The extended loops of ribosomal proteins L4 and L22 are not required for ribosome assembly or L4-mediated autogenous control

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ABSTRACT

Ribosomal proteins L4 and L22 both have a globular domain that sits on the surface of the large ribosomal subunit and an extended loop that penetrates its core. The tips of both loops contribute to the lining of the peptide exit tunnel and have been implicated in a gating mechanism that might regulate the exit of nascent peptides. Also, the extensions of L4 and L22 contact multiple domains of 23S rRNA, suggesting they might facilitate rRNA folding during ribosome assembly. To learn more about the roles of these extensions, we constructed derivatives of both proteins that lack most of their extended loops. Our analysis of ribosomes carrying L4 or L22 deletion proteins did not detect any significant difference in their sedimentation property or polysome distribution. Also, the role of L4 in autogenous control was not affected. We conclude that these extensions are not required for ribosome assembly or for L4-mediated autogenous control of the S10 operon.

Keywords: Autogenous control; polysomes; protein structure; rRNA

INTRODUCTION

Two-thirds of the mass of the bacterial ribosome is made up of rRNA. Not surprisingly, the crystal models of ribosomes give the impression of massive RNA structures decorated with small areas of proteins and offer convincing evidence that the peptidyl transferase activity of the ribosome is mediated by the RNA (Ban et al. 1999; Nissen et al. 2000). Nevertheless, ribosomal proteins (r-proteins) do affect the function of the ribosome, as is most clearly evidenced by mutations in r-protein genes that change rRNA structure (Gregory and Dahlberg 1999), ribosome sensitivity to antibiotics (Cundliffe 1980), the accuracy with which the ribosome translates the genetic code (Kurland et al. 1996), and translation arrest (Nakatogawa and Ito 2002). Furthermore, r-proteins may be involved in conformational switches in the ribosome during the translation process (Lodmell and Dahlberg 1997; Gao et al. 2003).

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High-resolution crystallographic studies of the 30S and 50S ribosomal subunits have revealed a remarkable structural feature of many r-proteins: a long terminal or internal extension, typically reaching from a globular domain positioned on the ribosome surface into the subsurface mass of the rRNA (Ban et al. 2000; Wimberly et al. 2000; Harms et al. 2001). Spectacular examples are the extended loops of L4 and L22, which reach all the way to the lumen of the peptide exit channel to form part of its lining (Ban et al. 2000; Nissen et al. 2000). The tips of these two proteins contribute to a constriction of the tunnel that might function as a gate to regulate the rate of translation (Nissen et al. 2000; Nakatogawa and Ito 2002). Moreover, mutations in the extensions of L4 and L22 result in resistance to the antibiotic erythromycin (Chittum and Champney 1994), an effect that correlates with changes in the ribosomal tunnel system (Gabashvili et al. 2001). Thus, the extensions of L4 and L22 could play a unique role in the function of the 50S subunit.

R-proteins also have roles outside of the ribosome. Some r-proteins (e.g., S10 and S4) associate with the transcription complex and help modify the processivity of the RNA polymerase (DeVito and Das 1994; Torres et al. 2001). Other r-proteins have a second function as regulators, autogenously inhibiting expression of their own (usually multicistronic) transcription units when they are produced in

excess of available binding sites on nascent rRNA molecules during ribosomal assembly (Zengel and Lindahl 1994; Keener and Nomura 1999). Autogenous control typically operates at the level of translation, but r-protein L4 in *Escherichia coli* and other γ proteobacteria regulates both transcription and translation of the 11-gene S10 operon (Yates et al. 1980; Zengel et al. 1980; Lindahl et al. 1983).

To gain insight into the role of L4's extension in autogenous control and in ribosome assembly and function, we constructed and analyzed mutant versions of L4 that lack part or all of the loop. Because the extension of L22 has been implicated together with L4's extended loop in a possible gating mechanism in the peptide exit tunnel (Gabashvili et al. 2001), we also constructed extension deletions in L22. We find that extensionless L4 still regulates the S10 operon, so the loop is not required for autogenous control. Furthermore, both L4 and L22 lacking their extensions are incorporated into normally sedimenting 50S ribosomal particles that can pair with 30S to form 70S ribosomes and accumulate in polysomes. These results indicate that the tentacles are not required for ribosome assembly and suggest that ribosomes assembled with an L4 or L22 protein lacking substantial parts of their extensions can still interact with 30S subunits and mRNA.

RESULTS

Regulation of the S10 operon by L4 loop-deletion mutants

We constructed three deletions in the *E. coli* L4 r-protein gene designed to remove progressively increasing portions of the extended loop. The smallest deletion, L4–Δloop1, was modeled on a spontaneous deletion in the L4 gene of *Bacillus stearothermophilus* that results in erythromycin resistance (V. Kruft, pers. comm.). The extent of these deletions is shown in the linear amino acid sequence in Figure 1A, and on a model of the *E. coli* L4 protein in Figure 1B. These deletions were introduced into an L4 gene carried by a plasmid where the gene is under control of an arabinose-inducible promoter. To facilitate later purification of the protein, the mutations were also introduced into L4 genes encoding either a 6-histidine (His6) or a Strep-tag sequence at the C-terminal end. We observed no effect of the C-terminal tags on L4 activity.

To test the ability of the mutant proteins to regulate the S10 operon, the mutant L4 genes were induced in a strain also harboring a plasmid with an S10′/′*lacZ* reporter gene downstream of the complete S10 leader (Fig. 2A). The presence of the S10 leader makes the reporter protein subject to transcription and translation control of the induced L4 protein. Figure 2B shows the reduction of S10′/′β-gal fusion protein synthesis in response to induction of L4 proteins with reduced or absent loops. The repressor effect of all of the deletion L4 proteins is indistinguishable from that of the wild-type L4.

Because L4 regulates both translation and transcription of the S10 operon, we also tested the ability of the deletion mutants to regulate target genes subject to only transcription or only translation control by L4 (Zengel and Lindahl 1996). Again, the mutants showed activity that was not distinguishable from wild-type L4 (data not shown). We conclude that the extended loop has no essential function in L4's role as a regulatory protein.

