Functional implications of ribosomal protein L2 in protein biosynthesis as shown by *in vivo* replacement studies

Monika ÜHLEIN*, Wolfgang WEGLOHNER†¹, Henning URLAUB* and Brigitte WITTMANN-LIEBOLD²

*Max-Delbrück-Centrum für Molekulare Medizin, Robert-Rössle-Str. 10, D-13125 Berlin, Federal Republic of Germany, and †Institut für Physiologische Chemie, Universitätskrankenhaus Eppendorf, D-20246 Hamburg, Federal Republic of Germany

The translational apparatus is a highly complex structure containing three to four RNA molecules and more than 50 different proteins. In recent years considerable evidence has accumulated to indicate that the RNA participates intensively in the catalysis of peptide-bond formation, whereas a direct involvement of the ribosomal proteins has yet to be demonstrated. Here we report the functional and structural conservation of a peptidyltransferase centre protein in all three phylogenetic domains. *In vivo* replacement studies show that the *Escherichia coli* L2 protein can be replaced by its homologous proteins from human and

archaeobacterial ribosomes. These hybrid ribosomes are active in protein biosynthesis, as proven by polysome analysis and poly(U)-dependent polyphenylalanine synthesis. Furthermore, we demonstrate that a specific, highly conserved, histidine residue in the C-terminal region of L2 is essential for the function of the translational apparatus. Replacement of this histidine residue in the human and archaeobacterial proteins by glycine, arginine or alanine had no effect on ribosome assembly, but strongly reduced the translational activity of ribosomes containing these mutants.

INTRODUCTION

In all living cells, translation of genetic information into proteins takes place at the ribosome. During the past three decades, extensive studies have been carried out to investigate the fundamental mechanisms of reactions involved in the translational process. However, our present knowledge of the central enzymic activity of the peptidyltransferase centre within the ribosome is still very limited, especially at the molecular level. It has been postulated for a long time that the peptidyltransferase activity is an integral part of the large ribosomal subunit [1] and the essential constituents have been narrowed down to domains IV and V of 23 S rRNA and a few ribosomal proteins, i.e. L2, L3, L4 and L23 [2]. During the initial phase of investigations the main enzymic activities of the ribosome were attributed to the proteins. Owing to the observation that RNA can exhibit catalytic activities [3] experimental activities were continued in the opposite direction [4,5]. It was then assumed that the ribosome has ribozyme-like activities, whereas the ribosomal proteins act only in stabilizing the conformation of functional rRNA sites within the ribosome. In addition, the unusually high conservation of the rRNA structures argued for a reflection of functional conservation throughout evolution. In contrast, comparisons of ribosomal proteins from different organisms showed relatively low sequence similarities indicating a large disparity of selective pressure for the conservation of the primary structure of individual proteins. To date it is not quite clear whether rRNA alone or rRNA together with ribosomal proteins contribute to peptidyltransferase reaction within the ribosome. Therefore it is challenging to identify the components involved in this central process. Among ribosomal proteins, L2 has been strongly implicated in protein synthesis [6–8]. Furthermore, L2 is highly conserved between species [9], which also indicates a functional importance of this protein in protein biosynthesis. In the present paper we report a functional replacement of the *Escherichia coli*

L2 protein by its homologous proteins from human (HumanL8) and the archaeobacterium *Haloarcula marismortui* (HmaL2). Further, we are able to show that a specific, highly conserved histidine residue within the C-terminal region of protein L2 is an essential component in protein biosynthesis by applying *in vitro* mutagenesis.

EXPERIMENTAL

Cloning and expression of the ribosomal proteins in *E. coli*

The coding region of ribosomal protein L2 from *H. marismortui* [10] and human ribosomal protein L8 [11] were obtained by PCR amplification, using genomic DNA of *H. marismortui* and a λ gt10 human brain cDNA library (Clontech, Palo Alto, CA, U.S.A.) respectively. Primers complementary to the 5' and 3' ends of the coding sequence of the ribosomal genes were synthesized using a commercial DNA synthesizer. The 5' end of each of the primers included appropriate restriction sites, followed by a 15–20-base region that was complementary to the genes. In particular, in the oligonucleotide complementary to the 5' end of the gene, the initiator ATG codon was part of an *Nco*I site. For the primer complementary to the 3' end of the L2 gene, the stop codon was followed by a *Bam*HI site, and in the case of the gene of humanL8 the restriction site was *Eco*RI. The translational stop codon in the inserts provides the expression of the target proteins without C-terminal fusions. The purified amplification products were cloned into pET-vectors, which carried a *T7lac* promoter (Novagen, Madison, WI, U.S.A.). The coding region for the L2 protein was introduced into pET-11d containing an ampicillin-resistance marker, whereas the gene of humanL8 was ligated into pET-24d, which contains kanamycin as the selective marker. The resulting plasmids were used to transform BL21(DE3)LysE (Novagen), and the recombinant proteins were expressed after induction with isopropyl β -D-

