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Initiation complex formation on *Euglena* chloroplast 30S subunits in the presence of natural mRNAs

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ABSTRACT

An *in vitro* system has been developed that allows the formation of translation initiation complexes with *Euglena* chloroplast 30S ribosomal subunits and natural mRNAs. For these experiments two regions of the *Euglena* chloroplast genome have been cloned behind the T7 transcriptional promotor and the corresponding RNAs synthesized *in vitro*. These mRNAs are capable of forming initiation complexes with chloroplast 30S subunits in the presence of fMet-tRNA and *E. coli* initiation factors. Deletion of the normal translation start site results in a message that is no longer recognized by the chloroplast subunits suggesting that the correct AUG initiation codon on the mRNA is being selected by the small ribosomal subunit. Initiation complex formation with the chloroplast 30S subunits is specific for chloroplast mRNAs and mRNA from the phage MS2 is not active in this system.

INTRODUCTION

Protein biosynthesis in chloroplasts is not well understood but certain aspects of translation in this organelle resemble the prokaryotic system more closely than the eukaryotic cytoplasmic system. Chloroplast and prokaryotic ribosomes are both about 70S in size and both systems use the formylated initiator tRNA. Three elongation factors from *Euglena* chloroplasts have been purified and have been shown to be functionally interchangeable with their prokaryotic counterparts (1−3). In addition, two chloroplast initiation factors from *Euglena* have been identified and partially characterized (4,5). The factor designated IF-3_{chl} facilitates ribosome dissociation and promotes initiation complex formation. IF-3_{chl} is active both on chloroplast and *E. coli* ribosomes (4). IF-2_{chl}, promotes the binding of the initiator tRNA to the small subunit. It is active only on chloroplast ribosomes (5).

At the present time there is little information on the nucleotide sequences which specify the start signal for protein synthesis on chloroplast mRNAs. However, an examination of putative translation start sites on *Euglena* chloroplast mRNAs indicates that many initiation signals do not contain the Shine/Dalgarno sequences which are so prevalent in prokaryotic systems. In an effort to begin the analysis of the translation start signals in chloroplast mRNAs we have developed a system that allows the assembly of an initiation complex on chloroplast ribosomal subunits. This work presents the first *in vitro* system for the formation of translation initiation complexes with natural mRNAs from an organelle system.

MATERIALS AND METHODS

Materials—Vector pTZ18R and T7 RNA polymerase were obtained from U.S. Biochemical Corporation. Plasmid pNEO and nucleotides were from Pharmacia. Plasmid $pEGCE_N$ which contains the EcoN fragment of the *Euglena* chloroplast genome cloned into pBR322 was a gift from Dr. E. Stutz, University of Neuchatel, Switzerland. Calf intestine alkaline

phosphatase, and yeast and *E. coli* tRNA were from Boehringer Mannheim Laboratories. Restriction enzymes were from International Biotechnologies Inc (IBI), Bethesda Research Laboratories (BRL) or Boehringer. T4 DNA ligase was from IBI or New England Biolabs. RNasin was from Promega Biotec. GTP and poly(A,U,G) were from P-L Biochemicals. Phosphoenolpyruvate, pyruvate kinase, creatine phosphate, creatine kinase and L-amino acids were from Sigma Chemical Company. Synthetic oligonucleotides, 25mer CGACTC-ACTATAGTATAAATAACTG (oligo 1) and the 32mer CGACTCACTATAGTAT-AAATAACTGTAAAGTG (oligo 2) were prepared in the Department of Microbiology and Immunology or the Lineberger Cancer Research Center at the University of North Carolina at Chapel Hill. [^{3}H]UTP and [^{35}S]methionine were from New England Nuclear.

Plasmid Constructions — The construction of plasmid pRbcN (Fig. 1A) was carried out in several steps. The chloroplast genome of *Euglena gracilis* klebs var. *bacillaris* Cori (*Euglena* B) was digested to completion with PstI. The 10 kb fragment was isolated, digested with EcoRI and the 948 bp EcoX fragment carrying the first exon, the first intron and part of the second exon of the gene for the large subunit of ribulose bisphosphate carboxylase (*rbcL*) was cloned into the EcoRI site of plasmid pTZ18R. The 222 bp XbaI/SspI fragment of EcoX was cloned into pTZ18R which had been cut with XbaI and HincII. A PstI fragment encoding part of the neomycin resistance gene from the plasmid pNEO was cloned in frame 3′ to the chloroplast derived sequence. Heteroduplex deletion mutagenesis (6) using oligo 2 was used to position the T7 transcriptional promotor adjacent to the region encoding the 5′ untranslated leader region of the *rbcL* mRNA (7). The plasmid pRbcNΔ is identical to pRbcN except that the 5′ leading sequence and 24 nucleotides from the first exon of *rbcL* have been deleted. This construct was obtained as a product of the deletion mutagenesis using oligo 1.