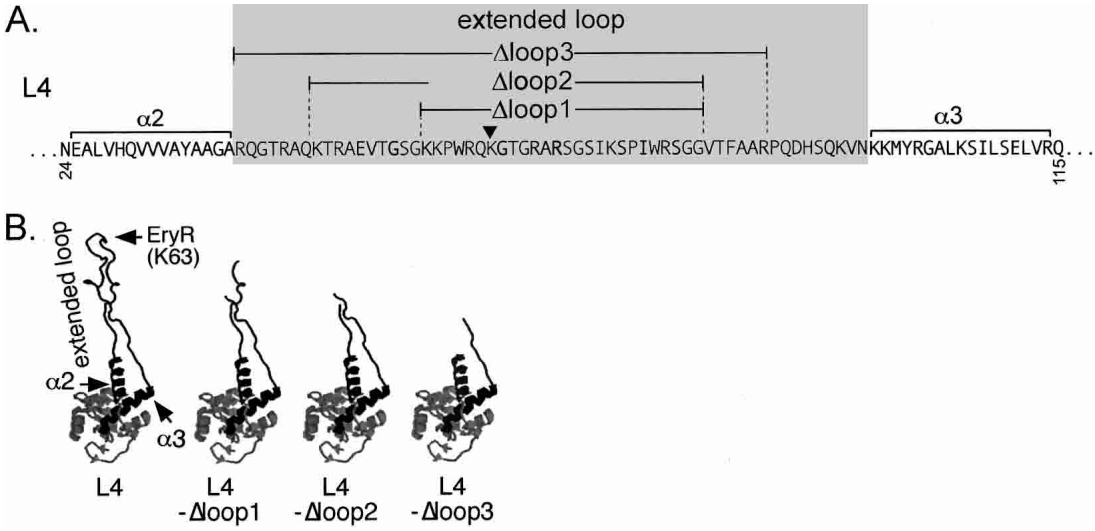

FIGURE 1. Structure of r-protein L4. (*A*) Sequence of the extended loop region of the *E. coli* r-protein L4 (201 amino acids total). The gray box indicates the region corresponding to the disordered structure in free L4 from *T. maritima* and extended loop in ribosome-bound L4 from *Haloarcula marismortui*. The extents of the deletions are shown. (▼) Amino acid which, when changed to glutamic acid, confers erythromycin resistance to *E. coli*. (*B*) Model of *E. coli* L4 drawn with RasMol (Bernstein 2000). The portions of the extended loop removed in the deletion mutants are indicated.

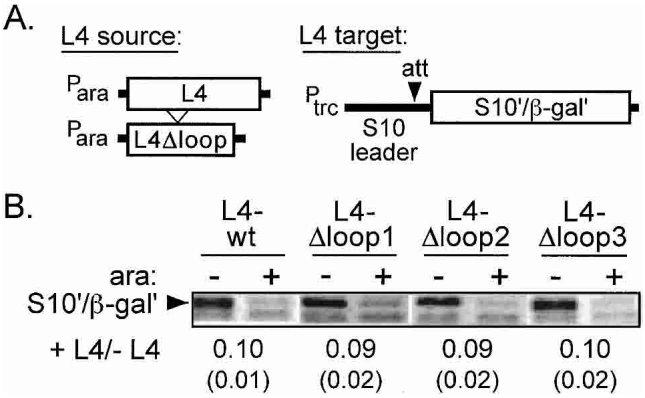

FIGURE 2. Autogenous regulation by wild-type and mutant L4 proteins. (*A*) Maps of plasmids. The source plasmid carries the L4 gene under control of an arabinose-inducible promoter. The target plasmid contains the S10 leader and S10′/′*lacZ* fusion gene under control of an IPTG-inducible promoter. (*B*) Effect of L4 oversynthesis on S10′/′β-gal synthesis. Cells induced with IPTG (−) or IPTG and arabinose (+) were pulse-labeled with [^{35}S]methionine and analyzed by gel electrophoresis and PhosphorImager quantitation. The effect of a given L4 on fusion protein synthesis (+L4/−L4) was calculated as described in Materials and Methods. Values shown are the average of all experiments for a given L4, whether or not it had a tag (n = 4–8). Standard deviations are in parentheses.

Incorporation of L4 loop-deletion proteins into ribosomal particles

We next investigated the effect of the deletions on the incorporation of L4 into ribosomes. Cells were harvested after induction of the plasmid-borne L4 gene, and a crude ribosomal preparation was produced by centrifugation of a whole-cell lysate. The content of L4 in the crude ribosomes was evaluated by Western analysis. Because the extension penetrates deep into the core of the 50S subunit and makes extensive contact with 23S rRNA, we expected that the deletion proteins would be defective in ribosome incorporation. To our surprise, the content of L4 in the crude ribosomal pellet was not noticeably affected by the deletion of the extension (Fig. 3).

To test whether the mutant L4 proteins were stably associated with the ribosomes, we salt-washed the ribosomes in the crude ribosome pellet. This procedure, consisting of diluting the crude ribosomes in buffer containing 1 M ammonium chloride and repelleting the ribosomes by centrifugation, is traditionally used to distinguish between true ribosomal proteins and proteins associated with ribosome as contaminants or accessory translational factors. Western analysis of the crude and salt-washed ribosomes, using an antibody specific to L4 or the relevant C-terminal tag, revealed no significant loss of any of the extension-deletion L4 proteins during ribosome purification, even after two consecutive high-salt washes (Fig. 3; data not shown). Thus, deletions of the extended loop do not affect the ability of L4 to be assembled stably into ribosomal particles.

Although it is not apparent from the Western blot shown in Figure 3, experiments in which we compared the abundance of mutant and wild-type L4 by probing with antibody to L4 showed that, after 2–4 doublings in the presence of arabinose, roughly one-quarter to one-half of the total L4 in ribosomes was mutant L4.