Abbreviations used: HmaLn, 50 S ribosomal subunit protein *n* of *Haloarcula marismortui*, which is similar to protein Ln of *Escherichia coli*; HumanL8, 50 S ribosomal subunit protein L8 of human, which is similar to protein L2 of *Escherichia coli*; IPTG, isopropyl β -D-thiogalactopyranoside; RP, reversed-phase; 2D, two-dimensional; 23 S rRNA, ribosomal RNA of the large ribosomal subunit; TP, total protein.

¹ Present address: B.R.A.H.M.S. Diagnostica G.m.b.H., Komturstasse 19–20, D-12099 Berlin, Germany.

² To whom correspondence should be addressed (e-mail liebhold@mdc-berlin.de).

thiogalactopyranoside (IPTG) for up to 3 h [12]. Aliquots of cell cultures were taken at various times, heated for 5 min at 95 °C in SDS/Tris sample buffer and analysed on a SDS/15 %-PAGE [13].

Ribosome preparation and analysis

Ribosomes (70 S), 50 S and 30 S ribosomal subunits and their total-protein (TP) portion were isolated from exponentially growing cells as described in [14,15]. To prove the incorporation of the expressed ribosomal proteins HmaL2 and humanL8, 100 µg of TP50 and TP70 (the total protein extracts of the 50 S and 70 S ribosomes) respectively were separated by two-dimensional gel (2D gel) electrophoresis as described in [16]. After Coomassie Blue staining, proteins were blotted on to PVDF membranes (Immobilon-P; Millipore, Bedford, MA, U.S.A.) as described in [17]. Areas corresponding to new spots were cut out and subjected to automated Edman degradation (Applied Biosystems Sequencer, model 477A; Procise, Foster City, CA, U.S.A.).

Generation of antibodies

In order to isolate the overexpressed protein for immunization, total cell extract was separated by preparative gel electrophoresis with a model 491 Prep Cell (Bio-Rad, Hercules, CA, U.S.A.). Fractions containing the recombinant protein were pooled and further purified by RP-HPLC on an HPLC Delta-Pak C4 column (Millipore Waters Chromatography, Milford, MA, U.S.A.). The gradient was 10 min isocratic elution with 2 % solvent B [propan-2-ol/acetonitrile (2:1, v/v) with 0.08 % trifluoroacetic acid] followed by a gradient of 2–50 % solvent B for 50 min. Solvent A was water with 0.1 % trifluoroacetic acid. Fractions were collected and freeze-dried. The purity of the material was confirmed by SDS/PAGE. Rabbits were injected subcutaneously with 400–600 µg of pure protein material suspended in complete Freund's adjuvant. Injections were repeated four times (3, 4, 5 and 10 weeks after the first immunization). The quality and titre of antisera were checked by immunoblotting [18,19] using the purified recombinant proteins.

In vitro mutagenesis

PCR-mediated *in vitro* mutagenesis of the genes *hmaL2* and *humanL8* was performed in a one-tube megaprimer-PCR reaction [20]. The codon for histidine-199 of *hmaL2* was replaced by the codons for arginine or glycine and the equivalent histidine-209 of *humanL8* was exchanged with glycine or alanine codons. The PCR products were purified and cloned into pGEM-3Z (Promega, Madison, WI, U.S.A.) to screen for mutations. After re-cloning into pET-11d and pET-24d respectively, the DNA sequences of the resulting mutants were confirmed for the presence of the introduced mutations and the integrity of the remaining sequence.

