Analysis of the Translational Initiation Region on the Euglena gracilis Chloroplast Ribulose-bisphosphate Carboxylase/Oxygenase (rbcL) Messenger RNA*

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The chloroplast mRNAs from Euglena gracilis fall into two classes. One group of mRNAs from this organelle contains a Shine-Dalgarno sequence 5' to the start codon, while the other group of mRNAs does not have a conserved sequence signal in the 5'-untranslated region. To investigate the start signals for E. gracilis chloroplast mRNAs that do not carry a Shine-Dalgarno sequence, 30 S initiation complex formation has been studied using a series of transcripts carrying the wild-type translational start site of ribulose-bisphosphate carboxylase/oxygenase (rbcL) or mutated derivatives of this site. Mutation of the start codon of the rbcL gene indicates that the chloroplast 30 S subunit is recognizing only the correct AUG codon. The analysis of the messages from a series of deletion mutants shows that a minimum of $\Delta 20$ residues 5' to the AUG codon is required for activity. Maximal activity requires the full 55-base leader sequence. Surprisingly, a transcript carrying the inverse complement of 48 bases in the leader is $\Delta 60\%$ as active as the wild-type message in promoting initiation complex formation. Introduction of a Shine-Dalgarno sequence in the 5'-leader increases the activity of the mRNA only $\Delta 1.4-2$-fold. The presence of an oligodeoxyxynucleotide containing a strong Shine-Dalgarno sequence does not significantly inhibit the formation of initiation complexes at the ribosomal subunit site. Similar results are obtained when initiation complexes are formed with initiation factors from either E. gracilis chloroplasts or Euglena gracilis.

During the past several years, progress has been made in understanding the mechanism of protein biosynthesis in chloroplasts. Chloroplast ribosomes are 70 S particles that consist of 30 S and 50 S subunits. Two chloroplast translational initiation factors have been purified from Euglena gracilis. Chloroplast initiation factor 2 (IF-2chl)1 promotes the binding of fMet-tRNA to 30 S ribosomal subunits (1). This factor is structurally complex (1, 2) and does not have significant activity on bacterial ribosomes (3). Chloroplast initiation factor 3 (IF-3chl) promotes the dissociation of ribosomes and facilitates initiation complex formation (4). IF-3chl is active on Escherichia coli ribosomes, but has many physical properties that distinguish it from the corresponding prokaryotic factor (5).

Little detailed information is available on the precise nucleotide sequences in chloroplast mRNAs that specify the start site for translation. In prokaryotes, a polypurine sequence (such as GGAGG) is centered ~10 nucleotides upstream of the start codon. This sequence (the Shine-Dalgarno sequence) hydrogen-bonds to a polypyrimidine sequence (CCUCC) near the 3'-end of the small subunit rRNA (16 S rRNA) during initiation complex formation. This rRNA-mRNA interaction facilitates the correct selection of the start codon by the 30 S subunit (6-8). In contrast, in the eukaryotic cytoplasmic system, 40 S ribosomal subunits carrying the initiator tRNA interact with the cap structure on the mRNA and, in the presence of appropriate initiation factors, scan the mRNA until it reaches the first AUG codon from the 5'-end, where initiation generally occurs (9). In higher plants, statistical analysis of putative start sites on chloroplast mRNAs indicates that some of these messages contain Shine-Dalgarno sequences 5' to the start codon (10). Other mRNAs do not appear to have an equivalent sequence. The results obtained with the chloroplast RNAs do not appear to have any equivalent sequence. The initiation of translation in the chloroplasts of Euglena gracilis has been complicated by the presence of Group III introns, which do not have a Shine-Dalgarno sequence. Although many chloroplast mRNAs do not appear to have an equivalent sequence, the AUG codon may be selected because it resides in a region of the mRNA that has little secondary structure, making it readily available for interaction with the 30 S ribosomal subunit (10).

The investigation into the nucleotide sequence signals in E. gracilis chloroplast mRNAs has been facilitated by the development of an in vitro system that measures the formation of initiation complexes (13). In this paper, we have examined the role of primary sequence information in initiation complex formation with the chloroplast mRNA encoding the large subunit of ribulose-bisphosphate carboxylase/oxygenase. Although many chloroplast mRNAs are polyadenylated, the message encoding the large subunit is monocistronic and has a 55-base 5'-untranslated leader. This mRNA does not contain a Shine-Dalgarno sequence and thus provides a model system in which to delineate the translational initiation signals recognized by ribosomes in one class of chloroplast mRNAs. In the accompanying paper (44), the effects of secondary structural

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1 The abbreviation used is: IF-2chl, chloroplast initiation factor 2.

