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Identification and Precipitation of the Polyribosomes in *Chlamydomonas reinhardi* Involved in the Synthesis of the Large Subunit of D-ribulose-1,5-bisphosphate Carboxylase[1]

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ABSTRACT

The size classes of polyribosomes involved in the synthesis of ribulose-1,5-bisphosphate carboxylase large subunit were determined by binding radioiodinated specific antibodies to polyribosomal preparations from *Chlamydomonas reinhardi*. Antibodies specific to the denatured large subunit and to the native enzyme bound primarily to small polyribosomes (N = two to five ribosomes). The binding of antibodies to small polyribosomes was unexpected since the large subunit is a large polypeptide (molecular weight 55,000) coded for by a corresponding large mRNA (12-14S). Control experiments showed that this unexpected pattern of antibody binding was not a result of messenger RNA degradation, "run-off" of ribosomes from polyribosomes, or adventitious binding of the completed enzyme to a selected class of polyribosomes. In addition, polyribosomes bearing nascent large subunit chains have been immunoprecipitated from small polyribosome fractions. A large RNA species that can direct the synthesis of large subunit *in vitro* was extracted from small polyribosomes.

In the accompanying paper (17), we have described the properties of the messenger RNA from *Chlamydomonas reinhardi* which directs the synthesis of the large subunit of ribulose-1,5-bisphosphate carboxylase in a protein-synthesizing system derived from *Escherichia coli*. In this paper, we have identified the polyribosomes from *C. reinhardi* which bear LS[2] nascent polypeptide chains by binding the appropriate ^{125}I-labeled antibodies to polyribosome preparations. These experiments are preliminary steps to the isolation of LS messenger RNA by immunoprecipitation of polyribosomes.

EXPERIMENTAL PROCEDURES

CELL CULTURES AND ENZYME PREPARATIONS

Two strains of *C. reinhardi* were used in these experiments. For polyribosome isolation, a cell wall mutant CW2 mt$^-$ (the gift of D. R. Davies) was used, and for RUBPCase isolation, a wild type 137 C$^+$ strain (from the collection of W. Ebersold) was employed. Cell cultures were grown as described in the previous paper (17).

RUBPCase and LS were purified to homogeneity as described previously (17). Purity of the enzyme preparation was assessed by electrophoresis on nondenaturing or SDS-polyacrylamide gels for RUBPCase and LS, respectively. Purified LS, used in subsequent experiments, was solubilized in 0.4% SDS, diluted, and stored in 0.04% SDS.

PURIFICATION AND RADIOIODINATION OF ANTIBODIES

Antibodies (IgG fraction) against native RUBPCase (anti-holo) and LS (anti-LS) were isolated from rabbit sera using standard procedures as described previously (17). Anti-holo IgG was further purified by affinity chromatography over a column of holoenzyme linked to cyanogen bromide-activated Sepharose 4B (20). Following the loading of anti-holo IgG, the column was washed with (*a*) 1 M NaCl, 0.1 M sodium acetate (pH 5.5); (*b*) 1 M NaCl, 0.1 M $NaHCO_3$ (pH 8.5); and (*c*) 2.5 M magnesium acetate, 0.05 M tris-Cl (pH 7.5). The final fraction (*c*) containing specific antibody activity was dialyzed exhaustively against 0.02 M sodium phosphate (pH 7) and stored at 4 C. Anti-LS IgG was further purified by affinity chromatography over a Sepharose 4B-LS-linked column. The column was washed with the same buffers as described above except that 1 M sodium acetate (pH 4) was substituted for 2.5 M Mg acetate. The final fraction containing the purified IgG was concentrated by ammonium sulfate precipitation and resuspended in a small volume of 0.15 M NaCl, 0.02 M sodium phosphate (pH 7).

Ten μg of each antibody preparation (affinity column purified anti-holo and anti-LS and preimmune IgG) were radioiodinated with ^{125}I by solid-state lactoperoxidase using the procedure of David and Reisfeld (7). Free ^{125}I was removed from the radioiodinated sample by chromatography on a Sephadex G-75 column equilibrated with 17 mM sodium phosphate (pH 6.3). The ^{125}I-labeled antibody preparation was purified from contaminating ribonucleases by passage through a DEAE-cellulose column (in a Pasteur pipette) in 17 mM sodium phosphate buffer (pH 6.3). The antibody, radioiodinated to approximately 10^6 cpm/μg protein, was stored at 4 C. Holoenzyme preparations were radioiodinated and purified in the same manner.

PREPARATION OF POLYRIBOSOMES

Pulse-labeling of polyribosome-bound nascent polypeptide chains with [^{3}H]arginine and the isolation of polyribosomes were carried out using techniques modified from Baumgartel and Howell (2).

