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Depletion of Free 30S Ribosomal Subunits in *Escherichia coli* by Expression of RNA Containing Shine-Dalgarno-Like Sequences

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We have constructed synthetic coding sequences for the expression of poly(α,L-glutamic acid) (PLGA) as fusion proteins with dihydrofolate reductase (DHFR) in *Escherichia coli*. These PLGA coding sequences use both GAA and GAG codons for glutamic acid and contain sequence elements (5′-GAGGAGG-3′) that resemble the consensus Shine-Dalgarno (SD) sequence found at translation initiation sites in bacterial mRNAs. An unusual feature of DHFR-PLGA expression is that accumulation of the protein is inversely related to the level of induction of its mRNA. Cellular protein synthesis was inhibited >95% by induction of constructs for either translatable or untranslatable PLGA RNAs. Induction of PLGA RNA resulted in the depletion of free 30S ribosomal subunits and the appearance of new complexes in the polyribosome region of the gradient. Unlike normal polyribosomes, these complexes were resistant to breakdown in the presence of puromycin. The novel complexes contained 16S rRNA, 23S rRNA, and PLGA RNA. We conclude that multiple noninitiator SD-like sequences in the PLGA RNA inhibit cellular protein synthesis by sequestering 30S small ribosomal subunits and 70S ribosomes in nonfunctional complexes on the PLGA mRNA.

Escherichia coli ribosomes initiate translation on most mRNAs by binding to Shine-Dalgarno (SD) sequences located approximately 6 to 9 nucleotides upstream of the correct initiation codon. The SD sequence is purine rich and is required in most cases for small ribosomal subunit (30S) binding to the mRNA. This binding occurs through base pairing with the anti-Shine-Dalgarno (anti-SD) sequence located in the 3′ end of the 16S rRNA. The minimal consensus SD sequence is GAGG or GGAG, but complementarity to the 16S rRNA can span up to 9 nucleotides to include UAAGGAGGU (5, 26, 27). Very few SD sequences have such high complementarity, so variation in the strength of the SD-16S rRNA interaction can be an important factor in determining the efficiency of translational initiation at start codons. For example, the SD sequence UAAGGAGG is four times more efficient in translational initiation than the shorter sequence AAGGA (22).

Several lines of evidence indicate that SD-like sequences that lack associated cognate initiation codons have an inhibitory effect on translation. Ribosomes interact directly with such noninitiator SD-like sequences in Qβ RNA in vitro (31), and oligonucleotides with strong complementarity to the anti-SD sequence in 16S rRNA inhibit fMet-tRNA^{Metf} -dependent formation of 70S ribosome initiation complexes with mRNA in vitro (9, 32). Furthermore, oligonucleotide analogues that mimic the SD sequence inhibit translation in cell extracts and also in vivo in permeable strains of *E. coli* (11, 18). Clusters of the rare AGG and AGA arginine codons resemble the SD consensus sequence, and it is well documented that overexpression of RNAs containing these sequences inhibits translation and cell growth (35). However, it is not clear whether this inhibition is due to competition with functional SD sequences in mRNA for binding to 30S subunits or to depletion of rare arginine-tRNAs or both. Ivanov et al. (16) showed that repeats of AGG codons inhibit translation even when the AGG clusters are inserted out of frame or outside of the coding sequence, suggesting that inhibition is due to the SD-like nature of the sequences. Indeed, AGGAGG can functionally replace a natural SD sequence in mRNA (1).

Other evidence indicates that inhibition by consecutive AGG triplets is related to their function as arginine codons. Inhibition is most pronounced when the AGG cluster is in the AGG reading frame and near the 5′ end of the coding sequence (23). Interestingly, this inhibitory effect is diminished or even abolished when the AGG codons are located in the middle of the gene (6) and is largely reversed by overexpression of tRNA$^{Arg/UCU}$ (7, 35, 36). These findings point to depletion of rare arginine-tRNAs by clusters of "hungry Arg codons" as the major cause of inhibition.

We previously constructed an artificial multimeric DNA coding sequence for the biological synthesis of poly(α,L-glutamic acid) (PLGA) in *E. coli* using the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible pQE15 expression system (33, 34, 37). The PLGA sequence was expressed as a tripartite fusion protein with an N-terminal His tag followed by 186 amino acids from the sequence for mouse dihydrofolate reductase (DHFR) and the PLGA sequence at the C terminus. The desired protein was produced on induction; however, yields were much lower than those obtained by us with many other artificial proteins composed of periodic repeat sequences.

There are two codons for glutamic acid, GAA and GAG, and the PLGA sequence contained both codons in proportion to their usage in highly expressed genes in *E. coli.* However, a coding sequence for PLGA that uses both Glu codons will

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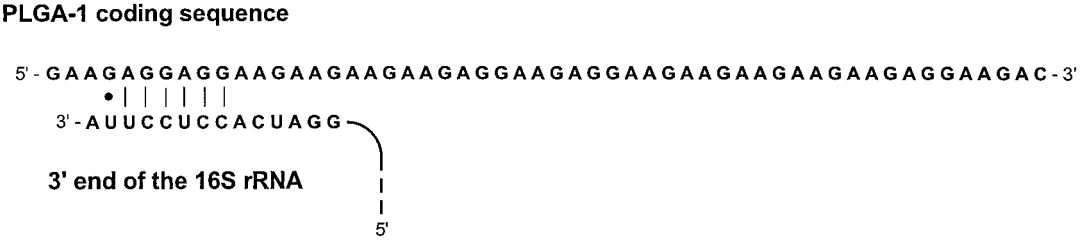


FIG. 1. Alignment between 16S rRNA and a strong noninitiator SD-like sequence PLGA RNA. The synthetic PLGA coding sequences used in our studies contain multiple regions capable of annealing with the anti-SD sequence in 16S rRNA. The most stable potential interaction is the 7-bp SD–anti-SD duplex shown. The specific PLGA coding sequence that we have analyzed in this study contains four tandem repeats of the sequence 5′-CGAAGAGGAGGAAGAAGAAGAAGAGGAAGAGGAAGAAGAAGAAGAAGAGGAAGA-3′, and therefore the PLGA RNA contains four copies of the 7-nucleotide SD-like sequence (underlined).

contain one or more SD-like elements, and these might interfere with expression. Indeed, the synthetic PLGA coding sequences used in our studies contain multiple regions capable of forming up to 7-bp duplexes with the anti-SD sequence in 16S rRNA; the stable 7-bp SD–anti-SD duplex is shown in Fig. 1. In addition to complicating PLGA expression, this situation has the potential to cause broader interference effects. Induction of the PLGA mRNA could interfere with translation of all cellular proteins by sequestering small ribosomal subunits on these SD-like sequences and perhaps also initiation factors and fMet-tRNA^{Metf}. However, the inhibitory effect associated with similar SD-like sequences formed by AGG clusters was moderated when they were located 76 or more codons downstream from the translation initiation site (6). This led us to anticipate that the inhibitory potential of the PLGA coding sequence might also be minimized by expressing it in the context of a gene fusion. To the contrary, several observations indicated that expression of the DHFR-PLGA fused gene is detrimental to the cell.