Plasmid pRps12/7 (Fig. 1A) containing the dicistronic region encoding chloroplast ribosomal proteins S12 and S7 was constructed by cutting the plasmid pEGCE$_N$ with DraI, isolating the 1057 base pair fragment and subcloning it into the HincII site of pTZ18R.

In vitro transcription—Prior to use in transcription reactions, 150 μg of plasmid was linearized using 240 units of HindIII in 600 μl digestion buffer. Following incubation for 2 hours at 37°C an additional 120 units of HindIII were added and digestion was continued for 16 hours. A small portion of the reaction mixture was analyzed on a 1% agarose gel to confirm that the digestion was complete. The remainder was extracted with phenol/chloroform and precipitated with ethanol prior to transcription. Preparative transcription reactions were carried out in reaction mixtures (780 μl) containing 40 mM Tris-HCl, pH 8, 5 mM dithiothreitol, 10 mM $MgCl_2$, 4 mM spermidine, 1 mM ribonucleoside triphosphates (except UTP which was supplied at a concentration of 0.8 mM and was present as [^{3}H] UTP diluted to a specific activity of 12−25 cpm/pmol), 0.01 mg/ml BSA, 125 μg of lineraized template DNA, 400 units RNasin and 3600 units of T7 RNA polymerase. Transcription reactions were incubated at 37°C for 30 min. Nucleic acid was extracted with phenol/chloroform and concentrated by ethanol precipitation. Transcription products were further purified by chromatography on a 5 cc column of Sephadex G50 (superfine) equilibrated in 25 mM Tris-HCl, pH 7.8, 3 mM EDTA and 0.02% diethyl oxydiformate.

Preparation of [^{35}S]formylmethionyl-tRNA, ribosomal subunits, initiation factors, E. coli S150 and MS2 RNA: [^{35}S]formylmethionyl-tRNA$_i$ was prepared from yeast tRNA as described (8). A high salt wash of *E. coli* ribosomes was prepared as described (9), concentrated by ammonium sulfate precipitation and dialyzed against 50 mM Tris-HCl,

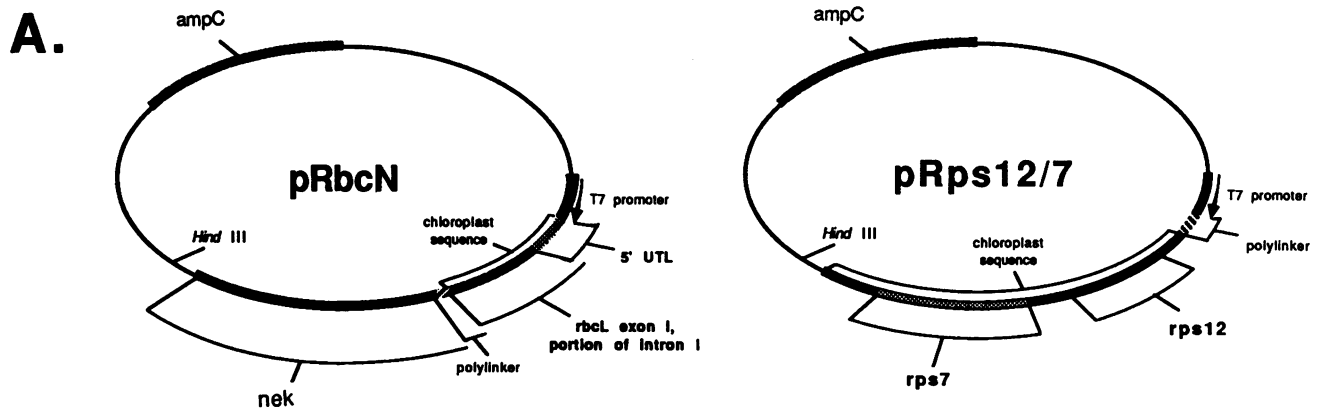
pH 7.6, 50 mM NH_4Cl, 1 mM EDTA, 1 mM dithiothreitol and 10 % glycerol before use. *E. coli* ribosomal subunits were prepared by the method of Suttle and Ravel (10) and the 30S subunits were activated prior to use (11). *Euglena* chloroplast ribosomal subunits were prepared as described (8). In certain experiments partially fractionated chloroplast ribosomes containing a mixture of subunits were used. *E. coli* was disrupted and a postribosomal supernatant (S150) was prepared as described (12,13). MS2 bacteriophage (ATCC 15597-B1) was grown in *E. coli* C3000 and the RNA was isolated as described (14).