Method A. CW2 cells (1-2 liters at 2-3 $\times$ 10^6 cells/ml) were incubated with 10 μg/ml cycloheximide and 50 μg/ml chloramphenicol for 3 min, poured over a 0.5 volume of crushed ice, and rapidly collected by centrifugation. All further manipulations were carried out at 0 to 4 C. The pelleted cells were washed once with 50 ml of 12.5 mM EDTA (pH 7.2) containing 100 μg/ml

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[2] Abbreviations: RUBPCase: ribulose-1,5-bisphosphate carboxylase; LS: large subunit of RUBPCase; SS: small subunit of RUBPCase; holoenzyme: native or undissociated form of RUBPCase; IgG: immuno γ-globulin; anti-LS: IgG specific for LS; anti-holo: IgG specific for holoenzyme; N: number of ribosomes in a polyribosome.

cycloheximide and 500 μg/ml chloramphenicol. Washed cells were centrifuged again and suspended in 10 ml of buffer A (25 mM tris-Cl [pH 7.5], 50 mM Mg acetate, 25 mM Na_2 EDTA, 200 mM KCl, 1 mM 2-mercaptoethanol, 10 μg/ml polyvinyl sulfate, 0.25 M sucrose). Cells were lysed by the addition of Nonidet P-40 (final concentration of 2%), and the cell lysate was clarified by centrifugation at 12,000*g* for 5 min. Five ml of the supernatant fluid were layered over 3 ml of buffer B (25 mM tris-Cl [pH 7.5], 10 mM Mg acetate, 200 mM KCl, 1 mM 2-mercaptoethanol, 10 μg/ml polyvinyl sulfate) containing 50% sucrose. Polyribosomes were collected by centrifugation for 2.5 hr at 40,000 rpm in a Beckman type Ti-50 rotor at 4 C. The pellet was washed three times with 2.5 ml of buffer A containing 2% Nonidet P-40. The pellet containing polyribosomes was suspended in buffer A.

Method B. Cells were harvested, washed, and lysed as in method A. The clarified lysate was immediately loaded onto 15 to 30% sucrose gradients in buffer B and sedimented at 40,000 rpm for 70 min in a Beckman SW 41 rotor at 4 C. The tubes were pierced and fractions collected.

ISOLATION AND FRACTIONATION OF RNA

Polyribosome fractions isolated by method B were pooled and precipitated by the addition of 3 volumes of cold 95% ethanol. The precipitated polyribosomes were washed with 1 ml of 50 mM sodium acetate (pH 5.2), 0.1 M NaCl (buffer D) with 0.1% SDS, reprecipitated by the addition of 3 volumes of 95% ethanol, and resuspended in 1 ml of the same buffer. RNA was extracted following the addition of 2 ml of phenol-chloroform-isoamyl alcohol (2:1:0.13) and the organic phase reextracted once more by the addition of 1 ml buffer D. RNA was precipitated by the addition of 3 volumes of 95% ethanol and stored under ethanol at −20 C.

The RNA was resuspended in 1 ml buffer D containing 0.1% SDS and loaded on 15 to 30% linear sucrose gradients in buffer D containing 0.01% SDS. Centrifugation was carried out for 16 hr at 35,000 rpm in a Beckman SW 41 rotor at 4 C. The tubes were punctured and fractions collected.

TRANSLATION ASSAY

RNA extracted from polyribosomes was tested for its ability to direct the synthesis of immunoprecipitable LS in an *E. coli* protein-synthesizing system. Both the translation system and immunoprecipitation procedures are described in the previous paper (17).

RESULTS

Purification and Specificity of Antibodies. Preliminary antibody-binding experiments in which polyribosomes were incubated with unpurified radioiodinated anti-holo and anti-LS (IgG fractions) were inconclusive. Little or no radioiodinated antibodies bound to polyribosomes. We purified anti-holo and anti-LS IgG fractions by affinity chromatography over holoenzyme- or LS-linked Sepharose columns, respectively. The purified fractions obtained from these columns were titrated by end point dilution using Ouchterlony immunodiffusion analysis. The highest titer anti-LS fraction, eluted with M sodium acetate (pH 4), showed an approximate 20-fold increase in specific activity over the bulk IgG fraction when reacted with SDS-solubilized LS (Fig. 1). The most active anti-holo fraction eluting with 2.5 M $MgCl_2$, 0.05 M tris-HCl (pH 7.5), showed about a 7-fold increase in specific activity when challenged with native holoenzyme in the same end point dilution assay.

In the Ouchterlony double diffusion analysis (Fig. 2), purified anti-holo reacted with native holoenzyme and denatured LS and SS. Precipitation lines against LS and SS displayed nonidentity. In contrast to the specificity of anti-LS observed in the previous paper (17), the purified anti-LS reacted only with denatured LS and not with the native holoenzyme. The absence of a reaction with holoenzyme is interesting but not unexpected since anti-LS was developed by the injection of denatured LS and purified on an affinity column bearing only this denatured polypeptide.

Binding of Radioiodinated Anti-Holo to Polyribosomes. We attempted to determine if the purified anti-holo would bind to nascent chains on polyribosomes obtained from *C. reinhardi* and if we could identify the polyribosome size classes to which the antibody was bound. Polyribosome preparations from *C. reinhardi* are a mixture of chloroplastic and cytoplasmic polyribosomes in which chloroplastic polyribosomes are a minority (~10%) species (2). Hence, if antibody-binding occurs, the binding could be to polyribosomes derived from either a cytoplasmic or a chloroplastic source.