Incorporation of L4 loop-deletion proteins into normally sedimenting 50S subunits

To further characterize the particles containing L4 mutant proteins, we sedimented crude ribosomes through low-magnesium sucrose gradients to separate 30S and 50S particles. As we have reported previously (Lindahl and Zengel 1979), L4-mediated repression of the S10 operon, which encodes 11 r-proteins, results in accumulation of incomplete ribosomal particles. Hence, arabinose induction of the L4–Δloop mutant proteins or wild-type L4 followed by sucrose gradient analysis of crude ribosomes generated A^{260} profiles with a shoulder on the 30S side of the 50S peak (data not shown). Also, Western analysis showed that the induced L4 proteins, mutant or wild type, were contained in particles smaller than 50S (data not shown).

To circumvent the complication of autogenous control, we "chased" the deletion proteins from incomplete particles

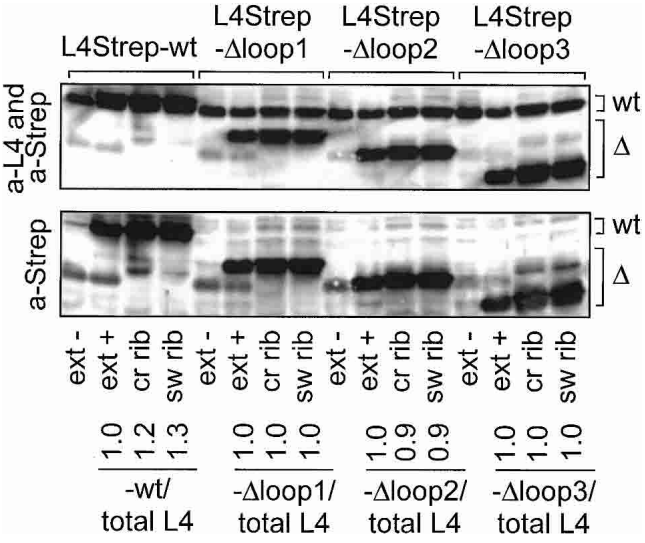

FIGURE 3. Western analysis of extracts and ribosomes from cells synthesizing the indicated L4 derivatives. Extracts from uninduced and induced cells (ext − and ext +, respectively) and crude and salt-washed ribosomes from induced cells (cr rib and sw rib, respectively) were fractionated on a 15% PAGE gel and subjected to Western analysis using antibodies to the Strep tag (a-Strep) followed by L4 antisera (a-L4). The relative amount of induced L4–Δloop protein was calculated from the anti-L4 Western by dividing the signal in the deletion protein by the total signal in deletion and wild-type (chromosome-derived) protein, and then normalizing the ribosome values to the induced extract value. The amount of induced wild-type L4 was calculated by dividing the signal from the Strep antibody (plasmid-derived L4) by the value from the L4 antibody (chromosome- and plasmid-derived L4), again normalizing to the extract value.

into normal ribosomes. After inducing the synthesis of plasmid-encoded extensionless or wild-type L4 proteins with arabinose for several generations, we washed the cells free of arabinose, resuspended them in fresh medium containing glucose, and allowed them to grow for one doubling in the absence of inducer. The chase was successful in eliminating the shoulder of <50S particles. More importantly, after the chase, the L4 proteins produced during the induction phase were found in what appear to be mature 50S particles (data not shown).

To analyze more carefully the sedimentation properties of ribosomes carrying the extension-deletion L4 proteins, we pooled the fractions from the 50S region of a low-magnesium sucrose gradient, pelleted the ribosomal particles, and centrifuged them again through a low-magnesium sucrose gradient, together with ^{3}H-labeled 50S isolated from uninduced cells carrying no L4 plasmid (these tracer 50S subunits contributed <10% to the total mass of ribosomes loaded on the gradient). The profile of the Strep-tagged wild-type and Δloop proteins, determined by Western analysis, matched both the A^{260} and the ^{3}H radioactivity profiles (Fig. 4A; data not shown). We conclude that deletions removing part or most of the extended loop still allow incorporation of the L4 protein into 50S ribosomes that are indistinguishable from normal ribosomes by sedimentation analysis.

L4 loop-deletion proteins are found in polysomes

Having shown that an L4 protein lacking the extended loop was incorporated into 50S subunits, we investigated whether the L4 loop-deletion proteins could be found in the polysomes. Crude ribosomes from chased cells synthesizing L4–Δloop1, -Δloop2, or -Δloop3 were sedimented through high-magnesium sucrose gradients. As seen in Figure 4B, the distribution of the extension-deletion L4 proteins was indistinguishable from the distribution of the chromosome-derived wild-type L4 protein: Most of the mutant L4 proteins were found in 70S particles, but a detectable amount was found in the polysome fractions of the gradient. These results suggest that 50S subunits with L4 derivatives lacking much or all of the extended loop are still capable of forming 70S couples, and those couples can associate with mRNA.

Analysis of ribosomal protein L22

As already mentioned, L4 is only one of a set of ribosomal proteins that have long extensions penetrating into the core of the ribosome. One other such r-protein is L22 (Ban et al. 2000; Nissen et al. 2000). Interestingly, the erythromycin-resistant protein (here called L22–Δery) has a three-amino-acid deletion in the extension of L22 (Chittum and Champney 1994) (Fig. 5). Hence, a small deletion in the loop still permits incorporation into active 50S ribosomal subunits. To determine whether L22 derivatives lacking more of the extension can be assembled into subunits, we made two additional deletions, removing about half (Δloop1) and nearly all (Δloop2) of the L22 extended loop (Fig. 5). The various L22 genes were then placed in the arabinose-inducible vector used for the L4 studies, with a Strep-tag at the C terminus. Because L22 has no regulatory properties, induction of the various derivatives should not have any complicating regulatory consequences.

