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Involvement of Helix 34 of 16 S rRNA in Decoding and Translocation on the Ribosome*

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Andrew Kubarenko[‡§1], **Petr Sergiev**[§], **Wolfgang Wintermeyer**[¶], **Olga Dontsova**[§2], **and Marina V. Rodnina**[‡3]

From the [‡]*Institute of Physical Biochemistry, University of Witten/Herdecke, 58448 Witten, Germany, the* [§]*Department of Chemistry, M. V. Lomonosov Moscow State University, 119899 Moscow, Russia, and the* [¶]*Institute of Molecular Biology, University of Witten/Herdecke, 58448 Witten, Germany*

Helix 34 of 16 S rRNA is located in the head of the 30 S ribosomal subunit close to the decoding center and has been invoked in a number of ribosome functions. In the present work, we have studied the effects of mutations in helix 34 both *in vivo* and *in vitro*. Several nucleotides in helix 34 that are either highly conserved or form important tertiary contacts in 16 S rRNA (U961, C1109, A1191, and A1201) were mutated, and the mutant ribosomes were expressed in the *Escherichia coli* MC250 Δ7 strain that lacks all seven chromosomal rRNA operons. Mutations at positions A1191 and U961 reduced the efficiency of subunit association and resulted in structural rearrangements in helix 27 (position 908) and helix 31 (position 974) of 16 S rRNA. All mutants exhibited increased levels of frameshifting and nonsense readthrough. The effects on frameshifting were specific in that −1 frameshifting was enhanced with mutant A1191G and +1 frameshifting with the other mutants. Mutations of A1191 moderately (~2-fold) inhibited tRNA translocation. No significant effects were found on efficiency and rate of initiation, misreading of sense codons, or binding of tRNA to the E site. The data indicate that helix 34 is involved in controlling the maintenance of the reading frame and in tRNA translocation.

Protein synthesis in bacteria entails four phases, initiation, elongation, termination, and ribosome recycling. The elongation cycle comprises three major steps. First, a complex of elongation factor Tu (EF-Tu)[4] GTP and aminoacyl-tRNA binds to the A site of the ribosome carrying initiator fMet-tRNA^{fMet} or peptidyl-tRNA in the P site. Once aminoacyl-tRNA is accommodated in the peptidyl transferase center, the peptide bond is formed rapidly, yielding deacylated tRNA in the P site and peptidyl-tRNA in the A site. The cycle is completed by translocation of the tRNA·mRNA complex catalyzed by elongation factor G (EF-G). During translocation, peptidyl-tRNA is displaced from the A site to the P site, whereas deacylated tRNA is moved from the P site to the E site and released from the ribosome. Following translocation, the ribosome enters another round of elongation or, when a stop codon is encountered, proceeds to termination.

The ribosome is a molecular machine that undergoes strictly coordinated movements during translation (1, 2). During decoding, the formation of the codon-anticodon duplex induces a conformational change at the decoding site and a global rearrangement of the 30 S domains, as shown by crystal structures of the 30 S ribosomal subunit (3, 4). Residues located in three 16 S rRNA helices, helix (h) 44, h18, and h34, change their position and establish interactions with the codon-anticodon complex (3). Conformational rearrangements of the 30 S subunit are important for the regulation of GTP hydrolysis by EF-Tu and, thereby, for tRNA selection (3–6). After aminoacyl-tRNA binding to the A site and peptide bond formation, the interaction of EF-G with the ribosome induces structural changes of the ribosome that are related to translocation, as suggested by cryoelectron microscopy (7–9) and kinetic measurements (10, 11). Although the resolution of the cryoelectron microscopy studies is generally not high enough to precisely identify the residues involved in the conformational changes, rearrangements seem to include an opening and closing of a putative mRNA channel (7, 8), opening the cleft between the head and the body of the subunit (9), movements of the 30 S relative to the 50 S subunit (7, 8), as well as a number of other conformational rearrangements (12, 13). Some of these movements involve regions of 16 S rRNA that constitute the A site, *i.e.* h34, h44, and h18.

H34 of 16 S rRNA is located in the head of the 30 S subunit and forms part of the decoding region. Genetic studies suggested that mutations in h34 have multiple effects on ribosome function (14). Mutations at positions 1054, 1057, 1058, 1199, and 1200 affected cell growth rate, ribosome synthesis, and ribosome incorporation into polysomes and led to enhanced readthrough of stop codons as well as +1 and −1 frameshifting (14, 15). The conformational flexibility of h34 seems to be important for translocation because the antibiotic spectinomycin, which binds to h34 and presumably interferes with its movements relative to h35 and h38, inhibits translocation (11, 16). In ribosome complexes with EF-G, which mimicked an

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[1] Supported by a Young Scientist INTAS Fellowship from the European Commission.

[2] To whom correspondence may be addressed. Tel.: 7-494-9328824, Fax: 7-495-9393181; E-mail: dontsova@genebee.msu.su.

[3] To whom correspondence may be addressed. Tel.: 49-2302-926205; Fax: 49-2302-926117; E-mail: rodnina@uni-wh.de.

[4] The abbreviations used are: EF-Tu, elongation factor Tu; EF-G, elongation factor G; IF, initiation factors; DMS, dimethyl sulphate; CMCT, 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide methyl-p-toluenesulphonate; h, helix; IC, initiation complex; WT, wild type.

early state of translocation, nucleotides 1054 and 1201 in h34 were protected from chemical modification, whereas no protections of these residues were found in the ground-state complexes before or after translocation (17).

The mechanism by which the mobility of h34 may modulate functions of the ribosome is not known. In the present work, we introduced mutations at positions that seemed likely to act as pivots for h34 movements (Fig. 1) and examined the effects of the mutations on decoding, maintenance of the reading frame, and translocation, as well as on the ribosome structure as monitored by chemical footprinting.

EXPERIMENTAL PROCEDURES

Buffer and Reagents—Buffer A was composed as follows: 50 mM Tris-HCl, pH 7.5, 70 mM NH_4Cl, 30 mM KCl, 7 mM $MgCl_2$. Experiments were performed at 37 °C if not stated otherwise. Chemicals were purchased from Roche Applied Science and Merck. IF1, IF2, IF3, EF-Tu, EF-G, f[^{3}H]Met-tRNA^{fMet}, [^{14}C]Phe-tRNA^{Phe}, and tRNA^{Phe}(Prf16/17) were prepared as described (10, 18–20).