Polyribosomes were isolated by sedimentation through a discontinuous sucrose gradient to separate polyribosomes from free holoenzyme and membrane fragments present in the lysate (method A). The polyribosome pellet was resuspended, incubated with radioiodinated anti-holo at 4 C for 2.5 hr, and sedimented in continuous sucrose gradients. $A_{260\ nm}$ and distribution of radioactivity in the gradients were determined and are shown in Figure 3A. There is a spread of ^{125}I-labeled anti-holo throughout the polyribosomes, but most of the radioactivity in the polyribosome region co-sediments with small polyribosomes (N = two to five). The major peak near the top of the gradient contains predominantly ribosomal subunits which have been dissociated in the high salt buffers (2). There is little radioactive label sedimenting in the subunit region that can be clearly distinguished from the bulk of ^{125}I-labeled material found at the top of the gradient. The binding of ^{125}I-anti-holo predominantly to polyribosomes containing two and three ribosomes is more clearly seen in Figure 3B in which the polyribosomes were sedimented for a longer time. In many repeats of this experiment, the general binding pattern to small polyribosomes was the same, except that the presence of a discrete peak(s) associated with small polyribosomes was not always clear.

To demonstrate that the ^{125}I-labeled anti-holo was truly bound to polyribosomes and that the sedimentation pattern of radioactive material depends upon intact polyribosomes, the polyribosomes were mildly digested with RNase following the antibody-binding step. In the resulting sedimentation pattern (Fig. 4), it can be seen that the polyribosomes are almost fully degraded and sediment as monoribosomes near the top of the gradient. No significant amount of ^{125}I-labeled material is found in the degraded profile around fraction No. 20 where most of the binding occurred in the equivalent undegraded profile (Fig. 3A). When ^{125}I-anti-holo is sedimented in the absence of polyribosomes, no labeled material is found in the equivalent polyribosome region of the gradient (data not shown). It is clear that ^{125}I-labeled anti-holo binds to polyribosomes and that the particular sedimentation pattern of the labeled antibody is due to this binding.

Binding of Radioiodinated Anti-LS to Polyribosomes. We attempted to determine whether a more specific probe, purified anti-LS, which reacts exclusively with denatured LS, would bind to nascent chains on polyribosomes. These experiments were performed exactly as those described in the previous section except that radioiodinated anti-LS was used instead of anti-holo. Just as in the binding with anti-holo, ^{125}I-labeled anti-LS is spread throughout the polyribosome profile with a concentration of labeled material in the smaller polyribosomes. In Figure 5 a prominent radioactively labeled peak is seen cosedimenting with small polyribosomes (N = two). As with anti-holo, many repeats of this experiment showed the same general binding pattern to small polyribosomes, but discrete radioactive peaks were not always seen.

As with anti-holo, the sedimentation pattern of ^{125}I-labeled anti-LS requires intact polyribosomes. When the polyribosomes