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elements on the recognition of this translational start site are explored.

EXPERIMENTAL PROCEDURES

Materials—General chemicals were purchased from Sigma. RNasin, HindIII, T4 DNA polymerase, and T7 RNA polymerase were purchased from Promega Biotec. The Sequenase™ version 2.0 kit, T4 polynucleotide kinase, calf intestine alkaline phosphatase, T7 DNA polymerase, and helper phage M13KO7 were obtained from U. S. Biochemical Corp. Deoxynucleobisulphate triphosphates and ribonucleotide triphosphates were from Pharmacia LKB Biotechnology Inc. α-32P-dATP (1200 C/ mmol) and (5,6-3H)UTP (43.3 Ci/mmol) were obtained from DuPont NEN. Type HA 0.45-μm nitrocellulose filter paper was purchased from Millipore Corp. The GeneClean™ DNA purification kit was from BIO 101, Inc. Yeast tRNA was from Boehringer Mannheim. Synthetic oligonucleotide primers for sequencing and mutagenic reactions (see Table I) were prepared in the Department of Pathology and of Microbiology and Immunology and at the Lineberger Cancer Center of the University of North Carolina at Chapel Hill.

Plasmid Preparation and Transcription—The preparation of the plasmid pRbcN has been described previously (13). A derivative of this construct (pRbcN X2) has had two XbaI sites introduced upstream of the start site of the RbcN open reading frame (13). A derivative of pRbcN X2 was prepared in which a large portion of the 5'-untranslated leader sequence was present as the reverse complement. Digestion of pRbcN X2 with XbaI released a 48-base pair fragment from the 5'-untranslated leader region. The 48-base pair fragment and the remaining large fragment of the plasmid were separated by electrophoresis on a 2% agarose gel. The large fragment was extracted from the gel using Whatman DE81-cellulose paper (16) and was ligated with the large fragment, which had been treated with calf intestine alkaline phosphatase (17). The ligation mixture was used to transform E. coli JM101 (18, 19). A derivative (pRbcN Xp) in which the 48-base pair fragment had been inserted in the reverse orientation was identified by DNA sequencing.

In vitro mutagenesis of the AUG start codon of the rbcL gene was carried out using oligonucleotide I (Table I). During synthesis, this oligonucleotide was doped at the positions designated N with 30% of the wild-type nucleotide and 67% of an equimolar mixture of the other 3 nucleotides. Deletions of portions of the 5'-untranslated leader region of the rbcL gene were carried out using oligonucleotides 2-5 (Table I). The insertion of a Shine-Dalgarno sequence (GGGAG) into the 5'-untranslated leader region and a partial deletion derivative of this construct were prepared using oligonucleotides 6 and 7. Site-directed mutagenesis was carried out according to the method of KunkeI et al. (20, 21) and McClary et al. (22). Mutants of interest were identified by DNA sequencing.

Prior to transcription, plasmids were prepared as described (17), further purified by centrifugation in CaCl2 gradients, and linearized by digestion with HindIII. In vitro transcription was carried out as described previously (13).

Preparation of Formylmethionyl-tRNA, Ribosomal Subunits, and Initiation Factors—[35S]Formylmethionyl-tRNA was prepared from yeast tRNA as described (20). A high salt wash of E. coli ribosomes was prepared as described (24), concentrated by ammonium sulfate precipitation, and dialyzed against 50 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol before use. E. coli initiation factors were partially purified by chromatography on DEAE-cellulose and phosphocellulose (24). Chloroplast 30 S ribosomal subunits from E. gracilis were prepared as described previously (25). IF-2,3 purified through the TSKgel DEAE-5PW HPLC column stage (1) and IF-3,3 purified through the TSKgel SP-5PW HPLC column stage (5) were kindly provided by Dr. Lan Ma and Qiong Lin (Department of Chemistry, University of North Carolina at Chapel Hill).