First, the growth rate of pQE15-PLGA$_x$-transformed *E. coli* cells (SG13009/pREP4) and the yield of the recombinant DHFR-PLGA fusion protein decreased as the number of $Glu_{17}Asp$ repeat units was increased incrementally from 3 to 6 (33). Second, the yield of DHFR-PLGA protein decreased as the level of IPTG induction was increased. Indeed, the highest yield of the DHFR-PLGA protein was obtained from cells grown to late stationary phase without IPTG induction (21). Further tests indicated that these effects were not due to toxicity of the PLGA protein, instability of the expression plasmid or the PLGA protein, or inefficient transcription of the target gene (V. Conticello, unpublished results). Finally, overexpression of tRNA$^{Glu/UUC}$ alone or in combination with glutamyl-tRNA synthetase did not significantly increase the accumulation of the DHFR-PLGA protein (21). Taken together, these preliminary results argue that the effects of DHFR-PLGA expression on cell growth and protein yield are most likely related to the cellular concentration of inhibitory elements in the PLGA coding sequence itself. Thus, increasing either the level of DHFR-PLGA transcription or the length of the multimeric PLGA coding sequence or both would exacerbate the inhibition. We hypothesize that the inhibitory elements are the SD-like sequences in PLGA RNA.

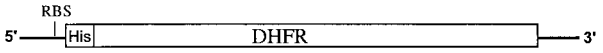
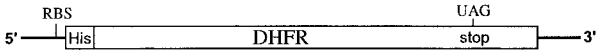
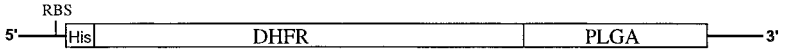
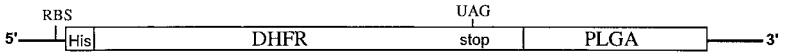
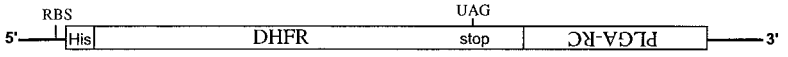
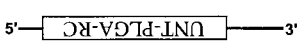
In this paper we present evidence that overproduction of untranslatable forms of PLGA RNA causes strong inhibition of cell growth and overall protein synthesis, apparently by sequestering ribosomal subunits in stable, nonfunctional complexes on the PLGA transcripts. These complexes resemble polyribosomes in that they contain both 16S and 23S rRNAs and sediment in the polyribosome region of sucrose gradients. However, unlike normal polyribosomes, these ribosome-PLGA RNA complexes are not dissociated by puromycin treatment. The puromycin-resistant complexes separate into ribosomal subunits during centrifugation in sucrose gradients containing small amounts of Mg^{2+}, although complexes between 30S subunits and PLGA RNA persist. Formation of stable but nonfunctional 70S ribosomes on SD-like sequences in the PLGA RNA indicates that subunit joining can occur efficiently in vivo in the absence of a cognate initiation codon and without fMet-tRNA^{Metf} bound in a puromycin-reactive site. These results have implications for understanding bacterial translation initiation and the poor translatability of mRNAs that contain noninitiator SD-like sequences.

MATERIALS AND METHODS

Bacterial strains and plasmids. For expression studies, strain BL21 [F$^-$ *ompT* *hsdS*$_B$ (r_B^- m_B^-) *gal dcm*], an *E. coli* B strain, was transformed with pREP4 (Qiagen), a low-copy-number plasmid that confers kanamycin resistance on the host strain and constitutively expresses the *lacI*^{q} repressor. Strain Top10F′ [F′ {*lacI*^{q} Tn*10*(Tet^r)} *mcr*A Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*Δ*M15* Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL*(Str^r) *endA1 nupG*] was similarly transformed with pREP4 and used as a strain for cloning plasmid constructs. Plasmid pQE15-PLGA$_4$ is a derivative of pQE15 (Qiagen), a low-copy-number plasmid that confers ampicillin resistance on the host strain. Plasmid pQE15-PLGA$_4$ carries the PLGA-4 coding sequence, which encodes four repeats of the $Glu_{17}Asp$ unit (33). (Note: Since PLGA-4 was the only PLGA sequence used in this study, PLGA will be used to designate PLGA-4). The PLGA sequence is expressed as a C-terminal fusion protein with mouse DHFR and an N-terminal His tag. Transcription of the target gene in pQE15 is driven by the strong T5 promoter and regulated by a double *lac* operator sequence (4). General procedures for recombinant DNA techniques, transformation, plasmid isolation, and gel electrophoresis were performed as described (25) or according to instructions provided by the manufacturers.

Plasmids constructed in this study. The expression constructs are summarized in Table 1. Plasmids were constructed for expression of untranslatable PLGA RNAs. First, a short DNA linker containing the amber stop codon in all three reading frames (5′ CTAGCTAGCTAG 3′) was inserted at the *Eco*NI site of pQE15 to form pQE15-stop. Since the *Eco*NI site is located 56 bp upstream from the 3′ end of the DHFR coding sequence in pQE15, the pQE15-stop construct expresses a truncated form of DHFR (DHFR-T), and sequences inserted immediately downstream from the stop codon linker will be transcribed upon induction with IPTG but the downstream RNA will not be translatable without a new translation initiation site. The ~250-bp *Bam*HI fragment containing the PLGA coding sequence from pET14xc (V. Conticello, unpublished results) was inserted at the *Bgl*II site in the polylinker region of pQE15-stop and transformed into Top10F′(pREP4). Plasmids with the PLGA *Bam*HI fragment inserted in

TABLE 1. Constructs prepared for this study[a]

Plasmid	Transcript	Protein (kDa)
pQE15		His-DHFR (24.0)
pQE15-stop		His-DHFR-T (20.8)
pQE15-PLGA		His-DHFR-PLGA (33.5)
pQE15-stop-PLGA		His-DHFR-T (20.8)
pQE15-stop-PLGA-RC		His-DHFR-T (20.8)
pQE15-UNT		n/a
pQE15-UNT-RC		n/a

[a] His, 6× His tag; RC, reverse complement; UNT, untranslatable; T, truncated; n/a, not applicable; RBS, ribosome-binding site.

both orientations were verified by restriction digest analysis. The plasmid pQE15-stop-PLGA had the PLGA coding sequence in the correct 5′−3′ orientation but downstream from the stop codon linker and is out of frame with respect to the upstream DHFR open reading frame. The construct in pQE15-stop-PLGA-RC (reverse complement) had the PLGA coding sequence inserted in the reverse orientation so that it would express the reverse complement of PLGA RNA. PLGA-RC RNA is CU rich and lacks any SD-like sequences. The plasmids were transformed into BL21(pREP4) for expression studies.