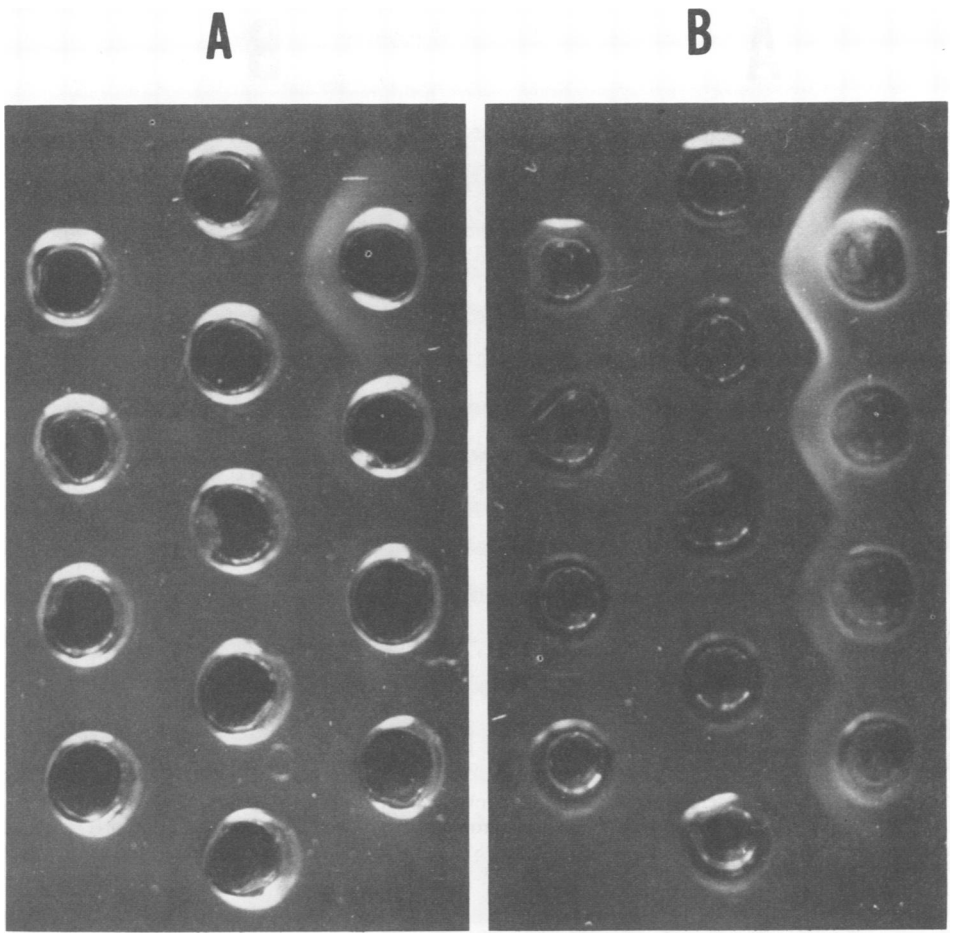

FIG. 1. Purification of anti-LS. Denatured LS (center vertical line of wells in each set, 4.25 μg of protein/well in 0.04% SDS) was reacted against 5 μl of 1/2 serial dilutions of anti-LS IgG before (A) and after (B) purification by LS-linked Sepharose affinity column chromatography. Dilutions are from top to bottom starting on the upper right well in each set. Initial concentrations of protein in the antibody preparations were 10.1 mg/ml (A) and 4.5 mg/ml (B).

were mildly digested with RNase following the antibody-binding step, little or no labeled material migrated into regions in which polyribosomes normally sediment (data not shown).

Binding of Radioiodinated Preimmune IgG to Polyribosomes. We investigated the possibility that binding of anti-holo or anti-LS to polyribosomes might be due to a nonspecific component present in the IgG fraction that might bind to *Chlamydomonas* polyribosomes. This possibility was discounted on the basis of two observations. First, preimmune rabbit serum (or IgG) shows no detectable precipitin lines when challenged with a *C. reinhardi* crude extract or with purified RUBPCase. Second, a preimmune IgG preparation was radioiodinated and incubated with isolated polyribosomes under the same binding conditions as described in previous sections. The sucrose gradient profile of the sedimented polyribosomes in Figure 6 shows that there is virtually no binding of ^{125}I-labeled preimmune IgG to polyribosomes. Therefore, the binding of ^{125}I-labeled material in the purified antibody preparations must be due to the specific interaction of the antibody with RUBPCase nascent chains.

We also attempted to determine if the binding of the labeled antibody to polyribosomes might be due to the adventitious binding of free RUBPCase to a selected class of polyribosomes. To test if free RUBPCase would bind to polyribosomes under the given isolation conditions, we incubated about 0.25 μg of ^{125}I-labeled holoenzyme with resuspended polyribosomes. The pattern obtained from sedimenting this mixture through a sucrose gradient is shown in Figure 7. It can be seen that nearly all of the radioactive holoenzyme is recovered at the top of the gradient, and that insignificant amounts of the highly radioactive enzyme co-sediment with polyribosomes. Certainly, there is no specific binding of free holoenzyme to small polyribosomes. The binding of specific antibodies to small polyribosomes does not seem to result from the prior, adventitious binding of RUBPCase to the polyribosomes.

Assessment of the Quality of the Polyribosome Preparation. The binding of ^{125}I-labeled anti-holo and anti-LS to small polyribosomes was a curious finding since LS is a relatively large polypeptide with a mol wt of 55,000 (16). It was expected that labeled antibodies would bind to a class(es) of larger polyribosomes (see under "Discussion").

We first considered that the binding of labeled antibody to small polyribosomes was artifactual, that the quality or intactness of the polyribosomes was adversely affected by our preparative procedures. Such an artifact could be generated in one of two ways—either by degradation of large polyribosomes to small, or by "run-off," *i.e.* normal or premature detachment of ribosomes from polyribosomes. We attempted to determine if either of these two problems prevailed.

With respect to degradation, there is little evidence in the optical profiles of polyribosome gradients (Figs. 3, 5, 6) that any gross degradation has occurred. When mild degradation is observed in occasional preparations, there is a substantial increase in material sedimenting near the top of the gradient and a rising optical profile from mid to light polyribosomes (from N = 10 to 2). The absence of degradation is backed by the experiment shown in Figure 8A where the distribution of [^{3}H]arginine-labeled nascent chains has been analyzed in polyribosomes profiles. These polyribosomes were isolated and treated in the same