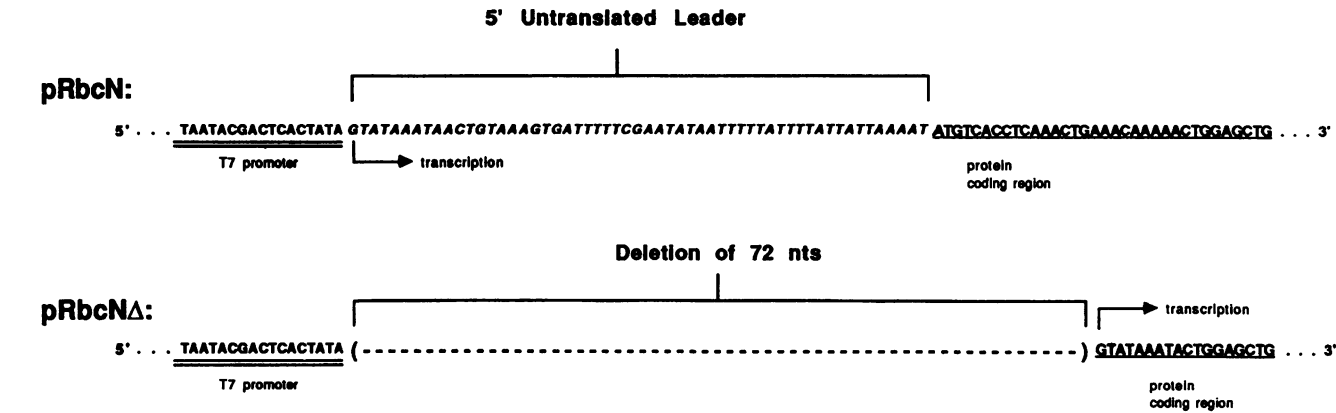
Initiation Complex Assays:—Reaction mixtures (50 μl) contained 50 mM Tris-HCl, pH 7.8, 30−80 mM NH_4Cl, 7−10 mM $MgCl_2$, 1 mM dithiothreitol, 0.25 mM GTP, 1.25 mM phosphoenolpyruvate, 0.06 units of pyruvate kinase, 25 units of RNasin, 28 μg of *E. coli* high salt wash as a source of initiation factors, 0.125 μg of poly(A,U,G) or the indicated levels of natural mRNA and 0.05−0.06 A_{260} units of chloroplast 30S subunits or 0.4 A_{260} units of a mixture of chloroplast ribosomal subunits. Incubation was for 15 min at 37°C when 30S subunits were used or at 28°C when a mixture of subunits was used. Reactions were terminated by the addition of 5 ml of cold buffer (50 mM Tris-HCl, pH 7.8, 80 mM NH_4Cl, 7−10 mM $MgCl_2$). Samples were filtered through nitrocellulose filters, the filters were washed twice with 5 ml of cold buffer, dried at 110°C and counted in 10 ml scintillation fluid containing 5 g of 2,5-diphenyloxazole per liter of toluene.

In vitro translation—Natural message programmed protein synthesis was carried out in a two-stage reaction. In the first stage initiation complexes were formed as described above in a final volume of 40 μl. At the end of the first incubation, 10 μl of the reaction mix was filtered through nitrocellulose membranes in order to determine the amount of initiation complex formed. In the second stage, an elongation mix was added providing the following final incubation conditions in a volume of 60 μl: 50 mM Tris-HCl, pH 7.8, 80 mM NH_4Cl, 14 mM $MgCl_2$, 6.5 mM creatine phosphate, 0.025 μg creatine kinase, 40 μg of *E. coli* S150, 30 μg *E. coli* tRNA, 2.5 mM ATP, 0.5 mM GTP, 0.05 mM amino acids and 0.23 A_260 *E. coli* 50S subunits. Incubation was continued for another 20 minutes at 37°C. The extent of protein synthesis was monitored by removing 30 μl of the mixture for hot 5 % trichloroacetic acid precipitation. To assure the dissociation of polypeptide from the hybrid ribosome, 0.3 mg of RNase A was added in most experiments to the remaining 30 μl of the reaction and incubated for 15 minutes at 37°C. Reactions were then analyzed by SDS polyacrylamide gel electrophoresis (15) on 17.5 % gels and translation products detected by fluorography (16).

RESULTS

Euglena chloroplast mRNAs are difficult to isolate intact and in large amounts. In order to investigate the formation of initiation complexes on these mRNAs, we have constructed RNA synthesis vectors that allow the *in vitro* synthesis of two mRNAs that carry translation start signals for several chloroplast genes. The first construct (pRbcN) contains exon I of the large subunit of ribulose bisphosphate carboxylase (*rbcL*) fused in frame to an internal portion of the neomycin resistance gene (Fig. 1A). The *rbcL* gene is one of the few *Euglena* chloroplast genes for which the 5′ end of the mRNA has been mapped (7) and was selected primarily for this reason. Because the *rbcL* gene in *Euglena* is complex (it contains 10 exons and 9 introns) we have fused a portion of the 5′ region of *rbcL* to an internal region of the neomycin resistance gene. The resulting mRNA synthesized *in vitro* contains the entire 55 base untranslated leader of the *rbcL* mRNA. The leader region of this mRNA


A.
ampC
pRbcN
T7 promoter
chloroplast sequence
Hind III
5' UTL
rbcL exon I, portion of intron I
polylinker
nek
ampC
pRps12/7
T7 promoter
Hind III
chloroplast sequence
polylinker
rps12
rps7