Plasmids were also constructed to express untranslatable PLGA and PLGA-RC RNAs but without the upstream DHFR coding sequence. To accomplish this, regions of the pQE15 plasmid were deleted in steps to remove the DHFR coding sequence and the flanking sequences necessary for translation. pQE15 was digested with *Bam*HI and *Bgl*II to release the entire DHFR coding sequence, leaving only the His tag coding sequence. The *Bam*HI fragment from pET14xc was inserted in both orientations between the *Bam*HI and *Bgl*II sites of pQE15, forming pQE15-Bam-PLGA and pQE15-Bam-PLGA-RC. Finally, a fragment containing the plasmid-encoded SD sequence, the ATG initiation codon, and the His tag coding sequence was deleted from pQE15-Bam-PLGA and pQE15-Bam-PLGA-RC by digesting plasmid DNA with *Eco*RI and *Bam*HI, filling in the 5′ overhangs with T4 DNA polymerase, and religating to form pQE15-UNT and pQE15-UNT-RC, respectively. These plasmids were transformed into BL21(pREP4) for expression studies.

The predicted sequence of the IPTG-inducible transcript from the UNT-PLGA construct in pQE15-UNT is 5′-AAUUGUGAGCGGAUAACAAUUUCACACAGAAUUGAUCCAUAUGGAAGAC(GAAGAGGAGGAAGAAGAAGAAGGAAGAGGAAGAAGAAGAAGAAGAGGAAGAC)$_4$GAAGAGUAAAUGCUCGAGGGAUCUUAAGCUUAAUUAGCUGAGCUUGGACUCCUGUUGAUAGAUCCAGUAAUGACCUCAGAACUCCAUCUGGAUUUGU-3′. The predicted sequence of the inducible transcript from the UNT-PLGA-RC construct in pQE15-UNT-RC is 5′-AAUUGUGAGCGGAUAACAAUUUCACACAGAAUUGAUCCUCGAGCAUUUACUCUUC(GUCUUCCUCUUCUUCUUCUUCCUCUUCCUCUUCUUCUUCCUCCUCUUC)$_4$GUCUUCCAUAUGGGAUCUUAAGCUUAAUUAGCUGAGCUUGGACUCCUGUUGAUAGAUCCAGUAAUGACCUCAGAACUCCAUCUGGAUUUGU-3′. The positions of the 3′ ends are based on the presence of the T_0 transcription terminator in pQE15. Although the UNT-PLGA RNA contains three AUG codons and one GUG and the UNT-PLGA-RC RNA contains two AUG codons and one GUG, none lies in a sequence context that resembles an efficient translation initiation site. Therefore, we consider these transcripts untranslatable.

Cell growth conditions. Cultures were grown at 37°C. Luria-Bertani (LB) medium plus ampicillin (100 μg/ml) and kanamycin (30 μg/ml) was used for most experiments. M9 medium plus 0.4% glucose and antibiotics was supplemented with either 0.25% Casamino Acids (CAA) or 0.1% 19-amino-acid mixture (minus methionine) and used for pulse-labeling experiments. For solid medium, agar was added to a final concentration ranging from 1.2 to 1.5%. LB agar containing 0.05 or 0.1 mM IPTG was used for studies of colony growth under inducing conditions.

In vivo protein synthesis. In vivo labeling of proteins with [^{35}S]methionine was carried out essentially as described by Zahn (35). Cells were inoculated from a colony growing on LB agar into M9 medium supplemented with 0.4% glucose and 0.25% CAA. Cultures were grown to a density of approximately 0.4 A_{600} and induced for target protein expression by addition of 0.05 to 1 mM IPTG. After 15 min, equal volumes of cells (1 ml) were harvested by brief centrifugation and resuspended in M9 medium plus 0.4% glucose and 0.1% 19-amino-acid mixture minus methionine. Labeling was carried out at 37°C for 5 min after the addition of 10 μCi of L-[^{35}S]methionine (>1,000 Ci/mmol; ICN) per ml. Labeling was stopped by addition of chloramphenicol to a final concentration of 2.5 mg/ml. Samples were placed on ice for 5 to 15 min, harvested by brief centrifugation, and frozen at −20°C overnight. To prepare cell lysates, the samples were thawed, resuspended in 50 μl of 0.1% sodium dodecyl sulfate (SDS), and incubated in a boiling-water bath for 10 min. The polypeptides in 15 μl of each sample were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the gel was stained with Coomassie brilliant blue, dried onto Whatman 3MM paper, and exposed to X-ray film for 1 to 7 days at −70°C.

Incorporation of [^{35}S]methionine into total cellular protein was determined by acid precipitation. Equal aliquots (4 μl) of the lysates from pulse-labeled cells were spotted onto Whatman 3MM paper and air dried. The filters were washed successively for 20 min each in cold 10% trichloroacetic acid (TCA), 5% TCA, and 100% ethanol. After air-drying, the filter paper was placed in a vial containing BioFluor scintillation fluid (NEN), and radioactivity was measured in a Beckman scintillation counter.

Sucrose gradient centrifugation of ribosomes. *E. coli* ribosomes were isolated and analyzed by sucrose gradient centrifugation as described (2, 19), with some modifications. Cells from a colony growing on LB agar were inoculated into 100 ml of M9 medium supplemented with 0.4% glucose and 0.25% CAA and incubated at 37°C until the cell density reached 0.4 to 0.6 A_{600} unit. To induce transcription of the target gene, the culture was incubated for 15 min in the presence of 0.5 mM IPTG. Chloramphenicol (0.34 mg/ml) was added immediately before harvesting to stabilize polyribosomes. To dissociate polyribosomes, chloramphenicol was omitted, and puromycin (0.5 mg/ml) was added 2 min before harvesting (15). Cells were harvested by pouring the culture medium into a 250-ml centrifuge bottle containing 100 g of crushed ice, followed by centrifugation in a GSA rotor at 5,000 rpm for 10 min. The cell pellet was resuspended in 1 ml of buffer 1 (60 mM HEPES-KOH [pH 7.8 at 0°C], 6 mM $MgCl_2$, 30 mM NH_4Cl, 4 mM β-mercaptoethanol, 16% [wt/vol] sucrose) and the suspension was transferred to a 1.5-ml microcentrifuge tube. The cells were collected by centrifugation at 4,000 rpm for 4 min in a microcentrifuge and resuspended in fresh buffer 1 (1 ml). The cells were lysed by addition of 15 μl of lysozyme (50 mg/ml in H_2O) and incubation on ice for 30 min. The sample was flash-frozen in liquid nitrogen, thawed in a 4°C H_2O bath, and stored overnight at −70°C. On the