Western analysis of crude ribosomal pellets showed that all of the Strep-tagged L22 proteins are assembled into ribosomal particles (Fig. 6). Therefore, like L4, the L22 pro-

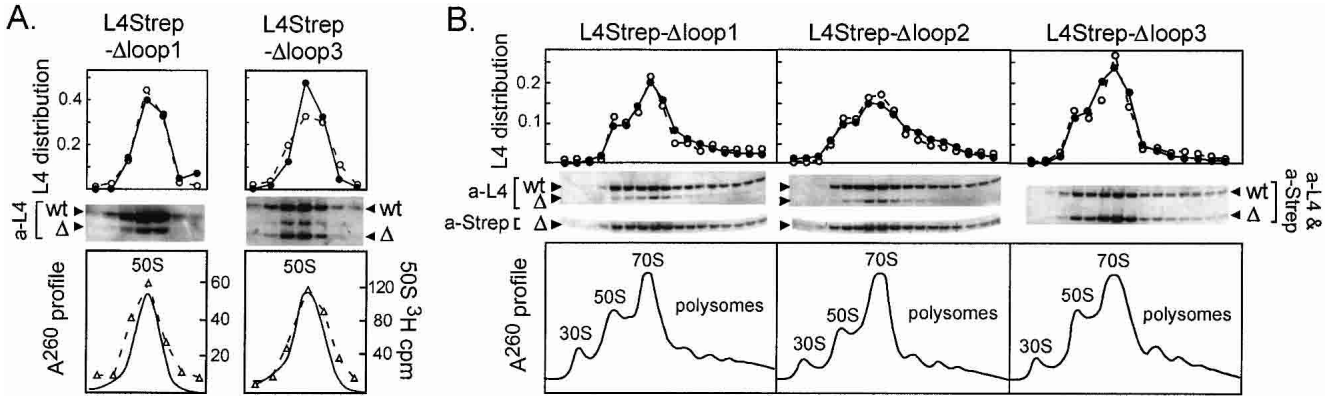

FIGURE 4. Sucrose gradient analysis of ribosomal particles containing L4 deletion proteins. (*A*) Distribution of L4 mutant proteins in 50S ribosomes. (*Bottom*) A^{260} profile together with a plot of the radioactivity in each fraction (indicated by open triangles and broken lines). (*Middle*) Western analysis using a 15% polyacrylamide gel and L4 antiserum. (*Top*) The signal in chromosome-derived L4 (wt) or in the indicated plasmid-derived extension-deletion protein (Δ) was calculated as described above. (○, broken line) Chromosome-derived L4; (●, solid line) plasmid-derived (mutant) L4. (*B*) Polysome gradient analysis. (*Bottom*) A^{260} profiles. (*Middle*) Western analysis of proteins from the collected fractions, probed with the indicated antibodies. (*Top*) For each fraction, the signal in chromosome-derived L4 (wt) or the indicated plasmid-derived extension-deletion protein (Δ) was divided by the total signal for the protein in all of the fractions of the gradient. (○, broken line) Chromosome-derived L4; (●, solid line) plasmid-derived (mutant) L4.

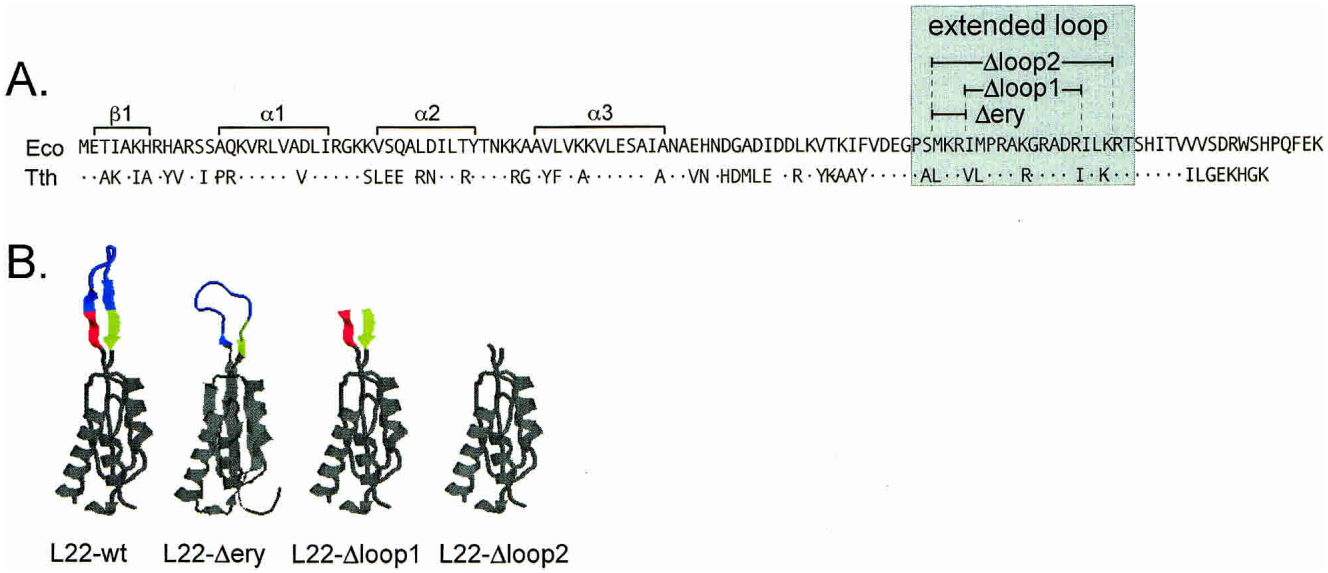

FIGURE 5. Structure of r-protein L22. (*A*) Amino acid sequence alignment of r-protein L22 from *E. coli* (Eco) and *Thermus thermophilus* (Tth). Amino acids identical in both proteins are indicated by dots in the *Tth* sequence. (*B*) Structure of L22 from *T. thermophilus* L4. The portions of the extended loop removed in the *E. coli* deletion mutants are indicated on the *T. thermophilus* structure in red (Δery), green (Δloop1), and blue (removed in Δloop2 together with amino acids indicated in red and green). The wild-type structure (Unge et al. 1998; PDB file 1BXE) and the structure of the L22–Δery mutant (Davydova et al. 2002, PDB file 1I4J) were drawn using RasMol (Bernstein 2000). The actual structures of Δloop1 and Δloop2 are not known; the deleted amino acids were removed from the wild-type L22 model.