Polysome isolation and sucrose-gradient analysis

E. coli cells harbouring the appropriate plasmids were grown in 100 ml of M9ZB [12] and induced with IPTG at an A_{560} of 0.1. After three doublings following induction, the culture was incubated for 2 min with chloramphenicol to a final concentration of 1 mM, harvested by centrifugation in 2 volumes of crushed ice, and lysed by the lysozyme-freeze-thaw method [21]. The lysate was separated by sucrose-gradient centrifugation [10–40 % sucrose in 20 mM Hepes-KOH (pH 7.5)/150 mM NH_4Cl /10 mM MgCl_2 /5 mM β -mercaptoethanol; Sorvall AH629 centri-

fuge, 18 500 rev./min. 14 h, 4 °C]. The gradients were fractionated, and the ribosomal components were pooled and pelleted by centrifugation (Beckman TLA-100.3; 100 000 rev./min., 2 h, 4 °C). In the case of mutant and wild-type HmaL2, 0.05 A_{260} units of 30 S and 50 S subunits, 70 S ribosomes, disomes and higher polysomes were separated by SDS/PAGE. Western blotting and immunostaining with the polyclonal antibody against HmaL2 were performed as described above. The polysomal fractions of cells expressing mutant and wild-type HumanL8 were separated by 2D gel electrophoresis as described above, and the presence of the L8 protein was verified by N-terminal-sequence analysis.

In vitro translation

Ribosomes were purified as described in [22]. Polyphenylalanine synthesis was performed according to [23] with slight modifications. The ionic conditions of the polyphenylalanine system were 20 mM Hepes/KOH (pH 7.8)/6 mM MgCl_2 /150 mM NH_4Cl /2 mM spermidine/0.05 mM spermine/4 mM 2-mercaptoethanol. Each reaction was carried out in a 120 µl total volume using 27 pmol of 50 S subunits from cells expressing the heterologous protein reconstituted with 54 pmol of 30 S subunits from *E. coli* MRE600, 40 µg of tRNA^{bulk} (*E. coli*), 100 µg of poly(U), 10 µl of tRNA-free S100 enzymes and 3 µg of pyruvate kinase in a solution containing 100 µM [^{14}C]phenylalanine (10 c.p.m./pmol), 1.5 mM ATP, 0.05 mM GTP and 5 mM phosphoenolpyruvate. As a control, native 50 S subunits from *E. coli* were reconstituted in the same manner. After incubation for 60 min at 30 °C, polyphenylalanine synthesis was determined by hot trichloroacetic acid precipitation. The number of phenylalanines incorporated was calculated per 70 S ribosome.

Amino acid analysis

Proteins derived from 70 S ribosomes and 50 S subunits were separated by 2D gel electrophoresis and the gels were blotted on to PVDF membranes as described above. The corresponding areas of the heterologous proteins and the endogenous *E. coli* L2 protein were cut out and hydrolysed in 5.7 M HCl with 7 % thioglycolic acid at 110 °C for 24 h [24]. For quantitative determination of the proteins, the amino acid compositions were analysed on an amino acid analyser (Model S 432; Sycam, Gilching, Germany) using *o*-phthalaldehyde post-column derivatization [25].

Computer analysis

The SwissProt database was used for sequence comparison by FASTA (Genetics Computer Group, University of Wisconsin, 1991) (GCG). Multiple sequence analysis was done with the programs PILEUP and PRETTYPLOT (GCG).

RESULTS

Protein L2 from the halophilic archaebacterium *H. marismortui* (HmaL2) and the human equivalent (HumanL8) were expressed in *E. coli* (Figure 1A) in order to examine their effects on assembly and activity within the *E. coli* ribosome. If the heterologous proteins share sufficient identical structural and functional properties, replacement of the endogenous *E. coli* L2 protein (EcoL2) should be possible. To test this, ribosomes from cells expressing HmaL2 and HumanL8 were isolated following standard procedures by ultracentrifugation and thorough washes three times in a sucrose cushion in order to remove overexpressed

Figure 1 Ribosomal proteins L2 from *H. marismortui* (HmaL2) and L8 from human (HumanL8) are incorporated into eubacterial ribosomes after overexpression in *E. coli*

(A) SDS/PAGE of total cell lysates before (0) and 1, 2 and 3 h after induction of expression with IPTG, showing the overproduction of HmaL2 and HumanL8 proteins. The identity of the overexpressed proteins was confirmed by N-terminal sequencing. Abbreviation: marker, molecular mass markers. (B) 2D gel electrophoresis of total protein from isolated 50 S ribosomal subunits (TP50) of cells expressing HmaL2 or HumanL8 (lower panels) in comparison with TP50 from uninduced cells (upper panels). Arrows denote the positions of *E. coli* L2 protein and the incorporated HmaL2 and HumanL8 proteins. Abbreviations: + 1 st dim —, first dimension; — 2 nd dim +, second dimension.