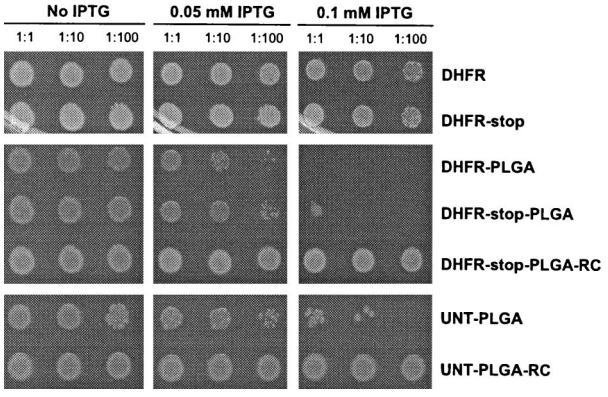


FIG. 2. Growth of PLGA-overproducing strains on IPTG-containing medium. Serial dilutions of BL21(pREP4) cells harboring pQE15 expression plasmids that specify the recombinant fusion proteins listed on the right were spotted onto LB agar containing 0, 0.05, or 0.1 mM IPTG. Cell suspensions were normalized to an A_{600} of 0.25, and 10-μl aliquots of the 1:1, 1:10, and 1:100 dilutions were spotted onto the plates. The plates were incubated at 37°C for 16 h.

following day, the sample was thawed in a 4°C water bath, and the cell debris was removed by centrifugation for 30 min at 4°C.

The concentration of RNA in the sample was estimated spectrophotometrically at 260 nm. An aliquot containing 10 to 20 A_{260} units of UV-absorbing material was diluted at least twofold into buffer 2 (60 mM HEPES-KOH [pH 7.8 at 0°C], 6 mM $MgCl_2$, 30 mM NH_4Cl, 4 mM β-mercaptoethanol) for loading onto a sucrose gradient. The sample (up to 2 ml) was layered on the top of a 10 to 40% (wt/vol) exponential sucrose gradient prepared in 20 mM HEPES-KOH (pH 7.5 at 0°C)–10 mM $MgCl_2$–150 mM NH_4Cl–4 mM β-mercaptoethanol. To dissociate ribosomes into subunits, the sucrose gradients contained 1 mM $MgCl_2$. The gradients were centrifuged in a Beckman SW27 rotor at 24,000 rpm for 6 h at 4°C. Gradient fractions (1 ml) were collected from the top of the gradient, and UV absorbance was monitored at 260 nm using a continuous-flow cuvette. The gradient fractions were stored at −20°C.

Hybridization analysis of RNA in sucrose gradients. DNA fragments encoding portions of the 16S rRNA, 23S rRNA, and PLGA RNA were isolated from plasmids and labeled with the BrightStar psoralen-biotin nonisotopic labeling kit (Ambion) as described by the manufacturer. Aliquots of the gradient fractions (10 to 30 μl) were diluted in 500 μl of RNA denaturing buffer containing 2.2 M formaldehyde, 50% (vol/vol) formamide, 10 mM MOPS (morpholinepropanesulfonic acid), 4 mM NaCl, and 0.5 mM EDTA (pH 7.0), heated at 65°C for 15 min, rapidly chilled in ice water, and adjusted to a final concentration of 10× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). (3). Equal volumes (100 μl) were spotted onto a prewetted GeneScreen Plus membrane (NEN Life Science) using a Bio-Dot microfiltration apparatus (Bio-Rad). Hybridization was carried out overnight at 65°C in 6.67× SSC–1× Denhardt's solution–10% dextran sulfate–1% SDS. Washing and detection were performed using the BrightStar BioDetect nonisotopic detection kit (Ambion). We have not shown that these hybridization conditions give a linear relationship between the intensity of the hybridization signals and the amount of each input RNA. Therefore, this dot blot hybridization analysis is intended to provide only qualitative information about the distribution of the three RNAs in the gradient fractions, not quantitative information about the relative amounts of the respective RNAs.

RESULTS

Inhibition of cell growth by the PLGA RNA. Cells harboring the DHFR-PLGA expression plasmids form small colonies on solid medium and grow slowly in liquid culture (33). We therefore asked whether expression plasmids containing either untranslatable PLGA or PLGA-RC (reverse complement) sequences would also confer this slow-growth phenotype. The PLGA-RC RNA is CU rich and lacks SD-like sequences. The results in Fig. 2 show that increasing the expression of PLGA-RNAs, either translatable (DHFR-PLGA) or untranslatable (DHFR-stop-PLGA and UNT-PLGA), progressively inhibited cell growth. There was no inhibition of growth in cells expressing the control RNAs (DHFR, DHFR-stop, DHFR-stop-PLGA-RC, or UNT-PLGA-RC). We conclude from these results that expression of mRNAs containing the PLGA coding sequence inhibits cell growth even when the PLGA sequence is untranslatable.

Translational blockage in cells expressing PLGA-containing RNAs. We performed pulse-labeling experiments to determine the effects of expression of the PLGA RNAs on cellular protein synthesis. Cells were incubated with 0, 0.1, 0.5, or 1.0 mM IPTG for 15 min and then pulse-labeled for 5 min with [^{35}S]methionine. The radiolabeled proteins in whole-cell extracts were analyzed by SDS-PAGE, followed by autoradiography of the dried gel. The results in Fig. 3A show that cellular protein synthesis is strongly inhibited by expression of either DHFR-PLGA or DHFR-stop-PLGA mRNAs but not by expression of either DHFR or DHFR-stop-PLGA-RC mRNAs. The radioactive bands corresponding to the DHFR-PLGA fusion protein and the truncated DHFR protein expressed from the DHFR-stop-PLGA construct are readily detectable in the samples from cells induced with 0.1 mM IPTG, but these bands are barely detectable in the samples from cells induced with 0.5 mM IPTG. By contrast, incorporation of [^{35}S]methionine into the proteins expressed from the DHFR and the DHFR-stop-PLGA-RC constructs increased dramatically as the concentration of IPTG was increased.

The effects of IPTG induction on the accumulation of the respective target proteins were similar to those seen in the pulse-labeling experiment. The intensity of the stained bands corresponding to DHFR-PLGA and DHFR-stop-PLGA polypeptides (indicated by arrows) decreased with increasing IPTG induction, and the opposite trend was observed for the DHFR and the DHFR-stop-PLGA-RC polypeptides (Fig. 3B). Note that the DHFR-PLGA and DHFR-stop-PLGA polypeptides are detectable by protein staining in the samples from cells induced with 0.5 and 1.0 mM IPTG even though these polypeptides showed very low incorporation of [^{35}S]methionine in the pulse-labeling experiment (Fig. 3A). A plausible explanation for this difference is that the material detected by staining was synthesized early in the induction period, when mRNA levels were low, and that synthesis of these polypeptides had ceased by the time [^{35}S]methionine was added after 15 min of induction.