tein is not dependent on the extension for incorporation into ribosomes. To test the stability of the proteins in ribosomal particles, we isolated salt-washed ribosomes. The Δery and Δloop1 proteins were also stably associated with ribosomal particles during the salt wash (Fig. 6). However, most or all of the L22Strep-Δloop2 protein appears to be lost during this step. Therefore, an L22 protein lacking almost all of the extension is incorporated efficiently into ribosomal particles, but appears to be released in the presence of high salt. Unfortunately, L22Strep-Δloop2 migrates very close to a cross-reacting band on the gel (x in Fig. 6), preventing reliable quantitation of this protein. Other gel conditions failed to separate the Δloop2 mutant from this contaminating band, which copurifies with ribosomal particles, so we focused on the L22–Δloop1 mutant.

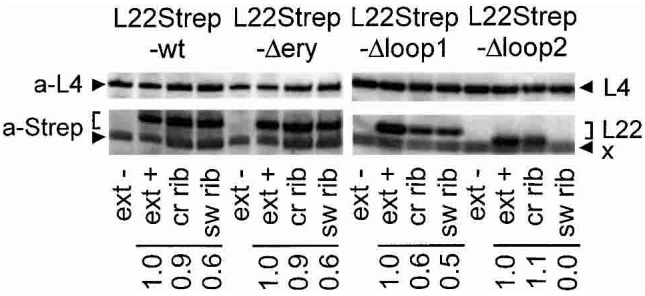

FIGURE 6. Western analysis of extracts and ribosomes from cells synthesizing L22 derivatives. Extracts from uninduced and induced cells (ext − and ext +, respectively) and crude and salt-washed ribosomes from induced cells (cr rib and sw rib, respectively) were fractionated on 10% Tricine gels and subjected to Western analysis using anti-sera to the Strep-tag on L22 (a-strep), followed by antisera to L4 (a-L4). The relative amount of induced L22 wild-type or mutant protein was calculated by dividing the signal in L22 (detected by anti-Strep) by the signal in the chromosome-derived L4 protein (detected by anti-L4). Resulting values for crude and salt-washed ribosomes were then normalized to the value in the corresponding induced extract. The band identified as x is a protein cosedimenting with ribosomal particles and recognized by the Strep-tag polyclonal antibody.

For a more detailed analysis of ribosomes containing L22–Δloop1, we centrifuged ribosomal pellets through sucrose gradients and analyzed by Western analysis the amount of L22 (and, as a control, L4) in each fraction. On a gradient separating 30S and 50S ribosomes, Strep-tagged versions of L22 wild-type and deletion proteins were found in the 50S region of the gradient, in a pattern indistinguishable from the chromosome-derived L4 pattern (data not shown). We purified the 50S subunits and showed that particles carrying the extension mutant protein had the same sedimentation pattern as normal 50S ribosomes (Fig. 7A). We conclude that the full L22 extension is not essential for incorporation into ribosomal particles with normal sedimentation properties.

To determine whether 50S ribosomes carrying the L22–Δloop1 proteins can associate with 30S subunits and form polysomes, we ran the crude ribosomes on polysome gradients. So that we could directly compare the distribution of the L22 wild-type and Δloop1 proteins, crude ribosomes from cells expressing the two proteins were pooled and cosedimented through the same gradient. The distribution of L22Strep-Δloop1 was indistinguishable from the distribution of wild-type L22Strep (Fig. 7B). Both proteins were