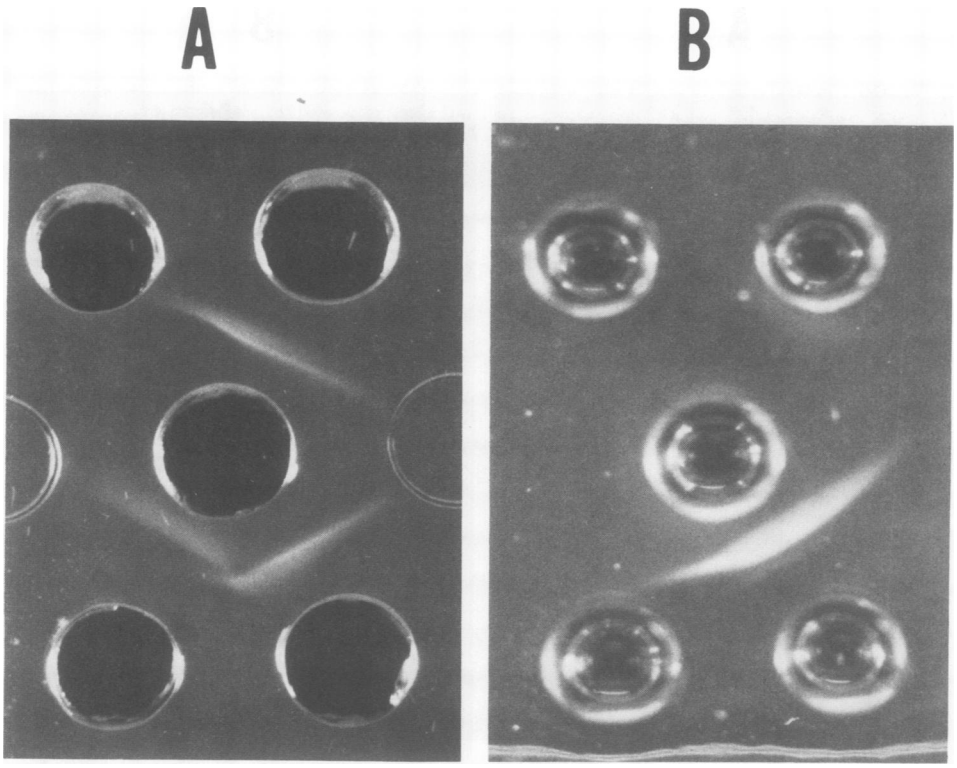

FIG. 2. Specificities of purified antibodies. Ouchterlony immunodiffusion reactions were carried out using (A) 5 μg of purified anti-holo or (B) 22.5 μg of purified anti-LS. Antibodies are in the center wells. Clockwise from upper left well in each set are: buffer E (20 mM $NaPO_4$ [pH 7], 0.15 M NaCl, 0.03% SDS), native holoenzyme , denatured LS, and denatured SS. All antigen wells contain 4.25 μg protein. Denatured subunits are solubilized in buffer E and native holoenzyme in buffer E minus SDS.

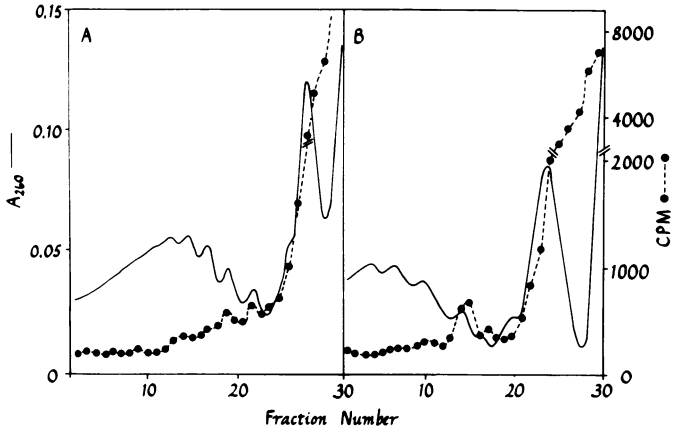

FIG. 3. Binding of ^{125}I-labeled anti-holo to polyribosomes. Polyribosomes were isolated by sedimentation from cell lysates as described under "Materials and Methods." The polyribosome pellet was resuspended in buffer A and 10 $A_{260\ nm}$ units were incubated with 3.1×10^5 cpm of ^{125}I-labeled anti-holo for 2.5 hr at 2 C. The mixture was layered onto a 15 to 30% sucrose gradient in buffer B and centrifuged for 70 min (A) or 120 min (B) at 40,000 rpm in a Beckman SW 41 rotor at 4 C. The gradients were analyzed optically using a spectrophotometer equipped with a flow cell. Ten drop fractions were collected and 0.2 ml of each fraction was spotted on glass fiber filters, dried, and counted in a scintillation counter.

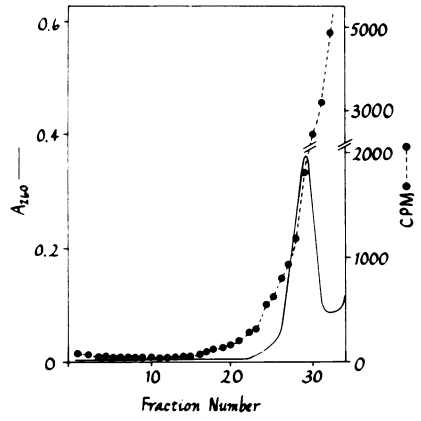

FIG. 4. Distribution of ^{125}I-labeled anti-holo in sucrose gradients containing RNase-treated polyribosomes. Ten $A_{260\ nm}$ units of a resuspended polyribosome pellet were incubated with 3.1×10^5 cpm of ^{125}I-labeled anti-holo for 2.5 hr at 2 C. Following antibody binding, RNase was added to 100 μg/ml, and the sample was incubated an additional 10 min at 37 C. The sample was layered onto a 15 to 30% sucrose gradient in buffer B and centrifuged for 60 min at 40,000 rpm in a Beckman SW 41 rotor at 4 C. The gradients were analyzed as in Figure 3.

way as those used in antibody-binding experiments. If extensive degradation has occurred, then a significant amount of the radioactive material representing nascent chains should shift to the lighter polyribosomes and the monoribosome region. (Monoribosomes bearing nascent chains do not release these nascent chains and will not dissociate in the high salt buffer conditions used in these gradients but will sediment along with normally dissociated ribosomal subunits.) However, little nascent chain radioactivity co-sediments with monoribosomes indicating that insignificant degradation of active polyribosomes has occurred. When polyribosomes are deliberately subjected to mild RNase treatment as shown in Figure 8B, then monoribosomes predominate in the optical pattern and the bulk of the radioactivity labeled nascent chains sediment with them.