Initiation Complex Formation—Reaction mixtures using a high salt wash of E. coli ribosomes as the source of initiation factors were prepared and analyzed as described previously (13), except that the concentration of NH4Cl was reduced to 40 mM. Reaction mixtures (50 μl) using the partially purified initiation factors were prepared as described (13) and contained 10 pmol of the indicated transcript, 1 unit of E. coli IF-1, and either E. coli initiation factors (0.12 unit of IF-2 and 0.3 unit of IF-3) or chloroplast initiation factors (0.3 unit of IF-2chl and 1 unit of IF-3chl). The units described here are as indicated previously (1, 5).

To test the ability of an oligonucleotide containing a Shine-Dalgarno sequence to inhibit initiation complex formation with various mRNAs, the indicated amount of the oligodeoxynucleotide dGGGAGUU, which is complementary to the sequence at the 3'-end of the chloroplast 16 S rRNA from E. gracilis, was hybridized to 0.06 μg of chloroplast 30 S subunits by incubation at 4°C for 16 h in 5 μl of buffer containing 25 mM Tris-HCl, pH 7.8, 50 mM NaCl, 1 mM EDTA, 10 mM MgCl2, 1 mM spermidine, and 20% glycerol. Following this incubation, the 30 S subunits were tested for the ability to participate in initiation complex formation as described (13) in reaction mixtures containing 5 pmol of the indicated mRNA.

Measurement of Off Rate of Translational Initiation Complexes—Reaction mixtures (50 μl) containing 5 pmol of the indicated message, 0.06 μg of chloroplast 30 S subunits, and 6 pmol of [35S]Met-tRNA, were prepared as described previously (13). After incubation for 10 min at 37°C to allow initiation complex formation, 100 pmol of nonradioactive Met-tRNA, in 10 mM potassium succinate, pH 6.0, were added, giving a final volume of 70 μl. Incubation was continued at 37°C, and at the indicated times, the reaction was terminated by the addition of 1 ml of cold stop buffer (50 mM Tris-HCl, pH 7.8, 40 mM NaCl, and 10 mM MgCl2). The amount of [35S]Met-tRNA remaining associated with 30 S complexes was determined as described previously (13).

RESULTS

Selection of Correct Translational Start Codon in RbcN Construct—In our previous work (13), a clone was prepared carrying the 5'-untranslated leader and exon 1 of the E. gracilis rbcL gene fused to a portion of the neomycin resistance gene under the control of the T7 transcriptional promoter. The mRNA transcribed from this vector (mRbcN) contains the entire 55-base untranslated leader of the rbcL gene (Fig. 1) and is identical to the in vivo RNA except for the presence of a 5'-terminal G residue that facilitates transcription by the T7 polymerase (26). This mRNA does not contain a Shine-Dalgarno sequence upstream of the start codon, but does have a very AU-rich 5'-untranslated leader region (Fig. 1).

The RbcN mRNA directs the formation of chloroplast translational initiation complexes (Met-tRNA-[35S]Met-30 S) in the presence of either E. coli or chloroplast initiation factors (15). Transcripts having deletions of the entire leader through the known AUG start codon are inactive in initiation complex formation, suggesting that the correct start site of this mRNA is being used for initiation complex formation (13). To verify this conclusion, the AUG start codon was mutated using saturation mutagenesis. A number of mutations in the putative AUG start codon were obtained, and two mutants were selected for additional study. The first of these mutants had three changes leading to a UAU codon in place of the original AUG start codon

### Table 1

| Plasmid   | Oligonucleotide          |
|-----------|--------------------------|
| pRbcN UAU | GAC CTGCAGT ATTTAAATAT  |
| pRbcN GAC | AAAATNNNTC ACGTCAACTG  |
| pRbcN GAC | TTTTTAATT ATTTTTTTTT AAAAAATAT CTCCAC |
| pRbcN Δ28 | CGACTCAGTA TAGATATATA TTTTTTTTT TATTATATA |
| pRbcN Δ40 | CTCACTCAGAT GATTTTTT CG  |
| pRbcN Δ21.40 | AAAATATGTT TTTTTTTTT |
| pRbcN SD | CAGCTCAGTA GATTTTTTT GGGATAAAA TATGCACC |
| pRbcN SDΔ20 | GACTCAGTA GATTTTTTT TGGG |

**Plasmid**
- pRbcN UAU
- pRbcN GAC
- pRbcN GAC
- pRbcN Δ28
- pRbcN Δ40
- pRbcN Δ21.40
- pRbcN SD
- pRbcN SDΔ20

**Oligonucleotide**
- CTTTTAATT ATTTTTTTTT AAAAAATAT CTCCAC
- CGACTCAGTA TAGATATATA TTTTTTTTT TATTATATA
- CTCACTCAGAT GATTTTTT CG
- AAAATATGTT TTTTTTTTT TT
- CAGCTCAGTA GATTTTTTT GGGATAAAA TATGCACC
- GACTCAGTA GATTTTTTT TGGG

**Remarks:**
- Table I lists the mutations that were tested for their ability to affect translation initiation complex formation.