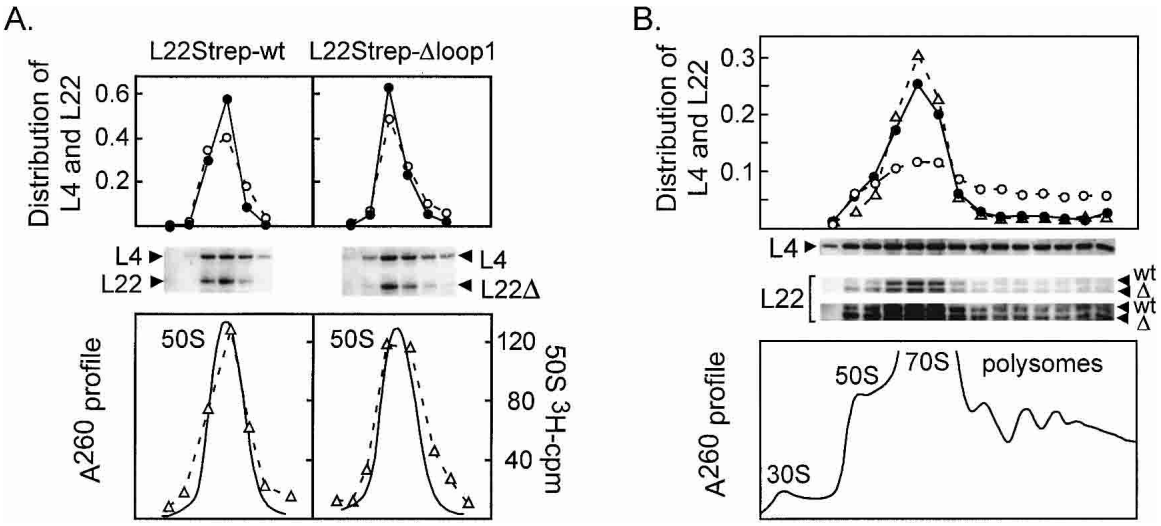

FIGURE 7. Sucrose gradient analysis of ribosomal particles containing wild-type or mutant L22 proteins. (*A*) Distribution of L22Strep and L22Strep-Δloop1 proteins in 50S ribosomes. Ribosomes enriched for 50S subunits were sedimented through a 10–30% sucrose gradient together with [^{3}H]uridine-labeled purified 50S ribosomes prepared from a control culture. (*Bottom*) A^{260} profile together with a plot of the radioactivity in each fraction (indicated by open triangles and dashed lines). (*Middle*) Proteins from the collected fractions were fractionated on a 15% PAGE gel and subjected to Western analysis using anti-L4 and anti-Strep antibodies. (*Top*) For each fraction, the signal in chromosome-derived L4 or in the indicated plasmid-derived L22 protein was divided by the total signal for the protein in all of the fractions of the gradient. (○, broken line) Chromosome-derived L4; (●, solid line) plasmid-derived L22. (*B*) Sucrose gradient analysis of polysomes containing wild-type or mutant L22 proteins. (*Bottom*) A^{260} profiles. (*Middle*) Proteins from the collected fractions were separated on a 10% Tricine gel and subjected to Western analysis using anti-L4 and anti-Strep. Two different PhosphorImager exposures of the anti-Strep Western are shown. (wt) Wild-type L22-Strep; (Δ) L22Strep-Δloop1. (*Top*) For each fraction, the signal in chromosome-derived L4 or in the indicated plasmid-derived L22 protein was divided by the total signal for the protein in all of the fractions of the gradient. (○, broken line) Chromosome-derived L4; (△, broken line) plasmid-derived wild-type L22; (●, solid line) plasmid-derived L22–Δloop1.

observed in the polysome fractions of the gradient, indicating that even in the absence of most of the extended loop of L22, the 50S subunits can participate in protein synthesis. Compared with the distribution of L4, however, both wild-type and L22 proteins appear to be shifted to the 50S/70S region of the gradient. We suspect that this is because many of the ribosomes that have assembled with the recently induced tagged L22 proteins have not yet had time to form polysomes (unlike the L4 experiments, the ribosomes were not prepared from "chased" cells). However, we cannot exclude the possibility that the Strep-tag itself may reduce the ability of 50S particles to enter the polysome fraction.

DISCUSSION

Many of the r-proteins in both small and large subunits of the ribosome contain extended loops and termini that are intertwined with the rRNA, often extending from globular surface domains to the inner parts of the ribosome (Ban et al. 2000; Brodersen et al. 2002). A number of r-protein mutations bestowing antibiotic resistance map to such extensions (Ban et al. 2000; Carter et al. 2000). The role of these extensions is not clear. Because they make many contacts with rRNA and often interact with more than one domain of the RNA, one role might be the stabilization of the proper rRNA tertiary structure (Ban et al. 2000; Wimberly et al. 2000; Brodersen et al. 2002). Besides their scaffolding role, the r-protein extensions might play a more active role in ribosome function. For example, the loops might be involved in promoting conformational changes in the ribosome during protein synthesis. This notion is supported by the change in rRNA structure caused by mutations in the loops of L4 and L22 (Gregory and Dahlberg 1999) and the effect of amino acid substitutions in L22 on the translation of *E. coli secM* (Nakatogawa and Ito 2002).

To begin understanding the functions performed by the loops of L4 and L22, we analyzed the effects of their deletion on regulation and assembly. In the case of L4, we observed that the protein functions normally as a regulatory protein even when virtually the entire loop is removed. This result was not entirely surprising, as our earlier genetic studies suggested that another region of L4 is involved in autogenous control (Li et al. 1996; Worbs et al. 2002). The surprising result was that much of the extended loops of L4 and L22 are apparently dispensable for assembly into 50S ribosomal particles.

The assembly of ribosomal subunits during L4 oversynthesis is complicated by the formation of incomplete particles resulting from repression of the chromosomal S10 r-protein operon. However, incomplete particles formed during unbalanced synthesis of ribosomal proteins were

converted into normal subunits during a chase period in which the inducer of the plasmid-borne L4 gene was removed. We have no way of knowing whether the incomplete particles formed during L4 oversynthesis are directly converted to 50S subunits or if the particles are degraded and the content of mutant L4 is reused during assembly of normal subunits. Regardless, no difference in conversion efficiency could be detected whether wild-type or extensionless L4 had been overproduced.

Ribosomal assembly is a highly interactive process in which binding of many proteins to the rRNA depends on prior binding of other proteins. L4 is one of just eight proteins that can bind efficiently to naked 23S rRNA (Nierhaus 1991). L22 is also an "early-binding protein", but it does not bind with high affinity to naked 23S rRNA; the initial step of L22 incorporation in the ribosome is strongly stimulated by the prior binding of several other proteins, particularly L4 (Nierhaus 1991). In the completed 50S subunits, both L4 and L22 make contacts with multiple domains in 23S rRNA (Ban et al. 2000; Harms et al. 2001; Worbs et al. 2002). However, when L4 and 23S rRNA are incubated together, the protein binds only to domain I (Stelzl et al. 2000), suggesting that the first interaction between L4 and 23S is the one between the globular part of L4 and a small region of domain I. L22 also binds initially to domain I. The remaining contacts, several of which involve the extended loops, evidently form later in assembly and are apparently made possible by rearrangements of the structure of the rRNA and the L4 and L22 extensions.

The 50S subunits containing L4 or L22 missing part, or all, of the extended loop cosedimented exactly with 50S particles prepared from an unperturbed control culture. Our results, therefore, imply that the extensions are not only dispensable for assembly of L4 and L22, but they are also unneeded for proper assembly of proteins that bind later in the 50S assembly pathway. Given that the extensions of L4 and L22 have multiple interactions with rRNA helices derived from several domains of 23S, it is interesting that, in the absence of many or most of these interactions, the rRNA can still fold into a tertiary structure that facilitates proper assembly of 50S subunits. Note, however, that for the larger loop deletion of L22 (L22–Δloop2), the protein appears to be removed from the ribosome by high salt, suggesting that, at least for this small r-protein, part of the extended loop is necessary for stable association. It will be interesting to determine how assembly steps can be bypassed during the incorporation of proteins missing all or part of the extended loop. Analysis of the effect of the loss of the extended loop on the assembly process may offer new insights into the mechanics of the construction of ribosomes.