Despite the apparent lack of polyribosome degradation in our preparations, it is possible that selective breakdown of polyribosomes bearing LS messenger RNA might have occurred. To

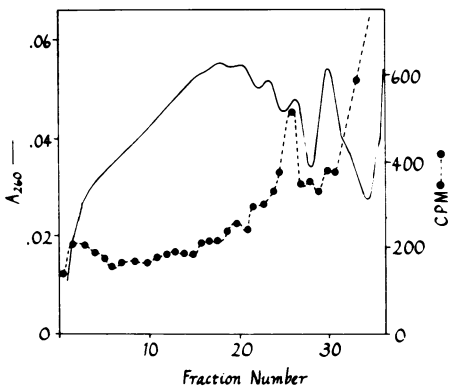

FIG. 5. Binding of ^{125}I-anti-LS to polyribosomes. The binding conditions were the same as described in Figure 3 except that 30 $A_{260\ nm}$ units of resuspended polyribosomes were incubated with 3×10^5 cpm of ^{125}I-labeled anti-LS. The incubated sample was layered onto a 15 to 30% sucrose gradient in buffer B and centrifuged for 70 min at 40,000 rpm in a Beckman SW 41 rotor at 4 C. The gradients were analyzed as in Figure 3.

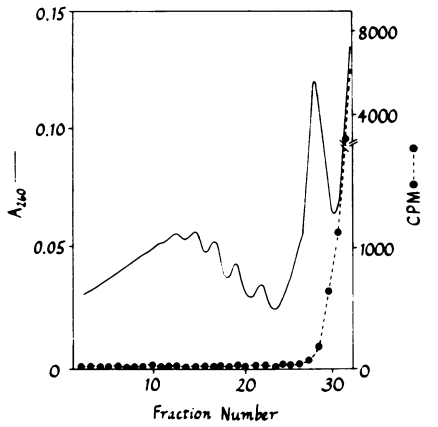

FIG. 6. Binding of ^{125}I-labeled preimmune IgG to polyribosomes. Ten $A_{260\ nm}$ units of a resuspended polyribosome pellet were incubated with 4.7×10^5 cpm of ^{125}I-labeled preimmune IgG for 2.5 h at 2 C. The mixture was layered onto a 15 to 30% sucrose gradient in buffer B and centrifuged for 70 min at 40,000 rpm in a Beckman SW 41 rotor at 4 C. The gradients were analyzed as in Figure 3.

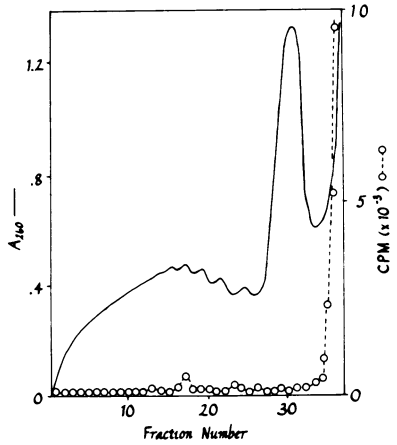

FIG. 7. Absence of binding of ^{125}I-labeled holoenzyme to polyribosomes. Ten $A_{260\ nm}$ units of resuspended polyribosomes were incubated with ^{125}I-labeled holoenzyme containing 2.6×10^5 cpm (5×10^6 cpm/μg) for 90 min at 2 C. The mixture was layered onto a 15 to 30% sucrose gradient in buffer B and centrifuged for 70 min at 40,000 rpm in a Beckman SW 41 rotor at 4 C. The gradients were analyzed as in Figure 3.

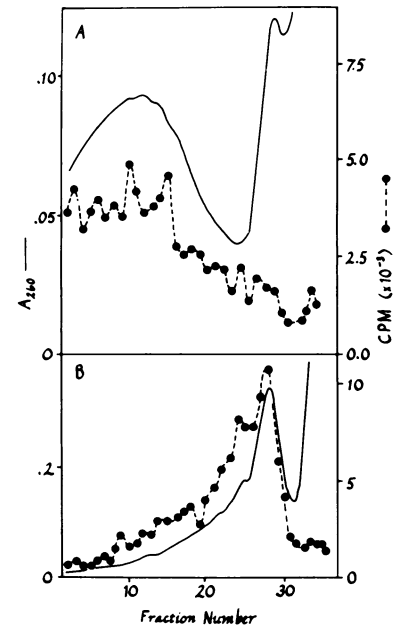

FIG. 8. Distribution of ^{3}H-labeled nascent polypeptide chains in sucrose gradients containing intact (A) and RNase-treated (B) polyribosomes. One liter of cells (1×10^6 cells/ml) was incubated with 0.1 μg/ml [^{3}H]arginine for 2 min. Cycloheximide and chloramphenicol were added to a final concentration of 10 μg/ml and 50 μg/ml, respectively, and the cells were poured immediately over ice. Polyribosomes were isolated by sedimentation from cell lysates as described under "Materials and Methods." Ten $A_{260\ nm}$ units of resuspended polyribosomes were layered onto each of two 15 to 30% sucrose gradients in buffer B. Ten μg of RNase was added to one gradient (gradient B), and the gradients were centrifuged for 60 min at 40,000 rpm in a Beckman SW 41 rotor at 4 C. The gradients were analyzed optically using a spectrophotometer equipped with a flow cell. Ten drop fractions were collected and each fraction was precipitated by the addition of cold trichloroacetic acid to 5%. The precipitates were collected on glass fiber filters and washed with 5% trichloroacetic acid and 95% ethanol. The filters were dried and counted in a scintillation counter.