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**Highlights:**
- The study investigates the role of the 5'-untranslated leader region in translation initiation.
- Various constructs were created to study the effects of mutations in this region.
- The results suggest that the correct start site of the mRNA is being used for translation initiation.
- The study employs a combination of in vitro and in vivo methodologies.

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**References:**
- For detailed citations, please refer to the original journal article.
(mRbcN UAU; Fig. 1). This message was tested for the ability to participate in initiation complex formation. As indicated in Fig. 2, mRbcN UAU is essentially inactive in this assay. This result strongly confirms the previous conclusion that initiation complex formation with chloroplast 30 S subunits and mRbcN is occurring at the correct translational start site (13). In addition, this result argues that the other 18 AUG codons present in mRbcN are unable to direct initiation complex formation with chloroplast 30 S subunits.

The second mutagenic construct tested also contained a 3-base change in the AUG codon, converting it to a GAC codon (mRbcN GAC; Fig. 1). This mutation created a new AUG codon just 2 nucleotides upstream of the original AUG codon. When mRbcN GAC was tested for the ability to promote initiation complex formation, it was 60% as efficient as the original mRbcN (Fig. 2). This result again argues that the AUG codon in the 5'-region of mRbcN is the codon selected for initiation complex formation by chloroplast 30 S ribosomal subunits. The somewhat reduced efficiency of mRbcN GAC compared with the wild-type mRNA probably reflects the different context immediately surrounding the start codon (AAUGA versus AUAGA). There are a number of cases in which the residues immediately surrounding the AUG codon affect the efficiency of initiation complex formation in bacterial systems (27, 28) and in the eukaryotic cytoplasm (29). Further mutagenesis of the nucleotides adjacent to the start codon will be required to examine the subtle effects of start codon context on the efficiency of initiation complex formation.

Effect of Length of Untranslated Leader on Initiation Complex Formation—In previous work (15), the 55-base 5'-untranslated leader of the RbcN construct was modified by mutagenesis to introduce two XbaI sites (mRbcN X2; Fig. 1). The mRNA transcribed from this construct is indistinguishable from the wild-type mRNA in assays of initiation complex formation (15). Deletion of the nucleotides between these XbaI sites in the leader results in the synthesis of a mRNA (mRbcN X49; Fig. 1) that contains only 10 nucleotides 5' to the normal start codon (9 nucleotides of the natural 5'-untranslated leader and a 5'-terminal G residue). This mRNA has little or no activity in initiation complex formation (Fig. 3) (15). There are two reasonable explanations for this observation. First, the 5'-untranslated leader may have essential sequence information that directs the chloroplast 30 S subunit to the AUG start codon. Second, there may be a minimal length in the 5'-region that is essential for ribosomal subunit binding. Since the 30 S subunit would be expected to cover 20 nucleotides upstream of the start codon, deletion of all but 10 nucleotides 5' to the initiation codon could result in a mRNA that cannot interact with the small subunit.

To examine the importance of the length and sequence of the 5'-untranslated leader of mRbcN, several additional deletion mutants were prepared. The first deletion mutation (mRbcN D20; Fig. 1) has the sequence from positions 55 to 21 deleted, leaving 20 nucleotides of the 5'-untranslated leader from the wild-type sequence and the additional G residue at the 5'-end. The mRbcN D20 mRNA has significantly reduced activity in initiation complex formation (Fig. 3). It promotes fMet-tRNA binding to 30 S subunits only about one-third as well as the normal mRNA. The second mutation (mRbcN D28; Fig. 1) has residues 55 to 29 deleted. This mRNA contains a 5'-untranslated leader with 28 residues from the wild-type sequence and the additional 5'-terminal G residue. This mRNA also shows a reduction in the ability to promote initiation complex formation (Fig. 3). Indeed, it is no more efficient than the mRNA with the 5'-untranslated leader that has 20 bases present. Another deletion mutation lacks residues 55 to 41 (mRbcN D40; Fig. 1). This mRNA has a 5'-leader containing 40 of the 55 residues of the wild-type sequence. However, it is only 30% as efficient as the full-length mRNA in promoting fMet-tRNA binding to 30 S subunits (Fig. 3). These observations suggest that the AUG start codon can be selected in the mRNA with only 20 residues
of the 5'-leader present. However, maximal activity in initiation complex formation requires the full 5'-untranslated leader.

