chloroplast DNA per chloroplast (or per cell) (20), 66 × 10^6 daltons of chloroplast DNA per cell is homologous to LS mRNA. If one uses a M_r of 0.42 × 10^6 for LS mRNA (and therefore 0.84 × 10^6 daltons for the LS gene), there are about 75 LS genes per chloroplast or per cell. Assuming a complexity of 127 × 10^6 daltons of DNA per chloroplast chromosome (unpublished observation), there is one LS gene per chromosome.

Isolation of a plasmid containing the LS gene

A large number of clones containing *C. reinhardi* chloroplast DNA linked to the plasmid pMB9 were isolated as described in *Materials and Methods*. Plasmid DNA was hybridized to LS mRNA (M_r 0.42 × 10^6), 16S rRNA, or 23S rRNA under the competition conditions described above, except that 400 μg of competitor RNA was used. One clone (clone 12) contained a chimeric plasmid that successfully hybridized with LS mRNA (Table 2). The plasmid DNA also hybridized extensively with 16S and 23S chloroplast rRNA. Several clones containing other *Eco*RI chloroplast DNA fragments did not hybridize to the LS mRNA or rRNA probes (Table 2). The chimeric plasmid in

clone 12 bore a single chloroplast DNA fragment, indicating that the LS gene and the 16S and 23S chloroplast rRNA cistrons indeed are found on the same restriction fragment piece.

DISCUSSION

In this paper, we have shown that the LS gene is located on the chloroplast genome. We have done so by hybridizing labeled LS mRNA to chloroplast DNA from *C. reinhardi*. Hybridization to saturation with ^{32}P-labeled LS mRNA to total chloroplast DNA indicates that there are about 75 LS genes per chloroplast or 1 LS gene per 127×10^6-dalton genome.

We have identified the chloroplast DNA *Eco*RI restriction fragment that hybridizes with LS mRNA. The DNA fragment has a M_r of 3.2×10^6 and is present in about 75 copies in the chloroplast (unpublished observation). The same chloroplast restriction fragment coding for the LS mRNA also contains portions of at least one set of 16S and 23S chloroplast rRNA genes. This has been shown by the hybridization of ^{32}P-labeled LS mRNA and 16S and 23S rRNAs to a single *Eco*RI restriction fragment obtained from *C. reinhardi* chloroplast DNA or from a chimeric bacterial plasmid. We do not know at present what proportion of the 16S and 23S rRNA molecules is encoded by this fragment (portions of genes coding for 16S and 23S rRNA are also found on fragments of 9.0, 2.3, and 0.4×10^6 daltons). However, this fragment does not contain the coding capacity for much more than the RNA species already assigned to it.

We have isolated labeled LS mRNA for use as a hybridization probe by immunoadsorption of small whole-cell polyribosomes to a specific antibody-affinity column. This procedure yields two nonribosomal RNA species, one with an apparent M_r of 0.42×10^6 and the other with a M_r of 0.29×10^6 (these species are recovered in about an equal molar ratio). The labeled RNA species of M_r 0.42×10^6 comigrates on polyacrylamide gels with an RNA obtained from bulk preparations (not immunoadsorbed) that stimulates LS synthesis *in vitro*. It also comigrates with the major RNA species found in the 12–14S region of a sucrose gradient in which we have previously shown LS mRNA activity (7). The function of the other nonribosomal RNA species (M_r 0.29×10^6) is not known. It has residual LS mRNA activity and may be a breakdown product of LS mRNA although its abundance does not vary significantly in different preparations. Preliminary experiments suggest that this species may be a breakdown product because it too binds to the same *Eco*RI fragment as the 0.42×10^6-M_r species (Fig. 2, lane 5) and it saturates the chloroplast genome to the same extent by hybridization (data not shown). We cannot easily determine from the translation product if the 0.29×10^6-M_r species is an LS mRNA fragment because we have been unable to synthesize the full LS polypeptide in the *E. coli* translation system using the 0.42×10^6-M_r species (7). Because of this difficulty, the immunoprecipitated translation product has been identified as LS from characteristic tryptic peptides (7).

We have proposed elsewhere that the LS mRNA and chloroplast rRNA are synthesized coordinately or at least at the same rate (21). The apparent juxtaposition of these genes may relate to their mutually high rate of transcription (21). The development of a chimeric plasmid containing only the LS gene will provide us with a hybridization probe with which to test this hypothesis.

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