To confirm that the PLGA coding sequence by itself inhibits translation, we examined protein synthesis in cells that had been induced to express UNT-PLGA transcripts, which lack the translatable DHFR sequence and a functional translation initiation site. Cells were induced and pulse-labeled as described for Fig. 3A except that the titration included additional concentrations of IPTG. For comparison, we repeated the pulse-labeling of cells expressing the DHFR-stop-PLGA mRNA. Inducing the expression of either UNT-PLGA RNA or DHFR-stop-PLGA mRNA caused nearly complete inhibition of cellular protein synthesis, whereas there was no such inhibition associated with induction of the UNT-PLGA-RC control RNA (Fig. 3C). As expected, there was no detectable translation product from the UNT-PLGA or UNT-PLGA-RC RNAs. Furthermore, incorporation of [^{35}S]methionine into acid-precipitable material was reduced by >95% when transcrip-

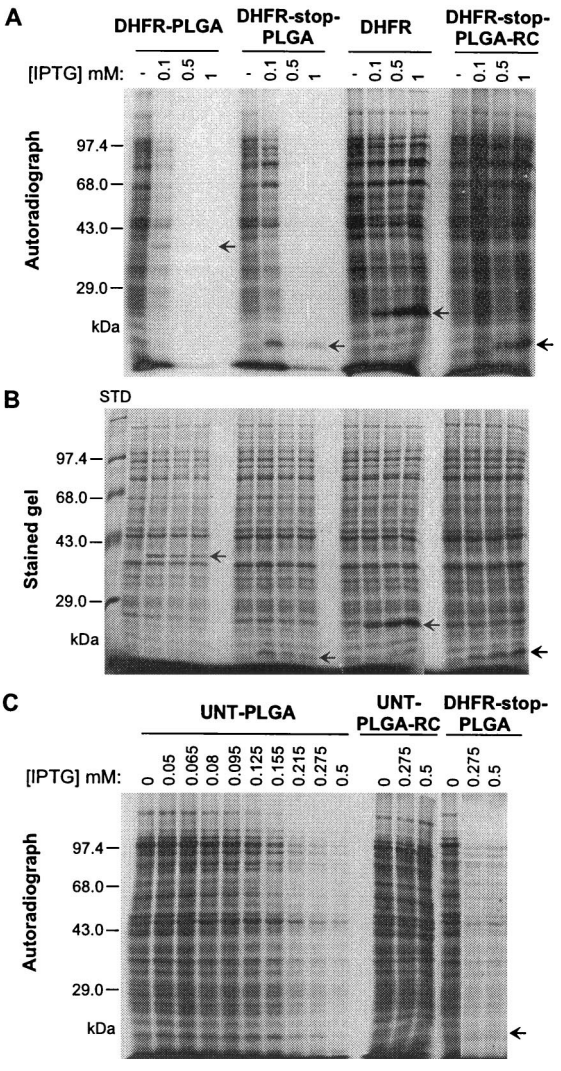


FIG. 3. Pulse-labeling of cells induced to express PLGA and PLGA-RC RNAs. BL21(pREP4) cells harboring either pQE15-PLGA, pQE15-stop-PLGA, pQE15, pQE15-stop-PLGA-RC, pQE15-UNT, or pQE15-UNT-RC (see Table 1) were incubated with 0 to 1.0 mM IPTG for 15 min and then pulse-labeled for 5 min with [^{35}S]methionine as described in Materials and Methods. The proteins in whole-cell extracts were analyzed by SDS-PAGE. (A and C) Autoradiographs of the dried gels; (B) gel stained with Coomassie brilliant blue. The RNA expressed in each sample is listed above the lanes in panels A and C, and the corresponding protein products are indicated by arrows. The positions and molecular masses (in kilodaltons) of polypeptide size standards are indicated on the left.

tion of PLGA-containing RNAs was induced with 0.5 mM IPTG (data not shown). These results are consistent with the notion that ribosomes interact directly with PLGA transcripts despite the lack of sequence elements required for efficient translation.

Sucrose gradient centrifugation of ribosomes from cells expressing PLGA RNAs. The possibility that ribosomes interact directly with untranslatable forms of PLGA RNA was examined by sucrose gradient centrifugation. Cells harboring the expression constructs for DHFR-PLGA, DHFR-stop-PLGA, and DHFR-stop-PLGA-RC were incubated for 15 min in the presence or absence of 0.5 mM IPTG, and then ribosomes were isolated and analyzed as described in Materials and Methods. The ribosomes from cells induced to express either DHFR-PLGA or DHFR-stop-PLGA mRNA had strikingly different sedimentation patterns in comparison to the ribosomes from uninduced cells and from cells induced to express the DHFR-stop-PLGA-RC mRNA (Fig. 4A). Specifically, expression of the PLGA RNAs caused a nearly complete disappearance of free 30S subunits, an increase in free 50S subunits, a decrease in 70S ribosomes, and the appearance of new peaks in the polyribosome region. These changes are consistent with binding of 30S subunits to the SD-like sequences in PLGA RNA, causing depletion of the pool of free 30S subunits. This would impair the formation of 70S ribosomes and increase the number of free 50S subunits. Each PLGA RNA molecule contains four SD-like elements spaced at 47-bp intervals. Since the footprint of the 70S initiation complex on mRNA is approximately 35 nucleotides (29), the PLGA RNA apparently can accommodate up to four 30S subunits. This offers a plausible explanation for the appearance of the new peaks in the polyribosome region of the gradients from cells expressing PLGA RNA.

To address more directly the interaction of ribosomes with the PLGA sequence, we examined the sedimentation properties of ribosomes from cells induced to express the UNT-PLGA or UNT-PLGA-RC RNA, which lack the translatable DHFR sequence. The gradient profile from cells expressing UNT-PLGA revealed changes in the distribution of 30S, 50S, and 70S particles (Fig. 4B) that were similar to those observed upon expression of the DHFR-PLGA or DHFR-stop-PLGA-mRNAs (Fig. 4A). However, instead of the complex A_{260} profile in the polyribosome region of the gradient for cells expressing DHFR-PLGA or DHFR-stop-PLGA mRNA (Fig. 4A), only two broad peaks were present in the polysome region when the cells expressed UNT-PLGA RNA (Fig. 4B). Presumably, the profile for the UNT-PLGA-expressing cells is less complex because the transcript is shorter and lacks the translatable DHFR sequence, but it is not clear why expression of UNT-PLGA RNA leads to formation of particles with sedimentation coefficients as large as 2-mer and 3-mer polysomes.