There are two possible interpretations for the observations described above. First, it is possible that the inherent length of the 5'-untranslated leader in the mRNA (55 base pairs) is an important determinant for providing maximal efficiency of initiation. A second interpretation is that the sequences between positions -40 and -55 are important as a translational enhancer. To distinguish between these two possibilities, two internal deletion constructs were prepared. In the first, nucleotides between positions -1 and -25 were deleted, providing a 31-nucleotide 5'-untranslated leader carrying the residues originally between positions -40 and -55. The mRNA prepared from this construct (mRbcN Δ1,25; Fig. 1) has only ∼30% of the ability of the wild-type mRNA to participate in initiation complex formation (Fig. 4). The second mutant carries an internal deletion from positions -21 to -40 in the 5'-untranslated leader (mRbcN Δ21,40; Fig. 1). The mRNA prepared from this deletion mutant contains a 35-residue 5'-untranslated leader from the rbcN message. This mRNA also shows a significant reduction in its ability to participate in initiation complex formation (mRbcN Δ21,40; Fig. 4). Thus, internal deletions in the 5'-untranslated leader cause a reduction in activity similar to that caused by the 5'-end deletions (Fig. 3). These results argue that the length of the 5'-untranslated leader is important for maximal activity of mRbcN in initiation.

Role of Primary Sequence of 5'-Untranslated Leader in Initiation—The results summarized above indicate that the full length of the 55-base leader of mRbcN is essential for maximal activity in initiation complex formation. However, these results do not clearly indicate whether there is essential sequence information in the 5'-untranslated leader that strongly influences the initiation process. Rather, the effects of the deletions summarized above appear to arise from changes in the length of the mRNA itself. To address the question of whether there are elements of essential sequence information in the leader, cassette mutagenesis was carried out to prepare a new mutant using the mRbcN X2 construct (Fig. 1) as the starting plasmid. The presence of the XbaI sites (positions -57 to -52 and -9 to -4) allowed the excision of a 48-base pair segment from the plasmid and its insertion in the opposite orientation (mRbcN XF; Fig. 1). Transcription of this plasmid results in the synthesis of a mRNA with a 5'-leader region that is the inverse complement of the mRbcN X2 sequence between positions -9 and -56. The 5'-untranslated leader from this construct has base pair changes at 24 out of 58 positions scattered throughout the leader region (underlined in Fig. 1) and has about the same percent A/U found in mRbcN X2. Interestingly, mRbcN XF is ∼60% as active as mRbcN when tested for the ability to participate in initiation complex formation (Fig. 5). These results suggest that the primary sequence of the 5'-untranslated leader region is not the major determinant in specifying a particular AUG codon as a translational start site.

Effect of Shine-Dalgarno Sequence in 5'-Untranslated Leader—As noted above, chloroplast mRNAs in E. gracilis fall into two major classes. The first class of mRNAs appears to have a Shine-Dalgarno sequence comparable to that observed in prokaryotic systems. The second class of mRNAs, including mRbcN, does not have a recognizable Shine-Dalgarno sequence. It was of interest to determine the effect of a Shine-Dalgarno sequence on the ability of mRbcN to promote initiation complex formation. To examine this question, oligonucleotide-directed mutagenesis was used to replace a short stretch of nucleotides between positions -7 and -11 with the strong E. gracilis chloroplast Shine-Dalgarno sequence (GGGAG). The transcript designated mRbcN SD (Fig. 1) was then prepared and tested for its ability to direct translation initiation. As indicated in Fig. 6, the presence of the Shine-Dalgarno sequence 5' to the AUG start codon enhances the ability of the mRNA to promote initiation complex formation ∼1.4-fold. This prokaryote-like initiation signal is thus only modestly helpful to the mRNA in enhancing initiation. This result is in stark contrast to the critical role played by the Shine-Dalgarno sequence in prokaryotes, in which it generally enhances initiation by 10-100-fold.