Mutagenesis, Ribosome Expression, and Purification—Mutations in 16 S rRNA were introduced by site-directed mutagenesis of an HindIII-SphI fragment of the *Escherichia coli rrnB* operon, coding for the 3′ half of 16 S rRNA, cloned into M13mp18 phage. The mutated fragment was cloned back into the pSTL102 plasmid. The XL1 strain of *E. coli* was used for all genetic manipulations and phage growth. For the preparation of uracil-containing single-stranded DNA for mutagenesis, strain CJ236 was used as a host for M13 phage. Strain MC250 lacking chromosome-encoded rRNA and encoding a streptomycin-resistance mutation in S12 as selection marker (21) was transformed by derivatives of the pSTL102 plasmid carrying the mutant 16 S rRNA genes. For the displacement of the prrnS12 plasmid originally present in the cells, transformants were grown on LB plates containing streptomycin. The loss of the prrnS12 plasmid was monitored by the loss of the kanamycin resistance encoded on the plasmid. The absence of wild-type rRNA in the cell was confirmed by primer extension analysis (22). Ribosomes were prepared as described (23) except that the concentration of $MgCl_2$ in washing buffers was 20 mM.

In Vivo Assays of Translational Fidelity—To check the effect of mutations on decoding *in vivo*, a set of lacZ-encoding plasmids (pSG) was used (24). *E. coli* strain MC127 carrying one of the pSG plasmids was transformed by one of the plasmids carrying a mutation in the 16 S rRNA gene or the control plasmid pSTL102. The activity of β-galactosidase was determined from at least three independent experiments as described (24).

Preparation of Ribosome and EF-Tu Complexes—70 S initiation complexes were prepared in buffer A by incubating ribosomes (1 μM) with f[^{3}H]Met-tRNA^{fMet} (1.5 μM) and mRNA (4 μM) in the presence of initiation factors IF1, IF2, and IF3 (1.5 μM each) for 60 min at 37 °C. mRNAs were derivatives of the 122-nucleotide mRNA m022 (23, 25) that included a Shine-Dalgarno sequence preceding the AUG start codon and UUU, UUG, or CUC as the second codon. Initiation complexes were purified and concentrated by centrifugation through 1.1 M sucrose cushions at 200,000 × *g* for 2 h on a Sorvall M120GX ultracentrifuge. To prepare pretranslocation complexes, ternary complex (see below) was added to the initiation complex, the incubation continued for 1 min at 37 °C to form a pretranslocation complex that was purified by ultracentrifugation as described above. Ternary complexes of EF-Tu·GTP and tRNA^{Phe}(Prf16/17) or [^{14}C]Phe-tRNA^{Phe} were formed by incubating EF-Tu (30 μM), EF-Ts (0.02 μM), GTP (1 mM), phosphoenol pyruvate (3 mM), pyruvate kinase (0.1 mg/ml), tRNA^{Phe}(Prf16/17) (10 μM), ATP (3 mM), L-phenylalanine (30 μM), and S100 fraction (2% v/v) as a source of Phe-tRNA synthetase and were purified by gel filtration as described (20, 26). To prepare pretranslocation complexes, EF-Tu ternary complexes were prepared in the same way except that precharged purified [^{14}C]Phe-tRNA^{Phe} (10 μM) was used and S100, ATP, and phenylalanine were omitted.

Biochemical Assays—The amount of [^{14}C]Phe-tRNA^{Phe} and f[^{3}H]Met-tRNA^{fMet} bound to ribosomes was determined by nitrocellulose filtration, applying aliquots of the reaction mixture to the filters (Sartorius), and washing with buffer A. Filters were dissolved, and radioactivity was measured in a QS361 scintillation mixture. Binding of the ternary complex to the ribosome was measured after a 1-min incubation at 20 °C. To quantify dipeptide formation, the reaction was quenched with 0.5 M KOH, and then the mixture was incubated for 30 min at 37 °C, neutralized, and analyzed by high pressure liquid chromatography on a RP18 column (Merck, Darmstadt) using a gradient of acetonitrile in 0.1% trifluoroacetic acid (26). Eluted fractions were analyzed for radioactivity by liquid-liquid scintillation counting. To induce translocation, EF-G was preincubated with 1 mM GTP for 15 min at 37 °C and added to the pretranslocation complex at the indicated concentrations. The extent of translocation (>85%) was determined by reaction with puromycin (1 mM puromycin, 10 s, 37 °C) (27).

Density gradients of 10–40% (w/v) sucrose were prepared in buffer A containing $MgCl_2$ at the indicated concentrations (7, 14, or 21 mM). 100 μl of 0.4 μM ribosome solution in the respective buffer were preincubated for 10 min at 37 °°C and loaded on top of the gradient. Centrifugation was carried out for 17 h at 21,000 rpm at 4 °°C (Beckman SW41 or SW28 rotors).

In Vitro Frameshifting and Misreading Assays—To measure the frequency of −1 frameshifting and misreading on mutant ribosomes, mRNA constructs of the following coding sequence were used: AUGUUUUUG and AUGUUGUUU. Initiation complexes (0.2 μM) were incubated with 0.4 μM ternary complex EF-Tu·GTP·[^{14}C]Phe-tRNA^{Phe} and 0.05 μM EF-G for 1 min at 20 °C. The efficiency of −1 frameshifting (E_{fs}) was calculated from the ratio of the tripeptide fMet-Phe-Phe to initiation complex (IC) formed on the AUGUUUUUG mRNA corrected for the −1 frameshifting frequency measured with wild-type ribosomes, $E_{fs} = ([\text{fMet-Phe-Phe}_{mutant}]/[IC_{mutant}])/([\text{fMet-Phe-Phe}_{WT}]/[IC_{WT}])$. The misreading level was calculated from the ratio of the dipeptide fMet-Phe to initiation complex formed on the AUGUUGUUU mRNA corrected for the misreading frequency of wild-type ribosomes, $E = ([\text{fMet-Phe}_{mutant}]/[IC_{mutant}])/([\text{fMet-Phe}_{WT}]/[IC_{WT}])$.

Kinetic Experiments—Fluorescence stopped-flow measurements were performed in buffer A on an SX-18MV spectrometer (Applied Photophysics) as described previously (6, 10). The fluorescence of proflavin was excited at 470 nm and measured

after passing KV 500 filters (Schott). With the apparatus used, time constants up to 500 s^{-1} could be measured. Experiments were performed by rapidly mixing equal volumes (60 μl of each) of ternary complex and ribosome complex at the indicated concentrations.