B.
5' Untranslated Leader
pRbcN:
5' . . . TAATACGACTCACTATA GTATAAATAACTGTAAAGTGATTTTTCGAATATAATTTTTATTTTATTATTAAAAT ATGTCACCTCAAACTGAAACAAAAACTGGAGCTG . . . 3'
T7 promoter
transcription
protein coding region
Deletion of 72 nts
pRbcNΔ:
transcription
5' . . . TAATACGACTCACTATA (----------) GTATAAATACTGGAGCTG . . . 3'
T7 promoter
protein coding region

is identical to that of the *in vivo* transcript except for the presence of a 5′ terminal G residue which was included to facilitate transcription by T7 polymerase (Fig. 1B). We believe that the 5′ untranslated region of this mRNA should contain all of the information necessary to direct the correct initiation of translation by chloroplast ribosomes. During the course of preparing pRbcN, a deletion of the 5′ untranslated region and of a portion of the coding region from exon I of the *rbcL* gene was also obtained (pRbcNΔ). RNA synthesized from this plasmid (Fig. 1B) lacks the normal chloroplast translation start site but contains the majority of the coding region including 18 AUGs. The second construct (pRps12/7) carries the coding regions for ribosomal proteins S12 and S7 (Fig 1A). These proteins are thought to be translated from a dicistronic mRNA (17). Although the exact 5′ end of this mRNA *in vivo* is not known, the present construct includes 44 bases 5′ to the start site for the S12 gene. This region would be expected to carry the majority of the sequence information required to specify the initiation site. None of the coding regions present on these mRNAs contains a Shine/Dalgarno sequence 5′ to the translation start codon.

Previous work (8) has indicated that translation initiation complexes carrying fMet-tRNA can be assembled on *Euglena* chloroplast 30S subunits using *E. coli* initiation factors and poly(A,U,G) as a message. In the present work we have extended these studies to examine the ability of chloroplast subunits to form initiation complexes on *in vitro* transcripts that contain start sites equivalent to those in natural mRNAs. As indicated in Fig. 2, fMet-tRNA binding to chloroplast 30S subunits can be observed in the presence of a mRNA containing the translation start site for the *rbcL* gene. As expected, little initiator tRNA binding is observed in the absence of message (data not shown). The amount of initiation complex formed is dependent on the amount of RbcN mRNA added and can reach levels comparable to those obtained in the presence of the synthetic mRNA poly(A,U,G). The binding observed is dependent on the presence of initiation factors which in this experiment were supplied as a high salt wash of *E. coli* ribosomes (data not shown). In independent experiments, similar fMet-tRNA binding was obtained with the mRNA encoding S12 and S7 (data not shown).

In order to gain some insight into whether the chloroplast 30S ribosomal subunits are selecting the correct initiation signal on the RbcN mRNA, we have tested the ability of the deletion derivative RbcNΔ to participate in initiation complex formation. This RNA lacks the 5′ untranslated region and 24 nucleotides of the normal coding region (Fig. 1B). The truncated mRNA contains 18 internal AUGs, but as indicated in Fig. 2, little initiation complex is formed when it is used in the fMet-tRNA binding assay. This observation suggests that the chloroplast 30S subunit is selecting the correct start codon during the formation of the initiation complexes under study here.

Natural mRNA-directed binding of fMet-tRNA to chloroplast 30S subunits is particularly sensitive to the monovalent cation concentration with optimal binding being observed at about 30 mM NH_4Cl (Fig. 3A). The formation of initiation complexes with synthetic mRNAs is not as sensitive to monovalent cations as is initiation complex formation with these natural mRNAs (8). However, the elongation phase of protein synthesis with *Euglena* chloroplast ribosomes displays a low monovalent cation optimum (18). The amount of binding observed is dependent on the Mg^{2+} ion concentration with optimal binding being obtained at about 7 to 12 mM Mg^{2+} (Fig. 3B).

Figure 1. A. Structures of the plasmids pRbcN and pRps12/7. B. Sequences of the mRNA-like strands of the transcription initiation regions of the pRbcN and its truncated derivative pRbcNΔ.

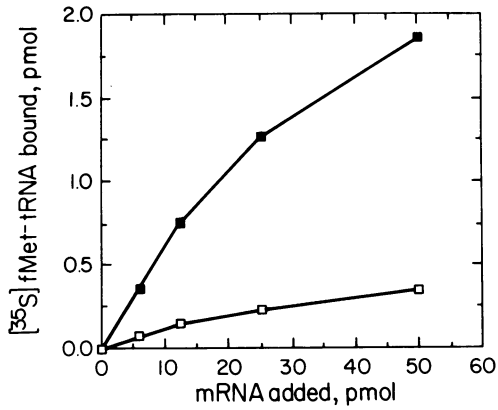


Figure 2. Initiation complex formation with RbcN mRNA and its truncated derivative. Reaction mixtures were prepared as described in Materials and Methods and contained the indicated concentration of mRNA derived from RbcN (−■−) or the deletion derivative RbcNΔ that lacks the *rbcL* translation start site (−□−).