To determine whether the presence of a Shine-Dalgarno sequence could offset the effect of a reduction in the length of the 5'-untranslated leader, a deletion of this construct was prepared that would direct the synthesis of a mRNA containing 20 nucleotides 5' to the start codon (mRbcN SDA20; Fig. 1). The Shine-Dalgarno sequence is still positioned between positions -7 and -11 in this transcript. As indicated in Fig. 6, the deletion derivative is ∼3-fold less effective in initiation complex formation compared with the full-length mRNA carrying a Shine-Dalgarno sequence. This reduction in activity is comparable to that observed with the normal RbcN mRNA and its deletion derivatives (Figs. 3 and 6). These results indicate that a long 5'-untranslated leader region is probably important for maximal efficiency in initiation whether or not a chloroplast mRNA contains a Shine-Dalgarno sequence.

Effect of Oligodeoxynucleotide Competition on Initiation
Complex Formation—In prokaryotes, the Shine-Dalgarno sequence in the mRNA hydrogen-bonds to the polypyrrimidine sequence located near the 3′-end of the 16 S rRNA. This interaction places certain constraints on the path of the mRNA along the small ribosomal subunit in the initiation complex. It was of interest to ask whether a mRNA that does not contain a Shine-Dalgarno sequence binds to the 30 S subunit in the same way as a mRNA having a Shine-Dalgarno sequence. In an effort to examine this question, the effect of an oligodeoxynucleotide that can bind to the polypyrrimidine sequence at the 3′-end of the small subunit rRNA on the ability of mRbcN to form initiation complexes was tested. This oligodeoxynucleotide contains a GGGAG sequence that can hydrogen-bond to the 3′-end of the E. gracilis chloroplast 16 S rRNA. Chloroplast 30 S ribosomal subunits were incubated overnight with increasing concentrations of the oligodeoxynucleotide to allow it to bind to the small subunit. The 30 S subunit-oligodeoxynucleotide complexes were then tested for their ability to form initiation complexes with mRbcN. Small subunits incubated under these same conditions overnight in the absence of the oligodeoxynucleotide retained >80% of their activity. As indicated in Fig. 7, a 100-fold excess of the Shine-Dalgarno oligodeoxynucleotide over 30 S subunits has almost no effect on the formation of initiation complexes with mRbcN. Preincubation of 30 S subunits with a 500-fold excess of the oligodeoxynucleotide results in a small reduction (<25%) in the ability of the 30 S subunits to participate in initiation complex formation with mRbcN. This observation suggests that this chloroplast mRNA can bind to the small subunit without any close interaction with the 3′-end of the 16 S rRNA. This conclusion is in agreement with the idea that there is no Watson-Crick hydrogen bonding between the rRNA and the 5′-untranslated leader region of chloroplast mRNAs that do not contain a Shine-Dalgarno sequence.

The effect of the oligodeoxynucleotide containing the Shine-Dalgarno sequence on the ability of mRbcN SD to form initiation complexes was also tested. This mRNA has a Shine-Dalgarno sequence equivalent to that present in the competing oligodeoxynucleotide. As indicated in Fig. 7, mRbcN SD is somewhat more efficient in initiation complex formation compared with the starting mRbcN message. In the presence of a 100-fold excess of the competing oligodeoxynucleotide, the ability of the Shine-Dalgarno sequence-containing mRNA is reduced essentially to the level observed with mRbcN. At higher concentrations of the competing oligodeoxynucleotide, mRbcN and mRbcN SD behave identically. These results suggest that the small enhancement in the efficiency of initiation complex formation with the Shine-Dalgarno sequence-containing mRNA probably arises from a direct hydrogen bonding between the small subunit rRNA and the Shine-Dalgarno sequence in the mRNA. The oligodeoxynucleotide appears to be able to compete with this rRNA-mRNA interaction, leaving mRbcN SD with an ability to form initiation complexes equivalent to that observed with the wild-type leader. These data again suggest that while mRbcN SD may take advantage of the Shine-Dalgarno sequence to increase the efficiency of initiation slightly, the leader of this mRNA has an intrinsic ability to direct initiation in the absence of any classical rRNA-mRNA hydrogen bonding. It should be noted that Canancano et al. (30) have observed that an oligodeoxynucleotide carrying a prokaryotic Shine-Dalgarno sequence inhibits the binding of an artificial message carrying a Shine-Dalgarno sequence to E. coli 30 S subunits more strongly than it inhibits the interaction of a corresponding mRNA lacking a Shine-Dalgarno sequence. Thus, the results obtained here are quite compatible with those observed in the bacterial system.