Particles containing L4–Δloop mutants or L22–Δloop1 can associate with 30S subunits to form 70S ribosomes. Moreover, these ribosomes retain the ability to enter the polysome fraction, suggesting that they can associate with mRNA. However, because these experiments were performed with a background of ribosomes containing chromosome-encoded wild-type L4 or L22, we cannot yet critically assess their activity in protein synthesis. For example, we cannot measure the peptide chain elongation rate or the accuracy of translation of ribosomes carrying the loop deletion proteins. Nevertheless, overexpression of the deletion L22 proteins had no effect on growth rate, indicating that these mutations have no dominant deleterious effect (data not shown). Equivalent experiments to investigate effects of deletion L4 proteins on growth rate could not be performed, because all deletion L4 proteins fully repress the S10 operon and thus stop growth.

The L22–Δery protein contains a deletion known to bestow erythromycin resistance. Arabinose induction of this protein (in the presence of the chromosome-encoded sensitive protein) results in a slight increase in resistance to the antibiotic, but induction of the L22–Δloop1 protein had no detectable effect on erythromycin resistance (data not shown). Perhaps this is not surprising, as L22-mediated erythromycin resistance may require a very specific alteration in the protein in order to change the shape of the peptide exit tunnel.

Further analysis of the roles of L4 and L22 requires isolation of mutant ribosomes without any wild-type particles. We anticipate that characterization of such 50S preparations will provide valuable information about the contributions of these two proteins to ribosome translation mechanisms.

MATERIALS AND METHODS

Strains and plasmids

Physiological experiments were done in *E. coli* K12 strain LL308 (Lindahl and Zengel 1979). DNA manipulations were done using DH5αF′. The *E. coli* L4 and L22 genes were cloned into plasmid pBAD18 (Guzman et al. 1995) (conferring ampicillin resistance), under the control of an arabinose-inducible promoter. The pBAD18–EcoL4 plasmids containing the wild-type *E. coli* L4 gene and a wild-type gene encoding a C-terminal 6-histidine tag (His6 tag) have been described (Worbs et al. 2002). The Strep-tag (IBA GmbH) sequence was introduced to the C-terminal end of L4 and L22 by a SOEing strategy (Ho et al. 1989). The L22–Δery gene was isolated by PCR amplification from the chromosome of erythromycin-resistant strain N281 (Chittum and Champney 1994). Other mutations were introduced either by QuikChange (http://www.stratagene.com) or SOEing (Ho et al. 1989) mutagenesis strategies.

For in vivo regulatory studies, the L4 plasmids were introduced into cells already carrying plasmid pACYC–S10′/′*lacZ* (Worbs et al. 2002), which contains the *E. coli* S10 leader and proximal 54 codons of the S10 gene fused in frame with a *lacZ* gene lacking the proximal 8 codons (Freedman et al. 1985); in this construct, expression of the fusion gene is under the control of the IPTG-inducible *trc* promoter.

In vivo regulatory studies

The ability of L4 to repress the S10′/′*lacZ* reporter protein was determined as described previously (Worbs et al. 2002; Zengel and Lindahl 2003). Briefly, cells carrying target and L4-source plasmids were induced with IPTG to turn on synthesis of the S10′/′β-gal fusion protein, or with IPTG and arabinose, to also induce expression of the mutant L4 protein. After 10 min, aliquots of both were labeled briefly with [^{35}S]methionine. Total cell extracts were fractionated by SDS–polyacrylamide gel electrophoresis, using a 7.5% (wt/vol) gel to resolve the β-galactosidase protein and a 12% or 15% (wt/vol) gel to resolve small proteins like L4 (22 kDa) and its deletion derivatives (Zengel and Lindahl 2003). Radioactivity in the S10′/′β-galactosidase (S10′/′β-gal) fusion protein bands was quantified using a Molecular Dynamics Storm PhosphorImager and normalized to the total amount of radioactivity in the lane (measured as the radioactivity in a box circumscribing the entire lane; Zengel and Lindahl 2003). The effect of L4 oversynthesis was calculated as the ratio between the normalized S10′/′β-gal value for a given strain in the presence of both IPTG and arabinose divided by the S10′/′β-gal value for the same strain in the presence of only IPTG.

Ribosome preparation

Crude ribosomes were prepared by sedimentation from whole-cell extracts as described previously (Worbs et al. 2002). Briefly, cells were grown in 200 mL of LB medium containing ampicillin (200 μg/mL). At OD_{450} = 0.1–0.4 (2–5 × 10^7 cells/mL), arabinose was added to 0.2%. Cells were harvested by centrifugation at OD_{450} = 1–2 and ribosomes were prepared using a procedure adapted from Korber et al. (2000). The cell pellets were washed once with 1 mL of Buffer A (20 mM HEPES-KOH at pH 7.5, 6 mM $MgCl_2$, 30 mM NH_4Cl, 6 mM β-mercaptoethanol), resuspended in 2 mL of Buffer A, and split into two 1-mL aliquots. Each aliquot received 100 μL of lysozyme (15 mg/mL). After 3 min incubation on ice, the samples were flash-frozen and stored at −80°C. The 1-mL lysozyme-containing extracts were lysed by slow-thawing in an ice-water bath. The resulting lysates were clarified by spinning at 22,000 rpm for 30 min in an MLA130 rotor in a Beckman Optima MAX Ultracentrifuge. The duplicate supernatants from each culture were then pooled and centrifuged for 4 h at 40K in the MLA130 rotor. The pellets were resuspended overnight in 200 μL of Buffer A, then centrifuged at 13K for 10 min at 4°C. The resulting supernatants contain what we refer to as 'crude' ribosomes. To prepare 'salt-washed' ribosomes, 150 μL of the crude ribosomes were mixed with 1.8 mL of Salt Wash Buffer (20 mM HEPES-KOH at pH 7.5, 30 mM $MgCl_2$, 1 M NH_4Cl, 6 mM β-mercaptoethanol), incubated for 1 h at 4°C, and then centrifuged in the MLA130 rotor for 4 h at 50K. The pellets were rinsed once with 200 μL of Buffer A, resuspended overnight in 100 μL of Buffer A, and centrifuged at 13 K for 10 min at 4°C. The resulting supernatants contain what we refer to as 'salt-washed' ribosomes.