Chemical Probing—DMS, kethoxal, and CMCT modifications were carried out for 10 min at 37 °C (28). Ribosomal RNA was purified by repeated phenol extractions, and modified sites were determined by primer extension with avian myeloblastosis virus reverse transcriptase (28) using a set of oligodeoxyribonucleotide primers spaced such as to cover the entire length of 16 S rRNA.

RESULTS

Choice of Mutation Positions and Construction of Mutants—Bases of 16 S rRNA were exchanged at positions C1109, A1191, A1201, and U961 of h34 (Fig. 1*A*) for the following reasons. C1109 is located in the loop between h34, h35, and h38. C1109 is highly conserved and was predicted to form a tertiary contact with the G933-C1384 base pair (29) in the neck region of 30 S subunit. In the high resolution crystal structures of the isolated 30 S subunit from *Thermus thermophilus* or the 70 S ribosome from *E. coli*, this tertiary contact is not present (3, 30). It is possible that the contact between C1109 and the G933-C1384 base pair is present in 70 S ribosomes or that it forms at an intermediate stage of translation. The mutation C1109G is expected to prevent the formation of the tertiary contact.

A1191 is a highly conserved nucleotide that is located at the junction of several 16 S rRNA helices, h34, and an element comprising h35/h36/h38, where the antibiotic spectinomycin binds (31). The structures of the *T. thermophilus* 30 S subunit (3) and the *E. coli* 70 S ribosome (30) suggest that A1191 may form hydrogen bonds with nucleotides in the vicinity, mainly G1068 and C1066, thereby stabilizing the structure of the decoding center. The three base replacements A1191C, A1191G, and A1191U that were tested are expected to affect the network of interactions in different ways (Fig. 1*B*).

In the crystal structure of the 30 S subunit (3), the non-conserved nucleotide A1201 forms hydrogen bonds with the highly conserved nucleotide U961, and the Watson-Crick base pair U961-A974 deduced from the secondary structure predictions (29) is not present. According to the crystal structure, the formation of a triple interaction A1201-U961-A974 seems possible, and this may affect the orientation of h34. Accordingly, three mutants were constructed, including the single mutations A1201U and U961A and the double mutation A1201U/U961A in which hydrogen bonds between the bases at positions 1201 and 961 should be restored, whereas the 961–974 base pair cannot be formed (Fig. 1*C*). It should be noted that somewhat different orientations of either A1201 or U961 were found in the two different 70 S *E. coli* structures reported recently (30); however, in these orientations, mutations of A1201 or U961 are also expected to alter the structure of the region.

Plasmids carrying mutations in h34 of 16 S rRNA in the *rrnB* operon were constructed in such a way that the C1192U mutation in 16 S rRNA that is carried by the original pSTL102 plasmid (32) was reverted to the wild-type C1192; the same reversion to the wild-type sequence of h34 was performed in the control plasmid. Two plasmids, pSTL102(C1192), which carries the A2058G mutation in 23 S rRNA, and pLK35, coding for wild-type 16 S rRNA, were used as controls. The plasmids were transformed into the *E. coli* strain MC250 that lacked all seven chromosomal rRNA operons and contained the gene for streptomycin-resistant S12 protein on the chromosome (21). The latter marker was essential for plasmid exchange. The mutation in S12 (K42R) confers streptomycin resistance due to the loss of a direct contact to the antibiotic (K45R in *T. thermophilus* (31)); the strain has essentially wild-type doubling times and no accuracy phenotype. The doubling times of strains expressing ribosomes with mutations at positions C1109, A1201, and U961 were essentially the same as of the parent strain, whereas mutations at position A1191 reduced the growth rate by about 30% (not shown).

Intersubunit Interactions—To test whether mutations in h34 altered the interactions between 30 S and 50 S subunits, we studied subunit association by sucrose gradient centrifugation at different Mg^{2+} concentrations. At the Mg^{2+} concentrations used (7, 14, or 21 mM Mg^{2+}), wild-type ribosomes were present as 70 S (Fig. 2), and the fraction of 30 S and 50 S subunits was negligible. Mutations at position C1109 and the double mutation A1201/U961 had no effect on subunit association. The single mutations A1191C and A1201U had a slight effect. The replacements A1191G, A1191U, and U961A impaired subunit association at low Mg^{2+} concentrations, and the formation of the respective 70 S ribosomes required elevated concentrations of Mg^{2+} (Fig. 2). 30 S subunits with the A1191G mutation were not fully associated to 70 S even at 21 mM Mg^{2+}. However, all mutant ribosomes were able to bind mRNA and fMet-$tRNA^{fMet}$, and the efficiency of initiation complex formation was about 80% in all cases (not shown). The rate of initiation complex formation, as studied by the fluorescence change of fluorescein attached to the 3′ end of the mRNA at position +12 from the initiator AUG codon, was not changed by any of the mutations (not shown).

As h34 is not directly involved in intersubunit contacts, the defects in subunit association caused by h34 mutations presumably originate from effects on the structure of regions at the interface that are distant from h34. To identify regions of 16 S rRNA that were affected by the mutations, the structure of 16 S rRNA was probed by chemical modification. Isolated 30 S subunits, 70 S ribosomes formed upon the addition of wild-type 50 S subunits to 30 S subunits, and the respective 30 S and 70 S initiation complexes were treated with DMS and CMCT, and the modification pattern was analyzed by primer extension (28). As ribosome reactivation is known to improve the functional activity of ribosome preparations, we compared ribosomes reactivated by heating for 1 h at 37 °C in the presence of elevated Mg^{2+} concentrations (20 mM) (33) with non-treated ribosomes. On the side of the 30 S subunit, h14, h20, h23, h24, h25, h27, h44, and h45 are involved in intersubunit bridges with the 50 S subunit. A number of these contacts can be monitored by chemical footprinting. Most of the protected nucleotides are found in five extended stem-loop structures around positions 700 (h23), 790 (h24), 900 (h27), and 1408–1495 (h44) (34). Position A908 in h27, which in wild-type 30 S initiation complexes was accessi-