Stability of Initiation Complexes Formed with Various mRNAs—The data provided above suggest that the 5′-untranslated leader of mRbcN contains essential information that directs the 30 S ribosomal subunit to the AUG start codon. The data also argue that a section of the leader >20 nucleotides is important for maximal initiation and that the addition of a Shine-Dalgarno sequence has a small enhancing effect on the ability of this mRNA to participate in initiation. There are several possible stages during initiation complex formation in which variations in the 5′-leader sequence might influence the initiation process. The formation of the initiation complex occurs by a rather complicated sequence of events, many of which may be important for efficient initiation (8). Changes in the 5′-untranslated leader might also lead to initiation complexes that are inherently less stable, resulting in a more rapid rate of dissociation. In an effort to determine whether the differences in the amount of initiation complex observed with several of the mRNAs used here resulted from changes in the rate of formation of the complexes, efforts were made to examine the time courses for initiation complex formation with a number of the mRNAs. However, the amount of initiation complex observed in every case had reached a maximal value within 2 min after assembly of the reaction mixtures and remained constant for ~20 min. Since the 2-min time point was the earliest one that could be measured accurately, potential changes in the rate of initiation complex formation could not be measured directly.
with the various mRNAs available. The data obtained indicate that the measurements reported here represent equilibrium values. Differences in the amount of initiation complexes observed with the various mRNAs probably are related to the equilibrium constants governing the formation of the various complexes.

Although it was not practical to obtain a direct measure of the forward rate constants for initiation complex formation, it was possible to gain some insight into the stability of the various complexes as measured by the off rate of the bound fMet-tRNA. To observe the off rate, 30 S initiation complexes were assembled with the appropriate mRNA and labeled fMet-tRNA. The reaction mixtures were then diluted in the presence of unlabeled fMet-tRNA, and the amount of labeled complex remaining as a function of time was measured. The unlabeled fMet-tRNA was added in a 17-fold molar excess to mask the unlabeled competitor (Fig. 8). With this mRNA, nearly half of the original complex is dissociated within 5 min, and <10% remains after 10 min of incubation. The stability of the complex formed with the mRbcN Δ20 deletion mutant is essentially the same as that formed with the natural mRNA. This observation suggests that it is the formation of the initiation complex rather than its dissociation that controls the amount of complex present at equilibrium. A similar analysis was carried out with mRbcN SD (Fig. 8). Again, the off rate observed is essentially identical to that found with mRbcN, indicating that the somewhat enhanced initiation complex formation observed with this mRNA probably arises from changes in the rate of formation of the complex rather than from a slower rate of dissociation of the complex once assembled.

**Role of Chloroplast Initiation Factors in Initiation Complex Formation**—The experiments described above were performed using chloroplast 30 S ribosomal subunits and initiation factors from *E. coli*. In general, it is believed that the recognition of the translational start site is carried out by the small subunit of the ribosome rather than by the initiation factors in prokaryote-type systems (7, 8). To make sure that the observations described above were not significantly affected by the use of the bacterial initiation factors, several of the constructs were tested for activity in initiation complex formation using IF-2 and IF-3 of *E. coli* rather than the crude *E. coli* initiation factor preparation. As indicated in Table II, initiation complex formation could be observed using the chloroplast initiation factors and mRbcN. When mRbcN SD was tested in this assay, an ~2-fold enhancement in activity was observed compared to that obtained with mRbcN (Table II). This result is essentially the same as that observed with the purified *E. coli* factors. Finally, the deletion derivatives mRbcN Δ20 and mRbcN SDΔ20 were tested and showed about the same -fold reduction in activity as observed in the previous experiments (Fig. 6 and Table II). These observations indicate that the results obtained with the bacterial factors are a direct reflection of the ability of the chloroplast translational system to use the mRNA derivatives tested here.