The initiation complexes detected above were analyzed on sucrose density gradients in order to determine whether they were forming on 30S subunits or on 70S ribosomes when both chloroplast ribosomal subunits were present in the incubation mixtures. As indicated in Fig. 4, essentially all of the fMet-tRNA binding observed with RbcN mRNA is present on chloroplast 30S ribosomal subunits. We believe that the failure to form 70S complexes in these assays probably results from the extreme fragility of the *Euglena* 50S ribosomal subunits which are difficult to isolate in an active form. Much lower binding is observed in the absence of mRNA and this material sediments in the 70S region of the gradient. This low level of binding probably represents an interaction of fMet-tRNA with the P-site on the ribosome.

Work with bacterial ribosomes from a variety of species suggests that the selection of the start signal on the mRNA is carried out by the small ribosomal subunit and not by

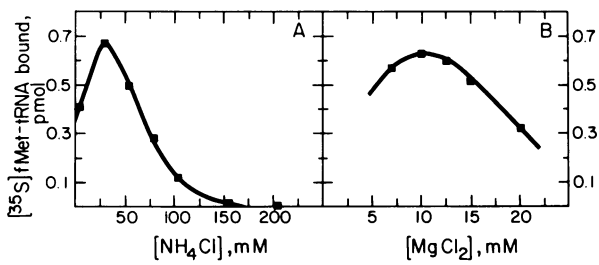


Figure 3. Cation optima for initiation complex formation with chloroplast ribosomes. A. Reaction mixtures containing 12 pmol of RbcN mRNA were prepared as described in Materials and Methods except that the concentration of NH_4Cl was adjusted as indicated. A blank representing the amount of binding obtained in the absence of mRNA (less than 0.1 pmol) has been subtracted from each value. B. Reaction mixtures containing 25 pmol of RbcN mRNA were prepared as described in Materials and Methods except that they contained the indicated concentrations of $MgCl_2$. A blank representing the amount of binding obtained in the absence of mRNA (0.02 pmol) has been subtracted from each value.

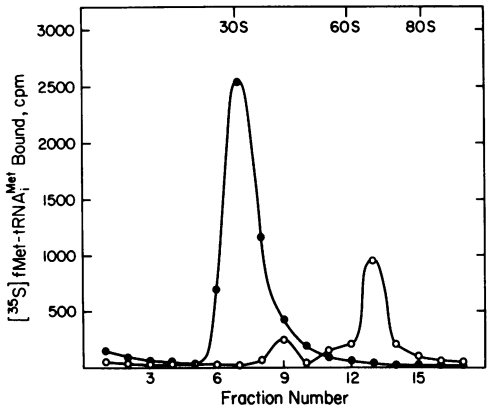


Figure 4. Sucrose gradient analysis of initiation complexes. Initiation complexes were formed as described in Materials and Methods using either 32 pmol of the RbcN mRNA (− ● −) or no mRNA (− ○ −) and were analyzed on 5 ml linear 10−30% sucrose gradients prepared the same buffer used for the reaction. After centrifugation at 36,000 rpm at 4°C for 3.5 hours, gradients were displaced with a 50% sucrose solution on an ISCO model 640 gradient fractionator, and the absorbance profile at 254 nm was recorded on an ISCO UA-5 absorbance monitor. Fractions (0.25 ml) were collected and filtered through nitrocellulose membranes and treated as described for the analysis of binding assays.

the initiation factors (19, 20). As indicated above, the formation of initiation complexes on chloroplast 30 subunits requires the presence of initiation factors which can be supplied from *E. coli*. In order to examine the specificity of the chloroplast 30S subunits, we have compared initiation complex formation on several mRNAs using chloroplast and *E. coli* ribosomes. As shown in Table I, chloroplast 30S subunits form initiation complexes efficiently with the RbcN and S12/S7 mRNAs which contain normal chloroplast translation start signals. However, these subunits are unable to form initiation complexes efficiently when the 5′ untranslated leader region including the normal start site has been deleted

Table I mRNA Specificity of Chloroplast and *E. coli* 30S Subunits

mRNA[a]	[35S] fmet-tRNA bound, pmol	
	Chloroplast 30S[b]	*E. coli* 30S[c]
poly(A,U,G)	1.73	1.07
MS2	0.05	0.61
S12S7	1.19	0.56
rbcN	0.98	0.53
rbcNΔ	0.06	0.37

[a] The amounts of mRNAs used were: poly(A,U,G), 12.5 μg; MS2 RNA, 27.6 μg; S12/S7, rbcN, rbcNΔ mRNAs, 20 pmol.

[b] Reaction mixtures (40 μl) were prepared as described in Materials and Methods and contained chloroplast 30S subunits. A blank representing the amount of binding obtained in the absence of mRNA (0.01 pmol) has been subtracted from each value.