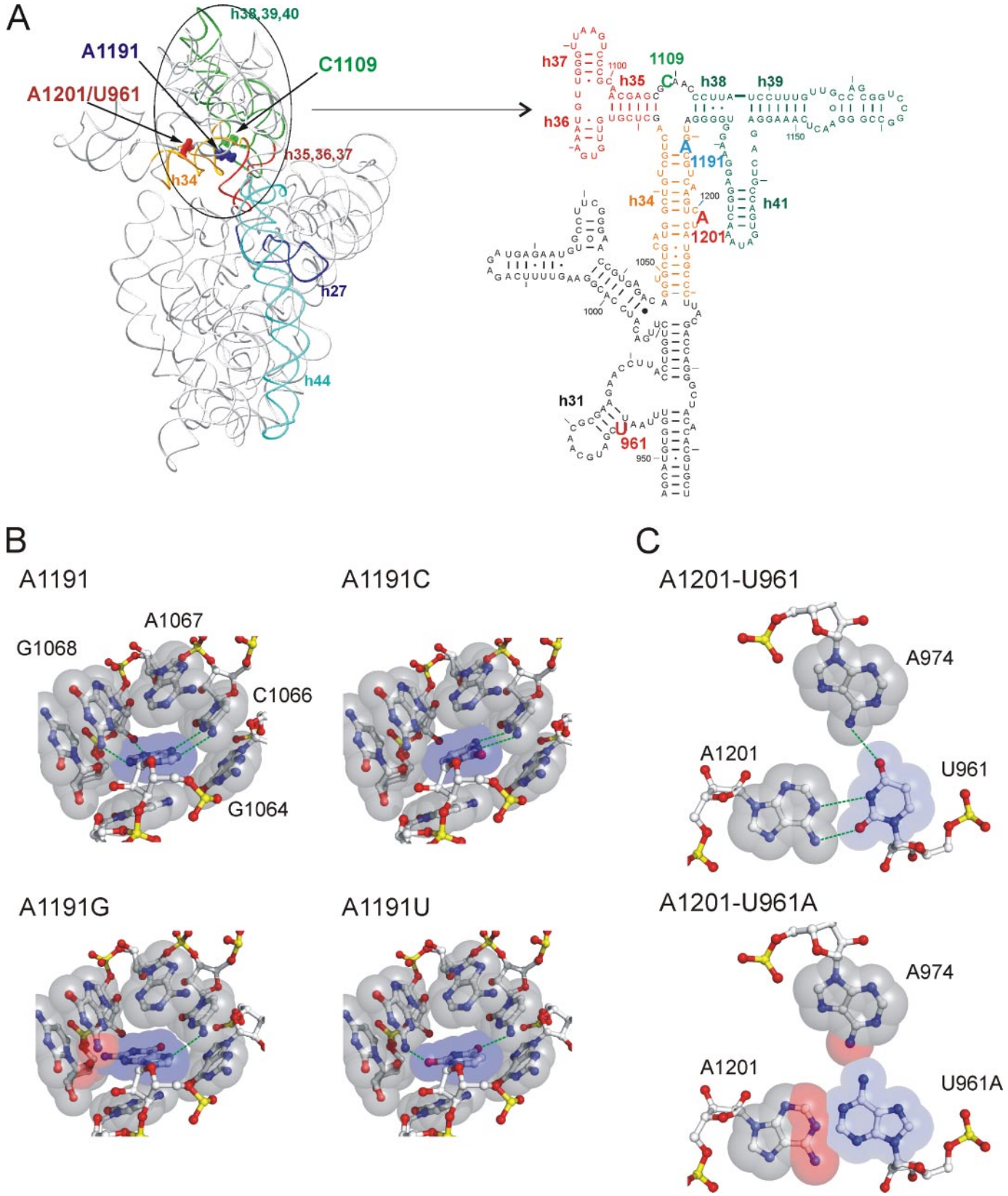

FIGURE 1. **Mutations in h34 of 16 S rRNA**. *A*, tertiary structure of 16 S rRNA in the 30 S subunit (*left panel*) and close-up of the secondary structure elements in the vicinity of h34 (*right panel*). *Orange*, H34; *green*, h38–40; *red*, h35–37; *dark blue*, h27; *cyan*, h44. Mutated positions are indicated. *B*, local surrounding of A1191 and presumed changes due to mutations. *Upper left*, surrounding of A1191 and interactions with G1068 and C1066. *Upper right*, replacement A1191C. *Lower left*, A1191G. *Lower right*, A1191U. According to the models, the replacement A1191G causes steric clashes (*pink*) with neighboring residues. *C*, interactions of A1201, U961, and A974 as in the crystal structure of the 30 S subunit (3) (*upper panel*) or modeled for the A961U mutant (*lower panel*), indicating steric clashes (*pink*) and a loss of hydrogen bonds. Models shown in *B* and *C* were produced by manually fitting the desired nucleotide into the crystal structure of the 30 S subunit (Protein Data Bank entry 1IBM (3)). The figures were produced using SwissPDB Viewer and PyMOL.

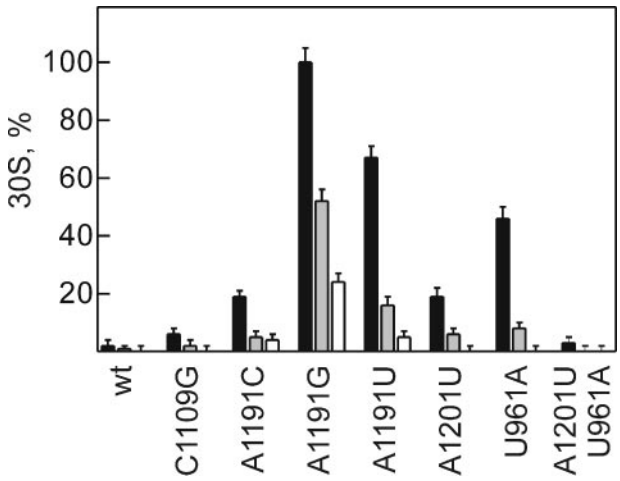

FIGURE 2. **Effect of mutations on subunit reassociation.** 30 S and 50 S subunits were incubated in buffer A at 7 mM (*black bars*), 14 mM (*gray bars*), or 21 mM (*white bars*) Mg^{2+} and analyzed by sucrose density centrifugation at the respective Mg^{2+} concentration. The amount of free 30 S subunits relative to the total amount of 30 S subunits is shown.

ble for DMS modification, was protected upon binding of the 50 S subunit (34), and this protection was abolished by the A1191G mutation (Fig. 3*A*). That mutant 30 S subunits efficiently formed 70 S ribosomes was further supported by the observation that residues A1413 and A1418 (Fig. 3*B*) as well as residues 700 and 790 (not shown) were equally well protected upon 70 S formation with wild-type and mutant 30 S subunits. Reactivities to other chemical reagents, kethoxal and CMCT, were not altered by the mutations in h34. These data indicate that mutations in h34 altered the structure or orientation of h27, thereby affecting intersubunit contacts and the efficiency of subunit association. Replacements A1201U, U961A, and A1201/U961 resulted in a strong enhancement of the reactivity of A974 against DMS (Fig. 3*C*), suggesting that tertiary interactions of these bases were disrupted by the mutations (possibly triple interactions between A1201, U961, and A974, as described above), and A974 was exposed by the resulting conformational change. The same modification patterns were observed upon reassociation of 30 S subunits with wild-type 50 S subunits or 50 S subunits from the pSRL102(C1192) strain that carried the A2058G mutation in 23 S rRNA (data not shown).