Puromycin treatment of cells induced to express UNT-PLGA RNA. To probe the nature of the ribosomal complexes that form on the UNT-PLGA RNA, we tested the sensitivity of polysome-like particles to treatment with puromycin. Puromycin treatment causes translation termination and dissociation of polysomes, so if ribosomes bound to UNT-PLGA RNA are capable of translation, they should be puromycin sensitive.

Cells were induced for expression of UNT-PLGA or UNT-PLGA-RC with 0.5 mM IPTG for 15 min and then incubated for 2 min in the presence or absence of puromycin (0.5 mg/ml). Ribosomes isolated from these cells were analyzed by sucrose gradient centrifugation as described for Fig. 4. In addition, the gradient fractions were probed for the presence of 16S rRNA, 23S rRNA, and PLGA RNA by dot blot hybridization. The A_{260} gradient profiles show that puromycin treatment caused nearly complete breakdown of the polysomes in uninduced cells (data not shown) and in cells induced for expression of the control UNT-PLGA-RC RNA (compare the profiles for UNT-PLGA-RC RNA in Fig. 4B and 5A). This demonstrates the effectiveness of puromycin treatment in causing the breakdown

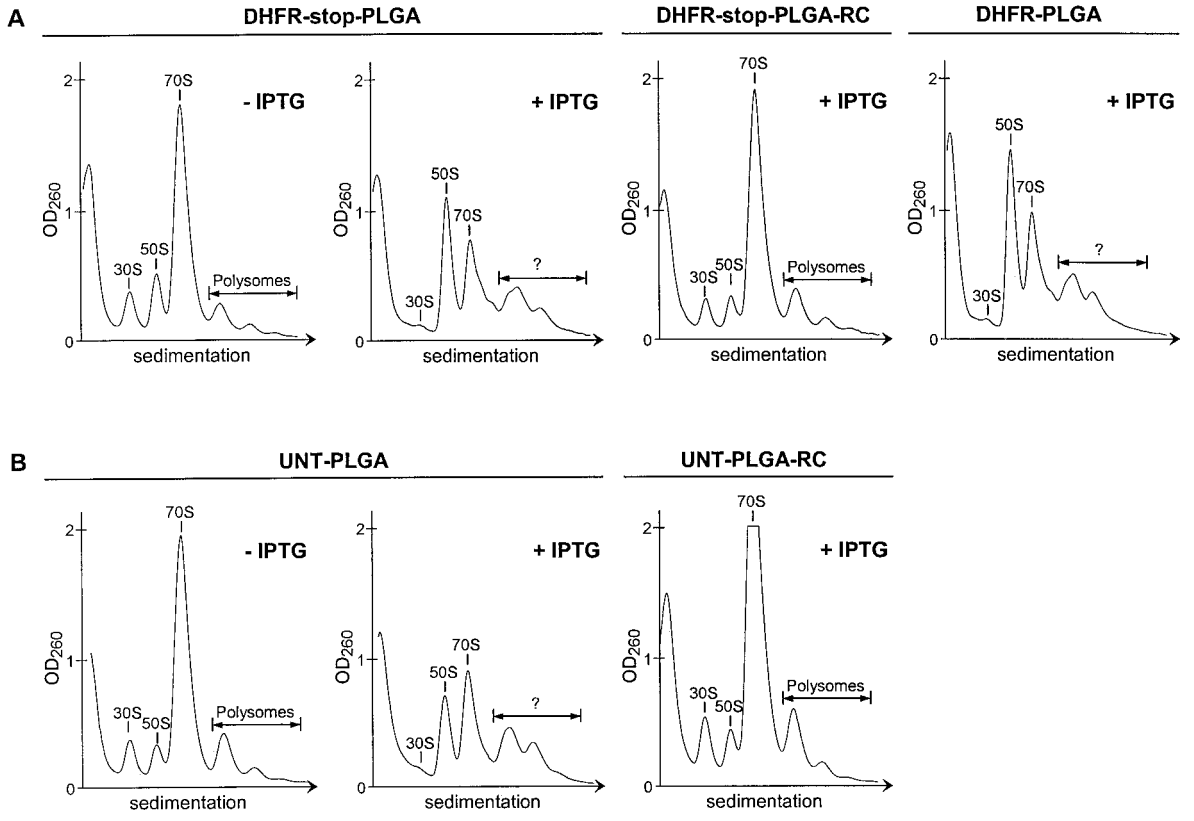


FIG. 4. Sucrose gradient centrifugation of ribosomes from cells expressing PLGA and PLGA-RC RNAs. Cells harboring the DHFR-PLGA, DHFR-stop-PLGA, or DHFR-stop-PLGA-RC construct (A) or the UNT PLGA or UNT-PLGA-RC construct (B) were incubated for 15 min in the presence or absence of 0.5 mM IPTG, and then ribosomal extracts were prepared and analyzed by sucrose gradient centrifugation as described in Materials and Methods. An aliquot containing 20 A_{260} units was layered onto each gradient (10 to 40% sucrose), and the gradients were centrifuged for 6 h at 24,000 rpm in a Beckman SW27 rotor. The gradients were harvested from the top, and the A_{260} was recorded using a flowthrough cuvette. The peaks corresponding to 30S subunits, 50S subunits, 70S ribosomes, and polysomes are labeled. Uncharacterized ribosomal complexes are indicated with a question mark. The RNA expressed in each sample is listed above the gradient profiles.

of normal polyribosomes under these conditions. Notably, puromycin treatment had no effect on the polysome region of the gradient from cells induced to express UNT-PLGA RNA (Fig. 5A), indicating that whatever the nature of the ribosome complexes that form on PLGA RNA, they are incapable of the puromycin reaction and are therefore probably not translationally active. This result supports our contention that the UNT-PLGA RNA is untranslatable.

As anticipated, the 16S rRNA and PLGA RNA were detected by dot blot hybridization in the polysome region of gradients from cells expressing UNT-PLGA RNA (Fig. 5A), but surprisingly, 23S rRNA was also present in these fractions, even when the cells had been treated with puromycin. Since it is unlikely that 50S subunits bind independently to the UNT-PLGA RNA, we interpret this as strong evidence that 70S ribosomes are formed on the untranslatable PLGA transcript. Furthermore, the qualitative distribution of the three RNA species did not change significantly when the cells expressing PLGA RNA were treated with puromycin. Thus, these 70S ribosomes must either have a blocked aminoacyl-tRNA-binding site (A site) or lack an aminoacyl-tRNA in the peptidyl-tRNA-binding site (P site) that can participate in a peptidyl-transferase reaction with puromycin. Note that the dot blot hybridization analysis was not designed to provide quantitative information about the concentrations of the three RNAs in the gradient fractions, and therefore the hybridization signals should not be used to draw conclusions about the ratios of the three RNAs in the samples.