[c] Reaction mixtures were prepared as described above except that they contained 80 mM NH_4Cl, 5 mM $MgCl_2$ and 0.17 A_{260} of activated *E. coli* 30S subunits. A minus mRNA blank (0.03 pmol) has been subtracted from each value.

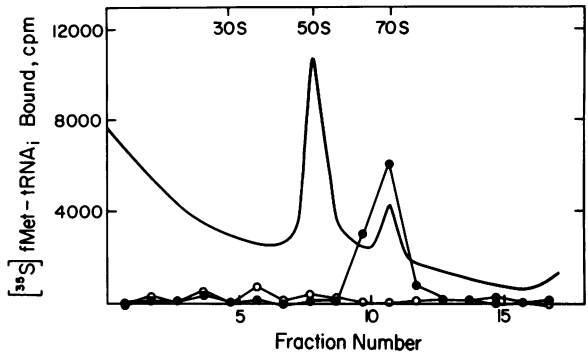


Figure 5. Formation of 70S initiation complexes with *Euglena* chloroplast 30S subunits and *E. coli* 50S subunits. Initiation complexes were formed with 29 pmol of S12/S7 mRNA (– ● –) or no mRNA (– ○ –) and 0.05 A_{260} units of chloroplast 30S subunits. After incubation for 15 minutes at 37°C, 0.23 A_{260} of *E. coli* 50S subunits were added and the ionic conditions were adjusted to 50 mM Tris-HCl, pH 7.8, 80 mM NH_4Cl and 14 mM $MgCl_2$. Samples were incubated for an additional 10 min at 37°C and then layered onto 5 ml linear 10–30% sucrose gradients prepared in the same buffer. Gradients were analyzed as described for Fig. 4.

(RbcNΔ). Furthermore, the chloroplast subunits have no activity with the RNA from bacteriophage MS2. These observations indicate that *Euglena* chloroplast small subunits are specific for the formation of initiation complexes with mRNAs that contain correct chloroplast translation start signals. In contrast, *E. coli* 30S subunits fail to discriminate strongly among the three chloroplast-derived messages and are presumably forming ribosome bound complexes at non-start signals. *E. coli* ribosomes are known to be rather nonspecific in their interactions with mRNAs from various sources (21).

As indicated above the initiation complexes formed on the chloroplast mRNAs are present on the small ribosomal subunit. We have not been able to form 70S initiation complexes with chloroplast subunits and we believe this problem lies with the 50S subunit which is quite labile. However, previous work (22) has indicated that *Euglena* chloroplast 30S subunits can form a hybrid ribosome with *E. coli* 50S subunits and that this hybrid ribosome is active in poly(U)-directed synthesis of polyphenylalanine. We have confirmed these early observations and have determined that the Mg^{2+} optimum for poly(U)-directed protein synthesis by these hybrid ribosomes is about 14 mM (data not shown). We have tested whether 30S initiation complexes formed with chloroplast S12/S7 mRNA could be transferred into 70S complexes in the presence of *E. coli* 50S subunits. As indicated in Fig. 5, sucrose gradient analysis indicates that 70S initiation complexes formed with natural mRNAs can be made with chloroplast 30S subunits and *E. coli* 50S subunits. No labeled fMet-tRNA is observed in the 70S region of the gradient in the absence of mRNA indicating that the formation of these 70S initiation complexes is dependent on the presence of a message (Fig. 5). If 30S subunits are omitted from these reaction mixtures, almost no binding can be detected (data not shown). This observation indicates that the formation of the 70S complexes shown here is dependent on the presence of the chloropalst small subunit.

The ability to form 70S initiation complexes on chloroplast mRNAs allowed us to ask whether the hybrid ribosome could elongate the polypeptide from the initiation site on the mRNA. Chloroplast 30S initiation complexes, formed in the presence of S12/S7 mRNA,

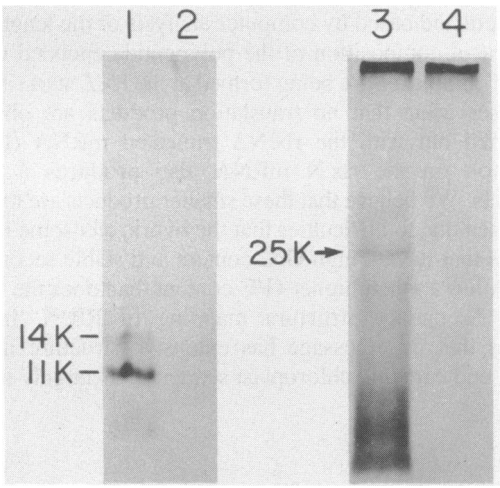


Figure 6. SDS-polyacrylamide gel analysis of *in vitro* translation products. Initiation complexes were formed and then chased into elongation reactions as described in Materials and Methods. Lane 1, S12/S7 mRNA (29 pmol). Lane 2, minus mRNA control. Lane 3, RbcN mRNA (25 pmol). Lane 4: RbcNΔ mRNA (25 pmol). A large amount of labeled material failed to enter the gel in lanes 3 and 4. These particular samples were not treated with RNase prior to electrophoresis and this material disappears upon nuclease treatment of the samples (data not shown).