Fidelity of Translation—The effect of mutations in h34 of 16 S rRNA on the fidelity of translation was first studied *in vivo*, using *E. coli* strains carrying plasmids coding for both mutant 16 S rRNA and β-galactosidase. These strains express a mixture of wild-type and mutant ribosomes; mutant ribosomes are efficiently incorporated into polysomes because the respective strains lacking the chromosomal rRNA operons are viable, and the efficiency of initiation complex formation is unchanged with mutant ribosomes (see above). The β-galactosidase reporter gene was modified in different ways such that the synthesis of the active enzyme could occur only upon +1 or −1 frameshifting, readthrough of a nonsense codon, or a missense error (24). β-Galactosidase activities were measured and related to that of the control strains carrying pSTL102(C1192) or pLK35; both plasmids gave identical activities designated in Fig. 4, *A* and *B*, as *WT*. Most of the mutations in h34 increased the levels of +1 frameshifting and stop-codon readthrough; the only exception was the A1191G mutant, which strongly stimulated −1 frameshifting and had no effect on nonsense suppression. Missense error frequencies were similar for all mutants except U961A, which was slightly more accurate.

To further substantiate −1 frameshifting with the A1191G mutant, the efficiency of −1 frameshifting was studied *in vitro* (Fig. 5). 70 S initiation complexes carrying f[^{3}H]Met-tRNA^{fMet} in the P site were prepared using an mRNA with the coding sequence AUGUUUUUG. Without −1 frameshifting, only the dipeptide fMet-Phe could be formed upon the addition of excess [^{14}C]Phe-tRNA^{Phe}, whereas in the case of −1 frameshifting, the tripeptide fMet-Phe-Phe should be synthesized. The extent of fMet-Phe-Phe tripeptide formation by misreading of the UUG codon was directly tested by using an mRNA with the sequence AUGUUG. As a positive control for tripeptide formation, an mRNA with the sequence AUGUUUUUU, coding for fMet-Phe-Phe, was used. In agreement with the *in vivo* data (Fig. 4*A*), the A1191G mutation in 16 S rRNA caused a significant increase in −1 frameshifting, whereas the A1191C or A1191U mutations had no effect (Fig. 5). The misincorporation of Phe on Leu codons *in vitro* was not changed appreciably (Fig. 5).

EF-G-dependent Translocation—The effect of h34 mutations on translocation was studied under single and multiple turnover conditions. The rate of single-round translocation was measured by stopped-flow, monitoring the fluorescence change of a proflavin reporter group in peptidyl-tRNA (27). Pretranslocation complexes containing fMet-Phe-tRNA^{Phe}(Prf16/17) in the A site and deacylated tRNA^{fMet} in the P site were rapidly mixed with excess EF-G, and the rate of translocation was measured. Experiments with mutant pretranslocation complexes were carried out at a saturating concentration of EF-G (1.5 μM) (10) (Fig. 6*A*). Mutations in h34 had little effect on translocation, with the exception of replacements at position A1191, which resulted in lower translocation rates. The effect was due to a decrease of the rate constant of translocation, rather than a decrease in EF-G affinity to the mutant pretranslocation complexes, as verified by measuring the concentration dependence of the translocation rate (Fig. 6*B*).

Multiple-turnover translocation experiments were performed in the presence of catalytic amounts of EF-G and excess of pretranslocation complexes. Mutations in h34 had no significant effect on the k_{cat}/K_m value of the multiple-turnover reaction (Fig. 6*C*). The rate-limiting step of the multiple-turnover reaction is a ribosome/EF-G rearrangement following mRNA-tRNA movement, referred to as relocking (10). Thus, mutations in h34 do not affect relocking of the ribosome after tRNA-mRNA translocation.

DISCUSSION

Effects of Mutations on 30 S Subunit Structure and Association with the 50 S Subunit—H34 is an important element of the head of the 30 S subunit, which has been implicated in a number of ribosome functions, including decoding (14, 15, 35), translocation (11, 17, 36) during the elongation phase of protein synthesis, and more recently, the initiation of translation (37). Reported effects of mutations in h34 on cell