If puromycin-resistant 70S ribosomes are formed on the PLGA RNA, then it should be possible to dissociate these complexes into individual 30S and 50S subunits under conditions of low Mg^{2+} concentration (19). To test this possibility, the three samples shown in Fig. 4B were analyzed by centrifugation in sucrose gradients containing 1 mM $MgCl_2$ instead of 10 mM $MgCl_2$. As shown in Fig. 5B, centrifugation under these conditions caused a disappearance of 70S particles and particles sedimenting in the polysome region and an increase in UV-absorbing material sedimenting under the peaks for 30S and 50S particles. However, the sample from cells induced to express UNT-PLGA RNA gave an unusually high normalized ratio of 50S A_{260} to 30S A_{260} (50S/30S = 3.6) in comparison to the more typical ratios obtained for uninduced cells (50S/30S = 1.1) and for cells induced to express UNT-PLGA-RC RNA (50S/30S = 1.0).

Dot blot hybridization analysis indicated that the high 50S/30S ratio obtained from cells expressing UNT-PLGA RNA

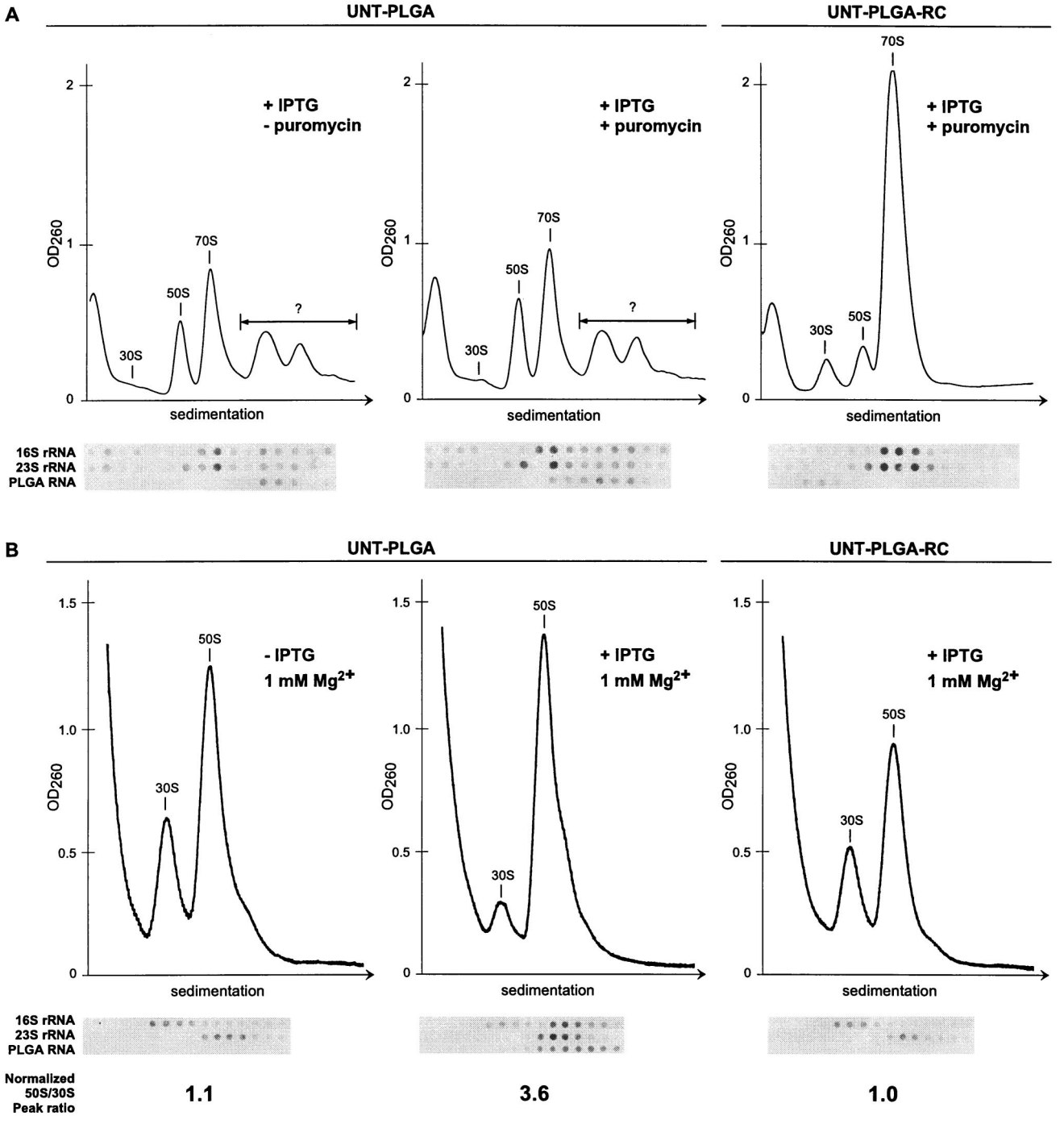


FIG. 5. Effect of puromycin treatment and low magnesium on the sedimentation of ribosomes from cells expressing untranslatable PLGA and PLGA-RC RNAs. Cells harboring the UNT-PLGA or UNT-PLGA-RC construct were incubated for 15 min in the presence or absence of 0.5 mM IPTG as indicated. (A) Cells were then incubated for 2 min in the presence or absence of puromycin (0.5 mg/ml). Ribosomal extracts prepared from these cells were analyzed by sucrose gradient centrifugation as described for Fig. 4. The presence of 16S rRNA, 23S rRNA, and PLGA RNA in gradient fractions was determined by dot blot hybridization. Aliquots (5 μl) from two consecutive fractions were combined, and RNA was extracted as described in Materials and Methods. The hybridization results are aligned below the ribosomal profiles and are displayed as composites of the separate hybridization reactions. (B) Ribosomal extracts from these cells were analyzed by sucrose gradient centrifugation as described for Fig. 4 except that 10 A_{260} units were loaded on each gradient and the gradients contained 1 mM Mg^{2+} to induce subunit dissociation. The presence of 16S rRNA, 23S rRNA, and PLGA RNA in gradient fractions was determined by dot blot hybridization as described for panel A except that RNA was extracted from 30-μl aliquots of each fraction. The relative amount of A_{260}-absorbing material sedimenting as 30S and 50S particles was determined by integrating under the peaks, and the 50S-to-30S peak ratios were normalized to that of the induced PLGA-RC control.

was due to sedimentation of 30S subunits under the 50S subunit peak. Specifically, both 16S rRNA and UNT-PLGA RNAs together with 23S rRNA were present in fractions isolated from the 50S peak. Apparently a 30S-PLGA RNA complex persists under conditions that promote dissociation of 70S ribosomes into individual subunits. The absence of particles sedimenting at approximately 70S in these gradients confirms that no 50S subunits remained associated with the 30S-PLGA RNA complex. Since binding of a single 30S subunit to the relatively short UNT-PLGA RNA would not generate a particle that sediments at approximately 50S, it appears that the most abundant form of the 30S-PLGA RNA complex contains at least two 30S subunits. An increase in the 50S/30S ratio was also observed in samples prepared from UNT-PLGA-expressing cells after only a 5-min induction with IPTG (data not shown), indicating that the stable 30S-PLGA RNA complexes start to accumulate relatively soon after induction begins. These results support our conclusion that 30S subunits form stable complexes on the internal SD sequences in the PLGA coding sequence.