**DISCUSSION**

In *E. coli*, several features of the mRNA appear to play an important role in translational initiation. The first and most striking feature is the Shine-Dalgarno sequence. Mutations in this region have a drastic effect on the efficiency of initiation (31–33). In addition, statistical analysis indicates that nucleotides -20 to +13 are not random, suggesting that this whole region has a modulating effect on the efficiency of initiation (32, 34, 35). Finally, it is clear that secondary structural features can play a critical role in initiation. Start sites with little or no secondary structure are generally much more efficient than those in which the Shine-Dalgarno sequence or AUG codon is base-paired (36, 37).

In this work, a number of mutants were constructed to delineate the region of the 5'-untranslated leader in the *E. gracilis* chloroplast *rbcL* mRNA that is important for specifying the AUG start codon. This mRNA does not have a Shine-Dalgarno sequence and hence cannot use the major bacterial determinant for specifying the start codon. The start site of the *rbcL* mRNA is probably specified by several features, all of which contribute to the selection process. One component that appears to be important is a 5'-untranslated leader of at least 20 nucleotides. This observation is quite reasonable considering data showing that the 30 S ribosomal subunit probably covers ~20 residues 5' to the start codon (38). These 20 residues do not have to represent immediate upstream sequences since an internal deletion mutant shows almost the same activity as 5'-end deletion mutants. The results presented here indicate that maximal initiation complex formation is dependent on the presence of the full-length 55-base leader sequence. This chloroplast mRNA may thus have two areas through which it contacts the 30 S subunit: one near the AUG codon and the other involving more distal regions of the mRNA. This observation is compatible with those obtained in prokaryotes, where it is believed that regions of the mRNA beyond the ribosome-binding site contribute to the interaction of the mRNA with the 30 S subunit (31, 36, 39). For example, Borisova et al. (40) observed that 53 nucleotides upstream of the AUG codon are required for initiation complex formation with the replicase cistron of MS2. It has been suggested (31) that during initiation, the 30 S subunit interacts with a larger region of the mRNA than is protected in the final complex formed. The ribosome-binding site is thus only a part of the true initiation region (41).

Details are not yet available on all of the precise contacts between the prokaryotic 30 S subunit and mRNA. However, it

![Fig. 8. Rate of dissociation of initiation complexes formed with various mRNAs. Initiation complexes were formed as described under "Experimental Procedures" using 5 \( \mu \)mol of mRbcN ( ), mRbcN SD ( ), or mRbcN Δ20 ( ). After formation of the complexes, unlabeled fMet-tRNA was added, and the amount of \( ^{35}S \)-fMet-tRNA remaining in 30 S initiation complexes was determined at the indicated times.](image-url)
has been suggested that the mRNA interacts with the 30 S subunit via a U-shaped trough or channel (42). It is likely that this channel is formed from parts of both the rRNA and the ribosomal proteins. In E. coli, at least two proteins (S1 and S21) along with several stretches of the rRNA have been implicated in facilitating the binding of the mRNA to the 30 S subunit. Recent evidence (42) indicates that the mRNA does not fill the entire channel, but, depending on its specific sequence and structure, makes contacts with groups lining the channel.

The analysis of the translational start site of the rbcL mRNA presented here argues that the leader region does not contain a specific sequence that is absolutely required for determining the start site of translation. For the E. gracilis chloroplast 30 S subunit, the major interactions between the mRNA and the subunit may involve the ribose phosphate backbone of the mRNA rather than the bases themselves. Thus, the overall length and structure (or lack of structure) of this region may be the essential features present. An analysis of the effects of structural elements in the mRNA on initiation is presented in the following paper (44).

Finally, it should be noted that the insertion of a strong Shine-Dalgarno sequence 5' to the start codon leads to only a modest improvement in the ability of the RbcN mRNA to participate in initiation complex formation. This result is quite different from E. coli messages, which show a much stronger requirement for the presence of a Shine-Dalgarno sequence in the mRNA (7). It is also compatible with the presence of two classes of mRNAs in E. gracilis chloroplasts, some having Shine-Dalgarno sequences and others lacking this sequence. The observation that the competing Shine-Dalgarno oligodeoxynucleotide overcame the slight advantage in initiation observed with the rRbcN SD argues that when a Shine-Dalgarno sequence is present, it probably results in a slightly altered placement of the mRNA in the RNA-binding trough on the 30 S subunit. This idea is compatible with the proposal that the RNA-binding channel is rather wide. Certain messenger RNAs may sit in this trough in a slightly different way than do other mRNAs (43).

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