meet this possible objection, we attempted to determine if large polyribosomes containing LS messenger RNA had been degraded into small polyribosomes. This was done by extracting RNA from small polyribosomes (N = two to five) accumulated from several sucrose gradients. The extracted RNA was separated into four separate size classes by sedimentation on a sucrose density gradient. The RNA from each size class was translated in an *E. coli* protein-synthesizing system, and the synthesis of LS was detected by immunoprecipitation. In Table I it can be seen that most of the specific LS mRNA activity is found in the gradient fraction of RNA sedimenting from 7 to 14S. In the accompanying paper (17), it was demonstrated that LS messenger RNA extracted directly from cells had a sedimentation coefficient of about 12 to 14S. It appears that LS mRNA derived from small polyribosomes is reasonably large.

Another possible explanation for the binding of anti-holo and anti-LS antibodies to predominantly small polyribosomes could be that during our isolation procedure some ribosomes might run-off LS mRNA, converting large polyribosomes into smaller ones without the degradation of the mRNA. We routinely isolate polyribosomes in the presence of both cycloheximide and chloramphenicol to prevent continued chain elongation and normal termination. Experiments in which the cells were exposed to 100 to 1000 μg/ml chloramphenicol for 30 min prior to polyribosome isolation (conditions which inhibit the *in vivo* synthesis of LS in our experiments) showed no significant change in the antibody-binding patterns (data not shown). We therefore con-

Table I. Translation of Immunoprecipitable LS by RNA Derived from Sucrose Density Gradient Fractions of Polyribosomes.

Experiment	RNA Sucrose Gradient Fraction	Acid Precipitable	Specific Immunoprecipitable[1]	% Immunoprecipitable
		cpm		cpm in fraction
a.	no RNA	5424	---	---
b.		2094	---	---
a.	>25S	9266	256	10
b.		7302	0	0
a.	14-25S	4782	118	5
b.		2758	11	2
a.	7-14S	9134	1438	57
b.		3368	523	90
a.	3-7S	5018	698	28
b.		4364	44	8
				100 100

[1] cpm precipitable by anti-LS minus cpm precipitable by preimmune IgG.

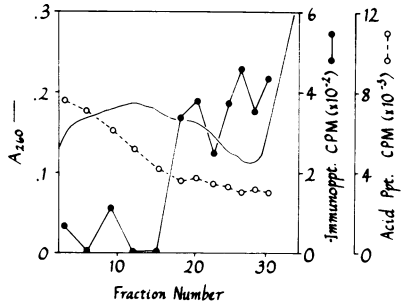

FIG. 9. Immunoprecipitation of polyribosomes with bound ^{3}H-labeled nascent polypeptide chains. 500 ml of cells (2×10^6 cells/ml) were incubated with 0.25 μCi/ml of [^{3}H]arginine for 2 min. [^{3}H]Arginine incorporation was immediately terminated by the addition of cycloheximide (final concentration 10 μg/ml), chloramphenicol (50 μg/ml), KCN, and NaN_3 (10 mM each), and the cells were poured over crushed ice. Polyribosomes were isolated according to method B. Ten drop fractions were collected and 10 μl of each fraction was acid-precipitated and analyzed as described in Figure 8. One hundred μl of selected fractions were analyzed for ^{3}H-labeled nascent LS chains by immunoprecipitation of polyribosomes with a double antibody technique using anti-LS and goat anti-rabbit IgG as described in the accompanying paper (17).

clude that ribosome run-off during polyribosome isolation is not the cause of antibody binding to small polyribosomes.

Immunoprecipitation of Polyribosomes Bearing Nascent Polypeptide Chains. As a next step to the antibody-binding experiments, we attempted to immunoprecipitate polyribosomes bearing LS nascent polypeptide chains. Polyribosomal associated nascent chains were labeled by pulse-labeling cells with [^{3}H]arginine. The labeled polyribosomes were sedimented in sucrose gradients, and polyribosome fractions throughout the gradient were immunoprecipitated with a double antibody system using anti-LS and goat anti-rabbit IgG. The pattern of total nascent polypeptide chain radioactivity (acid-precipitable cpm) and immunoprecipitable nascent chain radioactivity is shown in Figure 9. It is the striking observation that nearly all of the immunoprecipitable radioactivity is found in the small polyribosome fractions in accordance with the specific antibody-binding results described above. In the peak fractions, about 10% of the nascent chain radioactivity was immunoprecipitated.