For analysis of total-cell extracts, 1-mL aliquots of the 200-mL cultures were removed prior to arabinose addition and grown in parallel (uninduced extract, 'ext -'). Also, 1-mL aliquots were removed from the arabinose-induced culture immediately before harvesting (induced extract, 'ext +'). Both sets of samples were centrifuged and the pellets were resuspended in 200 μL of Laemmli sample buffer (Zengel et al. 2002), incubated for 2 min at 95°C, and stored at −20°C.

For the L4 chase experiments, cells were grown in 600 mL of LB plus ampicillin. At OD_{450} about 0.4–0.5, arabinose was added to 0.2%. At OD_{450} = 1–1.2, 400 mL were harvested by centrifugation and processed as described above. Cells in the remaining 200 mL were collected on a sterile cellulose acetate filter (0.22 μm, 72 mm diameter, Corning), washed 3 times each with 10 mL of prewarmed LB, and transferred to 400 mL of fresh LB ampicillin plus 0.2% glucose (to ensure shut-off the arabinose-inducible promoter). The cells were grown for one more doubling before harvesting as described above.

To prepare ^{3}H-labeled ribosomes, strain LL308 was grown at 37°C in 25 mL of AB minimal medium plus 0.2% glucose, 20 μg/mL each of 19 amino acids (no methionine), and 1 μg/mL thiamine. At OD_{450} about 0.2, 1 mL of cells was transferred to a disposable 50-mL tube (Corning) containing 10 μCi of [5,6–^{3}H]uridine (Amersham TRK410, 32 Ci/mmole, 1 mCi/mL), and maintained with shaking at 37°C. The remainder of the 25-mL culture was also continued. Forty minutes later, 10 mL of LB was added to the labeled cells, and 200 mL of LB was added to the 25-mL culture. When the larger culture reached OD_{450} = 1, each culture was spun down, resuspended in Buffer A (0.1 mL for radioactive culture and 1 mL for non-radioactive cells). They were then combined, pelleted, resuspended in 2 mL of Buffer A, and processed as for regular crude ribosome preparations (see above). The 50S subunits were then prepared by sucrose gradient centrifugation as described below.

Sucrose gradient centrifugation

Polysome sucrose gradients were prepared in Beckman SW40 tubes by layering sucrose solutions ranging from 10% to 50% in Buffer A (20 mM HEPES-KOH at pH 7.5; 6 mM $MgCl_2$; 30 mM NH_4Cl; 6 mM β-mercaptoethanol) (Korber et al. 2000). Lysates or crude ribosomes (~25 A^{260} units) were layered on top and centrifuged at 4°C for 3.5 h at 40,000 rpm. Fractions were collected using an ISCO sucrose gradient collector with a UV monitor. Aliquots of the fractions were either mixed directly with two volumes Laemmli sample buffer, or were precipitated with one-tenth volume 100% TCA and resuspended in 25 μl of 0.2 N NaOH and 100 μl of Laemmli sample buffer.

To prepare 50S subunits, crude ribosomal pellets (2.5 A^{260} units) were sedimented through sucrose gradients prepared as above, but with only 0.3 mM $MgCl_2$ and with sucrose ranging from 10% to 30%. Fractions from the region of the gradient containing 50S and residual 70S ribosomes were pooled, pelleted by centrifugation, and resuspended in Buffer A containing 0.6 mM $MgCl_2$. The ribosomes were then mixed with ^{3}H-labeled ribosomes, corresponding to 1/20–1/10 of the mass of the ribosomes from the experimental sample, and resedimented through the same type of gradient.

Western analysis

Proteins from ribosomes (0.1 A^{260} equivalence) or crude cell extracts (10 μl) were fractionated by 15% SDS–polyacrylamide gel electrophoresis (Laemmli 1970) or 10% Tricine gels (Schagger and von Jagow 1987) and then electroblotted to a PVDF nitrocellulose

membrane (Perkin-Elmer) according to standard procedures. Prestained molecular weight markers (Bio-Rad Low Molecular Weight) were included on each gel. For probing Strep-tagged proteins, the membrane was first incubated with a polyclonal rabbit antibody specific for the Strep-tag (IBA, diluted 1:4000). The secondary antibody was the goat anti-rabbit IgG (Bio-Rad, diluted 1:3000). The signal was detected using ECF Substrate for Western Blotting (Amersham) and scanning the membrane on a Storm PhosphorImager. In the second round, the primary antibody was a rabbit polyclonal antibody specific for *E. coli* r-protein L4 (a gift from M. Nomura, diluted 1:10000). The secondary antibody was an alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad, diluted 1:3000). Again, the signal was detected using the ECF Substrate for Western Blotting and scanning on the Storm PhosphorImager. We did not strip the blot after the first round because we found that the Strep-Tag antibody could not be removed. In some experiments, the L4 antisera preceded the anti-Strep antibody.

Molecular modeling of *E. coli* L4

Because no experimental atomic structure of *E. coli* L4 is presently available, we generated a homology model of the globular portion of the protein with the program Swiss-Model (Guex and Peitsch 1997), using the crystal structure of *Thermotoga maritima* L4 (Worbs et al. 2000) as the lead. Presently, only Cα-coordinates are available for L4 from *Deinococcus radiodurans*, whose crystal structure has been determined within the framework of the 50S subunit (Harms et al. 2001), thus revealing the trace of the extended loop. Least-squares superpositioning of the globular portion of the *E. coli* L4 model with the corresponding part of *D. radiodurans* L4 allowed transfer of these loop Cα-positions to the model. Side chains (according to the *E. coli* L4 sequence) and the missing atoms of the main chain were subsequently appended in optimized geometry with the generate option of CNS (Brünger et al. 1998). As the model was only used to visualize and guide mutations introduced into *E. coli* L4, we refrained from further modifying the model, for example, by energy minimization, as the extended loop conformation is expected to be stable only when in intimate contact with the rRNA.

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