Figure 2 *In vivo* incorporation of HmaL2 and HumanL8 into translationally active *E. coli* polysomes

(A) UV-absorption profile of the sucrose gradient showing the separation of ribosomal particles isolated from cells expressing ribosomal proteins. The elution profile was identical with that of uninduced *E. coli* cells (results not shown). Abbreviations: P, higher polysomes; D, disomes; 70S, 70 S ribosomes; 50S, 50 S subunits; 30S, 30 S subunits. (B) Detection of HmaL2 in polysomal and ribosomal fractions by immunostaining after overexpression. Note the presence of HmaL2 in higher polysomes as well as in the other ribosomal fractions. Abbreviations: ctr, control, using isolated ribosomal particles from uninduced cells; exp, isolated ribosomal fractions from cells expressing HmaL2. (C) 2D gel pattern of higher polysomes from cells expressing HumanL8. The arrow indicates the position of the incorporated HumanL8. The protein was also present in disomes, 70 S ribosomes and 50 S subunits but not in 30 S subunits (results not shown).

	160	170	180	190	200
Eco	GEVGNAEHMLRVLGKAGAR		WRGVREIVRG	TAMNPVDH	PHGGGE
Bst	GEVGNQEHMLVNIKGAGAR		WLGIREIVRG	SVMNPDH	PHGGGE
Spo	GLVAGGGRIIDKPIIKAGRA	YHKYKARN	QWERT	RGVAMNPVDH	PHGGGNEH
Human	GVVAGGGRIIDKPIIKAGRA	YHKYKARN	QWERT	RGVAMNPVDH	PHGGGNEH
Hma	GVVGGGGRIIDKPFV	KAGNKKHMKAR	GTKEW	NRGVAMNAVDH	PHGGGGR
Mva	GVVAGGGRIIDKPFV	KAGKHHSL	SAKAVAN	NRGVAMNAVDH	PHGGGGR

	210	220	230	239
Eco	...GRN	FG	KHPVTPWGVQTKGKKTRSNK	RTDKFIVRRRSK
Bst	...GRN	FG	KHPVTPWGVQTKGKKTRSNK	RTDKFIVRRRSK
Spo	QHVGCHSTTVF	RSAPGQK	VGLIAARRTG	LRLGAAAVEN
Human	QHVGCHSTTVF	RSAPGQK	VGLIAARRTG	LRLGAAAVEN
Hma	QHVGCHSTTVF	RSAPGQK	VGLIAARRTG	LRLGAAAVEN
Mva	QHVGCHSTTVF	RSAPGQK	VGLIAARRTG	LRLGAAAVEN

Figure 3 Sequence alignment of the C-terminal domains from members of the ribosomal protein L2 family (one-letter code)

The six sequences shown include representatives from each of the three lifeform Kingdoms, prokaryotes [*Escherichia coli* (Eco) and *Bacillus stearothermophilus* (Bst)], eukaryotes [*Schizosaccharomyces pombe* (yeast) (Spo) and *Homo sapiens* (human)] and archaea [*H. marismortui* (Hma) and *Methanococcus vannielii* (Mva)]. The numbering scheme refers to the *H. marismortui* sequence. Conserved residues are outlined. The conserved histidine residue chosen for *in vitro* mutagenesis is indicated by white letters in black outlines.

proteins that were not incorporated. These ribosomes were separated by sucrose-gradient centrifugation, and the protein composition of the ribosomal subunits was analysed by 2D gel electrophoresis. Both expressed proteins were found to be incorporated into 50 S ribosomal subunits from *E. coli* (Figure 1B). To show that replacement of the heterologous proteins had

really taken place, the quantities of the heterologous proteins in comparison with the endogenous proteins were determined by digital densitometry of the 2D gels and amino acid analysis. The results showed that about 50 % of the *E. coli* ribosome population contained the HumanL8 protein, whereas, in the experiment with the HmaL2 protein, up to 25 % of the endogenous L2 protein was replaced by HmaL2. The partial replacement of the endogenous EcoL2 protein by the overexpressed proteins resulted in a mixed ribosome population consisting of wild-type ribosomes and hybrid ribosomes which contained the HumanL8 or the HmaL2 protein respectively. To simplify matters, these mixed ribosome population will be designated in the following as hybrid ribosomes.