DISCUSSION

LS from *C. reinhardi* is a relatively large polypeptide (mol wt 55,000), and the mRNA coding for LS is correspondingly large sedimenting at about 12 to 14S (17). Based upon data from other systems, we had expected that polyribosomes containing mRNA coding for LS would be large (N = 14 to 16). For example, hemoglobin (mol wt 15,000, mRNA = 9S) is translated mostly on a small (N = five) polyribosomes (21). Various histones (mol wt 11,000-15,000 for H_{2a}, H_{2b}, and H_3; and 20,000 daltons for H_1; mRNAs = 8-9S) are translated on small (N = two to six) polyribosomes (4), and hen ovalbumin (mol wt 45,000, mRNA = 18S) is translated on large (N = nine to 10) polyribosomes (19) as is rat liver albumin (23). Chick embryo myosin (mol wt 200,000, mRNA = 26S) is translated on extremely large (N = 55 to 56) polyribosomes (14). Therefore, the observation that LS mRNA is translated on small (N = two to five) polyribosomes is not in correspondence with the ribosome-packing density of polyribosomes translating the mRNAs cited above.

We have excluded the possibility of polyribosome degradation by analyzing the nascent peptide chain radioactivity distribution in polyribosomes following a short [^{3}H]arginine pulse. Most nascent chain radioactivity was distributed over large polyribosomes with few cpm being found over monoribosomes. An even more convincing argument against the degradation of polyribosomes lies in the fact that large RNAs (in the 7-14S fraction) from small polyribosomes can direct the synthesis of immunoprecipitable LS in an *E. coli* protein-synthesizing system.

We have shown that the binding of antibodies to small polyribosomes was not generated by the run-off of ribosomes from the mRNA. Pretreatment of the cells with chloramphenicol (100-1,000 μg/ml) did not significantly change the pattern of binding. Other groups have noted that the addition of chloramphenicol to cells prior to polyribosome isolation both increases the proportion of chloroplast ribosomes found in polyribosomes (15, 16), and increases the size of polyribosomes (5, 18).

The binding of the specific antibodies to small polyribosomes can be explained if translation of LS occurs on chloroplast polyribosomes. In preliminary experiments, we have found that LS seems to be synthesized on chloroplast polyribosomes since radioiodinated anti-LS binds preferentially to 70S (chloroplast) monoribosomes. Several other laboratories have demonstrated that LS is probably synthesized on chloroplast polyribosomes. Criddle *et al.* (6) observed that the synthesis of LS, but not small subunit (SS), could be inhibited in barley by chloramphenicol but not by cycloheximide. Gooding *et al.* (11) found that peptides released from wheat chloroplast ribosomes by incubation with [^{3}H]puromycin could be immunoprecipitated by an anti-LS antibody but not by an anti-SS antibody. Blair and Ellis (3) demonstrated that isolated pea chloroplasts synthesize LS in a light-driven reaction. Hartley *et al.* (13) also found that RNA extracted from isolated spinach chloroplasts can direct the synthesis of LS in a heterologous translation system.

Thus, it is not surprising that if LS is translated in the chloroplast in *C. reinhardi*, it would be translated on sparsely packed polyribosomes. Chloroplast polyribosomes have been shown to be small in *C. reinhardi* (5, 8, 9, 18, 19). Electron micrographs of *C. reinhardi* chloroplasts show ribosomes in groups no greater than two to four (8–10). Chloroplast polyribosomes attached to thylakoid membranes after chloramphenicol treatment are small (N = five to six) (5, 18). Results from this laboratory indicate that chloroplast polyribosomes make up about 25% of small (N = two to seven) but only about 8% of large (N = eight to 25) polyribosomes (2). Polyribosomes derived from isolated chloroplasts of *Euglena gracilis* (1) and wheat (12) are also small (N = two to six). It is consistent with these observations that polyribosomes of chloroplast origin containing messenger RNA coding for LS in *C. reinhardi* are small, sparsely packed polyribosomes.

The synthesis of LS on small polyribosomes is of considerable consequence to the "tactics" by which the chloroplast protein-synthesizing machinery generates large quantities of this protein. LS synthesis on small, sparsely packed polyribosomes suggests that LS polypeptide chain initiation is rate-limiting. If the rate of polypeptide chain elongation is not usually high in the chloroplast, then the rate of LS synthesis on any single polyribosome would be expected to be low. To compensate for such a low rate of synthesis on a per ribosome basis, the chloroplast would have to engage a large population of polyribosomes in the exclusive synthesis of LS. To sustain a large chloroplast polyribosome population in LS synthesis would require high steady-state levels of LS messenger RNA. High steady-state levels could only be achieved by a rapid rate of LS messenger RNA synthesis coupled with a slow rate of messenger breakdown. How the chloroplast carries out selective transcription of the gene(s) coding for LS is an interesting question. That LS mRNA is one of the most abundant mRNA species in the chloroplast has been suggested by Hartley *et al.* (13) who have shown that spinach chloroplast RNA codes *in vitro* for almost the exclusive synthesis of LS.

In an operational sense, the observation that polyribosomes involved in LS synthesis are small is another important feature of the LS messenger RNA that will undoubtedly aid in its purification. In the accompanying paper (17), we showed that LS messenger activity had unique sedimentation characteristics and did not bind to oligo(dT)-cellulose. Techniques which exploit these properties should permit an extensive purification of LS messenger RNA from *C. reinhardi*.

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