DISCUSSION

A key step in the initiation pathway in bacterial protein synthesis is base pairing between the SD sequence in mRNA and the anti-SD sequence in 16S rRNA. It is thought that the 30S ribosome binds first to mRNA or to fMet-tRNA^{Metf}, forming binary complexes, either of which can serve as a precursor to the formation of a 30S-mRNA-fMet-tRNA^{Metf} ternary complex. Formation of the final 30S initiation complex probably involves a rate-limiting structural adjustment to allow annealing between the anticodon of fMet-tRNA^{Metf} and the initiation codon (12). The 30S initiation complex can then join with a 50S subunit to form the 70S initiation complex, which has puromycin-reactive fMet-tRNA^{Metf} bound in the P site. The initiation factors IF1, IF2, and IF3 ensure the efficiency and fidelity of this process. In particular, IF2 promotes specific binding of fMet-tRNA^{Metf} at the P site and formation of the 70S initiation complex, whereas IF3 is a fidelity factor that destabilizes 30S ternary complexes other than those containing fMet-tRNA^{Metf} and canonical initiation codons. In the mechanistic model proposed by Gualerzi and Pon (12), the initiation factors set kinetic screens that select "best-fit" 30S initiation complexes over other initiation-incompetent 30S ternary complexes, which are presumably unstable and dissociate into their individual components. This selection discriminates against aberrant 30S ternary complexes during formation of 70S complexes.

Several in vitro binding studies have shown that 30S subunits and 70S ribosomes can form relatively stable binary complexes with SD-like sequences in the absence of tRNA, initiation factors, or a cognate initiation codon (5, 13, 31). Presumably 30S subunits also interact in vivo with SD-like sequences that lack cognate initiation codons, but we are not aware of evidence that stable ribosome-RNA complexes are actually assembled on these sites. Ivanov et al. (16, 17) hypothesized that the inhibition of cellular protein synthesis caused by clusters of the rare AGG Arg codon is due to their similarity to the SD consensus sequence and competition with SD elements at functional mRNA initiation sites for 30S subunits. However, that study did not examine whether ribosomes form stable complexes on the AGG clusters in vivo. Other reports have noted that the translation inhibition associated with clusters of rare Arg codons could be partially due to their SD-like nature. However, this was not considered significant because overexpression of the *argU* (tRNA$^{Arg/UCU}$) gene suppresses the inhibition (28, 35). Moreover, models for prokaryotic translation initiation and start site selection predict that the presence of initiation factors and initiator tRNA in vivo should cause dissociation of nonfunctional complexes between 30S ribosomal subunits and SD-like sequences at noninitiation sites (12).

Our results clearly show that overexpression of either the translatable or the untranslatable PLGA coding sequence, which contains four copies of the strong SD-like sequence GAGGAGG, inhibits cellular protein synthesis and cell growth. As suggested by Ivanov et al. (16), this would be expected if the SD-like sequences compete effectively with bona fide initiation sites in mRNA for one or more of the factors required for translation initiation such as 30S subunits, fMet-tRNA^{Metf}, and initiation factors. High-level expression of PLGA transcripts depletes the cellular pool of free 30S subunits, indicating that at least this essential initiation component becomes limiting under these conditions. We have not determined whether the three initiation factors and fMet-tRNA^{Metf} are present in the ribosome complexes on PLGA RNA. However, since 30S ribosomal subunits are approximately sevenfold and threefold more abundant in the cell than the initiation factors and initiator tRNA, respectively (8, 14), it seems likely that the other initiation components become depleted along with 30S subunits. Further analysis will be needed to determine if this is the case.

The presence of 16S and 23S rRNAs in puromycin-resistant complexes on PLGA RNA (Fig. 5A) indicates that 70S ribosomes can assemble on this transcript even though it lacks a normal translation initiation site. It appears that the fidelity of the translation initiation process breaks down in cells burdened with a large excess of noninitiator SD-like sequences. Initiation factor IF3 is thought to destabilize 30S ternary complexes that do not have fMet-tRNA^{Metf} paired with a cognate initiation codon, thereby preventing premature joining of 50S subunits to imperfect 30S-fMet-tRNA^{Metf}-mRNA ternary complexes (12). Indeed, the autoregulation of IF3 translation in *E. coli* depends on the loss of fidelity in the formation of translation initiation complexes that occurs when IF3 levels are low, allowing translation initiation on the IF3 mRNA at an unusual AUU initiation codon (10, 24). Moreover, mutants deficient in IF3 exhibit increased translation initiation from several atypical initiation codons, including AGG and AAG (30). Although both AGG and AAG are present 6 to 9 nucleotides downstream from each of the four strong SD-like sequences in the UNT-PLGA transcript, it is unlikely that translation initiates efficiently at these sites because ribosomes bound to PLGA RNA are not puromycin reactive. We speculate that overexpression of PLGA RNA creates a situation in which there is insufficient IF3 to saturate the 30S subunits that bind to SD-like elements on the PLGA RNA. This would reduce the stringency of initiation complex formation and allow 70S ribosomes to assemble on PLGA transcripts.

La Teana et al. (20) reported that excess IF3 represses the cell-free translation of mRNAs with AUU initiation codons

but not of mRNAs using AUG. This suggests that increasing the level of IF3 in expression hosts could suppress the inhibitory effects associated with the overabundance of noninitiator SD-like elements in cells expressing PLGA RNA and possibly also in cells expressing mRNAs containing SD-like clusters of rare Arg codons. Although overexpression of the *argU* ($tRNA^{Arg/UCU}$) gene is known to improve in vivo translation of mRNAs containing rare Arg codons, this does not exclude the possibility that translation of these mRNAs could be augmented further by co-overexpression of IF3.

The artificial PLGA coding sequence that we have studied was constructed to reflect the codon bias of GAA and GAG Glu codons in highly expressed genes in *E. coli*. In retrospect, a PLGA coding sequence composed exclusively of GAA Glu codons would probably have been a better choice because it would lack strong SD-like sequences. It remains to be determined, however, whether such a coding sequence would improve the biological synthesis of the PLGA protein. Nonetheless, our results demonstrate the potential impact of noninitiator SD-like sequences on the expression of heterologous mRNAs in *E. coli*.

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