***In vivo* activity of the hybrid ribosomes**

To indicate whether hybrid ribosomes containing the heterologous proteins were translationally active *in vivo*, mRNA with two (disomes) or more translating ribosomes (polysomes) attached to it, were isolated from cells grown to mid-exponential phase and separated by sucrose-gradient centrifugation. A representative elution profile of the sucrose gradient is given in Figure 2(A). In the experiment shown in Figure 2(B), a polyclonal antibody against HmaL2 (which did not cross-react with EcoL2) was used to detect the archaeal protein in the different ribosomal fractions. The polysomal and ribosomal fractions of cells expressing HumanL8 were analysed by 2D gel electrophoresis and

Table 1 Effect of the mutated ribosomal proteins HmaL2 and HumanL8 on growth, ribosomal assembly and protein synthesis

Growth rates were determined as described in Figure 4. They were quantified, relative to *E. coli* cells expressing wild-type (wt) HmaL2 or HumanL8, using the following ratings: + + +, unchanged growth; + +, weak decreasing growth rate by about 10%; +, decreasing growth rate by about 30%; —, low growth rate (single colonies); — —, no growth detected. Ribosomal assembly of plasmid-encoded HmaL2 protein, HumanL8 and their mutants was determined as described for Figure 2; (+), incorporation; (—), no incorporation detected. The incorporation rate into 50 S subunits (given in percentages) was determined by quantitative densitometry of 2D gels and amino acid analysis. The activity in polyphenylalanine synthesis is given as a percentage of the efficiency of native ribosomes. Full activity (100%) was equivalent to the incorporation of 291 molecules of phenylalanine/ribosome. For details see the Experimental section. All listed data were averaged from four or more independent experiments.

Wild-type (wt)/mutant	Growth at:		Incorporation of mutant HmaL2 and HumanL8 protein in:			Polyphenylalanine synthesis (%)
	25 °C	37 °C	Polysomes	70 S ribosomes	50 S subunits [%]	
HmaL2 wt	+ + +	+ + +	(+)	(+)	25 ± 2	100 ± 1.5
HmaL2-Arg ¹⁹⁹	+	+	(—)	(+)	25 ± 3	81 ± 4.3
HmaL2-Gly ¹⁹⁹	+	+	(—)	(+)	25 ± 3	75 ± 1.8
HumanL8 wt	+ + +	+ + +	(+)	(+)	53 ± 2	100 ± 4.5
HumanL8-Gly ²⁰⁹	— —	—	(—)	(+)	53 ± 3	58 ± 2.3
HumanL8-Ala ²⁰⁹	— —	—	(—)	(+)	52 ± 2	55 ± 3.6

Figure 4 Effect of plasmid-encoded mutant HumanL8 on growth rate and sedimentation profile

(A) Growth rate at 25 °C of uninduced cells containing wild-type (wt) HumanL8 and mutated HumanL8 on agar plates containing 30 µg/ml kanamycin. (B) Colonies replated on agar plates containing 30 µg/ml kanamycin and 1 mM IPTG. No growth of the mutants could be detected in the presence of IPTG at 25 °C. (C) UV-absorption profile of the sucrose gradient from cells expressing mutant HumanL8. Note that the mutation gave an altered sedimentation profile compared with the wild-type one in Figure 2. No translationally active particles as polysomes and disomes could be obtained for further analysis. For abbreviations, see Figure 2(A).

the heterologous proteins were identified by N-terminal sequencing (Figure 2C). Strikingly, both proteins were incorporated into translationally active polysomes as well as into disomes, 70 S ribosomes and 50 S subunits. The absence of the heterologous proteins in the 30 S subunits proved that the incorporation is specific for the 50 S subunits (Figure 2B) and excludes the possibility that the heterologous proteins bind at a non-specific site on the ribosome.

Effect of mutant hybrid ribosomes on protein biosynthesis

Evidence from reconstitution studies [8] and chemical modifications and replacement studies of histidine residues within *E. coli* protein L2 [7,26] suggested that protein L2 may participate directly in protein synthesis. An alignment of the L2 protein family (Figure 3) shows that only one of the histidine residues is conserved between eubacteria, eukaryotes and archaeobacteria,

Figure 5 Effect of plasmid-encoded mutant HmaL2 in protein synthesis

(A) UV-absorption profile of the sucrose gradient of lysates from cells expressing mutant HmaL2-Gly¹⁹⁹. The elution profile is similar to the wild-type one in Figure 2. For abbreviations, see Figure 2(A). (B) Presence of mutant HmaL2-Gly¹⁹⁹ in ribosomes from *E. coli* as analysed by immunostaining. Note that the mutated protein does not take part in protein synthesis; no incorporation of the mutant protein into higher polysomes or disomes could be detected as compared with wild-type HmaL2 (Figure 2). HmaL2-Gly¹⁹⁹ is only present in 70 S ribosomes and 50 S subunits. The HmaL2-Arg¹⁹⁹ mutant showed an identical incorporation pattern (results not shown). For abbreviations, see Figure 2(B).