were converted into 70S initiation complexes by the addition of *E. coli* 50S subunits. Polypeptide elongation was then carried out in the presence of a postribosomal supernatant from *E. coli* which was used as a source of aminoacyl-tRNA synthetases and elongation factors. A portions of the reaction mixture was subjected to hot trichloroacetic acid precipitation to estimate the amount of the initiation complex that was chased into polypeptide and the remainder was subjected to SDS polyacrylamide gel electrophoresis in order to evaluate of the size and complexity of the products produced. In general, about 25−30% of the [^{35}S]fMet-tRNA could be recovered upon acid precipitation suggesting that a significant portion of the initiation complex can be driven into polypeptide. When initiation complexes formed with S12/S7 mRNA are elongated into polypeptide (Fig. 6, lane 1), two major products are observed having molecular weights of about 11 kD and 14 kD. We believe that these products represent translation initiation complexes formed at the start sites for S12 and S7 respectively. The SDS-PAGE products behave as if they are somewhat smaller than the predicted sizes of these proteins based on the gene sequences (about 14 kD and 18 kD). The apparent differences probably represent slightly anomalous gel migration behavior. There are no other open reading frames on this mRNA that could lead to large products in this size range. These results, thus, suggest that chloroplast 30S subunits are selecting the correct start site on the S12/S7 mRNA. Initiation complexes appear to have occurred at both the S12 and the S7 start sites but there appears to be more complex formed at the first cistron (S12) in the mRNA than at the second cistron. As expected, SDS-PAGE analysis indicates that in the absence of mRNA, no labeled bands can be observed following fluorography (Fig. 6, lane 2).

When initiation complexes formed with RbcN mRNA are similarly chased into polypeptide, a product of the expected size (25 kD) is observed (Fig. 6 lane 3). (The size

of this product was predicted based by computer analysis of the length of the open reading frame and the amino acid composition of the polypeptide encoded within it.) This result indicates that initiation complexes are being formed at the *rbcL* start site. This idea is further supported by the observation that no translation products are observed when similar experiments are carried out with the rbcNΔ truncated mRNA (Fig. 6, lane 4). The polymerization reaction on the rbcN mRNA also produces a number of smaller, heterogeneous, products. We believe that these smaller products are the result of incomplete chains which have arisen due to difficulties that the hybrid ribosome has carrying out chain elongation on mRNAs that have a high G/C content and stable secondary structures. The chimeric rbcN mRNA has a much higher G/C content than does the S12/S7 mRNA (57% compared to 31%). Secondary structure mapping of RbcN mRNA using reverse transcriptase indicates that this message has extensive structure in the coding region. However, the very 5′ end carrying chloroplast sequences is largely unstructured (data not shown).

DISCUSSION

The interaction between the ribosome and the start signal of the mRNA during the initiation of translation is an intriguing process. In prokaryotes most of the mRNAs contain Shine/Dalgarno sequences which facilitate the selection of the correct AUG codon during initiation complex formation. This sequence base pairs with a polypyrimidine sequence (CCUCC) near the 3′ end of the 16S rRNA during initiation (23). The sequence near the 3′ end of the 16S rRNA including the polypyrimidine region has been highly conserved among prokaryotes and is even found in the small subunit rRNAs from most plant chloroplasts (24). However, the 3′ end of the small subunit rRNA (17S) from *Euglena* chloroplasts (AACAACUCCC) is rather unusual (25). It contains a polypyrimidine sequence CUCCC which differs from the CCUCC sequence found most commonly in prokaryotes and in the 16S rRNAs from other chloroplasts. Furthermore, the polypyrimidine sequence in the *Euglena* rRNA actually terminates the chain rather than being located several nucleotides from the end. Binding studies with complementary oligonucleotides (25) indicate that the 3′ end of the 17S rRNA is exposed in the *Euglena* chloroplast 30S subunit and could potentially interact with complementary sequences in mRNAs.

We have examined known *Euglena* chloroplast translation start signals for the presence of potential Shine/Dalgarno interactions within 20 nucleotides upstream of the AUG start codon (Table II). Interestingly, many *Euglena* chloroplast mRNAs do not appear to contain traditional Shine/Dalgarno sequences. Among 20 of the *Euglena* transcripts whose sequences around the initiation codon are known, only 9 of them contain Shine/Dalgarno sequences. The deviation of the 3′ end of the small subunit rRNA in *Euglena* chloroplasts and the lack of Shine/Dalgarno sequences in many mRNAs suggests that the selection of the initiation signal on *Euglena* chloroplast mRNAs differs from the process found in prokaryotes and may also differ from that used in the translation of mRNAs from higher plant chloroplasts.