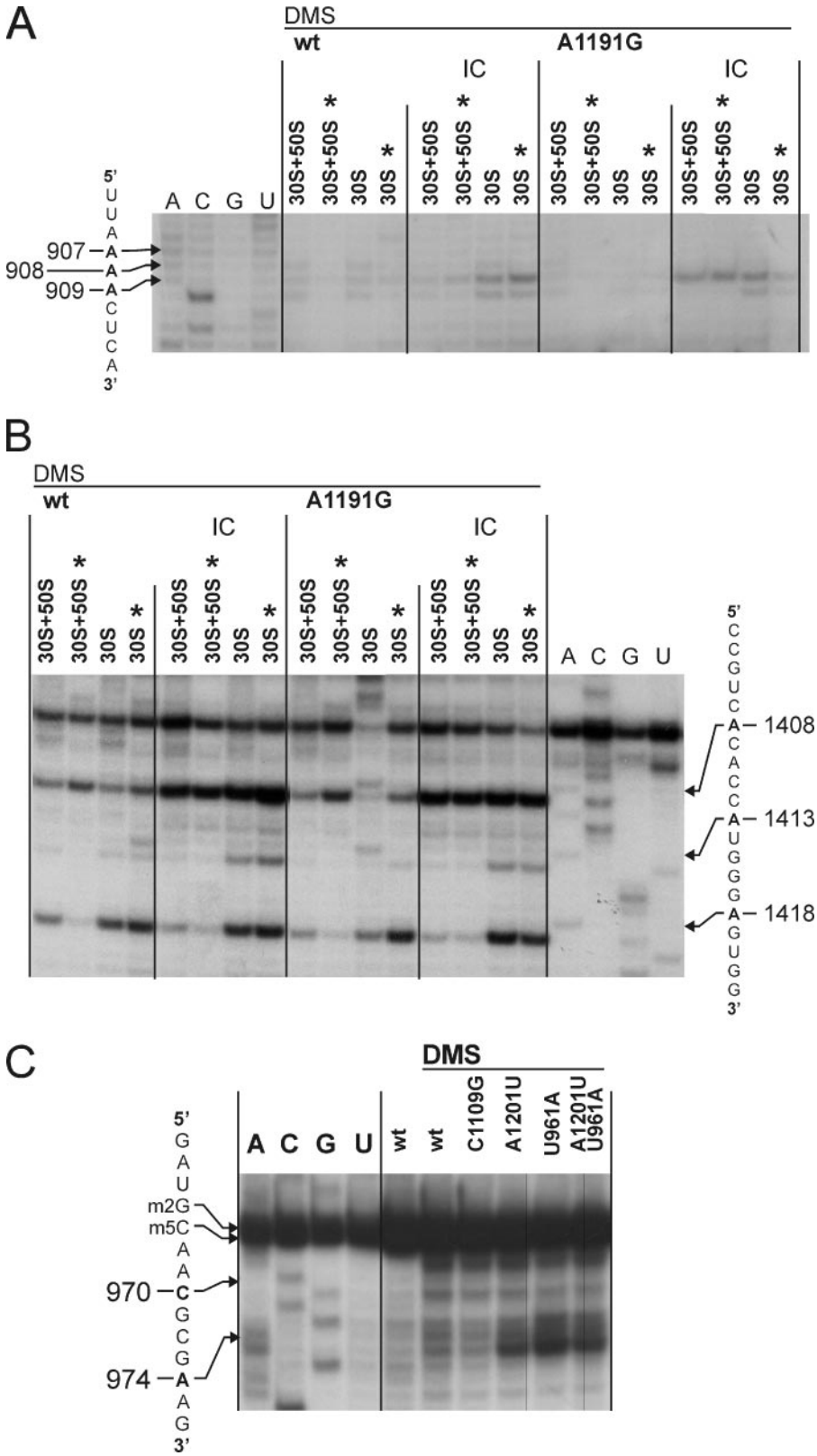

FIGURE 3. **Structural changes at the interface side of the 30 S subunits.** *A*, effect of the A1191G mutation on the DMS reactivity of h27. *B*, DMS reactivity of h44 in vacant WT or A1191 mutant ribosomes or the respective IC, as indicated. *Asterisks* indicate preincubation for 1 h at 37 °C at 20 mM Mg^{2+}. *C*, enhancement of A974 reactivity by mutations of A1201 and U961. *A*, *C*, *G*, and *U* indicate sequencing lanes. No stops at positions 907–909, 974, 1408, 1413, and 1418 on wild-type or mutant ribosomes were observed in the absence of DMS (data not shown).

growth ranged from strongly deleterious to insignificant (14, 37). Several mutations analyzed in the present study (replacements at positions C1109, A1201, and U961) had no effect on cell viability, and only mutations at position A1191 mildly retarded growth. Replacements of C1109 had essentially no phenotypic effects and will not be discussed in the following. The strongest effects were observed for the replacements of A1191, including effects on subunit association, decoding, and translocation. Mutations of U961 had slight effects on frameshifting and subunit association.

Replacements of A1191 inhibited subunit association. The effect must be indirect because h34 does not form an intersubunit contact. The inhibition of subunit association may be due to the structural rearrangement of h27 induced by the mutations in h34. As h34 and h27 are not adjacent in the tertiary structure of the 30 S subunit, the change in the orientation of h34 caused by h34 mutations has to be transmitted through other elements of the 30 S subunit. It is possible that the disruption of interactions of A1191 with G1068 and C1066 and/or steric clashes introduced by the mutations (Fig. 1) affect the position of the adjacent helices 35–37, which interact with h44 in the vicinity of the decoding center. H44, in turn, is packed against h27, which may further transmit the changes due to alterations in h34, resulting in structural changes manifested in the increased reactivity of nucleotide 909 in the initiation complexes formed with mutant 30 S subunits. Nucleotides G1064 and C1068 appear to have an important role in the communication between the two helices because modifications of G1064 and C1068 interfere with subunit association (38).

The effects of mutations of A1191 observed here may offer an explanation for the earlier observations of synergistic effects of mutations in h27 and in the pSTL102 plasmid, which has two mutations, C1192U

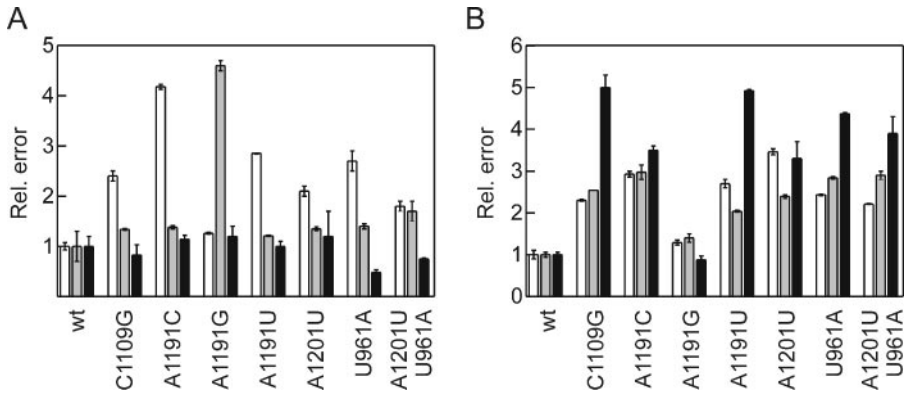

FIGURE 4. **Effect of h34 mutations on translation errors *in vivo*.** *A*, frameshifting (*white bars*, +1; *gray bars*, −1) and misreading (*black bars*) errors of mutant ribosomes relative to WT ribosomes set to 1. *B*, nonsense-codon readthrough (*white bars*, UAG; *gray bars*, UAA; *black bars*, UGA). For details, see "Experimental Procedures." *Rel. error*, relative error.