whereas other histidine residues within the N-terminal or central domain of L2 are not conserved throughout these three Kingdoms. Thus this highly conserved histidine residue was selected for *in vitro* mutagenesis in HmaL2 and HumanL8. His¹⁹⁹ in HmaL2 (corresponding to His²²⁹ of EcoL2; Figure 3) was changed to glycine and arginine (HmaL2-Gly¹⁹⁹ and HmaL2-Arg¹⁹⁹), and the equivalent His²⁰⁹ in HumanL8 was replaced by glycine and alanine (HumanL8-Gly²⁰⁹ and HumanL8-Ala²⁰⁹). The altered proteins were expressed in *E. coli*, and analysis of the isolated ribosomes showed the incorporation of these proteins within 70 S ribosomes and 50 S subunits (results not shown). The ribosomal protein pattern, as well as the relative amount of incorporation of the mutant proteins, was identical with that of wild-type HmaL2 and wild-type HumanL8, as determined by quantitative densitometry of the 2D gels and amino acid analysis of the proteins. The expression of the HmaL2 mutants in *E. coli* slightly affected the growth rate at 37 °C, and a reduced colony formation could be observed at 25 °C (see Table 1). In contrast, and most significantly, HumanL8-Gly²⁰⁹ and HumanL8-Ala²⁰⁹ expressed in *E. coli* showed a lethal phenotype (see Table 1). Uninduced cells containing plasmid-encoded wild-type and mutants HumanL8 grew equally well on agar plates containing 30 µg/ml kanamycin as a selective marker (Figure 4A). However, when IPTG (1 mM/ml) was included in the plates, in addition to kanamycin, so that expression of the HumanL8 protein was induced, the mutants were unable to form colonies, whereas expression of wild-type HumanL8 had no effect on the growth rate (Figure 4B).

To clarify whether these hybrid ribosomes containing the mutant proteins are also able to participate in the translation process, polysomes of *E. coli* cells expressing the mutant proteins were isolated as described above. Ribosomes as well as polysomes

could only be isolated for one generation after induction with IPTG, owing the lethal effect of both mutants of HumanL8 as mentioned above. Although 70 S ribosomes and 50 S subunits both contained mutant HumanL8 proteins (see above), the polysomal fraction obtained after sucrose-gradient centrifugation was clearly degenerated (Figure 4C; cf. Figure 2A), thus indicating the lethal effect of mutant HumanL8 in protein synthesis. In the case of HmaL2-Gly¹⁹⁹ and HmaL2-Arg¹⁹⁹, the elution profile of the sucrose gradient showed no significant difference from the wild-type HmaL2 (shown for HmaL2-Gly¹⁹⁹; Figure 5A; cf. Figure 2A). However, by immunostaining with a polyclonal antibody against wild-type HmaL2 of the different fractions, only the 70 S and 50 S fractions gave a positive immune response, showing that the mutant proteins were present, whereas neither HmaL2-Gly¹⁹⁹ nor HmaL2-Arg¹⁹⁹ could be detected within the polysomal fractions by immunostaining, demonstrating their absence in these translationally active particles (Figure 5B).

In vitro translation of the hybrid ribosomes

The 50 S subunits of all ribosome populations were isolated and assayed for *in vitro* poly(U)-dependent polyphenylalanine synthesis. The activity of the hybrid ribosomes was compared with that of native *E. coli* ribosomes (see the Experimental section). Table 1 shows that hybrid ribosomes containing either the HmaL2 or the HumanL8 protein were fully active in polyphenylalanine synthesis. These data confirm the results obtained *in vivo* and give direct evidence for the functional replacement of the EcoL2 protein by the heterologous proteins. Finally, 50 S subunits containing the mutant proteins were also tested for polyphenylalanine synthesis (Table 1). Hybrid ribosomes con-

taining HmaL2-Arg¹⁹⁹ were 81 % active, and hybrid ribosomes with incorporated HmaL2-Gly¹⁹⁹ were 75 % active as compared with hybrid ribosomes with the wild-type protein HmaL2. Hybrid ribosomes with incorporated HumanL8-Gly²⁰⁹ showed only 58 % activity, and the activity of hybrid ribosomes containing HumanL8-Ala²⁰⁹ was reduced to 55 %. Considering that these ribosomes consist of a mixed population containing either the endogenous EcoL2 or the mutant HmaL2 or mutant HumanL8 proteins respectively within the 50 S subunits, these values reflect the activities of the unmodified *E. coli* ribosomes.