Based on an exploration of the information listed in Table II, we would like to propose that the translational machinery in *Euglena* chloroplasts has two potential mechanisms available for the recognition of translational start signals. One class of start sites has a potential Shine/Dalgarno sequence. In these mRNAs the A/T content in the region up to −20 from the translation start is often less than 90% and the interaction between the 17S rRNA and the mRNA may be important to facilitate initiation complex formation. In the

Table II Translation Starts on *Euglena* chloroplast mRNAs

Gene	Shine/Dalgarno Sequence	% A/T −20 to the AUG	Comments	Reference
rps12	None to −20	90%		17
rps7	None to −20	90%		17
atpH	GGAG at −4 to −8	70%		26
tufA	None to −20	95%		27
orf206	None to −20	95%		27
psbE	GGGA at −9 to −12	85%		28
psbF	None to −20	75%	7 bp down from psbE-translationally coupled?	28
psbI	None to −20	100%		28
psbJ	GGGAG at −9 to −13	80%		28
rbcL	None to −20	100%		7
psbA	None to −20	95%		29
orf458	GUGGU at −13 to −17	85%		30
psaA	None to −20	90%		31
psaB	GGAG at −10 to −13	70%		31
rpl20	None to −20	80%	20% C, No G's	32
rpl23	UUGU at −10 to −13	85%		33
rpl2	None to −20	90%		33
rpl22	GUU at −12 to −14	85%		33
rpl19	UGUU at −12 to −15	95%		33
rps3	GUU at −10 to −12	85%		33

second class of initiation sites, no Shine/Dalgarno sequence is present. In these mRNAs the A/T content of the ribosome bound region 5′ to the start AUG is 90% or greater and this portion of the mRNA is probably quite unstructured. These start sites may, therefore, be readily accessible to the 30S subunit and allow initiation in the absence of a Shine/Dalgarno interaction. It is well known that the 30S subunit including the *Euglena* chloroplast small subunit (8) can bind easily to poly(A,U,G) in the absence of any Shine/Dalgarno interaction. This observation suggests that for a translation start site located in an A/T rich region, no Shine/Dalgarno type of interaction may be required to facilitate initiation complex formation.

There are two exceptions to the generalization suggested above: *psbF* and *rpl20*. The coding sequence of *psbF* is located only 7 nucleotides downstream from the termination codon of the *psbE* gene in *Euglena* and it is likely that *psbF* is translationally coupled to the translation of the upstream cistron. Under these conditions, ribosomes completing the translation of *psbE* would initiate translation at *psbF* without dissociation from the mRNA obviating the necessity for a Shine/Dalgarno sequence or for an A/T rich region 5′ to the AUG codon. Such translational coupling has been observed in prokaryotes (34) and has also been suggested to occur in higher plant chloroplasts (35). The second exception is *rpl20*. This cistron has only 80% A/T content 5′ to the start AUG; however, the remaining

20% consists entirely of C residues. The absence of any G's in this region might result in a low degree of secondary structure and allow the start AUG to remain accessible. In order to get some insight into this possibility the region from −50 to the start codon has been folded using the Wisconsin Genetics Computer Group (GCG) FOLD program run on a Vax computer. Computer modeling indicates that this region has very weak secondary structure with a ΔG of −1.2 kcal/mol. Thus, we believe that this region may be readily accessible to the 30S subunit allowing initiation in the absence of a Shine/Dalgarno sequence.

The *Euglena* chloroplast genome (36) itself is rather A/T rich (75%). In order to determine whether the high A/T content observed around a number of the translation start sites is simply a reflection of the A/T content of the genome, we have examined the A/T content of 48 AUG codons that, as far as we can tell, are present in mRNAs but are not used as start codons. The average A/T content in the region that spans the 20 nucleotides 5′ to these AUG codons is 68%. Of these sequences, less than 5% are located in regions of high (greater than 90%) A/T content just 5′ to the AUG. These few sites may be discriminated against by the ribosome through other unknown structural features.

Recently Ruf and Kossel (37) have examined the occurrence and spacing of ribosome recognition sites in the mRNAs of tobacco chloroplasts. They have observed that 90% of the mRNA species contain a sequence upstream of the respective initiation codon that can base pair with the 3′ terminal of the chloroplast 16S rRNA. However, the spacing observed between the potential ribosome recognition sequence and the initiation codon can be up to 25 nucleotides in a number of cases. This distance is much greater that the 7 to 12 nucleotide spacing generally observed in prokaryotic mRNAs (23). Bonham-Smith and Bourque (38) have examined close to 200 chloroplast sequences for possible Shine/Dalgarno regions and have observed that 40% of the mRNAs have Shine/Dalgarno sequences in a position that is comparable to those observed in prokaryotes. A number of other mRNAs appear to have Shine/Dalgarno sequences but they are located up to 100 nucleotides 5′ to the start AUG, well outside what one would predict for the ribosome bound region in standard models of ribosome:mRNA interactions. No Shine/Dalgarno sequence can be observed in 6% of the mRNAs examined. The possibility, thus, remains open that certain mRNAs in higher plant chloroplasts will also use an alternative mechanism for the recognition of the translational start signal.

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