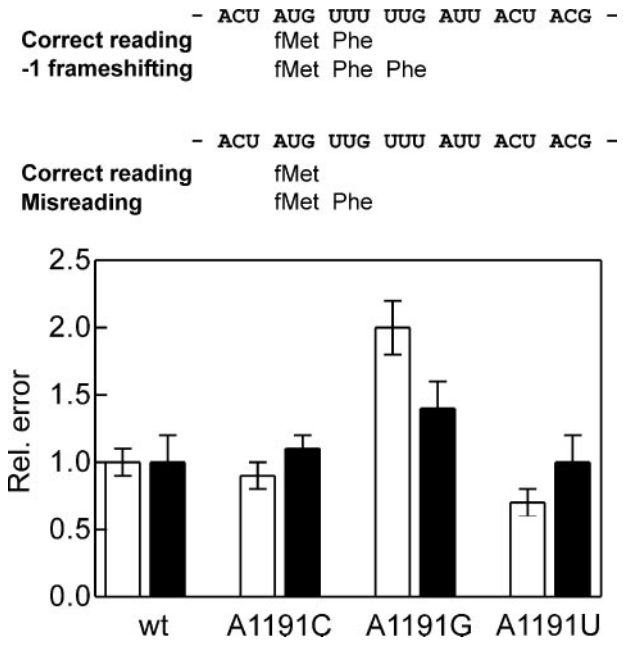

FIGURE 5. **Effect of A1191 mutations on frameshifting and misreading *in vitro*.** *Upper panel*, mRNAs used to measure −1 frameshifting and misreading. *Lower panel*, relative efficiency of frameshifting (*white bars*) and misreading (*black bars*). The respective error level found with WT ribosomes was set to 1.0. *Rel. error*, relative error.

in 16 S rRNA and A2058G in 23 S rRNA (39). In fact, if replacements of C1192 had similar effects as those of the adjacent A1191, then an effect on the conformation of h27 would be expected. In comparison, the second mutation carried by the pSTL102 plasmid, A2058G in 23 S rRNA, does not have a pronounced effect as identical chemical modification patterns were obtained with 50 S subunits expressed from the pSTL102 plasmid or in wild-type cells.

Subunit association was diminished by the U961A mutation but was restored in the double mutant A1201U-U961A, suggesting that the presence of a base pair at that position is important. In wild-type ribosomes, nucleotide A974, which may form a single hydrogen bond with U961, is protected from chemical modification by DMS. The protection is not easy to reconcile with the available structures of the 30 S subunit (3, 40) because the interactions between A974 and U961 do not involve N1 of A974, which is modified by DMS. On the other hand, A974 and U961 were predicted to form a Watson-Crick pair on the basis of sequence comparisons (29), and it is not clear whether the two alternative conformations may coexist in solution. Mutations of A1201, U961, or the double mutant restoring the 1201-961 interaction render A974 highly accessible to chemical modification, suggesting structural alterations in the vicinity of A974. Despite these conformational changes and defects in subunit association, none of the mutants tested was affected in the extent or rate of 70 S initiation complex formation.

Importance of h34 for Decoding—H34 is likely to be directly involved in the decoding process. Nucleotide U1052 was cross-linked to the 3′ end of the mRNA codon in the A site (41), whereas A1196 was cross-linked to mRNA residues +8 and +9 (42). According to the crystal structures of functional ribosome complexes, C1054 and A1196 are in contact with the mRNA-tRNA complex (3, 43), whereas other residues are not and may serve as a 3′-boundary for the A site (44, 45). Genetic studies examined the influence of mutations at a number of positions in h34 (1054, 1057, 1058, 1199, 1200, 1202, and 1203) on stop-codon readthrough and frameshifting (14), whereas mutations in positions studied in this work (1109, 1191, 1201/961) were not tested. Deletion of C1054 caused an increase in stop-codon readthrough (35). The C1200U mutation increased all kinds of translation errors, without affecting the growth rate. Substitution of G1057/G1058 with U/C significantly retarded cell growth and lead to a pronounced exclusion of the mutant ribosomes from polysomes; the growth inhibition correlated with increased stop-codon readthrough and both +1 and −1 frameshifting.

The present results are in agreement with the general notion that h34 is involved in frameshifting and nonsense readthrough (14). However, the mutations tested in the present work were very specific with respect to the direction (+1 or −1) of frameshifting, as would be expected for programmed ribosomal frameshifting (46), but in contrast to the effects of mutations tested previously, which showed a uniform enhancement of both types of frameshifting (14, 47). A particularly interesting observation is that the A1191G substitution causes −1 frameshifting, whereas A1191C and A1191U substitution result in +1 frameshifting. Importantly, mutations did not change the missense error frequency, indicating that mutant ribosomes were not deficient in their general ability to contribute to the accuracy of codon recognition. It should be noted that ribosomes used in the *in vitro* assays had an additional mutation in S12 (K42R) used as a selection marker for plasmid exchange in the MC250 strain, and it is in principle possible that this mutation attenuates the effects of h34 replacements. However, very similar