DISCUSSION

We have chosen L2 proteins from two distantly related organisms for functional replacement studies in order to investigate the specific role of ribosomal protein L2 in protein biosynthesis. Although protein L2 is among the most conserved ribosomal proteins throughout evolution [9,27], the proteins used in our experiments are rather divergent when compared with their *E. coli* counterpart. Protein L2 from the halophilic archaeobacterium *H. marismortui* (HmaL2) shows 36 % identical amino acids in comparison with the eubacterial L2 protein, and the human equivalent (HumanL8) only 30 % identity with its *E. coli* homologue. Despite these differences, both the archaeobacterial and human protein are able to replace their *E. coli* homologue. Interestingly, the HumanL8 protein was incorporated about twice as efficiently as was HmaL2. A possible explanation for the poorer incorporation of the HmaL2 protein could be that the halophilic L2 protein differs more in surface properties than the other L2 proteins, owing to its strong saline environment [28]. An indication for this is that the excess of HmaL2, which was not incorporated into the ribosomes, had formed insoluble products such as inclusion bodies. However, raising the salt concentration of the lysis buffer to 0.6 M NaCl results in soluble protein remaining in the supernatant after centrifugation of the cell lysate. The obvious necessity of high salt concentration for correct folding of the HmaL2 protein leads to a possible explanation for the poor incorporation of the HmaL2 protein into the *E. coli* ribosomes.

The hybrid ribosomes generated are fully active in protein synthesis *in vivo*, as shown by polysome analysis and *in vitro* translation. Here we show, for the first time, the incorporation of eukaryotic and archaeobacterial ribosomal proteins into eubacterial ribosomes *in vivo*. While the successful incorporation of non-eubacterial ribosomal proteins into *E. coli* ribosomes *in vitro* has also been described for other ribosomal proteins (e.g. L12 from *Sulfolobus solfataricus* [29] and maize (*Zea mays*) chloroplast L23 [30]), the functionality of these ribosomes has not been tested *in vivo*. Replacement studies were also performed with HmaL3 and HmaL23 overexpressed in *E. coli* [31]. Like L2, these proteins are essentially involved in ribosome assembly [32] and are also located in the proximity of the peptidyltransferase centre of the ribosome [33,34]. However, we could demonstrate that HmaL23 was not assembled into ribosomes at all. In contrast, HmaL3 was able to replace up to 35 % of the eubacterial L3 protein in the assembly process, but the resulting hybrid ribosomes were not active in protein biosynthesis *in vivo* and *in vitro* [31]. The protein was absent in polysomes and was only incorporated into 70 S ribosomes and 50 S subunits. Furthermore, the activity in polyphenylalanine synthesis was drastically reduced.

These findings point to the high functional conservation of protein L2. Our results are consistent with other data reflecting that protein L2 is an essential component within the ribosome. In various biochemical studies [6], protein L2 has been reported to

be a major constituent of the peptidyltransferase centre. Watanabe and Kimura [35] have shown, by protein-protection analysis, that the central domain of protein L2 contains the primary rRNA-binding site and that the N- and/or C-terminal region of L2 may be involved in peptidyltransferase activity. These data are supported by immunoelectron-microscopic analysis. Monoclonal antibodies raised against the N- and C-terminal domain of *E. coli* L2 protein inhibited polyphenylalanine synthesis and the association of the subunits [36,37]. Deletion of a region close to the C-terminus of protein L2 resulted in aberrant 50 S subunits which lack L16 completely and are unable to form 70 S ribosomes [38]. Moreover, Maden and Monro [1] proposed a correlation between the pH-dependence of the peptidyltransferase reaction and the involvement of the imidazole part of a histidine residue of a ribosomal protein. These results led to the hypothesis that a histidine residue, together with a carboxy group from a ribosomal component, is involved in both peptide-bond formation and peptidyl-tRNA hydrolysis, as has been described for the active centre of serine proteases [39]. Therefore studies were performed using histidine-specific photochemical oxidation. Modification of all histidine residues within proteins L2, L4 and L16, which are possible candidates for the peptidyltransferase activity, abolished the peptidyl transfer [26,40]. On the basis of reconstitution studies, L16 was excluded from these potential candidates necessary for peptidyltransferase activity [8].

Sequence alignment of the L2 protein family shows only one of the histidine residues conserved in pro- and eukaryotes as well as in archaea. The local region around this histidine residue is not part of the 23 S rRNA-binding domain [35]. It seems unlikely that mutations in this region will have deleterious effects on protein synthesis by perturbing the rRNA structure and/or the protein-rRNA interaction sites. To investigate the specific role of this histidine residue, we exchanged this amino acid in HmaL2 with glycine and arginine and in HumanL8 with glycine and alanine respectively. These side chains cannot substitute for the histidine imidazole group in a catalytic mechanism involving general acid-base catalysis [39]. We examined the incorporation of the mutant proteins into *E. coli* ribosomes as well as their effects on protein synthesis in *E. coli*. Strikingly, an amino acid exchange at this histidine residue in HumanL8 leads to a drastic reduction in growth rates and thus provides first evidence for the functional importance of this amino acid within the protein. Mutants of HmaL2 at the corresponding position also lowered the growth rate, but showed less drastic effects, owing to the difference in incorporation rate. Accordingly these ribosomes had wild-type-like sedimentation profiles. However, the analysis of polysomes of these *E. coli* cells showed no incorporation of the mutant HmaL2 protein in translationally active particles. Furthermore, the translational activities of these mutants containing hybrid ribosomes were clearly reduced in the poly(U) assay. To define the nature of the defect in protein synthesis, it is important to note that our mutations did not lead to an assembly defect of ribosomes and gave no alterations in protein composition of ribosomal subunits as analysed by sucrose-gradient centrifugation and densitometry of the 2D gels. These data are consistent with *in vitro* studies by Cooperman et al. [7]. Those authors showed that a mutation of His²²⁹ to Gln in EcoL2 completely abolished the activity of reconstituted 50 S subunits in polyphenylalanine synthesis. The reconstituted 50 S subunits also assembled into functional 70 S ribosomes. Additionally, they could show that the mutants bind to 23 S rRNA in the same manner as wild-type L2, indicating that the exchange of the His residue causes no general perturbation of L2 or the ribosome structure. Taking into account these *in vitro* data and our *in vivo*

data, an exchange of the highly conserved histidine residue within protein L2 in all three Kingdoms mainly affects the elongation process rather than translational initiation.

The altered growth characteristics in our studies could, in principle, reflect the impairment of protein synthesis. The HumanL8 mutations lead to a lethal phenotype, and the mutations in HmaL2 produced altered growth rates, although the cellular ribosomes contained a mixture of plasmid- and chromosome-encoded L2 protein. During protein biosynthesis, multiple initiations take place, with a new ribosome 'hopping' on to the 5' end of a molecule of mRNA almost as soon as the preceding ribosome has translated enough of the amino acid sequence to get out of the way. Ribosomes containing mutant L2 and mutant humanL8 respectively might block the translation process until the inactive ribosome releases the mRNA molecule and a new ribosome can hop on to the mRNA. The ribosome population of *E. coli* cells expressing mutant HumanL8 contained more functionally inactive ribosomes than the ribosome population of cells expressing mutant HmaL2, owing to the higher incorporation of HumanL8 into *E. coli* ribosomes. The probability that inactive ribosomes bind to the mRNA instead of functionally active ribosomes is higher in the case of cells expressing mutant HumanL8 than for cells expressing mutant HmaL2. The excess of endogenous EcoL2 protein, and therefore native ribosomes, could compensate for the defect of functional impairment in the mutant ribosomes of cells expressing variants of HmaL2.

The present results support the possibility that the highly conserved histidine residue is an essential part of the peptidyl-transferase catalytic centre. These results are in line with relevant literature data on the peptidyltransferase centre. Evidence for a participation of L2 in the translational process derives from its attachment site on 23 S rRNA. It has been shown by chemical and ribonuclease footprinting methods that L2 binds to the central part of domain IV on 23 S rRNA [33,41]. This domain, together with domain V of 23 S rRNA, is now generally accepted as being an integral and functional important part of the peptidyltransferase centre [42]. Intra-rRNA, inter-rRNA and tRNA-rRNA cross-linking experiments [43] and hydroxyl-radical footprinting experiments [44] indicated a very close proximity of L2 to potential functionally important components of the peptidyl-transfer reaction (e.g. acceptor end of tRNA, decoding region of the 16 S rRNA, growing peptide chain). In light of these data, protein L2 might be as important as the 23 S rRNA for the peptidyltransferase activity of the ribosome.

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