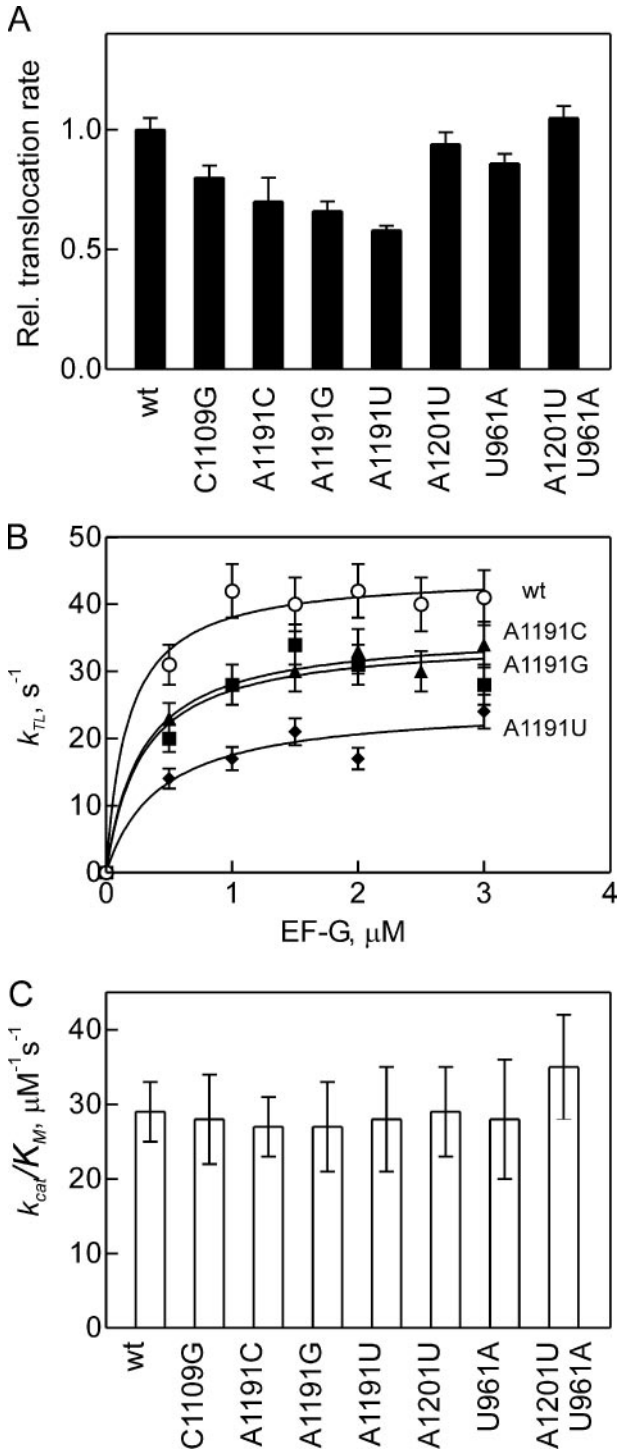

FIGURE 6. **Effect of h34 mutations on translocation.** *A*, single-round translocation. Fluorescence stopped-flow experiments were performed as described under "Experimental Procedures," using EF-G at 1.5 μM final concentration. Translocation rates are given relative to the rate measured with wild-type ribosomes (40 s^{-1}). *B*, concentration dependence of single-round translocation rates (k_{TL}) with wild-type (*open circles*), A1191C (*closed triangles*), A1191G (*closed squares*), and A1191U (*closed diamonds*) ribosomes. *C*, k_{cat}/K_m of multiple-turnover translocation measured at a catalytic concentration of EF-G (0.5 nM) and a subsaturating concentration of pretranslocation complex (0.2 μM).

effects on decoding were obtained *in vitro* and *in vivo* in strains that have wild-type S12. This indicates that the K42R mutation, which alone has no fidelity phenotype, has no synergistic effects with mutations in h34, in keeping with the fact that K42 is not directly involved in contacts with any other ribosomal protein or rRNA. Furthermore, the replacement of A1191 with G had a very different effect than replacements with C or U, supporting the notion that the mutations in h34, rather than synergistic effects, were responsible for the respective increase in −1 or +1 frameshifting.

The effects of the mutations can be explained by changes in (i) the interactions of h34 with mRNA and the presumed function of h34 as mRNA boundary (44, 45) and/or (ii) the stability of the mRNA-tRNA complex at the state at which frameshifting may occur (46). Because A1191 is located in the center of the junction formed by h34 and h35/36/38, mutations in h34 may not only change the local structure in this region but also the global structure of the 30 S subunit by altering the relative orientation of the head and the body of the subunit. This may change or abolish the interactions of ribosomal residues with the mRNA that are necessary to stabilize the mRNA-tRNA complex or prevent frameshifting. It can be expected that the changes introduced by A1191 substitutions with U or C are not as dramatic as those with G. Furthermore, the direction of the structural change may be different, thus explaining the opposite effects of A1191G and A1191C/U on frameshifting. Alternatively, an increase in −1 frameshifting was predicted to be correlated with the selective destabilization of the E site-bound tRNA (48). However, when the effect of A1191 replacements on the binding of tRNA to the E site was studied by monitoring fluorescence changes of $tRNA^{Phe}$ (Prf16/17) (49, 50), the rate of E site binding was not affected appreciably (data not shown). Thus, the ability of the mutants to promote frameshifting is not related to the interaction of the tRNA with the E site.

H34 in Translocation—Spectinomycin, an antibiotic that interferes with tRNA translocation (11, 36), binds to G1064, C1066, G1068, and C1192 in h34 at the junction between h34, h35, and h38 (40), and several mutations in h34 cause resistance to spectinomycin (16, 51, 52). It was suggested that spectinomycin inhibits translocation by interfering with the movement of the head relative to the body of the 30 S subunit (31). Kinetic analysis indicated a specific inhibition by the antibiotic of EF-G-induced tRNA movement in addition to a stabilization of peptidyl-tRNA in the A site, whereas the antibiotic did not interfere with the unlocking rearrangement of the ribosome that is induced by EF-G and precedes tRNA movement (11). This indicated that structural rearrangements around the binding site of spectinomycin in h34 following unlocking were required to allow tRNA movement. C1054 and A1201 in h34 are protected from base-specific chemicals in a pretranslocation ribosome complex with EF-G stabilized by thiostrepton (17), although the nucleotides are not in direct contact with the factor (53). This functional complex is believed to be an early intermediate of translocation (18), and therefore, a rearrangement in h34 seems to be required for tRNA movement.

The present data show that mutations of A1191 reduce the rate of tRNA movement. It is not known when the contacts between h34 and mRNA are released during translocation and whether the release is synchronous with the disruption

of other 30 S-mRNA (and tRNA) contacts. If the A1191G mutation leads to an enhancement of mRNA-h34 interactions, it may impede ribosome sliding along the mRNA during translocation. Such a model would imply a certain probability of disruption of mRNA-tRNA interactions. Assuming that tRNA can move independently of mRNA, the changes in h34-mRNA interactions would account for both increased frameshifting and slower translocation on mutant ribosomes. However, because the effects of A1191 substitutions on translocation rates do not correlate with frameshifting efficiencies, the simplistic model involving the enhancement or reduction of h34-mRNA interactions appears unlikely. The similarity of the effects of replacements at different positions suggests that the flexibility rather than the precise orientation of h34 has an influence on translocation. The modest magnitude of the effect (<2-fold) is consistent with the notion that restricting the mobility of h34 does not affect the rate-limiting unlocking step (11) and suggests that a later step is affected. The rate constant of tRNA movement on wild-type ribosomes is much higher than that of unlocking and cannot be measured (10). This may be different with ribosomes carrying h34 mutations. Thus, the effect of h34 mutations on translocation is attributed to an inhibition of a structural rearrangement of the 30 S subunit that is required for tRNA movement to take place. Apparently, the effect is large enough to shift the rate-limiting step of translocation on mutant ribosomes from unlocking to a rearrangement that is coupled to tRNA movement.

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